Isolation and Characterization of *Clostridium perfringens* Bacteriophages and Optimization of Electro-Transformation Parameters for *Clostridium difficile*

Thesis submitted for the degree of

Doctor of Philosophy

at the University of Leicester by

Ali ABDULKAREEM Ali

Department of Infection, Immunity, and Inflammation

University of Leicester, Leicester, UK

September 2017

Abstract

Isolation and Characterization of *Clostridium perfringens* Bacteriophages and Optimization of Electro-Transformation Parameters for *Clostridium difficile*

By
Ali Abdulkareem Ali

Clostridium perfringens (*C. perfringens*) is responsible for a variety of diseases in humans and animals. *Clostridium difficile* (*C. difficile*) is the leading cause of antibiotic-associated diarrhoea. The available treatments for infections caused by both pathogens are not effective. Bacteriophage (phage) therapy is a promising treatment strategy to tackle and treat any infections or diseases caused by *C. perfringens* or *C. difficile*. The aim of this study was to isolate bacteriophages that infect *C. perfringens* and characterize them, and to optimize electroporation parameters for *C. difficile* with the end goal being to be able to genetically modify *C. difficile* phages.

Five phages that infect *C. perfringens* were isolated and characterized from environmental samples. Two phages were sequenced and annotated, one was found to be a Podovirus and the other a Siphovirus. The Podovirus is a strictly lytic phage that does not possess any undesired genes such as the transduction gene, antibiotic resistance gene, and toxin genes.

The endolysin of the Podovirus was cloned, expressed, and purified. The muralytic activity of the enzyme was confirmed by a zymogram. This endolysin has the ability to completely lyse 85.7 % of the tested *Clostridium perfringens* strains.

A series of electroporation experiments were carried out using many experimental settings and varying parameters in order to deliver engineered phage and plasmid DNA genome to *C. difficile*. The electroporation refractory *C. difficile* R076 was treated with a cysteine protease inhibitor E- 64 and lysostaphin to facilitate electroporation. E-64 was able to reduce the thickness of the *C. difficile* surface layer proteins. The treatment with lysostaphin resulted in cell lysis. Unfortunately, all the attempts made to optimize the electroporation protocol for *C. difficile* were unsuccessful as no cells were transformed. However, the experimental observations provide a strong foundation for further work to develop an effective electro-transformation protocol for *C. difficile.*

Acknowledgements

I would like to gratefully acknowledge my supervisor Professor Martha Clokie for her continued guidance and support throughout my PhD project. Her invaluable encouragement and patience during the practical work and the writing up period were crucial in allowing me to complete this project. I would also like to thank the Iraqi ministry of higher education and scientific research for funding my project.

Furthermore, I would like to thank my friends Ananthi, Anisha, Ahmed, Bander, Depesh, Faizal, Guillermo, Jaspreet, Janet, Jinyu, Lamiaa, Mohammed, Neda, Peter, Saroa, Thekra, Tolis, Wafaa and everyone in the phage laboratory for the friendly atmosphere that they created and for their continuous support.

Among all my friends, I would particularly like to thank Christopher Turkington for sharing his experience with me, and for the unforgettable jokes and discussions we had while we were working late. Also, a big thank you to the expert technician Natalie Allcock of the Transmission Electron Microscopy unit for her helpful training and assistance.

Finally, I want to thank my family and my friends back home for their support and reassurance during my studies here at Leicester. Special thanks go to my wife for her understanding and patience.

Contents

List of Figures

List of tables

List of abbreviation

APS Ammonium persulfate

BA Brucella agar

BHI Brain heart infusion

BLAST Basic local alignment search tool

BRIG Blast Ring Image Generator

CFU Colony forming unit

CM Cytoplasmic membrane

EB Electrical breakdown

FA Fastidious Anaerobic

GIT Gastro-intestinal tract

GST Glutathione S-transferase

HMW High molecular weight

LB Lysogeny broth

LMW Low molecular weight

MOI Multiplicity of infection

MW Molecular weight

NE Necrotic enteritis

ORF Open reading frames

PBS Phosphate buffered saline

PCR Polymerase chain reaction

PEG Poly-ethylene glycol

PFGE Pulsed field gel electrophoresis

RAST Rapid Annotation using Subsystem Technology

REA	Restriction endonuclease analysis
SDS	Sodium dodecyl sulfate
SLP	Surface layer protein
SM	Sodium magnesium
SMP	Sodium magnesium phosphate
TBE	Tris-Borate-EDTA
TE	Tris-EDTA
TEM	Transmission electron microscope
TEV	Tobacco Etch Virus
TGY	Trypticase glucose-yeast
TS	Tryptose-sulfate
TSC	Tryptose-sulphate Cycloserine
TY	Tryptose-yeast

Chapter 1

Introduction

Chapter 1. Introduction

1.1 Background

1.1.1 *Clostridium difficile* (*C. difficile*)

C. difficile is the leading cause for bacterial-derived nosocomial antibiotic-associated diarrhoea globally. It is both a health and economic burden with a high morbidity and mortality rate associated with *C. difficile* infection (CDI), along with a high cost incurred for antibiotic treatment, relapses, and prolonged hospitalisation (Segar *et al.*, 2017, Barkin *et al.*, 2017, Messick *et al.*, 2017). One of the reasons for the success of *C. difficile* infection is that it produces spores that can resist common antiseptics used to decontaminate surfaces. These spores are able to retain their infectivity for months and spread CDI by the faecal-oral route or through patients touching contaminated surfaces, including the hands of healthcare staff (Joshi *et al.*, 2017, Ananthakrishnan, 2011). In general, CDI starts following antibiotic treatment for another condition that results in dysbiosis. Dysbiosis is a term that describes the imbalance of the microbiota inside or on the body, such as the intestinal microbiota. The impaired functional and structural conditions of the intestinal microbial community allow *C. difficile* to establish the infection (Bibbo *et al.*, 2014, Carding *et al.*, 2015).

The current treatment of CDI is the use of antibiotics. Antibiotic treatment has many disadvantages such as the high cost of certain antibiotics, the fact that this therapy is only partially effective, and does not prevent infection from reoccurring (Surawicz *et al.*, 2013, Winston *et al.*, 2016, Liubakka *et al.*, 2016, Nayak *et al.*, 2017). Moreover, the development of multiple-antibiotic resistance mechanisms by *C. difficile* necessitates the identification of adjuvant or alternative treatments (Hutton *et al.*, 2017). One of these is faecal transplantation, an inexpensive and effective treatment for recurrent CDI (Brumbaugh *et al.*, 2017).

To address this resistance, the interest in using phage therapy against CDI has been renewed. Phage therapy provides local dose amplification and targets the pathogen in a highly specific manner. However, no strictly lytic *C. difficile* phages have been described yet, thereby making it difficult to exploit *C. difficile* phages for therapeutic purposes. Lysogenic phages are considered unsuitable for therapy, particularly due to their potential

roles in horizontal gene transfer (transduction ability) (Hargreaves *et al.*, 2014, Nale *et al.*, 2016b).

The potential advantages of integrase defective *C. difficile* phages is that given that they are not able to integrate with the bacterial chromosome, they could, in theory, proceed with lytic growth and destroy the *C. difficile* cells. On the other hand, the disadvantage of the integrase gene deletion is that the phage is no longer able to persist as a prophage integrated in *C. difficile* chromosome. Consequently, no lysis will occur when the prophage free *C. difficile* spores germinate, as it does not have a prophage which can switch to lytic growth.

1.1.2 *Clostridium perfringens* (*C. perfringens*)

C. perfringens causes a wide range of diseases in both humans and animals. Similar to other members of the *Clostridium* genus, it produces spores that spread the infections (Talukdar *et al.*, 2017). In England and Wales, *C. perfringens* causes 80-95% of gas gangrene cases and has been involved in human gastroenteritis outbreaks every year between 1992 and 2013 (Public health England, 2013). Gas gangrene is a serious traumatic and post-surgical wound infection that becomes fatal if it is not treated at the correct time (Sudharsanan *et al.*, 2017, Harmsen *et al.*, 2016). Globally, *C. perfringens* also causes massive economic losses by causing broiler chickens enteritis. This disease occurs when intestinal health is impaired, which allows *C. perfringens* to flourish and produce toxins. Broiler chicken enteritis has a financial implication of $6 billion a year (Broom, 2017).

The gas gangrene infection is treated by the resection or surgical debridement of the infected part of the body and antibiotic administration (Harmsen *et al.*, 2016, Wormald *et al.*, 2016). Both human enteritis and broiler chicken enteritis are treated with antibiotics, however, new antimicrobials are particularly needed due to the development of multiple-antibiotic resistance by *C. perfringens.* Ideally, the antimicrobials which have no influence on the useful commensals of the host's gut microbiota are the ones required to treat *C. perfringens* infections (Skrivanova *et al.*, 2016, Gaucher *et al.*, 2017). Thus, any therapy that can lyse vegetative cells as soon as spores start to germinate into vegetative cells will prevent the infection in an effective manner.

Phage therapy has been used to control infections with *C. perfringens*. Many *C. perfringens* phages have been isolated; some of these are temperate, such as phages

Φ3626 and Φ8533 (Zimmer *et al.*, 2002a), ΦCP39O and ΦCP26F (Seal *et al.*, 2011), ΦS9 and ΦS63 (Kim *et al.*, 2012), and phiSM101 (Nariya *et al.*, 2011). Others are virulent *C. perfringens* phages, such as ΦCP24R (Morales *et al.*, 2012), ΦCPV1 (Volozhantsev *et al.*, 2011), and ΦCPV4 & ΦZP2 (Volozhantsev *et al.*, 2012). The lytic *C. perfringens* phages have proved to be effective against *C. perfringens in-vivo* (Miller *et al.*, 2010, Caly *et al.*, 2015). Few studies have focused on the use of the phage endolysin instead of using the whole phage to overcome the complications that may accompany phage therapy (Caly *et al.*, 2015), such as the endolysin of Φ3626 (Zimmer *et al.*, 2002b).

The isolation and characterisation of new phages helps in understanding their diversity and how they interact with their host. It can also broaden the knowledge of their biology, thereby potentially opening new research fields. The activity of phages and phage-derived endolysins can be further explored when new phages are isolated, and when new phage cocktails are formulated. In order to treat a particular pathogen effectively, there is the requirement for phages with wide host ranges and strong lytic activity. Therefore, the isolation of new phages is important in order to increase the collection of phages and therefore be able to target more strains of particular pathogenic species.

1.2 Hypothesis

C. difficile phages can be designed to have superior properties to their parent wild type phages. In theory, integrase defective phage will only grow in a lytic manner and lyse the infected *C. difficile* cells. A construct that is able to disrupt *C. difficile* toxin genes can also be inserted in the phage genome; this improvement can be beneficial for therapeutic purposes.

C. perfringens phages can be isolated from the environment and be useful for therapeutic purposes. Phage endolysins can also be useful in the control and prevention of *C. perfringens* related infections. Moreover, phage-derived endolysins can be used to control or prevent *C. perfringens* growth.

1.3 Research Aims

The first major aim of this project was to optimise the electroporation parameters for *C. difficile* in order to allow for the genetic manipulation of *C. difficile* phages. The second aim was to isolate and test the effectiveness of *C. perfringens* phages and their endolysins as antimicrobials to treat *C. perfringens* infections. The major objectives needed to fulfil these aims are listed below; all were attempted and are described in the relevant chapters, while both the context and background are introduced in this chapter.

1. Empirically optimizing electroporation parameters to transform *C. difficile* with engineered phage nucleic acids. (Chapter 6)
2. Treatment of *C. difficile* with high concentrations of cysteine protease inhibitor and lysostaphin to diminish the surface layer protein and facilitate electroporation. (Chapter 6)
3. Isolate and characterize *C. perfringens* from environmental samples including soil, animal faeces, and sediments. (Chapter 4)
4. Induce phage from the newly isolated *C. perfringens* strains and also exploit them as a host to isolate lytic bacteriophages. (Chapter 4)
5. Characterize the isolated phages according to their host-infectivity, morphology and genome characteristics. (Chapter 4)
6. Clone, express, and purify the *C. perfringens* phage endolysin, and determine the effect of this enzyme on the growth of *C. perfringens* strains. (Chapter 5).

Chapter 2

Literature review

Chapter 2. Literature review

2.1 Introduction to *C. difficile* and *C. perfringens*

2.1.1 *C. difficile* and *C. perfringens* history

The *Clostridium* bacterial genus consists of Gram-positive, anaerobic spore-forming rod species that are spread throughout the environment. Most of these species are non-pathogenic saprophytes. However, the most known potent toxins are produced by five members of this genus including *C. difficile* and *C. perfringens* (Johansson, 2006, Milton *et al.*, 2017, Orrell *et al.*, 2017, Watts *et al.*, 2017).

C. difficile was first isolated in 1935 and called *Bacillus difficile.* It was first identified as the etiological agent of antibiotic-associated pseudomembranous colitis in 1978 and is currently a leading cause of nosocomial infection with a high rate of morbidity and mortality (Hall *et al.*, 1935, Bartlett *et al.*, 1978, Liubakka *et al.*, 2016).

C. perfringens was first isolated in 1892 from a gas gangrene wound and named *Bacillus aerogenes capsulatus*; this was changed to *Bacillus perfringens*, then *Clostridium welchii* and is now known as *Clostridium perfringens.* It causes many animal and human diseases (Johansson, 2006, Li *et al.*, 2013).

2.1.2 *C. difficile* and *C. perfringens* infections and pathogenicity

The ubiquitous presence of *C. perfringens* and *C. difficile* spores in the environment and as a commensal in the intestine of mammals, birds and reptiles may account for the high rate of transmissibility. Additionally, the pathogenesis and disease of these organisms initiates upon contact, ingestion or disruption of normal gut flora as a result of antibiotic use for the treatment of infections other than CDI. (García *et al.*, 2011, Hensgens *et al.*, 2012, Dubberke *et al.*, 2011, Sekulovic *et al.*, 2016, Goldenberg *et al.*, 2017).

Both pathogens incur both a health and economic burden, causing animal diseases along with hospital and community acquired human infections with a high rate of morbidity and mortality (Buzby *et al.*, 1997, Abou Chakra *et al.*, 2014, Collery *et al.*, 2016, Hwang *et al.*, 2012, Keessen *et al.*, 2013, Vaishnavi *et al.*, 2011, Vernacchio *et al.*, 2006, Carter *et al.*, 2014, Winston *et al.*, 2016).

C. difficile causes hospital and community acquired antibiotic-associated diarrhoea that range from mild diarrhoea to deadly pseudomembranous colitis with high rates of morbidity and mortality (Hwang *et al.*, 2012, Mattila *et al.*, 2013, Carter *et al.*, 2014). The bacterium *C. difficile* produces three types of toxins; A, B, and the binary toxin AB (Sun *et al.*, 2015). In addition to pseudomembranous colitis, *C. difficile* can cause extra-intestinal infections in anatomic areas both near and away from the colon. Infections in the vicinity of the colon area include peritonitis, intra-abdominal abscesses, and abdominal wound infections. Bacteraemia, brain abscess, and foot infection (chronic osteomyelitis) are infections found in areas that are distal from the colon (García-Lechuz *et al.*, 2001).

Between 2007 and 2011, *C. difficile* has caused approximately 10,258 deaths in England and Wales, and was a participating factor in another 12,687 deaths. Most CDI deaths (91%) occurred in elderly people (85 years of age and over) (Porter *et al.*, 2015). Tables 1 and 2 show CDI infections and the registered deaths caused directly by it, and as an underlying disease.

Between January 2013 and February 2017, *C. difficile* caused outbreaks in France, as a nosocomial infection in elderly people and as a community acquired infection in young adults. Before that, in 2011, *C. difficile* resulted in about half a million infections in the U.S with 29,000 deaths; a similar rate was recorded in Europe (Lessa *et al.*, 2015, Leffler *et al.*, 2015, Cassir *et al.*, 2017).

C. perfringens causes a variety of diseases for human and animals (Table, 3), all of which depend on the organisms prolific ability of being able to produce approximately 18 toxins. These diseases include tissue toxicity diseases, enteritis and enterotoxaemia (Li *et al.*, 2013, Ferreira *et al.*, 2016). *C. perfringens* can be classified into five groups; A, B, C, D and E (Table,4), this depending on the ability of being to produce one or more of the main four toxins produced by this pathogen, these being alpha (α), beta (β), epsilon (ε) and iota (ι) (Petit *et al.*, 1999, Dave, 2017).

C. perfringens causes 80-95% of gas gangrene cases in England and Wales. It has also been the cause of many human gastroenteritis outbreaks between 1992 and 2013, with an average of 14.28 outbreaks per year (2-38). The reported cases in these outbreaks ranged between 23-748 infections (Public health England, 2013). Although the illness lasts for only one day, it has a serious effect on infants and elderly people and results in diseases

such as intestinal necrosis, haemorrhaging and perforation of the intestine at varying levels (Kosloske *et al.*, 1985).

C. perfringens is the etiologic agent of necrotic enteritis (NE) in chickens, which has emerged as a significant economic problem in the poultry industry (Abid *et al.*, 2016). Large amount of research has described the use of microbes and microbial products including bacteriophages and its endolysin as novel alternatives for the treatment and prevention of NE (Caly *et al.*, 2015, Seal, 2013).

Number of deaths caused by CDI mentioned by gender																		
Years	1999	2000	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016
Male	18	:	24	36	24	33	34	51	145	187	148	134	98	69	69	52	39	33
Female	38	:	40	53	59	55	70	119	254	274	233	234	138	86	108	59	69	46
Number of deaths caused by CDI as underlying cause mentioned by gender																		
Male	11	:	12	19	10	19	22	29	59	83	72	55	51	32	34	18	13	12
Female	20	:	17	28	36	35	39	64	118	120	129	129	67	50	57	24	37	20

Table 1. Number of deaths caused by *C. difficile* infection in Wales between 1999 and 2016. The non-residents and neonates deaths are excluded from these data. Source: https://www.ons.gov.uk/peoplepopulationandcommunity/birthsdeathsandmarriage

Date	April to June 2007	July to September 2007	October to December 2007	January to March 2008	April to June 2008	July to September 2008	October to December 2008	January to March 2009	April to June 2009	July to September 2009	October to December 2009
Total CDI	16,864	13,419	12,248	12,967	10,883	8,948	7,907	8,357	6,854	6,407	6,002
Date	January to March 2010	April to June 2010	July to September 2010	October to December 2010	January to March 2011	April to June 2011	July to September 2011	October to December 2011	January to March 2012	April to June 2012	July to September 2012
Total CDI	6,341	5,981	5,909	4,984	4,833	4,967	4,994	4,350	3,711	3,656	3,870
Date	October to December 2012	January to March 2013	April to June 2013	July to September 2013	October to December 2013	January to March 2014	April to June 2014	July to September 2014	October to December 2014	January to March 2015	April to June 2015
Total CDI	3,756	3,412	3,386	3,671	3,298	3,007	3,449	3,979	3,366	3,398	3,652
Date	July to September 2015	October to December 2015	January to March 2016								
Total CDI	4,011	3,534	2,942								

Table 2. Quarterly counts of CDI in England for patients aged 2 years and over between 2007 and 2016 (Trust apportioned cases only).
Source: https://www.gov.uk/government/statistics/clostridium-difficile-infection-annual-data

Type	Major toxin(s)	Human disease(s)	Animal disease(s)
A	Alpha-toxin	Human myonecrosis (gas gangrene).	Gas gangrene in sheep, cattle and horses. Yellow lamb disease in sheep.
	Alpha-toxin, CPE	Human food poisoning, non-food born GI diseases.	Enteritis in dogs, pigs, horses, foals and goats.
	Alpha-toxin, NetB	Not reported.	Necrotizing enteritis in chickens.
	Alpha-toxin, CPB2	Not reported.	Possible enteritis in pigs, possible enterocolitis in horses.
B	Alpha-toxin Beta-toxin Epsilon-toxin	Not reported.	Necrotizing enteritis and enterotoxaemia in sheep, cattle and horses.
C	Alpha-toxin Beta-toxin	Human enteritis necroticans.	Necrotizing enteritis and enterotoxaemia in pigs, lambs.
D	Alpha-toxin Epsilon-toxin	Not reported.	Enterotoxaemia in sheep, goats and cattle.
E	Alpha-toxin Iota toxin	Not reported.	Enteritis in rabbits, lambs and cattle.

Table 3. Animal and human diseases caused by *C. perfringens*. The figure shows the main human and animal diseases caused by *C. perfringens* and the main toxin(s) produced by each toxin type (Li et al. 2013).

	Toxin produced			
Type	Alpha	Beta	Epsilon	Iota
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

Table 4.*C. perfringens* toxin-types.Type A produces alpha toxins. Type B produces alpha, beta and epsilon toxins. Type C produces alpha and beta toxins. Type D produces alpha and epsilon toxins, and type E produces alpha and iota toxins (Li et al. 2013).

The following section will give an overview of the types of diseases associated with *C. perfringens*. Type A *C. perfringens* causes fatal septicaemia and malignancy in immunocompromised patients (Cochrane *et al.*, 2015, Landi *et al.*, 2017). Also caused by type A strains is gas gangrene, which is the most serious form of *C. perfringens* human infection (Kumar *et al.*, 2017). Food poisoning is caused by *C. perfringens* as a result of the production of the theta toxin, and the enterotoxin (CPE) respectively (Hifumi *et al.*, 2013, Nagahama, 2013, Yelland *et al.*, 2014). Type A *C. perfringens* can also cause haemorrhagic diarrhoea in dogs, and necrotizing enteritis in foal (Gohari *et al.*, 2017).

The *C. perfringens* minor β2-toxin is a variant of the main *C. perfringens* beta toxin, and is responsible for the enterotoxaemia syndrome in both humans and animals (Serroni *et al.*, 2017). Some type A and type C strains of *C. perfringens* produce this toxin (Lee *et al.*, 2014, Lee *et al.*, 2012). The β2-toxin helps in the toxin typing of type A variants of *C. perfringens* along with the CPE toxin (Finegold *et al.*, 2017).

Necrotic enteritis toxin B-like (NetB) is a pore-forming toxin considered as a virulence factor responsible for the pathogenesis of necrotic enteritis (NE). NetB producing type A *C. perfringens* causes NE in chickens (Mishra *et al.*, 2017).

Type B and C strains of *C. perfringens* that are able to produce delta toxins (β-barrel-pore-forming toxins) cause a lethal intestinal infection in animals (Seike *et al.*, 2017).

Type B and D *C. perfringens* strains cause neurological disorders in animals, human brain lesions (multiple sclerosis) and livestock enteritis/enterotoxaemia as a result of epsilon toxin production (Wioland *et al.*, 2014, Rumah *et al.*, 2013, Li *et al.*, 2017).

The type C strain of *C. perfringens* that produces beta toxins causes necrotic enteritis in mammalian species (Bhatia *et al.*, 2014). On the other hand, type E *C. perfringens* causes haemorrhagic enteritis in calves as a result of the production of α and ι toxins (Billington *et al.*, 1998).

C. perfringens can also cause fulminant pancreatic sepsis and fatal liver abscesses (Stuckle *et al.*, 2017, Cherenfant *et al.*, 2009). Additionally, only one case of spondylodiscitis caused by *C. perfringens* has ever been reported (Seller *et al.*, 2016).

2.1.3 Toxin mechanism of action

C. difficile produces three toxins; toxin A (TcdA), toxin B (TcdB), and the binary toxin CDT which is produced by certain *C. difficile* strains associated with more severe CDI (fulminant colitis) (Smits *et al.*, 2016, Nakamura *et al.*, 2014). TcdA and TcdB are large glucosyltransferase toxins that are similar in structure and activity. Their primary structure consists of a two-domain, single-chain proteins which have the ability to attach to and penetrate the host cell and catalyze the transfer of glucosyl to the cellular cytoskeleton actin-regulatory GTPases. Subsequently, GTPases are de-activated, resulting in a disorganization of the cell cytoskeleton and thus eventually leading to cell death (Stiles *et al.*, 2014, Just *et al.*, 2005, Young *et al.*, 2014, Chandrasekaran *et al.*, 2017). Similarly, CDT is an ADP-ribosyltransferase that consists of the CDTa enzymatic domain, and CDTb which is the cell-binding domain. The latter is involved in receptor mediated endocytosis and the enzyme will catalyse the ribosylation of globular actin once it is inside the cell resulting in cell death (Gerding *et al.*, 2014).

The *C. perfringens* Alpha toxin is a phospholipase consisting of two domains - a membrane binding domain and a catalytic domain that disrupts the membrane integrity and cause cytolysis and cell death (Sakurai *et al.*, 2004, Manni *et al.*, 2017). The beta toxin also has a binding and an intra-membrane catalytic domain, and acts by causing a pore in the cytoplasmic membrane (Seike *et al.*, 2016). The epsilon toxin is another pore-forming toxin with a much more powerful membrane destruction ability than the alpha and beta toxin, as it has three catalytic domains which can form an oligomeric pore on the cell surface through membrane-associated proteins. However, the epsilon toxin's

exact mechanism of action is not completely understood (Popoff, 2011, Stiles *et al.*, 2013, Khalili *et al.*, 2017). The iota toxin has a binding-translocation and catalytic domain; it is an ADP-ribosyl-transferase that acts similarly to the *C. difficile* toxin (Chellapandi *et al.*, 2016, Papatheodorou *et al.*, 2016).

2.1.4 *C. difficile* and *C. perfringens* treatment

The treatment of CDI is recommended by medical guidelines to be stratified depending on the severity of the infection. Metronidazole is used for mild to moderate disease states, thereafter if metronidazole gives a poor outcome, vancomycin is administered (Surawicz *et al.*, 2013, Löfmark *et al.*, 2010, Khanna *et al.*, 2017). Although fidaxomicin has been used for CDI treatment, it is effective only when used at the early phase of infection, as patients with previous or prolonged CDI do not respond to it. Moreover, fidaxomicin does not prevent CDI relapses. Another disadvantage is the high cost of fidaxomicin pills; they cost $2800 which is significantly higher than vancomycin which costs $680 and metronidazole which cost $22 for a 10-day treatment (Spiceland *et al.*, 2016, Surawicz *et al.*, 2013). CDI treatment options are limited and new medication is urgently required (Postma *et al.*, 2015). In fact, most recently, cadazolid has been suggested to treat CDI (Caspers *et al.*, 2017). Moreover, faecal transplantation (FT) is an inexpensive and effective method to treat recurrent CDI. However, the presence of an underlying bowel disease attenuates its success (Brumbaugh *et al.*, 2017). Additionally, despite the fact that the use of probiotics (microbial preparations) for the prophylaxis against CDI has been suggested, it is not recommended by the recent clinical practice guidelines (Goldenberg *et al.*, 2017). Finally, the use of anti-TcdA and anti-TcdB antibodies to neutralize *C. difficile* toxins has been shown to be effective in the reduction of CDI recurrence in a pre-clinical model of CDI (Hernandez *et al.*, 2017).

The disadvantages of antibiotic treatment include partial effectiveness, the high cost of treatment associated with certain antibiotics, the high levels of CDI relapse after successful antibiotic treatment, the need for faecal microbiota transplantation to prevent relapse of CDI and the development of antibiotic resistance (Surawicz *et al.*, 2013, Winston *et al.*, 2016, Liubakka *et al.*, 2016, Nayak *et al.*, 2017). It is apparent that a novel therapy is needed to decrease, replace or compliment the use of antibiotics in the treatment of CDI. Phage therapy has shown promising potential as an antibacterial to treat CDI (Ivarsson *et al.*, 2015, Hegarty *et al.*, 2016, Hutton *et al.*, 2017). The details of the use of phage therapy to treat CDI are reviewed in section 2.2.4.

There is only one treatment available for human *C. perfringens* gas gangrene; the resection and prolonged multiple-antibiotic treatment upon the early diagnosis of infection. This management can save a patient's life (Wormald *et al.*, 2016). Examples of these treatments include resection arthroplasty, pancreatectomy, resection of infected soft tissue, above the knee resection of lower limb and long period antibiotic administration (Garcia-Jimenez *et al.*, 2016, Cherenfant *et al.*, 2009, Carretero *et al.*, 2016, Ying *et al.*, 2013). Chicken NE caused by *C. perfringens* is currently treated by antibiotics (Jayaraman *et al.*, 2017). Vaccination against the NetB *C. perfringens* toxin have also proved efficient in the prevention of NE, as 55% of orally vaccinated chickens remained healthy (Mishra *et al.*, 2017).

2.1.5 Genotyping of *C. difficile* and *C. perfringens*

Genotyping of *C. difficile* strains helps in the understanding of their epidemiology and pathogenesis. Ribotyping is currently used, while pulsed field gel electrophoresis (PFGE) and restriction endonuclease analysis (REA) have been used before. Ribotyping of *C. difficile* strains is an important process which allows for the discrimination between them. Particular ribotypes cause more severe CDI than others; for instance, The R027 ribotype causes a more severe disease than others, and is involved in the worse clinical outcome (Huber *et al.*, 2013). Ribotyping also allows for the identification of the genetic relatedness between *C. difficile* strains which are isolated from different areas and sources. For example, *C. difficile* R078 is found in different geographic areas and can be isolated from a variety of animal species. It has a high genetic relatedness with the human *C. difficile* R078, which may suggest a zoonotic potential for this lineage (Alvarez-Perez *et al.*, 2017). Nevertheless, whole genome sequencing provides more information than ribotyping (Huber *et al.*, 2013).

Determining *C. perfringens* strains' genetic variability helps with the comprehension of the pathogenicity and epidemiology of virulent and non-virulent isolates (Lacey *et al.*, 2016). Many methods have been described for *C. perfringens* typing, like multi-locus sequence typing, bacteriocin typing, serotyping, bacteriophage typing, plasmid profiling, ribotyping and macro-restriction with pulsed-field gel-electrophoresis, and multi-locus variable-number tandem repeat analysis (MLVA) (Lacey *et al.*, 2016, Mahony, 1974, Stringer *et al.*, 1980, Yan, 1989, Eisgruber *et al.*, 1996, Schalch *et al.*, 2003). Toxin typing has both epidemiological and diagnostic importance, as the pathogenicity, the destruction of cells and lesions produced are connected with the type of toxins produced.

In both early and recent *C. perfringens* genotyping studies, the presence of one or more toxin genes has been used to type *C. perfringens* (Sawires *et al.*, 2005, Li *et al.*, 2017, Silva *et al.*, 2016, Afshari *et al.*, 2015, Khan *et al.*, 2014, Cooper *et al.*, 2013, Oakley *et al.*, 1953, McDonel, 1980, Songer, 1996, Razmyar *et al.*, 2014). Nevertheless, other methods have different purposes and benefits. The multi-locus variable-number tandem repeat analysis, and multi-locus sequencing typing are usually used to evaluate the genetic relationship between bacterial strains. Bacteriocin typing is used to identify certain strains in outbreak conditions, while serotyping is useful for diagnostic and vaccination studies. In addition, bacteriophage typing is used to distinguish between *C. perfringens* strains, and plasmid profiling is used to determine the connection between strains that have caused outbreaks. Finally, ribotyping is used to distinguish between strains and for diagnostic purposes (Zhu *et al.*, 2017, Pitt *et al.*, 1995, Gillis *et al.*, 2017, Yan, 1989, Eisgruber *et al.*, 1996, Durovic *et al.*, 2017). The toxin type of a given *C. perfringens* strain announces the potential pathogenicity and disease that it causes. However, the susceptibility to phage infection cannot be related to the toxin type as it does not reveal the genetic relationship (differences or relatedness) between *C. perfringens* strains.

2.2 Introduction to Bacteriophages

2.2.1 Bacteriophages

Bacteriophages (phages) are viruses that infect and lyse bacteria (Figure, 1). They are the most numerous organism and are ubiquitous in the environment (Carvalho *et al.*, 2017). About 96% of existing bacteriophages are tailed phages that belong to the *Caudovirales* order, which includes three families: *Myoviridae* (contractile tail), *Podoviridae* (short tail) and *Siphoviridae* (non-contractile long tail) (Kaliniene *et al.*, 2017). Filamentous, cubic, and polymorphic phages belong to the same order and are classified into 10 small families. They comprise of about 3.6% of the existing bacteriophages. Figure 2 shows examples of phage morphologies (Ackermann, 2006, Orlova, 2012).

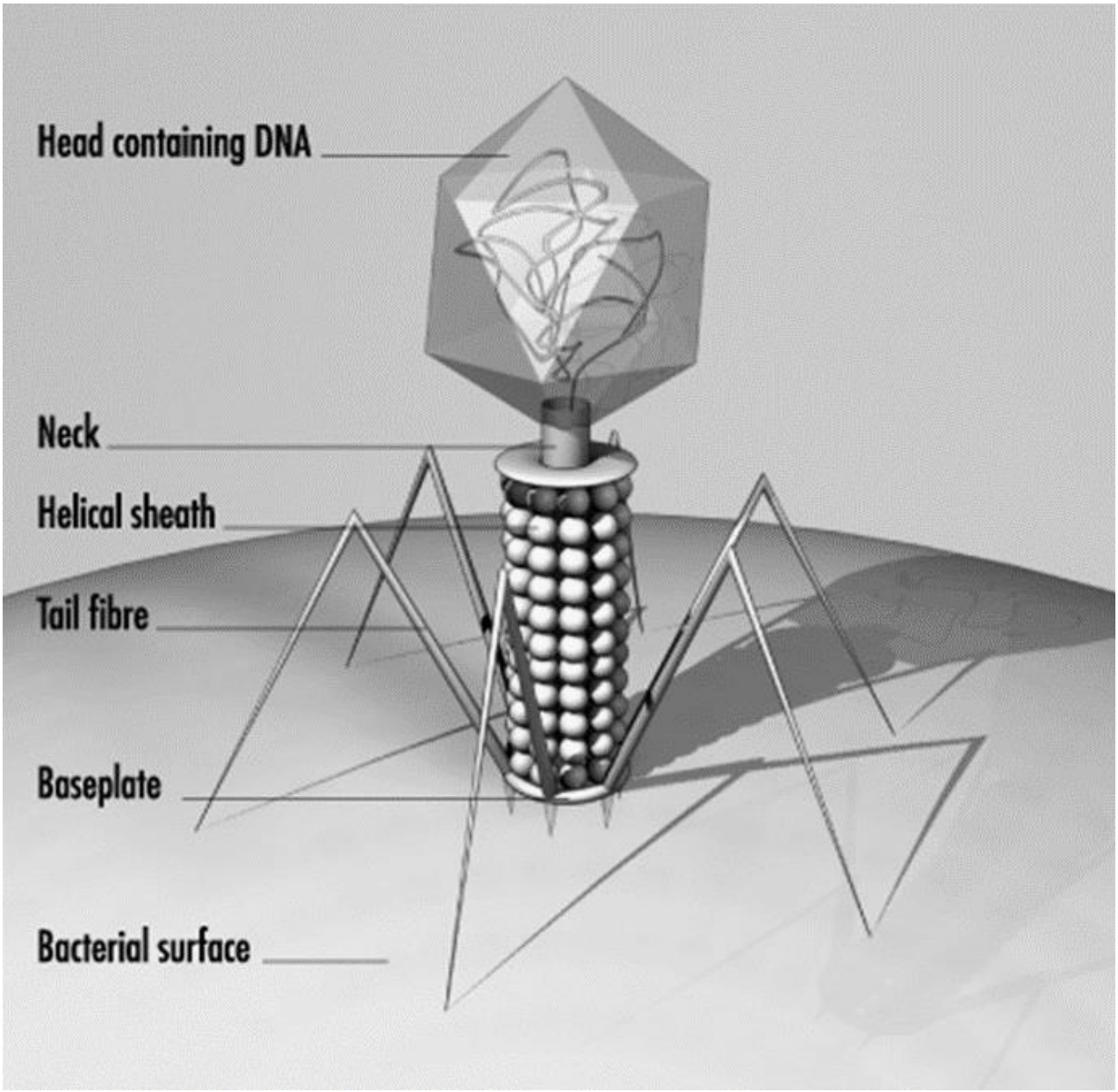


Figure 1. Diagram of bacteriophage (myovirus) structure.

(Adapted from http://www.bacteriophagetherapy.com)

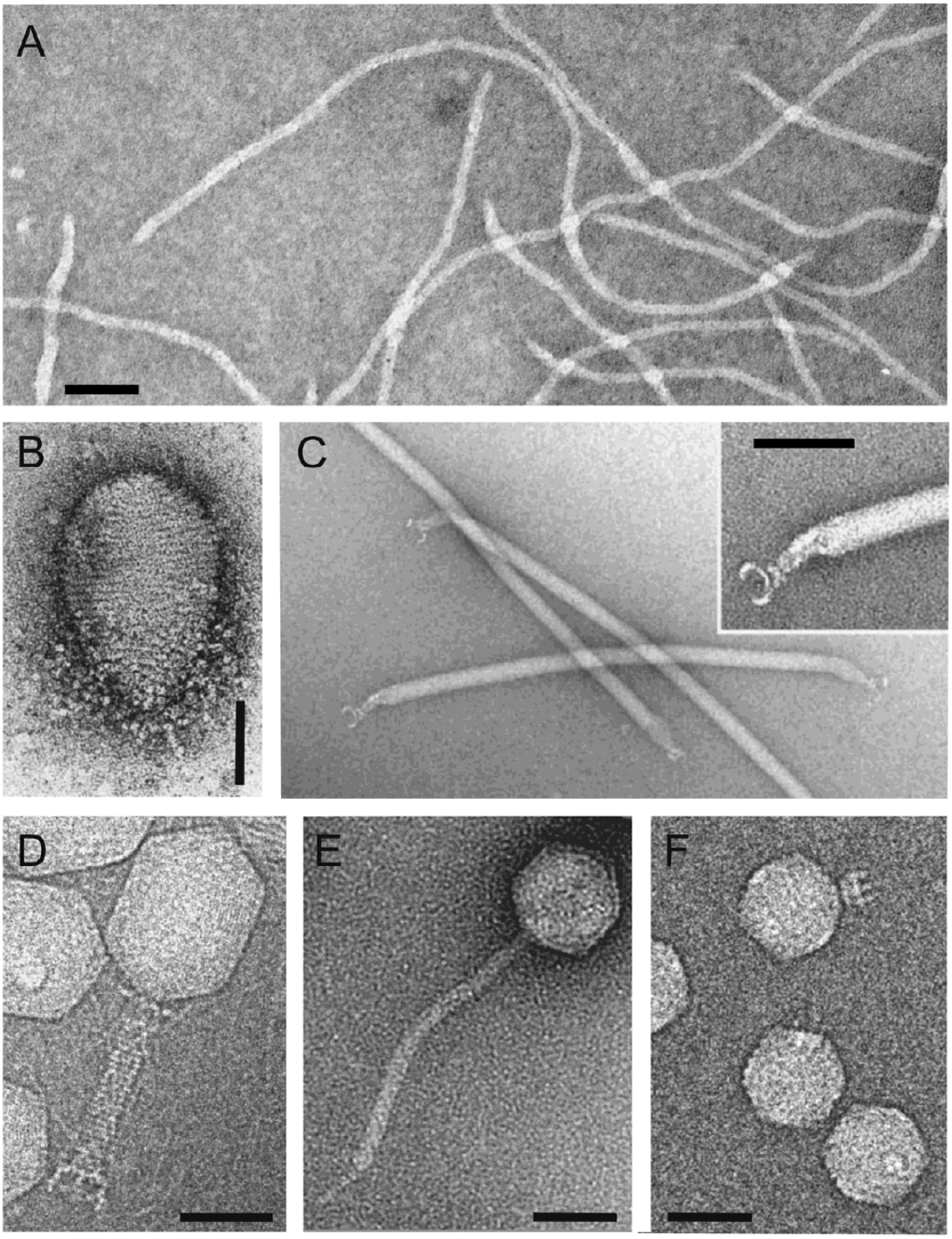


Figure 2. Morphology of different bacteriophages, scale bars of 50nm. A) A filamentous phage belonging to *Inoviridae*. B) Droplet shaped phage from the *Guttaviridae*. C) *Acidianus* filamentous phage belonging to the *Lipothrixviridae*. D) Phage T4 from the *Myoviridae* family. E) Phage CPP1 belonging to the *Siphoviridae* family. F) Phage P22 belonging to the *Podoviridae* family (Orlova, 2012).

2.2.2 Phages life cycle

Phages have two life cycles; the lytic cycle which ends by the lysis of the bacterial host cell and the lysogenic cycle, where the phage nucleic acid is integrated in the bacterial chromosome and persists till the cell is lysed (Figure, 3) (Campbell, 2003).

Both phage lifecycles start with the phage recognition of and binding to the bacterial cell. This reversible adsorption process is achieved by the receptor-binding proteins that protrude from the distal part of phage's tail as tail-spikes or tail-fibres. Some of these fibres have enzymatic activity and are able to depolymerise the polysaccharides of the bacterial cell wall (Prokhorov *et al.*, 2017). This reversible binding is followed by a base plate structural re-arrangement that triggers the contraction of tail-fibres. Binding becomes irreversible once the tail-fibres contract and bend toward the host cell wall, and the proximal region binding to the membrane structures on the host cell surface. The infection proceeds to the next step with the ejection of the phage nucleic acid, starting with the penetration of the tail tube into the host cell periplasm. During penetration, the virus uses a variety of lysins and carbohydrates de-polymerases. A transmembrane channel for phage DNA delivery is formed after the tail-fibre fuses with the host cytoplasmic membrane. Phage DNA is then ejected into host cytoplasm through this channel (Latka *et al.*, 2017, Hu *et al.*, 2015).

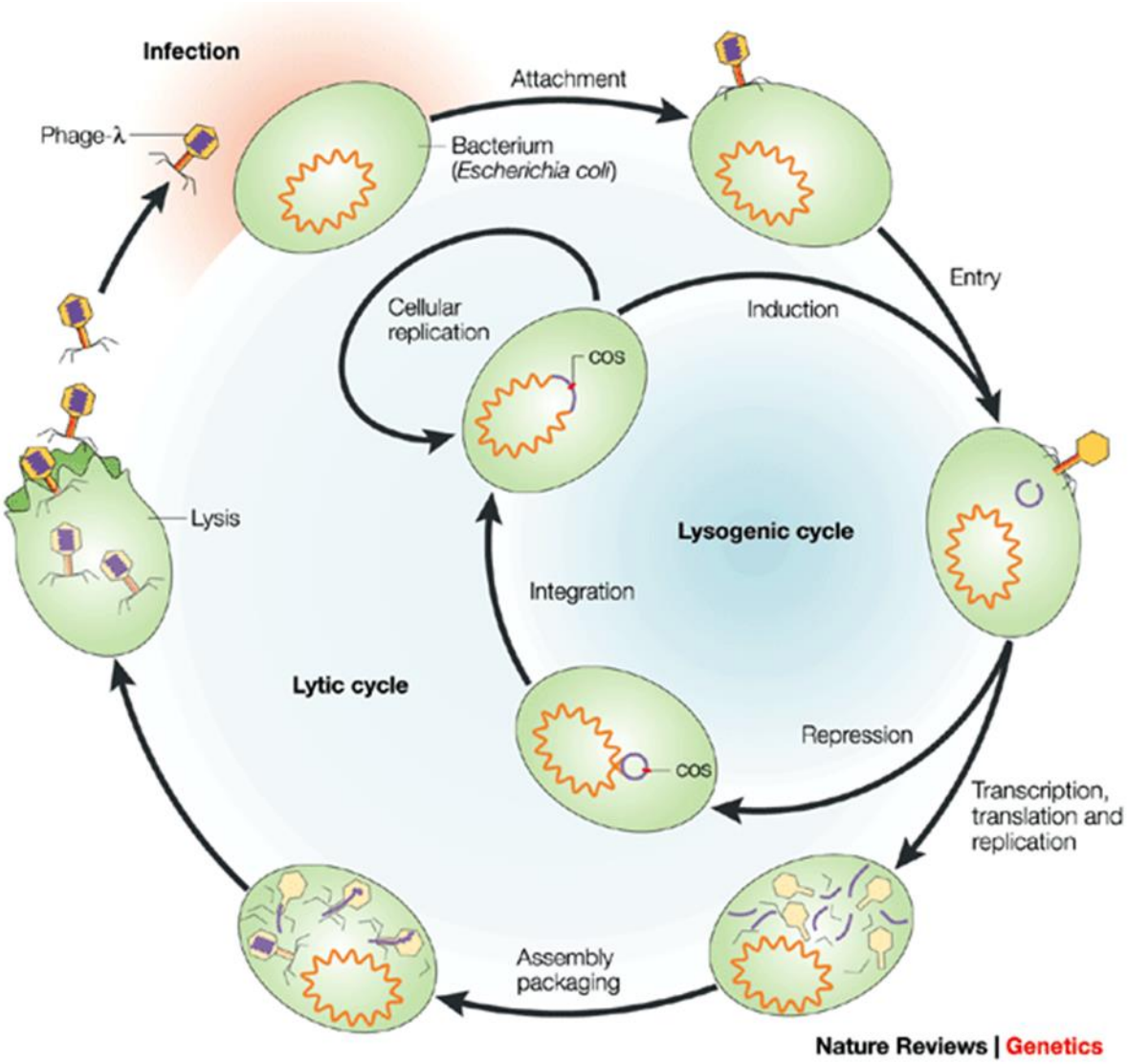


Figure 3. The two possible phage lifecycles (Campbell, 2003). Both phage life cycles start with the attachment of the phage to the bacterial surface. Penetration of phage's nucleic acid follows, and one of the two life cycles start at this point. Phage nucleic acid will either integrate into the bacterial chromosome and start the lysogenic life cycle, or hijack the host cellular machinery to produce new phages and eventually lyse the host cell, the latter being the lytic life cycle.

The phage choice of lytic or lysogenic growth can depend on the population density. Lytic growth is favoured when the host population is high, while lysogenic growth is dominant when the number of host cells is low and a large number of phages are infecting the same host cell. However, the mechanism of this growth pattern is unknown (Zeng *et al.*, 2010). In the case of the temperate lambda (λ) phage that infects *E. coli*, it can grow in a lytic manner and cause cell lysis producing hundreds of virus progeny. Alternatively, it can switch to lysogenic growth which starts once phage DNA is integrated into the bacterial chromosome. The regulation of the lysogenic state and the switching to lytic growth for the λ phage is controlled by the repressor *cl* gene and the adversary *cro* gene. The *cl* gene halts the expression of bacteriophage genes and maintain the lysogenic state. Conversely, the *cro* gene promotes the expression of the lytic growth genes (Oppenheim *et al.*, 2007).

The rate of prophage induction varies according to the surrounding environmental conditions. Prophages can switch to lytic growth when the host is under stress, such as that caused by exposure to ultra violet light or other DNA damaging chemicals or stimuli. The mechanism involved in this process is termed the SOS response, where the RecA protein cleaves the lexA repressor to activate a variety of DNA repair strategies. RecA protein can also cleave the *cl* repressor and provoke lytic growth (Figure, 4) (Gandon, 2016, Ptashne, 2004).

The prophage induction can be spontaneous in the absence of an external inducing factor. This phenomenon participates in bacterial evolution and fitness. The integration of prophages and the subsequent activity of prophages, prophage-like elements, cryptic prophages, and phage remnants within the host cell genetic circuitry has been shown to be involved in bacterial evolution. These mobile genetic elements can carry and transfer a variety of virulence and fitness genes by horizontal gene transfer. For example, the cholera and shiga toxins can be carried and transported by prophage release (Nanda *et al.*, 2015, Ventura *et al.*, 2009, Waldor *et al.*, 1996, Neely *et al.*, 1998, Brown *et al.*, 2006).

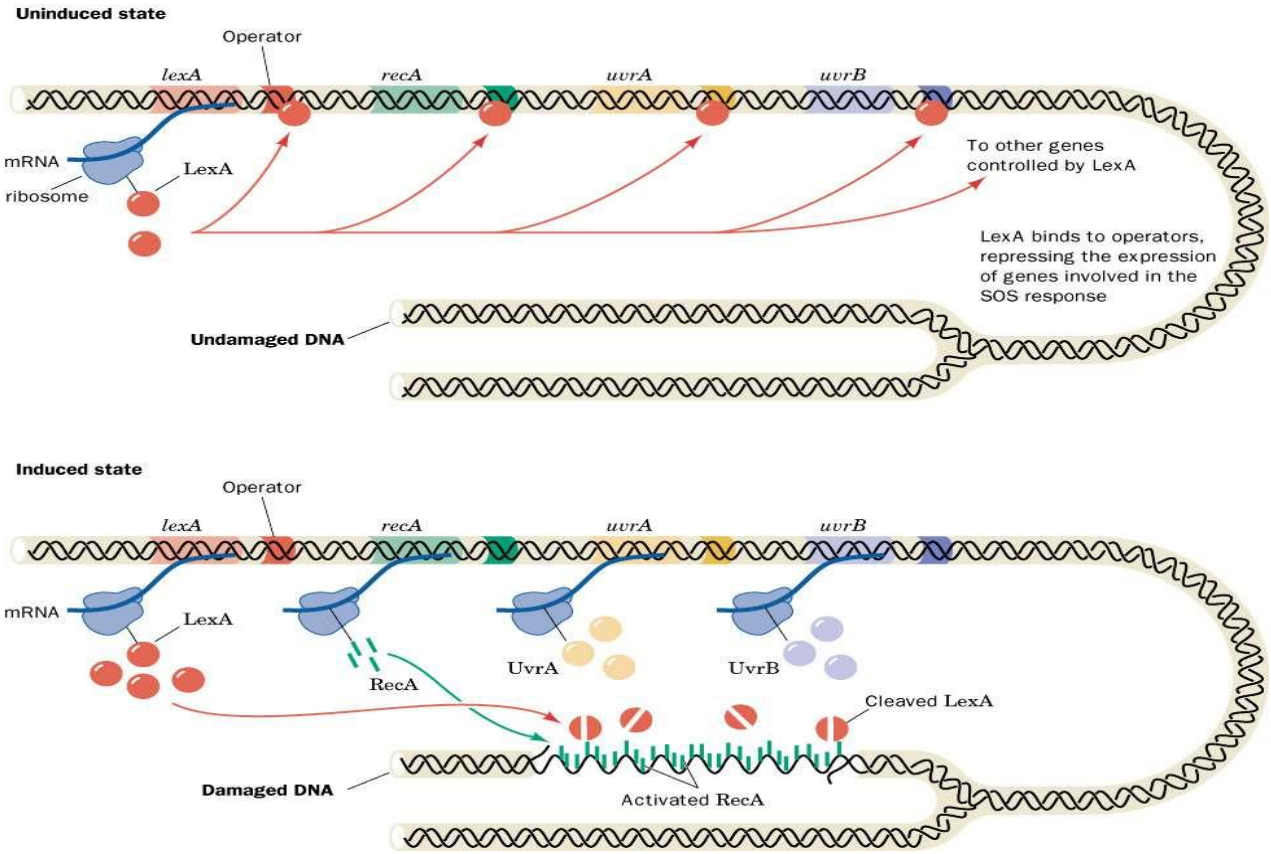


Figure 4. RecA-dependent SOS response. SOS response is a DNA repair system, which involves approximately 40 genes. When there is no DNA damage, these genes are repressed by the presence of the LexA repressor bound in their operators (upper part of the figure). In the case of DNA damage, the RecA protein is expressed and cleaves the LexA, resulting in the expression of SOS genes. Adapted from www.esf.edu/chemistry/nomura/fch532

2.2.3 Uses of phages

Since their discovery, phages have been used in the control of bacterial population and as a bacterial transformation tool (Abedon, 2018). For instance, the use of the gateway cloning system allows the cloning of multiple DNA fragments of different sizes in parallel using the same recombination enzyme. The system depends on the highly specific excision and integration reactions of phage λ both out of and into the *E. coli* genome (Reece-Hoyes *et al.*, 2018). Examples of biocontrol include the use of phages to control the growth of bacteria that cause potato spoilage and to treat *Salmonella enterica* (Choi *et al.*, 2017, Wei *et al.*, 2017). Following this, the use of phage was expanded to include using phage-derived enzybiotics, such as the use of the phage endolysin which is able to lyse the phytopathogen *Agrobacterium tumefaciens* (Attai *et al.*, 2017).

Phages have also been used for diagnostic purposes; many phages were engineered to detect the presence of particular pathogens by inserting a reporter bioluminescence or fluorescence genes. Thus, visual bioluminescence or fluorescence will appear upon the infection of a pathogen. These reporter phages have been used to detect *E.coli* 0157:H7 (Hall *et al.*, 2012), where the *E. coli* 0157:H7 reporter phage was able to detect titres as low as 5 CFU/ml in the tested samples (Zhang *et al.*, 2016). *Mycobacterium tuberculosis* has also been detected from septum samples directly by a reporter phage (Jain *et al.*, 2012). Moreover, glass slide agglutination tests were successfully developed for the detection of *Campylobacter jejuni* and *Campylobacter coli* using the phage receptor-binding proteins (Javed *et al.*, 2013).

Phages have also been used for drug delivery. The Clustered Regularly Interspaced Short Palindromic Repeats (CRISPER-cas9) gene was successfully inserted in phage genome so as to treat multi-antibiotic resistant *Staphylococcus aureus.* The inserted CRISPER-cas9 gene was programmed to target and cleave the antibiotic resistance genes in a sequence specific manner. It was also programmed to destroy the *Staphylococcus* plasmids that carry the antibiotic resistance gene to prevent the spread of antibiotic resistance to non-virulent *Staphylococci*. This method allows the manipulation of complex bacterial communities in a sequence specific manner (Bikard *et al.*, 2014).

Phage display technique involves the bio-engineering of phage genomes to express a variety of peptides in their capsid (Carnazza *et al.*, 2010). This technology has been used for the prevention of oral diseases, such as the blocking of *Streptococcus gordonii*

adherence to the salivary pellicle (Cukkemane *et al.*, 2014). It has also been used for the production of nanobodies against *Acinetobacter baumannii* for immunotherapy (Payandeh *et al.*, 2014). A variety of constructs can be inserted into phage genomes; the resulting phage-expressed peptides will have a high binding specificity and affinity to many ligands (O'Sullivan *et al.*, 2016). Consequently, phage display technology is applicable in many disciplines, such as the dispersion of clinically important biofilm like *E. coli* (Lu *et al.*, 2007, Ivanova *et al.*, 2017), vaccine delivery to combat bacterial and viral pathogens (Gao *et al.*, 2010), and increased agriculture and animal yield (Carvalho *et al.*, 2017, Ivanova *et al.*, 2017).

2.2.4 Phage therapy

Phage therapy is the use of phages to treat bacterial infections. The emergence of multiple antibiotic resistant pathogens continues, and the absence of new antibiotic to treat these pathogens have urged researchers to look for other treatment options. Phage therapy is a promising treatment option, particularly since phages have been used in the treatment of bacterial infections since their discovery in 1915 (Dufour *et al.*, 2017). After the discovery and success of antibiotics, phage therapy was ignored in the west while it was continually used in Eastern Europe and the former Soviet Union. However, the progressive development of multiple antibiotic-resistance by bacteria together with the banning of certain antibiotics and the insufficient development of novel antimicrobials has revived interest in phage therapy (Nakonieczna *et al.*, 2015, Carvalho *et al.*, 2017, Chanishvili, 2012). Lytic phages have the potential to be used to treat bacterial infections with multi-drug resistance as they lyse bacterial host cells at the end of their life cycle (Cisek *et al.*, 2017).

Phages are harmless to humans and animals as they do not infect human and animal cells, but only bacteria, in a highly specific manner, thus phages can be selected to target pathogens and not the commensal organisms within the human flora (Carvalho *et al.*, 2017). Additionally, they are non-toxic, self-replicating and can disperse through biofilms (Szafranski *et al.*, 2017). Lastly, the process of bacteriophage production is cost-effective, fast and simple in comparison with antibiotics (Loc-Carrillo *et al.*, 2011).

However, there are difficulties brought about by the use of phages as a therapeutic agents, including the clearance of phages by both innate and adaptive immune response, especially for intravenously delivered phages (Cisek *et al.*, 2017). The transmission of

virulent genes by horizontal gene transfer is another disadvantage, such as the transfer of shiga stx-1 enterotoxin gene between *E. coli* strains by phage transduction, and the transfer of tetracycline resistance gene to *Enterococcus gallinarum* (Petridis *et al.*, 2006, Mazaheri Nezhad Fard *et al.*, 2011). Temperate phages can incorporate CRISPR genes in their genome and transfer it to new bacterial cells, providing the new lysogens with the ability to resist further infection with another phage, such as *C. difficile* phiCF5. This phage encodes a spacer in the CRISPR arrays that has identical matches to *C. difficile* 630 prophages phiCD38-2 and phiCD6356 (Hargreaves, 2013). Furthermore; phage virulence genes can encode for additional toxins and turn non-pathogenic strains to pathogenic ones (Saunders *et al.*, 2001). Some strains of different bacterial species depend on a particular phage-encoded toxin to cause a specific disease; for example the shiga toxin producing *E. coli, Clostridium botulinum, Corynebacterium diphtheriae, and Vibrio cholerae. Other bacterial species harbour a large number of prophages, each of which encodes a fitness or virulence factor that contribute to the lysogens fitness (Brüssow et al., 2004).* Similar to antibiotics, phages can cause fast and enormous bacterial destruction, an adverse immune response in the human body may occur as a result of the release of bacterial cell wall fragments (endotoxin) such as the immunogenic lipopolysaccharides of Gram-negative bacteria, which can then cause toxin shock (Górski *et al.*, 2012, Hagens *et al.*, 2004, Rosenfeld *et al.*, 2006). The high specificity of phage tropism towards bacterial strains can be considered as both an advantage and disadvantage of phage therapy. Although phages target a particular strain of bacteria, which does not affect the other commensal flora, they are unlikely to infect all the strains of a given species. Therefore, a combination of phages (phage cocktails) are is required (Loc-Carrillo *et al.*, 2011, Hyman *et al.*, 2010a).

The power of phage therapy in the treatment of infections with multi-drug resistance bacteria has been demonstrated in several animal models and humans (Henry *et al.*, 2013, Nilsson, 2014). They have been suggested to treat a variety of bacterial infections as listed; a clinical trial with mice gave a promising result with regards to treating pulmonary infection with *Burkholderia cepacia* (Semler *et al.*, 2014), treatment of localized and systemic infections caused by *Staphylococcus aureus,* and infant meningitis caused by *Cronobacter sakazakii* (Tamariz *et al.*, 2014), additionally infections caused by *Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Enterococcus faecalis, Actinomyces naeslundii, Veillonella spp., Neisseria spp., Lactobacillus spp. and*

Streptococcus spp. have been shown to be counteracted with the use of phages (Szafranski *et al.*, 2017). The use of phages has also been suggested for the control of food-borne diseases such as enteric infection caused by *Campylobacter jejuni* and *Campylobacter coli* (Kittler *et al.*, 2014). Phage therapy has yielded promising results for the treatment of infections of *Psuedomonas aeruginosa* (Sahota, 2016), for the prevention of *Listeria monocytogenes* infection by the biocontrol of this bacteria in both water and food (Leverentz *et al.*, 2003, Guenther *et al.*, 2009), and for the treatment of adherent invasive *E. coli*, but has been less encouraging for *E. coli* O157:H7 (Galtier *et al.*, 2017, Sabouri *et al.*, 2017).

2.2.5 *C. difficile* & *C. perfringens* and phage therapy

Gastro-intestinal tract (GIT) infections with multi-antibiotic resistant bacteria poses a serious health problem. The resistance to and the ability of antibiotics to alter intestinal normal flora promoting the onset of disease has driven research to look for alternative treatments. Many *in-vivo* studies have demonstrated that phage therapy has potential to treat such infections, including *C. difficile* and *C. perfringens*. However, it worth noting that a high titre of phage taken orally should be delivered to the infection site as the phage titre can be reduced by the patient's gastro-intestinal tract and immune response (Nakamura *et al.*, 2014, Petit *et al.*, 1999, Tenover *et al.*, 2012, Osman *et al.*, 2013, Zelasko *et al.*, 2017).

In contrast to aerobes, little research efforts by comparatively fewer researchers has been done on *C. difficile* phages in comparison with their counterparts that infect other pathogenic bacteria. This fact is a consequence of the technical obstacles that generally accompany work with anaerobes and particularly with *C. difficile* (Hargreaves *et al.*, 2014). However, the treatment of *C. difficile* infection (CDI) with phage in an artificial colon environment system revealed that the viability of *C. difficile* vegetative cells can be significantly reduced compared with the untreated system. Consequently, the production of toxins TcdA and TcdB was also reduced. Moreover, the lysogenization of phiCD27 used in the system can downregulate the expression of *C. difficile* pathogenicity locus as a result of phage expressed repressor proteins' effect. Although the total number of aerobic and anaerobic normal flora of the phage treated system was not significantly reduced compared with the antibiotic treated artificial system, *C. difficile* was not completely terminated from the phage treated system (Meader *et al.*, 2010, Meader *et al.*, 2013, Govind *et al.*, 2009). Moreover, phages can prevent *C. difficile* colonisation in the

Galleria mellonella larvae intestine and highly reduce *C. difficile* biofilm formation. Additionally, the prophylactic oral administration of phage cocktails to *Galleria mellonella* larvae could prevent the disease when the larvae was challenged with *C. difficile*. Phage treatment has also reduced the colonization of *C. difficile* and subsequently elongated the life span of artificially infected larvae, as confirmed by assessing the larvae-survival rate and colonization levels. Moreover, the effect of *in-vitro* phage addition before and after biofilm formation was assessed using turbidity test, viable count, and scanning electron microscopy. The phage addition could prevent biofilm formation when added before the bacteria. Phages could also penetrate the already established *C. difficile* biofilm (Nale *et al.*, 2016a). Lastly, the use of phage cocktails *in-vitro* to control *C. difficile* growth has shown a potential for the treatment of CDI (Nale *et al.*, 2016b).

Similarly, little is known about *C. perfringens* phages and their *in-vivo* effects on *C. perfringens*-associated diseases. However, the use of phage has been suggested to control *C. perfringens*-associated enteritis in animals in countries that complied with terminating the use of antibiotics as nutrition additives, where necrotic enteritis began to cost about 2 billion dollars a year (Seal, 2013, Stanley *et al.*, 2014). The oral admission of phage can control necrotic enteritis in broiler chickens and significantly decrease the mortality rate (Miller *et al.*, 2010).

2.2.6 Phages endolysin

Phage-derived endolysins are peptidoglycan hydrolases enzymes that are expressed during the late phase of the phage lytic life cycle (Maciejewska *et al.*, 2017, Stoffels *et al.*, 2017). They have been successfully used as an alternative for antibiotics in the treatment of both Gram-negative and Gram-positive bacteria for many reasons. These include their ability to react with both the vegetative cells and the germinating spores, their distinct independent activity mode and the lack of their parents specific bacterial tropism, which give them a wider target range of strains within the same bacterial species. They exhibit a high level of bacterial lysis at considerably low concentrations within a short time. Moreover, the development of endolysins resistance is rare, as peptidoglycan is essential to the survival of bacteria, which make these enzymes a propitious anti-microbial agent. More recently, endolysins have been immobilized and incorporated into nanoparticles, so as to be used on surfaces as an anti-microbial (Van Tassell *et al.*, 2017, Oliveira *et al.*, 2015, Wu *et al.*, 2017, Yang *et al.*, 2012, Thandar *et al.*, 2016).

Endolysins have been shown to be effective against Gram-positive bacteria (Gerstmans *et al.*, 2016) like *Enterococcus faecalis* (Swift *et al.*, 2016), *Bacillus anthracis* (Yang *et al.*, 2012), *Streptococci* (Schmelcher *et al.*, 2015), *Bacillus cereus* group and *Clostridium genus* (Nakonieczna *et al.*, 2015).

Endolysins have been used for the treatment of wound bacterial infections (Abouhmad *et al.*, 2016), *Vibrio parahaemolyticus* (Wang *et al.*, 2016), multi-drug resistant *Acinetobacter baumannii* (Thandar *et al.*, 2016), *Paenibacillus larvae* (Oliveira *et al.*, 2015), the control of biofilm forming pathogens (Kaistha *et al.*, 2016) and *Streptococcus pneumoniae* (Diez-Martinez *et al.*, 2015).

Endolysin can also be used for DNA extraction purposes. They have been successfully used to prepare a *Staphylococcus aureus* DNA sample for real-time PCR. Endolysins were shown to take and take less time than DNA extraction using the lysostaphin method, thereby accelerating the detection of *Staphylococcus aureus* by PCR (Hu *et al.*, 2016).

The endolysin derived from the *C. difficile* phage ΦCD27 is considered as a candidate medication for the treatment of CDI, as it is able to lyse *C. difficile in-vitro* (Dunne *et al.*, 2014). Similarly, the endolysin derived from phages that infect *C. perfringens* has shown potential towards most of the *C. perfringens* strains and the high activity and specificity of the enzyme indicate that it can be developed to control *C. perfringens* growth (Tamai *et al.*, 2014a, Gervasi *et al.*, 2014a). Details about *C. perfringens* phages endolysins are reviewed in section 5.1.3.

2.2.7 The structure of endolysins

The endolysins encoded by phages that infect Gram-positive bacteria are usually between 250-400 amino acids in length. The majority of them have a modular structure consisting of a C-terminal binding domain which determines the host specificity of the enzyme and an N–terminal hydrolase domain that cleaves the peptidoglycan layer of bacterial cell wall (Figures 5 and 6). However, the endolysin encoded by the prophage of *Streptococcus dysgalactiae subsp. equisimilis SK1249* has a cell wall binding domain located between two enzymatically active domains (Fischetti, 2008, Oechslin *et al.*, 2013, Dunne *et al.*, 2016).

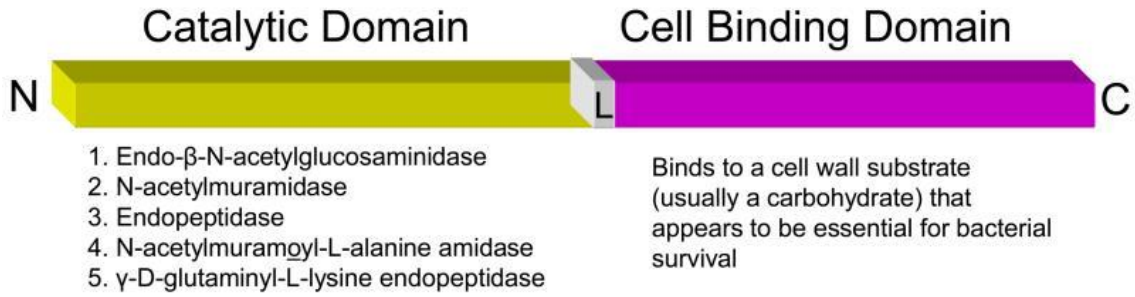


Figure 5. Basic structure of endolysins of phages that infect Gram-positive bacteria. Most of these lysins consist of a C-terminal cell wall binding domain (right) and an N-terminal enzymatically active domain connected by a short linker. According to the type of bond that the catalytic domain is able to cleave, these endolysins are divided into five types (left) (Fischetti, 2008).

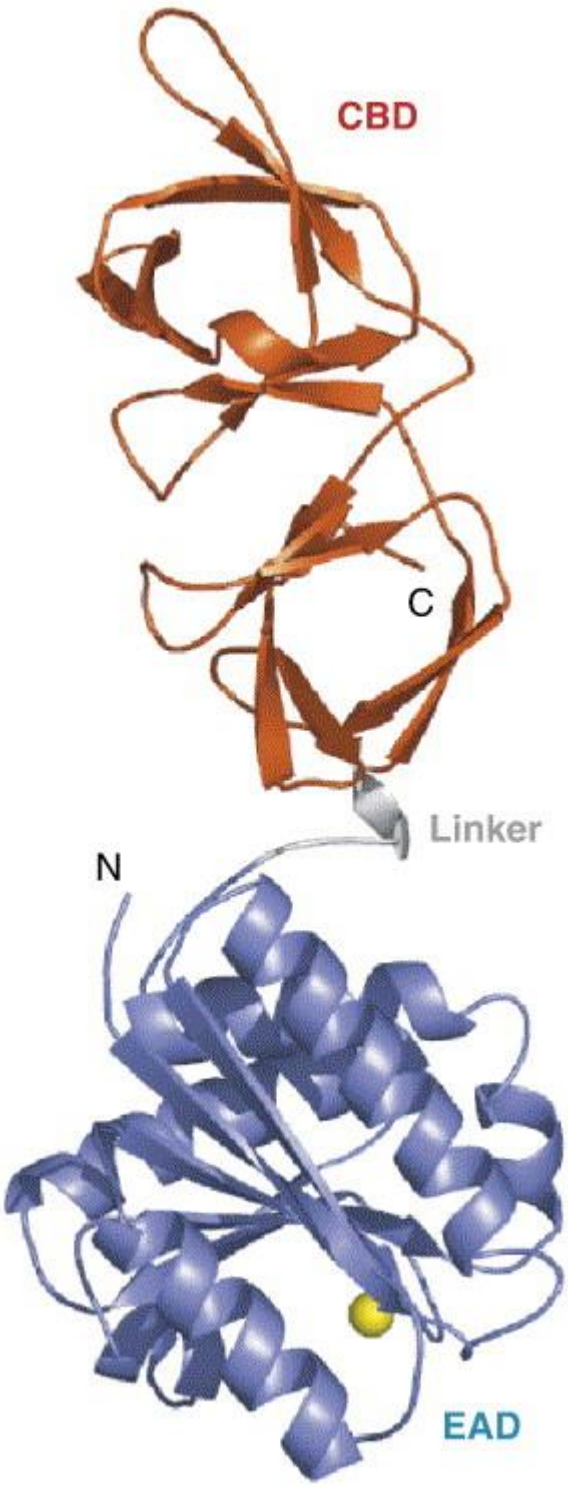


Figure 6. Chrystal structure of endolysin of Listeria monocytogenes temperate phage PSA. The cell wall binding domain (CBD) of the PSA endolysin is shown in orange (upper part) and the enzymatically active domain (EAD) is shown in blue (lower part). A small linker (L) connecting CBD & EAD is shown in grey. The yellow sphere is the catalytic Zn^{2+} (Korndörfer et al., 2006).

In contrast, endolysins encoded by phages that infect Gram-negative bacteria are smaller (150-200 amino acids) and usually lack the cell wall binding domain, comprising of an active hydrolase domain only (Cheng *et al.*, 1994). However, in the case of Gp110 of a *Salmonella* phage, there is an uncharacterised domain at the C-terminal of the enzyme with unknown functions (Rodriguez-Rubio *et al.*, 2016). Another exception is found with the endolysins of two *Pseudomonas aeruginosa* phages KZ144 and EL188, which have a modular structure of an N-terminal cell wall binding domain and a C-terminal catalytic domain. This structural module is the opposite of the Gram-positive endolysins' modular structure (Briers *et al.*, 2007). In another study, the crystal structure of endolysin of the *Salmonella typhimurium* phage SPN1S showed that the enzyme consists of both a large and small domain. The latter two modular domains have a groove between them with a possible binding site in it (Park *et al.*, 2014).

2.2.8 Zymography

Zymography is a method by which the enzymatic activity of a protein can be detected, identified and even quantified with the use of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS_PAGE) and the suitable substrate embedded in the gel. A number of clinically important enzymes are routinely detected by zymography (Wilkesman *et al.*, 2017), such as the matrix metalloproteinase (MMP) activity (Nyati *et al.*, 2017), the identification and confirmation of the presence of cysteine protease 30 in the *Tritrichomonas foetus* parasite (Gould *et al.*, 2017), and the bacteriolytic activity of LytFM proteins produced by the house dust mite *Dermatophagoides pteronyssinus* (Tang *et al.*, 2017). It is thus unsurprising that this method has recently received a large amount of interest (Wilkesman *et al.*, 2017).

Zymography has also been used to determine the muralytic activity of bacterial and phage derived proteins. For example, it has confirmed the peptidoglycan hydrolase activity of resuscitation promoting factors that are involved in the re-activation of dormant Mycobacterium marinum (Iakobachvili, 2015), and the bacteriolytic activity of the putative endolysin derived from *Enterococcus faecalis* phage (Uchiyama *et al.*, 2011).

2.2.9 Modified phages and phage engineering

Genetic engineering has been used to modify bacteriophages and improve their properties, increase their lytic action and overcome the obstacles which accompany the use of some natural phages as a therapeutic agents (Krylov, 2001). The *in-vivo* and *in-vitro* studies on the modified temperate and virulent phages have further emphasized the preference of using them over antibiotic (Moradpour *et al.*, 2011).

Various techniques have been used for phage engineering, including homologous recombination which has been used to delete, insert, and replace genes within the phage genome, such as the insertion of the luxAB gene in the genome of *Listeria monocytogenes* phage for diagnostic purposes. However, this method only allows for the targeting of one loci at a time due to low recombination rates (Pires *et al.*, 2016, Loessner *et al.*, 1996). Bacteriophage recombineering of electroporated DNA (BRED) is another method employed to engineer phage genomes, and has been used to engineer *Mycobacterium spp.* phages, *E. coli* phages, and *Salmonella enterica* phages (Marinelli *et al.*, 2008, Fehér *et al.*, 2012, Shin *et al.*, 2014). Moreover, *in-vivo* recombineering using phage lambda recombinase has been used to engineer *E. coli* phages (Oppenheim *et al.*, 2004, Sharan *et al.*, 2009).

Clustered regularly interspaced short palindromic repeats and their associated *cas* gene (CRISPR-Cas) system has been used to enhance the selection of recombinant T7 *E.coli* phage after the homologous recombination process. In brief, the unessential 1.7 gene was deleted by homologous recombination. Then, the lysate (mixture of recombineered and wild type T7 phages) was propagated on an *E. coli* strain that harbours the required constructs to encode the modified CRISPER-Cas spacer components. The modified CRISPER-Cas was able to target and cleave the 1.7 gene. Thus, only the recombineered phage was able to grow. In this method, the CRISPER-Cas was used for the counter selection of recombineered phage (Kiro *et al.*, 2014).

In-vitro re-factoring of phage genomes has been used to re-design the phage T7 genome. The overlapping region between genetic segments was eliminated and the genes were divided into six sections (Chan *et al.*, 2005). Whole genome synthesis and assembly from synthetic oligonucleotides is another way of *in-vitro* phage genome engineering. *E. coli* phage phiX174 (~6kB genome) was *in-vitro* synthesized and electroporated back to *E. coli* in order to recover the modified phage (Smith *et al.*, 2003).

Saccharomyces cerevisiae (*S. cerevisiae*) was used instead of bacterial cells for the *in-vivo* recombineering of *E. coli* phages genomes, as the recombineering processes was toxic for *E. coli* and due to the fact the genomes had a large size. In this method, the phage genome is delivered to the yeast using a *S. cerevisiae*-bacteria shuttle vector and maintained stably there. Then, it can be engineered using a plasmid which carries a homologous sequence. *E.coli* phages T3 (38208bp) and T7 (39937bp), and *Klebsiella* phage K11 (41181bp) were modified using this method (Ando *et al.*, 2015, Lu *et al.*, 2012). Again, this process also requires the extraction of the recombineered phage genome, and deliver it back to the propagation bacterial host.

The constraints of the three methods is the difficulty of manipulating a large-sized phage genome *in-vitro*, and the need for highly competent bacterial cells and a highly effective transformation protocol, as both the *in-vitro* modified and the yeast recombineered phage nucleic acid has to be delivered back into bacterial host cells so as to obtain a viable phage progeny. This explains why *E. coli* and *Pseudomonas aeruginosa* phages are nearly the only phages that have been engineered in this way (Pires *et al.*, 2016).

2.3 *Clostridial species* and molecular studies

2.3.1 Background

The following section offers an overview of the molecular studies carried out on *Clostridial species* until now. Most of these studies have focused on the transformation of different *clostridium* species in the context of anaerobic production or anaerobic degradation, such as the production of ethanol by *Clostridium autoethanogenum* from factories' waste fluids (Liew *et al.*, 2017), the production of butanol by *Clostridium saccharoperbutylacetonicum* (Herman *et al.*, 2017), bio-mass degradation (Hahnke *et al.*, 2014), the production of ethanol, hydrogen, and different types of acids (Ellis *et al.*, 2014, Peterson *et al.*, 2014), fatty acid production (Cheng *et al.*, 2014) and the production of acetone and butanol (Cooksley *et al.*, 2012). The study of the genes that regulate these processes has been another discipline of focus (Bengelsdorf *et al.*, 2016).

Most of these industrial modifications are dependent on the knock-in of genes by either a single crossover event or by disrupting the coding sequence using the ClosTron. These genes utilize different substrates and metabolic pathways to give the same product. Thus, the mutant bacterial strain will be left with the desired metabolic pathway of production.

In the 90s the number of generated mutants of *Clostridial* species was disappointingly low (Heap *et al.*, 2009a). The genetic manipulation of *C. difficile* was hindered by the lack of a mature transformation system. However, the first specific *C. difficile* expression vector was developed in 2002 (Purdy *et al.*, 2002), and was followed by the construction of ClosTron, which facilitates the direct mutagenesis in a variety of *Clostridium* species (Heap *et al.*, 2007, Heap *et al.*, 2010).

Thereafter, the construction of nine *Clostridium-E. coli* shuttle vectors was accomplished. These shuttle plasmids have a variety of replicons and selection markers separated by restriction sites. A total of 400 plasmids can be constructed using different combinations of these components. These constructs are suitable for certain hosts and applications (Heap *et al.*, 2009b).

2.3.2 Conjugation

Bacterial conjugation is a process of a conjugative plasmid DNA transfer by cell to cell contact from a donor bacterial cell to a recipient one. For example, a donor *E. coli* cell specific F pili is responsible for the formation of the conjugation-bridge. F pili is encoded by the conjugative F plasmid. The plasmid DNA transfer starts at a definite point in the plasmid DNA and proceeds in a linear manner (Figure, 7) (Holmes *et al.*, 1996, Birge, 2006). Conjugation with donor *E. coli* has been used to transform *C. difficile* strains with DNA of ClosTron, semi-suicide, and expression plasmids (Faulds-Pain *et al.*, 2013, Heap *et al.*, 2007, Heap *et al.*, 2009b). *C. difficile* strains differ widely in their conjugation efficiency, with *C. difficile* R20291 being refractory to plasmid transfer by conjugation. However, the heat treatment has improved the conjugation efficiency of *C. difficile* R20291 (up to 10^{-4} trans-conjugant/µl of plasmid DNA) (Kirk *et al.*, 2016).

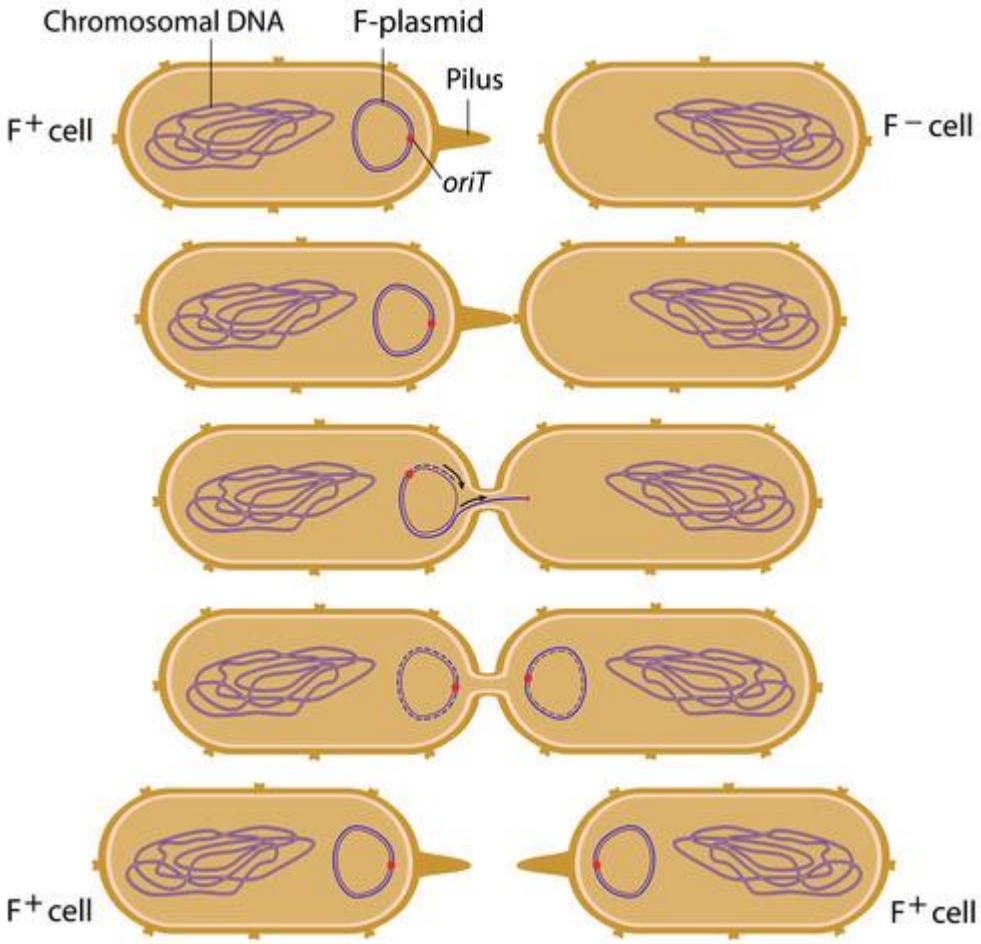


Figure 7. Schematic diagram of bacterial conjugation. The cell in the upper left is a donor F^+ cell, the origin of transfer oriT is shown as a red dot on the F plasmid. Conjugation starts when the donor cell attaches to the recipient cell by the F pili and forms a DNA transfer bridge. One strand of the conjugative plasmid is then transferred to the recipient cell in a linear fashion. Following this, the recipient cell will synthesize the complementary strand of the plasmid and will become an F^+ cell. Adapted from http://science-tuition.co.uk/wp-content/uploads/2013/11/bacterial_conjugation.jpg.

2.3.3 Transposon mutagenesis

Transposon are mobile DNA units able to insert and excise themselves in another DNA molecule. They are also able to catalyze DNA inversion or deletion reactions (Birge, 2006). They are self-replicating genetic elements, which participate in the dissemination of antibiotic resistance genes by horizontal gene transfer. Transposons are transferred from cell to cell rapidly, and the process of transfer is much like the one of *E. coli* F^+ plasmid (Harper, 2010). Transposon mutagenesis has been used to create a large number of mutants in the hyper-virulent *C. difficile* R20129 using the conjugative transposon Tn*916* (Mullany *et al.*, 2012, Dembek *et al.*, 2015).

2.3.4 The ClosTron

Group II introns are ribozymes; a self-splicing RNA-protein complex (RNP) (Costa *et al.*, 2016). TargeTrons are a genomic knockout tool based on the mobile group II introns taken from *Lactococcus lactis.* TargeTron can de-activate the gene by insertion, in a site-specific manner (Liu *et al.*, 2015).

ClosTron is a *Clostridium* TargeTron. Simply, the ClosTron construct includes two parts, in addition to the fundamental components of any plasmid such as an antibiotic marker, origin/s of replication, multiple cloning site, and origin for transfer (Figures 8 and 9). The first part is a PCR mutated *Lactococcus lactis* group II intron gene that has the target gene recognition sequence (homologous sequence) attached. The second part is a reverse transcriptase gene. The first part produces a single strand of RNP (spliced group II introns and the recognition sequence), which recognises and inserts itself into the bacterial chromosome inside the gene to be knocked-in. The second part expresses the reverse transcriptase and builds the complementary DNA. Thus, the gene is then permanently knocked-in (Kuehne *et al.*, 2012, Heap *et al.*, 2009b).

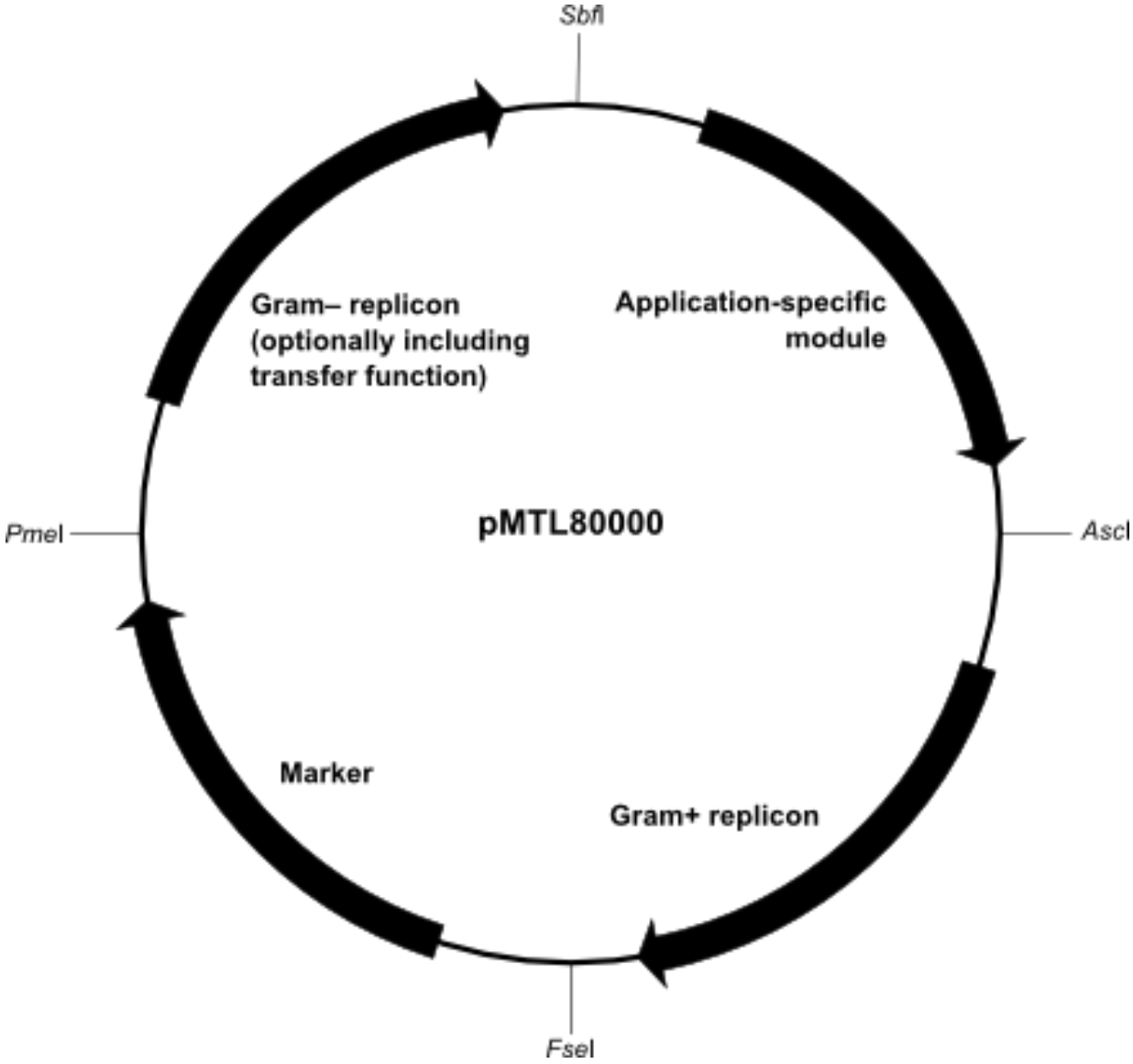


Figure 8. Basic structure of the ClosTron. The ClosTron consists of four basic parts separated by a restriction site. The application specific module (modified group II intron and reverse transcriptase genes), Gram-positive and Gram-negative origins of replication, with optional origin of transfer, and a selection marker (antibiotic resistance gene). Adapted from http://clostron.com/pMTL80000.php.

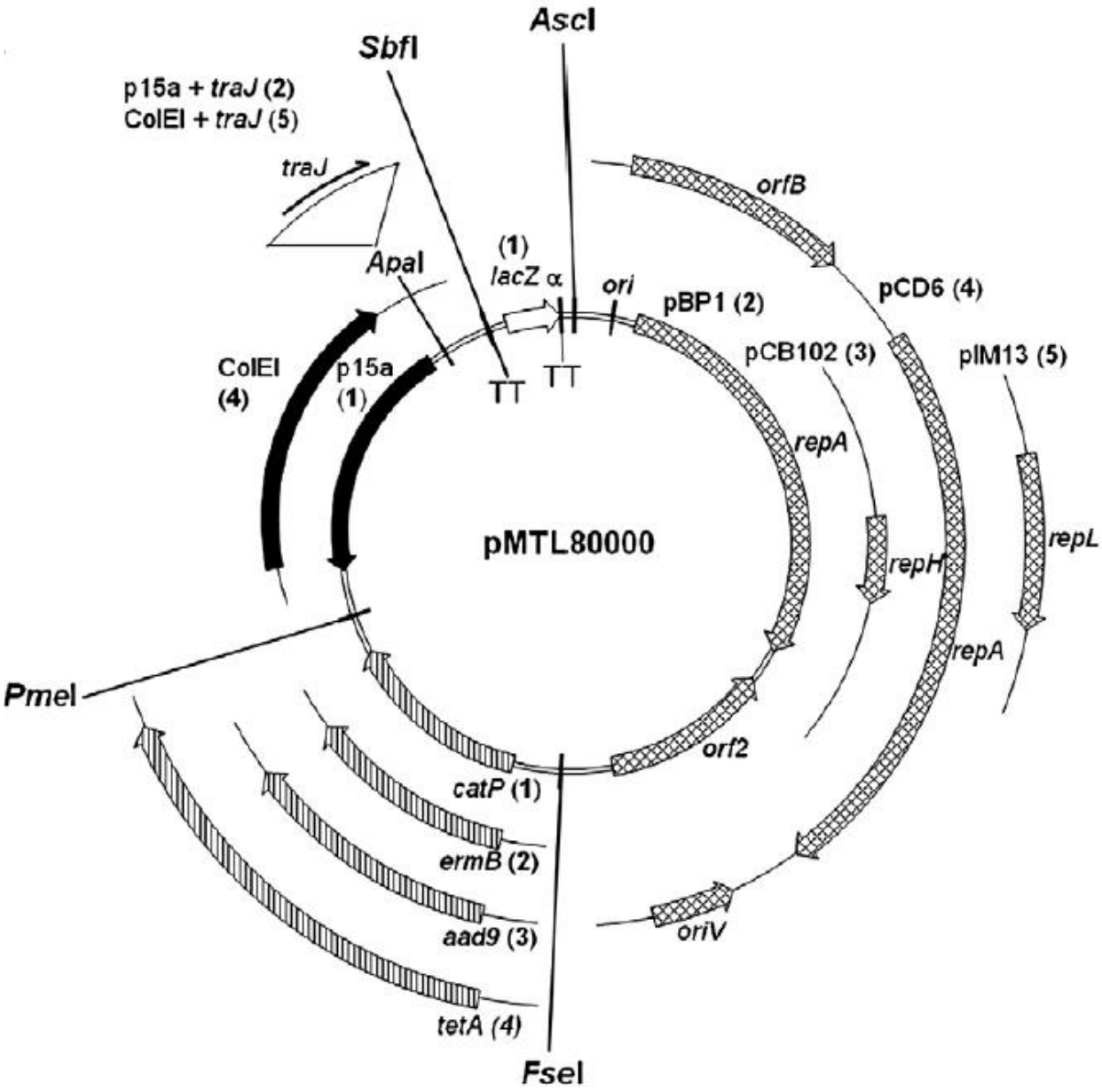


Figure 9. Schematic diagram of a modular plasmid pMTL80000. Most of the available modules are shown in this diagram. Four restriction sites are separating each module, allowing the construction of many *Clostridium-E. coli* shuttle plasmids which have different host range and functionality (Heap et al., 2009b).

In 2010, the host range of the ClosTron was increased to include *C. beijerinckii* and *C. difficile* ribotype 027 "epidemic strain" and can currently transfer DNA to *C. difficile*, *C. acetobutylicum, C. beijerinckii, C. botulinum, C. perfringens, C. sporogenes, C.*

pasteurianum, C. ljungdahlii, C. autoethanogenum and even *Geobacillus thermoglucosidasius* (Heap *et al.*, 2010, Minton *et al.*, 2016).

2.3.5 Homologous recombination

Homologous recombination (HR) is a process by which DNA sequence is exchanged between two double stranded DNA molecules that have homologous sequences of DNA, such as two copies of the same chromosome or any DNA molecules that share the same sequences. HR is involved in the double stranded DNA repair by correcting the errors that occur during DNA replication, like correcting damaged replication forks. During meiosis, HR generates new combinations of DNA sequences to ensure genetic variability in the off-spring. HR's mechanism is dependent on a recombinase enzyme that catalyzes the recognition of homologous sequences and the subsequent homologous end-joining reaction (Alberts *et al.*, 2002, Li *et al.*, 2008). The HR based mutagenesis laboratory technique has become an important and widely used genome editing method (Mohanta *et al.*, 2017, Carter, 1986).

Aside from the gene knock-in integration of whole plasmids and the ClosTron, system another method is homologous recombination-based gene excision. Until 2011, almost all the *clostridium spp.* mutations by homologous recombination was achieved by a single crossover event using a suicide plasmid where the whole plasmid backbone was incorporated in the bacterial chromosome. Examples of these mutations include the inactivation of the sorbitol gene in *C. acetobutylicum* (Wilkinson *et al.*, 1994), the formation of butyrate and acetate by *C. acetobutylicum* (Green *et al.*, 1996), the inactivation of regulator genes in *C. difficile* (O'connor *et al.*, 2006), and the silencing of *spoIIE* and *sigF*, *sigG*, and *sigE* genes in *C. acetobutylicum* (Bi *et al.*, 2011, Jones *et al.*, 2011, Tracy *et al.*, 2011). Only a few *C. perfringens* mutants were created by double crossover, such as the excision of *C. perfringens* alfa and theta toxins (Awad *et al.*, 1995).

After 2011, double crossover events were accomplished for *C. difficile*. This process is firstly dependent on the creation of a uracil auxotrophic and fluoroorotic acid (FOA) resistant mutant. This facilitates the manipulation of the target genes by homologous recombination where the heterologous *pyrE* gene from *C. sporogenes* is used as a counter selection and negative selection marker. The second step is the restoration of uracil prototrophy by the repair of the *pyrE* gene (Ng *et al.*, 2013, Minton *et al.*, 2016).

2.3.6 Electroporation

2.3.6.1 Definition and hypothesis

The process of increasing cytoplasmic membrane permeability to macromolecules and ions by using short intensive electric pulses is called electroporation. Introducing DNA into a cell by applying an electric pulse on it is also called electroporation (Rubinsky, 2010, Bennett *et al.*, 2014). However, the exact mechanism by which the increase in membrane permeability occurs is not known. This phenomenon has been studied extensively through the use of computer simulation (molecular dynamic simulations). The aforementioned research revealed that the presence of free ions can strengthen the transmembrane pulse and improve the electroporation process. Having said that, these free ions can also hinder pore resealing (Sridhara *et al.*, 2014, Nickoloff, 1995).

The electroporation hypothesis posits that a strong, short electric pulse causes the transmembrane voltage $U_{(t)}$ (where U is the voltage magnitude and (t) is the elapsed time of the voltage (µSec.-mSec) in the membrane). The transmembrane voltage can in turn cause a rearrangement (breakdown) in the cytoplasmic membrane (CM) which results in the formation of pores also referred ot as "temporary aqueous pathways", for the hypothesized CM fluctuation, where the outer layer of the CM is invaginated into the membrane to form a hydrophobic pore (Nickoloff, 1995, Chizmadzhev *et al.*, 1979, White, 1978). The hydrophobic pore is suggested as an instant precursor for the hydrophilic pore, while the latter is considered as the first pore with a radius of 1 nm minimum, which allows the passage of ions and molecules (Abidor *et al.*, 1979, Litster, 1975, Taupin *et al.*, 1975, Weaver, 1993). The second hypothesized pore structure is a composite pore, containing one protein or more at the pore's verge (Weaver *et al.*, 1992). The last pore structure is also a composite pore but with a charged large molecule incorporated in the hydrophilic pore (Figure, 10) (Tsoneva *et al.*, 1990).

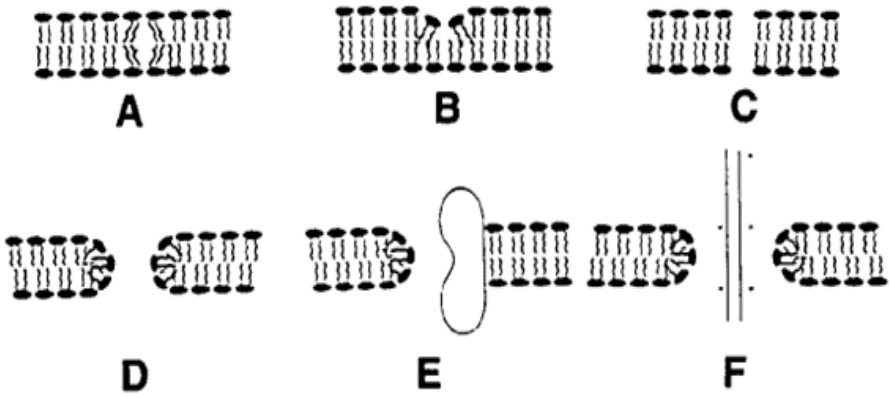


Figure 10. Hypothetical structure of pores formed by electroporation. A. Membrane fluctuation. B. Invagination of the outer layer of cytoplasmic membrane. C. Hydrophobic pore, the immediate precursor of the hydrophilic pore. D. Hydrophilic pore that allows the passage of ions and molecules. E. Composite pore with protein molecule at the inner edge. F. Composite pore with macromolecule passing through.

2.3.6.2 Factors that affect electroporation

Successful macromolecule delivery by electroporation depends on pulse density, pulse duration, number of applied pulses, type of nucleic acid used for transformation and how it interacts with the CM and on bacterial strain (Rols *et al.*, 1998, Jirásková *et al.*, 2005, Li, 2008, Ackermann *et al.*, 2001, Wagstaff *et al.*, 2016). Many studies have emphasized the role of the Gram-positive bacterial cell wall as a physical barrier confronting the passage of DNA by electroporation, and have described a successful transformation protocol after a cell wall treatment, such as that of *Lactococcus lactis* after treatment with lysozyme (Powell *et al.*, 1988). Many electroporation studies on *Clostridium* revealed that the characteristic of the strain to be electro-transformed is a crucial condition to the success of electroporation (Ackermann *et al.*, 2001).

The *C. difficile* cell wall consists of a thick de-acetylated peptide-glycan (PG) layer that has unique cell wall glycopolymers (CWGs) bounds to the surface layer (S-layer) protein lattice surrounding the whole PG (Figure, 11). The S-layer is composed of SlpA and an additional 28 proteins (Brüggemann *et al.*, 2009, Kirk *et al.*, 2017a). There are two distinct proteins in the S-layer which, a high molecular weight (42–50 kDa) protein and a low molecular weight (22–38 kDa) protein (Cerquetti *et al.*, 2000). The above-mentioned cell wall complexity and thickness represent a physical barrier that confronts the passage of DNA by electroporation.

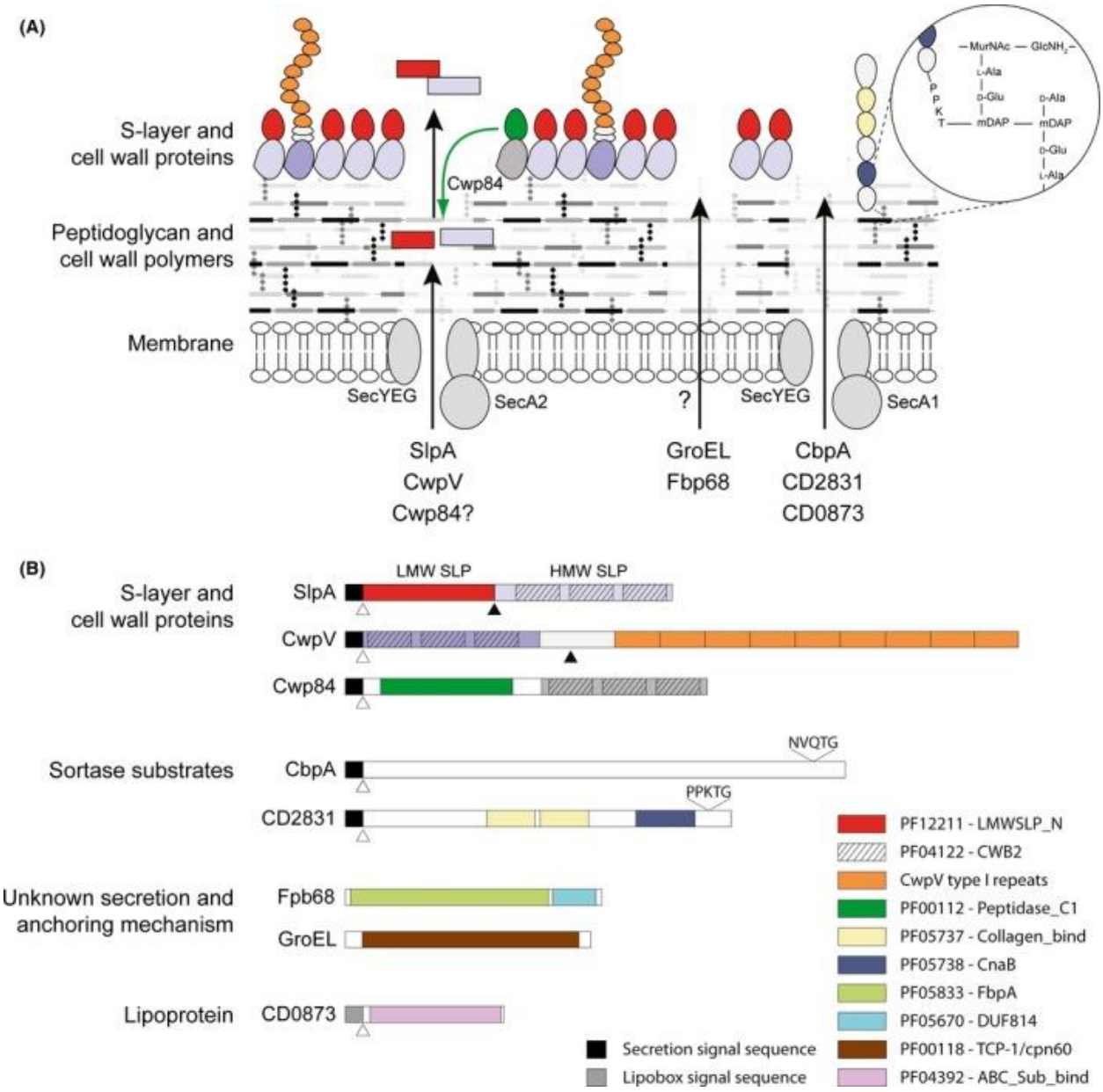


Figure 11. Complexity and thickness of *C. difficile* cell envelope. A. *C. difficile* cell envelope. The outer layer is decorated by the cell wall proteins family. SlpA, S-layer, and cell wall binding proteins are shown at the top, while the peptidoglycan and the glycopolymers can be seen in the middle. The plasma membrane is shown beneath the peptidoglycan layer. B. Organization of the proteins domains shown in A. Black boxes refer to the secretion signals of N-terminal. The black arrows refer to the post-secretion cleavage sites of the SlpA and CwpV. The sorting motifs location and sequence are shown above CD2831 and CbpA. (Kirk et al., 2017a).

The electric pulse has two roles in electroporation. Firstly, it causes the formation of pores, secondly, it provides a regional impose to transport molecules and ions through the pore. Depending on the cell size, a range of 1-10 kV/cm is required to cause electrical breakdown (EB) to the CM of isolated cells (Nickoloff, 1995). As EB starts, nonspecific molecular and ionic exchange between the inner cell and the surrounding medium occurs. Whether cells can survive these events or not depends on cell kind, cell volume (the ratio between extra to intracellular magnitude), field intensity (can cause cell rupture) and medium chemical structure (Nickoloff, 1995, Weaver *et al.*, 1996).

2.3.6.3 Advantages and applications

Electroporation has many advantages compared with chemical and biological methods; it is relatively easier, faster, more efficient, and can be used to transform a wide range of different cell types (Chang *et al.*, 1992, Jordan *et al.*, 2008, Deng *et al.*, 2017).

Electroporation has become widely used in biotechnology to deliver a variety of molecules (DNA, RNA, proteins and chemicals for therapeutic purposes) into different cell types (Bacteria, fungi, mammalian and plant cells) (Coletti *et al.*, 2017, Yang *et al.*, 2017, Prpar Mihevc *et al.*, 2017, Han *et al.*, 2017, Jaeger *et al.*, 2017, Liu *et al.*, 2017).

2.3.6.4 Disadvantages

The disadvantages of electroporation include the short lasting effects (nanoseconds) and intensive pulses that lead to the electroporation of the cellular organelles. It has been noticed that the endocytotic vesicle membrane of B16 F1 mouse melanoma cells became permeable when exposed to such a pulse. The endocytotic vesicle releases lucifer yellow and uptakes propidium iodide as a result of the penetration of the electric field into the cell interior (Napotnik *et al.*, 2010). This effect takes place if the dielectric permittivity of the organelle membrane is lower than that of the cell membrane, or the conductivity of the organelle interior is higher than that of the cytosol, as is the case with mitochondria (Kotnik *et al.*, 2006).

The disadvantages also include the high cost of certain electroporator devices. The electroporation applications in molecular biology require a wide range of “pulse amplitude, pulse duration, number of pulses, pulse repetition rate, and pulse shape”. Such a device is difficult to obtain and usually costs a lot of money (Reberšek *et al.*, 2011).

2.3.6.5 Clostridium species and electroporation

Electroporation has been successfully used to transform different *Clostridium* species with plasmid DNA. Different transformation efficiencies (TE) were obtained for each species, and for each plasmid within the same species. For example, *Clostridium thermocellum* with TE of 1.5×10^3 CFU/μg, 1.0×10^3 CFU/μg, and 0.85×10^3 CFU/μg for pNW33N-adh, pNW33N-pdc, and pNW33N-pdc-adh plasmids respectively (Kannuchamy *et al.*, 2016), *Clostridium pasteurianum* with TE of 1.2×10^2 CFU/μg (Kolek *et al.*, 2016), *Clostridium beijerinckii* with TE of 7.44×10^3 CFU/ μg (Oh *et al.*, 2015), *Clostridium ljungdahlii* with TE of $3.8\pm0.2 \times 10^3$ with pMTL82151 and $3.1\pm1.8 \times 10^3$ with pMTL83151 (Leang *et al.*, 2013), and many others.

Only one report has described a protocol to transform *C. difficile* by electroporation. Ackermann *et al*. (2001) were able to transform one non-toxigenic *C. difficile* strain out of eight. However, the optimized electroporation parameters of this protocol are not reproducible with other *C. difficile* strains, and the success rate is 82% with the same strain. It is clear that this fact is due to the complex stratified structure of the thick *C. difficile* envelope reviewed in section 2.3.4.2.

During the optimization of the electroporation of *C. difficile* ribotypes 076 and 220, an attempted was made to obtain the *C. difficile* strain used in Ackermann's study. Although authors were contacted by email, there has not been any reply.

Chapter 3
General methods

Chapter 3. General Methods

3.1 Media, buffers, solutions, and enzymes

3.1.1 Media

All media, buffers and enzymes used throughout this study are listed in Appendix 1. Prestige Medical Autoclave, England was used to autoclave all the media and buffers at 121ºC, 15psi (1 bar) for 15 minutes.

3.2 Bacterial strains

3.2.1 *C. difficile* source and culture methods

C. difficile ribotypes 220 and 076 were kindly provided by Prof. Clokie. *C. difficile* ribotype 220 harbours the CDHM1 and CDHM3 bacteriophages as prophages. These two phages are able to infect and lyse the relatively important clinical *C. difficile* strains, including the hypervirulent R027 (Hargreaves, 2013). *C. difficile* ribotype 076 is susceptible to infection with many bacteriophages, and was used as a factory to propagate the bacteriophages.

C. difficile was routinely cultured on a blood agar plate (Appendix, 1). This plate was then incubated at 37ºC for 24-48 hours under anaerobic conditions; 5-10% Hydrogen, 10% Carbon dioxide and 80-85% Nitrogen inside an anaerobic chamber (miniMACS anaerobic workstation) (Don Whitely Scientific, UK).

Typically, from the blood plate, one colony was inoculated in to 5ml of pre-reduced Fastidious Anaerobic broth (FA) (Appendix, 1) inside a bijou tube (Sterilin, UK) and incubated anaerobically overnight (8-16 hours) at 37ºC. The growth curves of *C. difficile* ribotypes 076 and 220 were already done separately by (Rashid, 2016) and (Hargreaves, 2013) respectively. The next day, 500µl of FA culture was inoculated in 50ml of pre-reduced brain heart infusion BHI broth. After the BHI culture reached the log phase (0.2-0.3 OD at 550nm), it was used for spot tests, plaque assays, CDHM1 phage propagation and for the preparation of *C. difficile* cells for electroporation.

3.2.2 *C. difficile* cryogenic stock

1.4ml of FA overnight culture was centrifuged at room temperature for 5minutes at 13,000xg (Eppendorf, Germany). Glycerol taken from a cryo-preservation vial (Abtec,

UK) was used to re-suspend the pellet after decanting the supernatant. The suspension was dispensed back to the cryo-vial and stored at -80ºC.

3.2.3 *C. perfringens* source and culture methods

3.2.3.1 Sampling for the isolation of C. perfringens and C. perfringens phages

Environmental soil samples and faeces samples from a variety of animals and soils were collected from different sites in and around the Leicester area, as well as from Iraq (three samples only). 60 Samples were collected from Melton Mowbray farms, Ansty (Gorse Hill City Farm), Bradgate Park, Desford (Tropical Birdland), Blaby, Lutterworth Road farms, Astley (Walton Lodge Farm), Water Mead Park and Abbey Park, in Leicestershire (United Kingdom). 21 out of the 81 samples were kindly provided by Julian Clokie (soil, pigs, dogs and duck faeces).

Environmental faecal samples were obtained from chickens (n=9), cows (n=5), sheep (n=5), horses (n=5), rabbits (n=4), a turkey (n=1), a fish (n=1), ducks (n=9), parrots (n=3), dogs (n=3), sediment (n=2), ostriches (n=2), a lamb (n=1), deer (n=2), a swan (n=1), an unknown sample taken from the side of the road (n=1) and soil samples (n=6).

The pigs faeces (n=7), soil (n=2), and duck sample (n=3) were kindly provided by Julian Clokie. Other soil samples (n=9) were kindly provided by Mahananda Chutia (Appendix, 5). Samples were stored at 4ºC until processing. Nine *C. perfringens* strains, which were kindly provided by Prof. Martha Clokie and which were isolated from outside Leicestershire, 69 *C. perfringens* strains were isolated out of the 81 collected samples (Appendix, 5).

3.2.3.2 Isolation of C. perfringens strains

C. perfringens strains were isolated by enrichment culture in tryptose-sulphate cycloserine (TSC) broth supplemented with 400µg/ml cycloserine (Appendix, 1) (Erickson *et al.*, 1978).

Approximately 1cm^3 of faeces or soil samples were suspended in 10ml of (TSC) broth and incubated anaerobically overnight at 37ºC. The culture was then centrifuged at 3000xg for 5minutes (Beckmann coulter, UK) at room temperature, and 10µl of the supernatant was streaked on to TSC agar (Appendix, 1) and incubated anaerobically overnight at 37ºC.

The next day, half of one distinct large black colony was used for the amplification of the *C. perfringens* specific 16s rDNA gene by PCR. The other half was used to prepare a cryogenic stock. It was suspended and thoroughly mixed using a pipette in 100µl of ultra-pure distilled water and boiled at 99ºC for 10minutes. The mixture was left for 5minutes to cool down, then centrifuged at 16000xg for 5minutes. The supernatant containing the bacterial DNA was used as a template for PCR. A pair of specific primers for *C. perfringens* 16s rDNA (Skånseng *et al.*, 2006) (Appendix, 3) was used to confirm that the isolated strain is *C. perfringens*. The PCR reaction mix's volume was 25µl and contained: DNA template, 0.4µM of each primer, 250µM of dNTPs, 1X buffer, and 0.5 unit/25µl of BioTaq polymerase (Bioline, UK). Cycler conditions were: initial denaturation step at 95°C for 5minutes, 35 cycles of 95°C for 30seconds, 56°C for 20seconds, 72°C for 1minute, and a final extension step of 72°C for 5minutes. The 260bp PCR product was visualised on a 1% agarose gel (Fisher scientific, UK) prepared in 1X TAE buffer, stained with medori green DNA stain (Geneflow, UK), and a 1kb or 100bp DNA ruler (New England Biolabs, UK) was used to determine the product's size. Electrophoresis was carried out in a 1X TAE buffer by using 90volts for one hour (Bio-Rad, UK). Gel images were taken using a gel dock (Bio Rad, UK).

Amplification of bacterial universal 16s rDNA was performed for the colonies, which were repeatedly negative for the *C. perfringens* specific 16s rDNA. A pair of primers for universal bacterial 16s rDNA amplification (Turner *et al.*, 1999) (Appendix, 3) was used. The PCR reaction mix's volume was 25µl, and contained: DNA template, 0.2µM of each primer, 250µM of dNTPs, 1X buffer, and 0.5 unit/25µl of BioTaq polymerase (Bioline, UK). Cycler conditions were: initial denaturation step at 95°C for 5minutes, 30 cycles of 95°C for 30seconds, 55°C for 90seconds, 72°C for 2minutes, and final extension step of 72°C for 10minutes. The PCR product was purified from the gel using a mini-elute Kit (Qiagen, UK). The product was sent for sequencing to GATC Biotech. When the sequence was returned, a BLAST search against the NCBI sequence collection was carried out using the nucleotide blast software available online at:

(https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome).

3.2.3.3 C. perfringens culture and cryogenic stock preparation

The other half of the same isolated colony which was confirmed by *C. perfringens* specific 16s rDNA PCR to be *C. perfringens* strain was inoculated in a pre-reduced tryptose sulphate (TS) broth (Appendix, 1) and incubated anaerobically for 2 - 2.5 hours at 37ºC (Figure, 33 in section 4.4.9) to prepare a cryo-stock. 1.4ml of TS broth culture was centrifuged at room temperature for 5 minutes at 13,000xg (Eppendorf, Germany). Glycerol taken from a cryo-preservation vial was used to re-suspend the pellet after decanting the supernatant. The suspension was dispensed back into the cryo-vial and stored at -80ºC.

C. perfringens was routinely cultured on a tryptose-sulphate agar (TSA) plate (Appendix, 1). The plate was incubated at 37ºC for 12-24 hours under anaerobic conditions.

TS broth was used to prepare a liquid *C. perfringens* culture for both a plaque assay and spot test. A loospful of the colony was inoculated in a pre-reduced TS broth. The exponentially growing bacteria was ready after 2-2.5 hours (Figure 33 in 4.4.9). The CFU after 2.5 hours was around 10^8 CFU/ml, reaching 10^9 CFU/ml after about another hour and began declining after the fifth hour post inoculation. The reason for using TS broth instead of Tryptose-Yeast extract is that *C. perfringens* grows much faster in the former. It was not used for experiments requiring optical density calculation because of the black colour produced at the early stage of *C. perfringens* growth. Therefore, the optimized tryptose- yeast-extract (TY) broth (see 4.3.1.4) was used instead as it doesn't develop the black colour.

3.2.4 *E. coli* strains and culture methods

E. coli DH5α In-Fusion® HD Cloning (Clontech, USA) was used to amplify the backbone of plasmids pTML82151 (Figure, 12) and pMTL84151 (Figure, 13), which were used in the optimization of the electro-transformation of *C. difficile* R220. pTML82151 is a semi-suicide vector for *C. difficile*. It has a Gram-negative origin of replication in *E. coli* and a Gram-positive origin of replication derived from *C. pasteurianum*, which allow the replication of the plasmid at a very low level inside *C. difficile*. Plasmid pMTL84151 is a *C. difficile* specific expression plasmid that possesses the native pCD6 promotor (Heap *et al.*, 2009b). *E. coli* DH5α has also used to amplify pLEICS-02, which has the recombinant endolysin gene of the CPAP1 phage. The

expression of recombinant endolysin was performed in *E. coli* BL21 DE3 (Agilent Technologies, UK).

E. coli strains were grown in Luria Bertani agar (LB agar) and lysogeny broth (LB broth) (Appendix, 1). *E. coli* DH5α that harbours the pMTL82151or pMTL84151 was streaked from a cryo-stock on LB agar supplemented with 30µg/ml chloramphenicol (Appendix, 1) and incubated aerobically at 37°C overnight. The plate could be stored at 4°C and used for up to two weeks. A loopful of colonies were inoculated in 10ml of LB broth supplemented with 30µg/ml chloramphenicol, and incubated aerobically overnight with agitation at 220rpm using the Innova® 44 (New Brunswick scientific, USA) shaker incubator. The following day, the amplified plasmid was extracted as described in section 3.11.2 and used for *C. difficile* transformation.

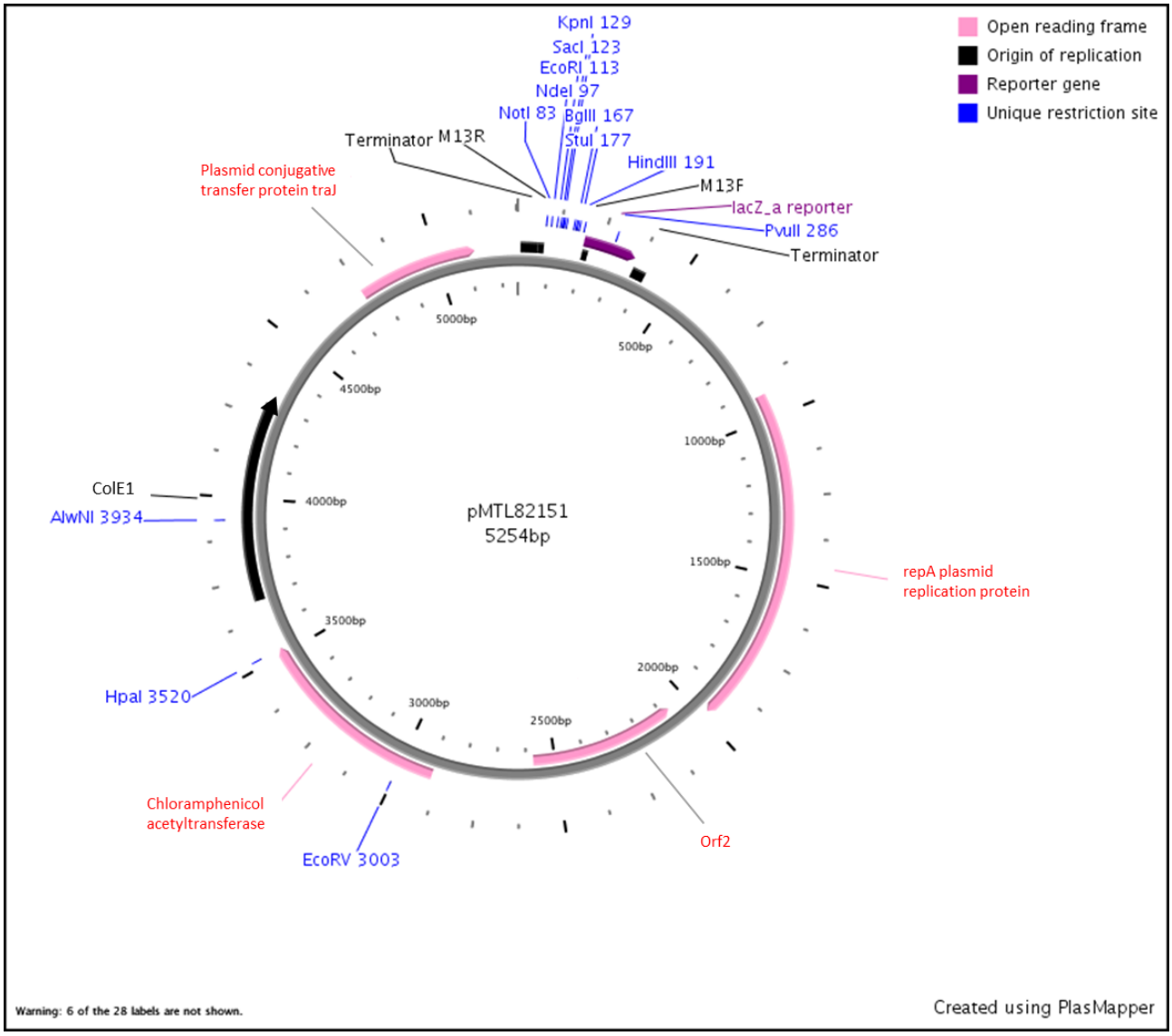


Figure 12. The pMTL82151 plasmid map. The plasmid map was generated using the PlasMapper software (Dong et al., 2004). The pMTL82151 is an *E. coli – C. difficile* shuttle plasmid. It is 5254bp in size and a chloramphenicol/thiamphenicol resistance marker.

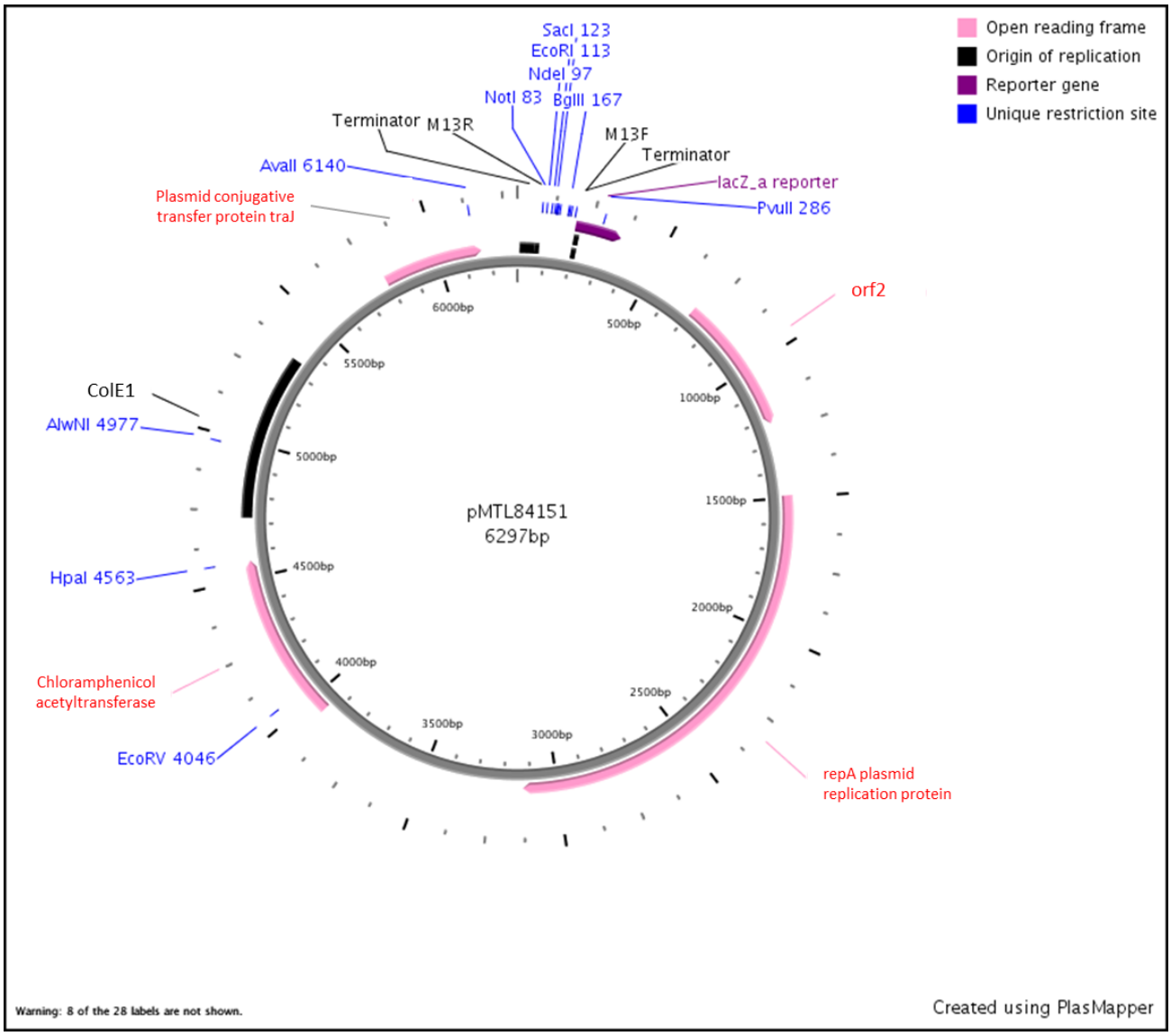


Figure 13. A pMTL84151 plasmid map. The plasmid map was generated using the PlasMapper software (Dong et al., 2004). The pMTL84151 is an *E. coli – C. difficile* shuttle plasmid. It is a C. difficile specific expression plasmid that possesses the native *C. difficile* pCD6 promotor. It is 6297bp in size and has a chloramphenicol/thiamphenicol resistance marker.

E. coli DH5α that harbours the pLEICS-02 was streaked from a cryo-stock on LB agar supplemented with 150µg/ml of carbenicillin (Appendix, 1) and incubated aerobically overnight. The plate could be stored at 4°C and used for up to two weeks. loopful of colonies were inoculated in 10ml of LB broth supplemented with 150µg/ml carbenicillin and incubated aerobically overnight with agitation at 220rpm using the Innova® 44 (New Brunswick scientific, USA) shaker incubator. The following day, the amplified plasmid was extracted as described in 3.11.2 and used for the transformation of *E. coli* BL21.

3.3 *C. perfringens* genotyping (toxin typing)

3.3.2 *C. perfringens* toxin typing

C. perfringens strains were toxin-typed using multiplex PCR as described by (van Asten *et al.*, 2009). Six pairs of specific primers that amplify the 5 essential *C. perfringens* toxins, plus the Beta 2 toxin (Appendix, 3) were used to detect the presence of one or more toxin and classify the isolated strains accordingly (van Asten *et al.*, 2008, van Asten *et al.*, 2009, Baums *et al.*, 2004b, Meer *et al.*, 1997). A volume of 1µl of the extracted DNA was used as a template for PCR. The primers were used at a final concentration of 0.2µM, with the exception of the Beta 2 primers, whose concentration was 0.4µM. The PCR mix of (Qiagen, UK) was ready to use. The multiplex PCR was carried out using the following conditions; 15minutes initial denaturation step at 95°C, 40 cycles of 30seconds denaturation at 94°C, 90seconds annealing at 53°C, 90seconds extension at 72°C, and 10minutes final extension at 72°C. The reaction was repeated using a 57°C annealing temperature. A 3% agarose gel (BioLine, UK) was used to help separate between the amplicons, as the difference in size between them ranged from 52 to 109bp. 1µl of distilled water was used as a negative control. A New England BioLab 100bp DNA gene ruler and Bioline 1kb GeneRuler were used to determine the PCR products' size.

All the *C. perfringens* strains tested by multiplex PCR were negative for beta, iota and epsilon toxins. A separate PCR reaction was carried out to detect the presence of these toxin genes by using three pairs of primers (Appendix, 3) (Gharaibeh *et al.*, 2010, Perelle *et al.*, 1993, Kadra *et al.*, 1999).

3.4 Colony forming unit CFU

CFU counts were performed as described by (Lee, 2008) with some modification. Serial dilutions (down to 10^{-8}) of bacterial liquid culture were prepared using a TY broth in a 96 wells microtiter plate. 10µl of each dilution was spotted in duplicate on a

segmented 3.7% BHI agar 120mm square plate, allowed to dry and incubated anaerobically overnight at 37ºC.

The next day, CFU was calculated as separate colonies became visible; to do this, the following equation was used:

$$CFU/ml = the\ average\ colonies\ count\ of\ duplicate\ 10\mu l\ spots X 100 X dilution\ factor$$

3.5 *C. perfringens* growth curve

One *C. perfringens* colony was inoculated in 50ml of pre-reduced TY broth and incubated anaerobically overnight at 37°C. The next day, 500µl of the liquid culture was added to 49.5ml of pre-reduced TY broth. Both CFU and OD at 550 nm were taken at the inoculation time. A colony forming unit was performed and O.D at 550nm (Multicell changer, Jinway) was taken at different time points. These time points were 1, 2, 3, 4, 5, 6, 12, and 24 hours. 50 ml of TY broth was used as a blank.

3.6 Phage related methods

3.6.1 *C. difficile* phages CDHM1 and CDHM3

These phages were kindly provided by Prof Martha Clokie. Both phages are temperate, long tail myoviruses and have a 54kb DNA genome. CDHM3 is able to infect and lyse hypervirulent *C. difficile* strains such as R027 and R078, while CDHM1 can be easily propagated to a high titre.

3.6.2 *C. perfringens* phages

Five phages were isolated from environmental samples collected during this project (Table, 5).

Phage lab name	Isolation CP strain	Name	Isolation source
F42	CP42	CPAP1	Cow faeces
F43	CP40	CPAS1	Cow faeces
F44	CP40	CPAS2	Cow faeces
F56	CP56	CPAS3	Turkey faeces
F65	CP65	CPAP2	Soil sample

Table 5. The Isolated *C. perfringens* phages. Five phages which are able to grow on their indicator strains are listed below.

3.7 Phage cryo-stock preparation

The isolated purified phage lysates were mixed with a 50% glycerol solution at a ratio of 1:1 (25% final glycerol concentration) inside a cryo-vial and stored at -80ºC. The final PFU/ml in the cryo-stock was: CPAP1 5 X 10^{10}, CPAP2 8 X 10^{5}, CPAS1 1 X 10^{6}, CPAS2 7 X 10^{6}, and CPAS3 4 X 10^{5}.

3.8 Spot test (determining phage stock titre)

A spot test (small drop plaque assay) (Mazzocco *et al.*, 2009) was used with modifications to determine the phage concentration (plaque forming unit PFU/ml). 500µl of exponentially growing *C. perfringens* was mixed with 8ml of TY soft agar and calcium-magnesium salt (Appendix, 1), and poured onto a 120mm square 1% TY agar plate. The plate was left to solidify for five minutes. Serial dilutions of the phage preparation were made (10^{-1}-10^{-8}) and 10µl was spotted in duplicate on the overlay agar. The plate was incubated overnight anaerobically at 37ºC. The next day, the PFU/ml was determined by the following equation:

$$PFU\ /ml\ = the\ average\ count\ of\ duplicate\ 10\mu l\ spots\ X\ 100\ X\ dilution\ factor$$

3.9 Plaque assay

A plaque assay (double agar layers) (Kropinski *et al.*, 2009), with a few modifications, was used to propagate and get a high *C. perfringens* phage titre, which is required for further experiments. 500µl of exponentially growing *C. perfringens* host was mixed with

250µl of lysate of the phage to be concentrated in a 15ml falcon tube. One tube was prepared for each 120mm square plate; usually, 20 plates were prepared at a time. Then, 4ml of Tryptose-yeast extract TY soft agar and 4ml of salt solution (overlay agar) (Appendix, 1) were added to the tube. This tube was then inverted a few times and poured onto 120mm square 1% TY agar plate (Oxoid, UK). The double layered plates were left to dry for 5minutes in order to allow them to solidify, after which they were incubated anaerobically at 37ºC for between 9-15 hours. For the circular 90mm plates, 200µl of culture was mixed with 100µl of phage lysate and 1.5ml from both the soft agar and salt was added.

The plates were scraped after the incubation was over using an L shaped scraper into 50ml falcon tubes and were incubated at 4ºC for more than 5hours. The tubes were centrifuged at 12000xg for 5minutes and the supernatant that contained the phage was filter-sterilized using a 0.22µm filter (Miller ®Millipore, UK). The resulting phage lysate was titrated by a spot test and stored at 4ºC for further use.

3.10 Phage concentration

3.10.1 Phage concentration by ultra-centrifugation

Phage lysate was cleaned by tube dialysis in order to remove any remaining media. It was then aliquoted in 5ml inside dialysis membranes and immersed in a beaker with 2litres of sodium magnesium buffer (SM) pH 8 (Appendix, 1). The beaker was placed on a magnetic stirrer overnight at 4ºC. The buffer was replaced once during the incubation.

The following day, the phage lysate was transported to 6 tubes and ultra-centrifuged at 117,000xg for three hours at 4ºC (Sorvall-Discovery, Hitachi). The tubes were taken out of the centrifuge and the upper part of the lysate was aspirated with extreme care, as vibration can re-mix the packed phages. Approximately 1ml of the lysate was left in each tube. The phage titer was determined by a spot test (counting plaque forming unit PFU) and stored at 4ºC to be used in further experiments.

3.10.2 Phage concentration by polyethylene glycol (PEG)

Phage lysate was concentrated by PEG as described by (Nale *et al.*, 2012), with some modifications, after which it was transported to a 50ml falcon tube. Sodium chloride was dissolved in the phage lysate to achieve 1M, and after one hour of incubation in ice, the tubes were centrifuged at 3500xg for 10minutes and the supernatant was filtered using

0.22µm filter into a new tube. 10% w/v of PEG8000 (Fisher scientific, Belgium) was gradually added with continuous stirring until it dissolved. The treated lysate was incubated overnight at 4°C. During incubation, the PEG was precipitated and the clean phage remained in the solution.

The next day, the tube was centrifuged at 15000xg for 20minutes at 4°C, and the supernatant was discarded. The pellet was re-suspended with 1ml of SM buffer and 1ml of chloroform was added. The tube was then centrifuged at 4000xg for 15minutes at 4°C. The top aqueous solution was collected, filtered using the 0.22µm filter, and the phage titre was determined by a spot test.

Alternatively, the chloroform washing step was not performed. Instead, the pellet was re-suspended in SM buffer and transferred to a 2mL sterile tube. 2M KCL V:V was added, after which the mixture was incubated on ice for 20minutes. The tube was centrifuged at 12,000xg for 10minutes at 4°C in order to precipitate the PEG solution slowly, while the free phage remained in the supernatant. Finally, the supernatant with clean, concentrated phage was pipetted into a new tube (Colombet *et al.*, 2012).

3.10.3 Phage DNA extraction

Phage DNA was extracted by the phenol/chloroform/isoamyl alcohol method using the ultra-centrifugation concentrated phage lysate. 1ml of the lysate was transported into an eppendorf tube. 12.5µl of 1M magnesium chloride was added, after which the tube was gently mixed. The mixture was treated with 10µl of 30mg/ml DNaseI and 2µl of 100mg/ml RNase H (New England Biolabs, U.K). The tube was then briefly vortexed and incubated overnight at 37^0C.

The following day, the mixture was filtered with a 0.22µm filter into a new tube. Universal bacterial 16s rDNA PCR was performed to detect any contamination with the bacterial genome (Appendix, 3). The following reagents were added in this exact sequence; 40µl of 0.5M Ethylene Di-amine Tetra-acetic Acid (EDTA), 5µl of 10mg/mL proteinase K (Fisher Scientific, UK), and 50µl of 10% Sodium dodecyl sulphate (SDS). The mixture was vigorously vortexed and incubated at 55^0C for one hour; the tube was vortexed twice, every 20 minutes during the incubation. Then, an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) was added and the tube was inverted several times and centrifuged at 15000xg for 15minutes at 4^0C. The aqueous layer on top

containing the DNA was retained, and the addition of an equal volume of phenol/chloroform/isoamyl alcohol and centrifugation was repeated.

The aqueous layer was transported into a new tube and the DNA was precipitated by the addition of two volumes of ice-cold isopropanol and 1:10 volume of 3M sodium acetate pH 7.5. The tube was incubated in ice for 30minutes and centrifuged at 21000xg for 20minutes at 4^0C. The entire liquid was removed carefully using a pipette and 1ml of 70% ethanol was added to wash the pellet. Following this, the isopropanol was carefully removed, and the DNA pellet was briefly air-dried and dissolved in 50µl of 5mM Tris-acetate pH 8-8.5. DNA concentration was determined using a Nano-Drop 1000 spectrophotometer (Thermo-scientific, USA), and stored at -20^0C.

3.10.4 Determination of DNA concentration by Qubit

The concentration of DNA preparations for sequencing was determined using a Qubit 3 Fluorometer (Invitrogen, Malaysia). The DNA sample was diluted 200 times and two standards (high 10ng/µl and low 0ng/µl) were used to calibrate the machine. After that, the DNA sample reading was taken. The following equation was used:

$$DNA\ concentration \frac{ng}{\mu l} = the\ value\ given\ by\ the\ Qubit\ X\ \frac{200}{Sample\ volume}$$

3.11 Protein related work

3.11.1 Cleaning PCR products

PCR products for both sequencing and cloning were cleaned up using ISOLATE II PCR and a gel kit (Bioline, UK). The silica membrane of the cleaning column can bind DNA in the presence of a chaotropic agent. The manufacturer instructions were followed to clean the PCR product. Briefly, a 2:1 ratio of binding buffer to PCR product was used (80µl of binding buffer was added to 40µl the PCR product) and the mixture was transported to a silica column placed in a 2ml collection tube. Then, it was centrifuged (eppendorf 5418, Germany) at 11000xg for 30seconds at room temperature, the flow-through was discarded and 700µl of the washing buffer supplemented with the kit was loaded to wash the silica membrane. Another centrifugation was carried out at 11000xg for 30seconds at room temperature, after which the flow-through was decanted. The silica membrane was dried by centrifugation at 11000xg for 1minute at room temperature. The PCR products were eluted using the elution buffer provided with the kit; 30µl of elution buffer was added directly onto the silica membrane and the column was incubated for

1minute at room temperature. Then, it was centrifuged at 11000xg for 1minute at room temperature. The cleaned DNA was stored at -20^{0}C for further use.

3.11.2 Plasmid extraction

Plasmids for both protein expression and optimization of *C. difficile* electro-transformation parameters were extracted using the GeneElute Plasmid Miniprep Kit (Sigma-Aldrich, USA). Briefly, 1-5ml of recombinant *E. coli* cells harboring the plasmid of interest were grown in LB broth (3ml for pLEICS-02, 5ml for pMTL82151, and 3ml for pMTL84151) and harvested by centrifugation at 12,000xg for 1minute at room temperature. The supernatant was decanted and the pellet was re-suspended with 200µl of re-suspension solution supplemented with RNase provided with the kit. The mixture was vortexed until it became homogenized, as incomplete suspension of cells causes poor plasmid recovery. The mixture was transported to a 1.5ml micro-centrifuged tube and the cells were lysed by the addition of 200µl of the lysis solution provided by the kit. The mixture was immediately mixed after the addition of the lysis buffer with 6 to 8 gentle inversions, until it became viscous and clear. This had to be done immediately, as prolonged alkaline lysis can permanently destroy plasmids.

The cell debris was precipitated by the addition of 350µl of the Neutralization/Binding solution provided with the kit. The mixture was mixed with 4 to 6 inversions and the debris were pelleted by centrifugation at 12000xg for 10minutes at room temperature. Consequently, proteins, chromosomal DNA lipids, and cell debris were in the cloudy viscous precipitate, while the plasmid was in the clear supernatant.

During the 10minutes of the last step centrifugation, the binding column was inserted in a 2ml micro-centrifuge tube and prepared with the addition of 500µl of column preparation solution that maximizes the binding with the plasmid. Then, it was centrifuged at 12000xg for 30-60seconds and the flow-through was discarded. The clear supernatant from the previous step was loaded in the prepared column and centrifuged at 12000xg for 30-60seconds, and the flow-through was once again discarded. The column was washed twice with 500µl and 750µl of both the optional washing and the washing solutions respectively.

Thereafter, the column was transported to a fresh 2ml tube and the plasmid was eluted with the appropriate elution liquid (biology grade distilled water for the electro-transformation experiment). The extracted plasmid was stored at -20^{0}C for further use.

3.11.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

The analysis of proteins was carried out by running them through a 12% SDS-PAGE and subsequently staining them with Coomassie brilliant blue (Fisher Bioreagents, Belgium) in order to visualize them. A pre-stained protein standard P7712 (New England Biolabs, UK) was used alongside with the proteins to confirm their sizes. SDS-PAGE was used to visualise the effect of the cysteine protease inhibitor treatment on *C. difficile* surface layer proteins in comparison with untreated cells. It was also used to visualise the recombinant endolysin of phage CPAP1, and in a zymography experiment where 0.2% of a peptidoglycan substrate was embedded in the resolving gel. The SDS-PAGE was conducted as described by (Fagan *et al.*, 2010).

3.11.3.1 Preparation of resolving and stacking gels

A resolving gel (12% polyacrylamide) and stacking gel (5% polyacrylamide) were prepared as described by (Fagan *et al.*, 2010). The resolving gel consists of 3.4ml distilled water, 2.5ml of 1.5M Tris–HCl (ChemCruz, USA) pH 8.8, and 4ml of 30% (W/V) Acrylamide/Bis-acrylamide Protogel (National Diagnostics, England), 100µl of 10% sodium dodecyl sulphate (SDS) (Fisher scientific, USA), 50µl of 10% ammonium persulfate (APS) (BioRad, USA), and 10µl of N, N, N', N'-Tetramethylethylenediamine TEMED (Sigma, China). The stacking gel consists of 833µl of 30% (W/V) Acrylamide/Bis-acrylamide Protogel (National Diagnostics, England) 50µl of 10% SDS (Fisher scientific, USA), 25µl of 10% APS (BioRad, USA), and 5µl of TEMED (Sigma, China). Both gels were prepared at the same time; the APS and TEMED were added before pouring the gel.

The gel casting apparatus (BioRad, USA) was assembled as instructed by the manufacturer. The resolving gel was prepared and 4ml was pipetted between the glass plates of the gel casting apparatus (two gels were prepared each time). Thereafter, 0.5ml of 96% ethanol was overlaid on each of the gels, so as to exclude the oxygen and give a straight top edge for the resolving gel. The gel was left to polymerise for half an hour. After that, the ethanol was poured out and the gel was briefly allowed to dry. Then, the APS and the TEMED were added to the stacking solution and mixed briefly before the mixture was pipetted onto the resolving gel so as to fill the remaining space between the casting glasses. A comb was gently inserted to make the sample loading space. The gel was left for half an hour to solidify and be ready for use. Finally, it was placed in a Mini-PROTEIN Tetra system running tank (BioRad, USA).

3.11.3.2 Preparation of protein samples for electrophoresis

A 5X loading buffer (Genecopoeia, USA) consisting of 250mM Tris·HCl, pH 6.8, 10% SDS, 30% v/v Glycerol, 10 mM DTT, 0.05% w/v bromophenol blue was used to prepare the samples for electrophoresis. 5µl of the 5X loading buffer was added to 20µl of the protein sample in a small eppendorf tube, and 15-25µl of the mixture was loaded in the gel, depending on the comb used.

3.11.3.3 Running the protein

The 10X running buffer in which the gel was to be immersed during electrophoresis consists of: 1.92M glycine (Fisher Bioreagents, USA), 250mM Tris-base (Fisher Bioreagents, Belgium), and 1% SDS (Fisher scientific, USA). The running buffer was diluted with distilled water to 1X before it was used. The gap between the two gels was filled with 130ml of running buffer, and the electrophoresis tank was filled to the marked level of two gels within the gel tank (500ml of the running buffer were added).

After the pre-stained protein standard and protein samples were loaded, the electrophoresis tank was connected to the power unit PowerPac basic (Bio-Rad, USA). The electrophoresis was performed at 160volt for 1 hour.

3.11.3.4 The staining and de-staining of the SDS-PAGE gel

After the electrophoresis was completed, the gel was placed in a plastic container and washed with distilled water twice. During each wash, the gel was immersed into distilled water as much as possible, and heated in the microwave for 30seconds until the water became warm. The washing steps were performed to get rid of the SDS.

The gel was then stained with Coomassie brilliant blue stain. The stain was added to a point where the gel was fully immersed (approx. depth of 1.5cm). The gel was then left overnight on a rocker shaker (Joint Biomedical workshops, University of Leicester) at room temperature. The next day, the stain was removed and the gel was washed with distilled water (without heating), until there was no stain coming out of the gel. Lastly, it was de-stained overnight with distilled water rather than a de-staining buffer, while being left on a rocker shaker at room temperature.

Chapter 4

Isolation and characterization of *C. perfringens* phages

Chapter 4. Isolation and Characterization of *C. perfringens* phages

4.1 Introduction

4.1.1 Rationale behind the environmental isolation of *C. perfringens* phages

Although very little work has been carried out on phages that target *C. perfringens,* there is a clear need for novel antimicrobials to treat this pathogen. Despite the standard care that includes surgery and antibiotic treatment, *C. perfringens* human infections are still associated with high mortality rate (Hifumi *et al.,* 2018). Usually, phages are found in high abundance in the environment that their bacterial hosts inhabit (Suttle, 2005). *C. perfringens* spores are found throughout the environment, and *C. perfringens* vegetative cells are present inside animal intestines as both commensals and pathogens (Pruteanu *et al.,* 2013, Aziminia *et al.,* 2016). Most of the isolated *C. perfringens* phages were obtained from environmental samples; for instance ΦCP39O and ΦCP26F were isolated from chicken offal washes and chicken faeces (Seal *et al.,* 2011), ΦCP24R was isolated from raw sewage water (Morales *et al.,* 2012), ΦCPV1 was isolated from chicken faeces (Volozhantsev *et al.,* 2011), and ΦCPV4, ΦZP2, and ΦCP7R were isolated by screening sewage water, faeces, and soil samples (Volozhantsev *et al.,* 2012). *C. perfringens* phages are likely to be isolated from environmental samples such as soil and animal faeces.

4.1.2 *C. perfringens* toxin typing

The pathogenicity of *C. perfringens* and the tissue destruction caused by this pathogen correlates with the type of toxin that it produces. The prevalence of different types of *C. perfringens* strains can also be monitored by the detection of the toxin genes that they possess. Thus, toxin genotyping has both epidemiological and diagnostic importance (Razmyar *et al.,* 2014, Yonogi *et al.,* 2016), and during the last two decades, it has been used for the genotyping and detection of *C. perfringens* strains (Brzychczy-Wloch *et al.,* 2017, Chukwu *et al.,* 2016, Sabry *et al.,* 2016, Park *et al.,* 2015, Gurjar *et al.,* 2008, Badagliacca *et al.,* 2010, Baums *et al.,* 2004a, Petit *et al.,* 1999, van Asten *et al.,* 2009, Yoo *et al.,* 1997). The other *C. perfringens* genotyping methods have been previously used are illustrated in section 2.1.5.

4.1.2 Host ranges of phages

In order to decide whether a phage is a therapeutic candidate, it must be able to infect and lyse a wide range of strains that belong to the same pathogenic species. However, more characterisation and insight is required to make this decision (Hyman *et al.*, 2010b).

There is significant variation in phage host ranges. Certain phages can infect one strain only, the isolation strain, some phages can infect a few bacterial strains within the same species, while other phages have the ability to infect many species, some of which belong to different genera (Ross *et al.*, 2016).

Given that *C. perfringens* infection often begins from the location of new traumatic or surgical wounds, or in-patients with cancer (immune-compromised), urogenital and gastrointestinal problem after contamination with the spores (Finsterer *et al.*, 2007, Hugelshofer *et al.*, 2012), it is important to screen the isolated phages against the environmental *C. perfringens* strains, as they are the source of infection.

4.1.3 Phage one-step growth curve

The lifecycle of lytic phages has multiple sequential steps; the adsorption of phage onto a susceptible bacterial cell, phage nucleic acid injection (or entry), the latent period during which the phage proteins and nucleic acid are synthesized and assembled, and the virion release after cell lysis. Thus, the virulent phage life cycle has three phases; adsorption, a latent period, and phage release. The number of phages released per one cycle is termed as the burst size, and the time elapsed during all these steps is known as the phage generation time (Hyman *et al.*, 2009). The temperate phages' lifecycle differs from the lytic cycle in that temperate phages are able to integrate and insert their genomes into the bacterial chromosome. Thus, the phage DNA remains in the bacterial chromosome as a prophage and replicates along with it, allowing the host cell to survive in a lysogenic state. The prophage leaves the host chromosome under particular conditions and proceeds with the lytic cycle (Griffiths, 2002).

The determination of a given phage growth kinetic can be considered as a baseline indicator for its therapeutic potential and how effective it would be to control the growth of the host bacterial population. The phage burst size and the time required for the phage to cause cell lysis can be determined by a one-step growth curve. It also helps to assess the phenotypic differences that may occur between phage strains on the same host cell,

as related phages can have markedly different lytic efficiency on the same host bacteria (Anfasa *et al.*, 2017).

4.1.4 Phage cocktails

Often, single phage treatments only reduce the bacterial growth rate, and fail to completely lyse bacterial cells due to the development of phage resistance. To overcome this, phage cocktails can be used to increase the efficacy of phages in reducing pathogen growth both *in vitro* and *in-vivo*. For example, *C. difficile* phage cocktails have proved to be highly effective both in delaying the appearance of infection symptoms in-vivo and completely lysing their host in-vitro (Nale *et al.*, 2016b).

However, it is worth noting that the efficiency of phage cocktails is dependent on the phages and the specific pathogen under study. For example, phage cocktails have shown to be highly effective against *Pseudomonas aeruginosa* and *Staphylococcus aureus*, whereas cocktails of phages that target some *Escherichia coli* and *Proteus spp.* strains have been found to be ineffective. This may in part be due to the small number of phages used in the cocktails; for this reason, more phages need to be isolated and added to the phage library (Bernasconi *et al.*, 2017, Ozkan *et al.*, 2016).

4.2 Aims of the study

In order to isolate and characterise phages that infect *C. perfringens*, the following aims were followed:

1. Isolate *C. perfringens* environmental strains.
2. Toxin type the isolated *C. perfringens* environmental strains.
3. Isolate *C. perfringens* free phages.
4. Induce the temperate phages from *C. perfringens* strains.
5. Characterise the isolated phages to determine:
    a. Morphology by visualising under Transmission Electron Microscope.
    b. Genome size by Pulsed-Field Gel Electrophoresis (PFGE)
6. Determine the therapeutic prospect of the isolated phages by:
    a. Comparing the host range against the isolated *C. perfringens* strains.
    b. Exploring the physiological properties such as the burst size and the latent period.
    a. Sequencing the genome of isolated phages that show potential as a therapeutic agent, annotating the sequence and looking for the presence of virulence genes, transduction genes and other undesired genes.

4.3 Material and methods

4.3.1 Phage isolation

4.3.1.1 Isolation of free phages from environmental samples by enrichment culture

Free phages were isolated by culture enrichment of the environmental samples of soil and faeces. To do this, ~1cm^3 of faeces or soil sample was suspended in 10ml of TS broth supplemented with 400µg/ml cycloserine (Oxoid, UK). 100µl of exponentially growing TS broth culture of *C. perfringens* (Figure 33 in section 4.4.9), 50µl of 0.8$MgCl_2$, and 0.2$CaCl_2$ were added and incubated anaerobically overnight at 37ºC.

The next day, the culture was centrifuged at 5000xg for 5 minutes at room temperature. Then, the supernatant was filtered through 0.22µm (Millipore, UK) and used in a spot test against the isolated *C. perfringens* strains. Whenever a plaque appeared, the upper soft layer of the double layer was scraped and suspended in SM buffer and incubated overnight at 4ºC. The next day, it was centrifuged at 5000xg for 5minutes, filtered using a 0.22µm filter, and finally used in a plaque assay to purify the phage.

4.3.1.2 Temperate phage induction

In order to isolate temperate phages that have virulent activity on *C. perfringens* strains, 17 *C. perfringens* strains were induced using both mitomycin C (M) (Fisher Scientific, UK) and norfloxacin (N) (Sigma Aldrich, UK), as described by (Raya *et al.*, 2008, Nale *et al.*, 2012, Mahony, 1977), with a few modifications. 500µl of exponentially growing TS broth culture of *C. perfringens* strains was added to 50ml of a pre-reduced TY broth. Mitomycin C or norofloxacin were added to a final concentration of 3µg/ml as recommended by (Mahony, 1977). The tubes were then incubated anaerobically for 8 hours at 37ºC. Thereafter, they were centrifuged at 3500xg for 10minutes and the supernatant was filtered through 0.22µm filters and stored at 4ºC. Part (20µl) of the supernatant was visualised under transmission electron microscope to look for the presence of phages. The remaining supernatant was used to test for the presence of temperate phages with lytic ability on 27 lawns of *C. perfringens* strains, using both spot tests and plaque assays. Both norfloxacin and mitomycin C controls were used; serial dilutions of 3µg/ml of each antibiotic were prepared and added to bacterial growth on plates, along with the induction lysate.

4.3.1.3 Phage plaque purification

Individual plaques were picked and suspended in 500µl of TY broth inside an eppendorf tube. This tube was then incubated for 5 hours at 4ºC before being centrifuged at 5000xg for 5minutes and filtered through a 0.22µl filter. The filtered phage was purified a further round by plaque assay. In total, five rounds of purification were carried out, after which a clonal stock of the purified phage was prepared.

4.3.1.4 Optimization of tryptose-yeast extract medium

Following the isolation of the first stable phage, CPAP1, the model system was used to further optimise the tryptose-yeast extract-peptone medium system. A new double-layer medium without sulphur was required to carry out both a spot test and plaque assay. Then the composition of Tryptose-yeast extract-peptone agar, broth and soft agar was optimized (Table, 6).

Component	Tryptose-yeast extract agar in 1 litre (grams)	Tryptose-yeast extract broth in 1 liter (grams)	Tryptose-yeast extract soft agar in 1 liter (grams)
Tryptose	15	15	15
Yeast-extract	5	5	5
Pepton	5	5	5
Sodium Chloride	5	5	5
Bacteriological Agar	20	-	4
Sodium-Thioglycolate	-	1	-

Table 6. Composition of optimized tryptose-yeast extract medium.

4.3.2 Phage Transmission Electron Microscopy (TEM)

4.3.2.1 Washing phage lysate with ammonium acetate

1.5ml of phage lysate was centrifuged at 21000xg for 1 hour at 4°C. Then, 1.4ml was gently aspirated and 1 ml of ammonium acetate (0.1M, pH 7.5) was added to the 100µl left from the lysate. This washing step was repeated three times. Following this, the sample was stained and examined by electron microscope.

4.3.2.2 TEM examination

Negative staining with 0.1% uranyl acetate was used to visualise phages by TEM. Electron microscope grids (Agar scientific, UK) were coated with 0.25% pioloform and left for 1hour to dry. They were then carbon coated and glow discharged (Quorum Technology, UK) for 30seconds in the presence of argon gas to make the carbon upper layer hydrophilic.

After preparing the grids, 4µl of the phage sample was placed on the carbon side of the coated grids and allowed to stand for 2-5minutes. Then, any un-adsorbed sample was removed via removal with filter paper. Immediately after blotting the sample away, the grids were washed with two drops of distilled water and blotted to remove the excess water. Thereafter, 10µl of the uranyl stain was added in two instalments and left to stand for a few seconds. The grid was blotted again to remove the excess stain. The grids were examined by TEM (JEOL JEM-2100, UK) under 80kv voltage, and images were taken with a Gatan 2kx2k digital camera and iTEM software. Between 30 to 40 fields were examined in each preparation (Van Regenmortel *et al.*, 2000).

4.3.3 Pulsed-Field Gel-Electrophoresis (PFGE)

4.3.3.1 Preparation of plugs

The approximate phage genome size was determined using PFGE. 100µl of 10^{11} PFU/ml phage stock was incubated overnight at 37°C with 1.4µg/ml DNaseI (New England Biolabs, U.K.) and 3µg/ml RNase H (New England Biolabs, U.K.). Agarose plugs of phage were prepared by adding 40µl of 2% w/v warm molten agarose (Lonza, U.K.) dissolved in 0.5X Tris-Borate-EDTA (TBE) at pH 8 to a 40µl of nuclease treated phage stock in an eppendorf tube. They were then mixed and transported into a plug mould. The plugs were left to set for half an hour at room temperature and then incubated for 3hours at 4°C. They were then digested by incubating with 1ml of lysis buffer (100mM EDTA, 100mM Tris-HCl, 1% SDS and 0.5mg/ml proteinase K (Fisher Scientific, UK), pH 9.0) overnight in a 55°C water bath. The next day, the plugs were washed three times in a 1XTris-EDTA buffer (TE) pH 8.0 to remove excessive lysis buffer.

4.3.3.2 PFGE procedure

Pulsed-field certified agarose gels 1% (BioRad, USA) were prepared by dissolving 2.5g of agarose in 250ml of 0.5X TBE buffer in a beaker and heating this in a microwave.

The agarose was left to cool down and poured into an assembled PFGE tank-comb and allowed to solidify. Then, the plugs were carefully taken out of the TE buffer and inserted in the PFGE gel wells. 2mm of PFEG markers (New England Biolabs, UK) were inserted into one of the gel's empty wells. All the wells were sealed with more agarose. A Bio-Rad CHEF-DR® III System PFGE (BioRad, USA) machine was filled with 2litres of 0.5X TBE buffer and the run was performed with the following conditions; 6v/cm for 16 hours at 14ºC, initial switch time 5 seconds, final switch time 13seconds, and at a 120degree angle.

The following day, the tank was carefully removed and the gel was gently placed on to the backplane into staining tray. The gel was flooded with 200ml of 0.5XTBE buffer with ethidium bromide (1µg/ml) for 30minutes on a rocker. Thereafter, the gel was visualised under ultra violet light using a Syngene box (Syngene, UK).

4.3.4 Phage host range

The host range of the isolated *C. perfringens* phages was determined by using spot tests (3.8) against 42 environmental *C. perfringens* strains (CP1 to CP42 strains). Only the neat and first dilution of each phage stock 10^{-1} was used. It was also tested against two *C. difficile* strains (ribotypes 076 and 220). Whenever a clearance was observed, further plaque assays with dilutions up to 10^{-6} were used to observe single plaques.

4.3.5 Phage one-step growth curve

A multiplicity of infection (MOI) of 10 was used for the virulent phage CPAP1 one-step growth curve, the other sequenced phage is temperate. The following equation was used to calculate the MOI:

$$\text{MOI (for equal volumes)} = \frac{\text{Number of phages}}{\text{Number of bacteria}}$$

One-step growth experiments were conducted as described by Ellis and Delbruck (1939) with some modifications. In brief, 1ml of 10^{10} PFU/ml phage lysate was added to 9ml of exponentially growing *C. perfringens* (10^{8} CFU/ml) (see 4.4.10) in a 15ml falcon tube. 1ml of SM buffer was added to the control tube. The test and control tubes were incubated anaerobically at 37ºC for 15minutes. The tubes were then centrifuged at 3500xg for 5minutes and the pellet was then re-suspended in a pre-reduced TY broth and 10µl was immediately taken (three replicates) for a plaque assay. Thereafter, plaque assay was carried out at time points of 20, 30, 40, 50, 60, 70, 80, 90, 100, and 110 minutes. The

average of plaques numbers at each time point was used to draw the one-step growth curve.

The burst size was determined as described by (Hyman *et al.*, 2009). In brief, the highest PFU/ml obtained at the end of the growth curve (post-rise) was divided by the pre-rise PFU/ml count (the baseline during the latent period).

The burst size per infected cell was also calculated using the increase in phage numbers at each time point (relative concentration). The actual PFU/ml count at each time point was divided on the baseline PFU/ml to obtain the relative concentrations. Then, the relative concentration of each time point was plotted against that point. The burst size per infected cell was the value that meets the peak of the curve on Y axis. The protocol is available online at:

http://www.sas.upenn.edu/LabManuals/biol275/Table_of_Contents_files/13-PhageGrowth.pdf .

4.3.6 Phage genome sequencing and annotation

The genomes of three phages, CPAP1 (DNA concentration 4.3ng/µl), CPAS1 (15.7ng/µl) and CPAS2 (6.2ng/µl) were sequenced using Miseq Illumina. The reads were trimmed using the sickle software and assembled using the SPAdes software. The prediction of open reading frames (ORFs) was done using PROKKA. All this work was kindly carried out by Dr Andrew Millard of the University of Leicester. The Rapid Annotation using the Subsystem Technology server (RAST) available online at http://rast.nmpdr.org/rast.cgi was also used to confirm the PROKKA prediction of the ORFs. The function of predicted ORFs was assigned using the basic local alignment search tool (BLAST) available online at https://blast.ncbi.nlm.nih.gov/Blast.cgi. Moreover, the Blast Ring Image Generator (BRIG) was used to draw the circular map of each genome; these figures were kindly produced by Dr. Nathan Brown of the University of Leicester. Finally, pairwise sequence alignment (protein) was used.

The genome of CPAP1 was originally found to be in two contigs, one of which is very short (418bp). The blast of this short sequence gave 99% similarity to the *E. coli* gene. However, foreign genes can be incorporated into a phage's genome and remain integrated. Four primers were used to fill the gap and close the contigs (Appendix, 3). These primers were designed to have a very similar annealing temperature, then, one PCR

run was conducted using the same cycler conditions. The PCR reaction mix's volume was 50µl, containing: DNA template, 0.5µM of each primer, 200µM of DNTPs, 1X high fidelity buffer, and 1 unit/50µl of Phusion high fidelity polymerase (New England Biolabs, UK). Cycler conditions were: initial denaturation at 98°C for 30 secs, 35 cycles of 98°C for 10secs; 59°C for 30secs; 72°C for 90secs, with a final extension of 10min at 72°C.

No PCR product was obtained, as the short sequence was indeed shown to contain a contaminating DNA piece that did not belong to the phage CPAP1 genome. In order to make sure that the second small contig (*E. coli* sequence) did not belong to CPAP1, a pair of primers (Appendix, 3) was designed to amplify it using the phage nucleic acid as template. The PCR reaction mix's volume was 50µl, and contained: phage DNA as a template, 4µM of each primer, 250µM dNTPs, 3mM MgCl2, 1X PCR buffer and 0.5 unit/50µl of BioTaq polymerase (Bioline, UK). Cycler conditions were: initial denaturation at 95°C for 5mins, 30 cycles of 95°C for 30seconds, 55°C for 30seconds, 72°C for 60seconds, with a final extension of 10mins at 72°C. The PCR gave no product, confirming that the second contig did not belong to the phage genome.

The genome of CPAS1 was found in one of the contigs. The sequence result of CPAS2 genome revealed that there was a mixture of two phages in the sample. Although six rounds of plaque purification were carried out for CPAS2, it was still mixed with another phage. Further plaque purification work is required for CPAS2 in order to separate the two phages, after which the nucleic acid can be extracted and the two genome sequenced.

4.4 Results

4.4.1 Isolation of environmental *C. perfringens* strains

C. perfringens environmental strains were isolated by enrichment culture as described in section 3.2.3.2. 69 *C. perfringens* strains were isolated out of the 81 environmental samples. The *C. perfringens* isolation rate from all collected samples was high (85.2%). All *C. perfringens* colonies on TSC agar had a characteristic distinct black colour, as a result of sulphur reduction (Figure, 14). The colony morphology is circular with entire or undulated margins and elevated (convex) centre. However, the black colour was not enough to confirm that the isolated strains were *C. perfringens* because in addition to this species, *Proteus Spp*., *Pseudomonas Spp*., *Enterococcus Spp.*, and *Bacillus Spp.* can all form black colonies on TSC agar. Thus, it was essential to confirm results by the amplification of *C. perfringens* specific16s rDNA region and the analysis of the PCR product (~270bp) (Figure, 15). The black colonies which were repeatedly negative for the *C. perfringens* specific 16s rDNA were analysed by the amplification of the bacterial 16s rDNA region and analysis of the PCR product (~1800bp) (Figure, 16).

C. perfringens was not found in faecal samples taken from adult pigs, rabbits, and fish (seven pig faecal samples, four rabbit faecal samples, and one fish faecal sample were tested). *C. perfringens* strains were isolated from soil samples along with faecal samples from chickens, sheep, cows, horses, dogs, ducks, lambs, ostriches, deer, swans, turkeys and parrots.

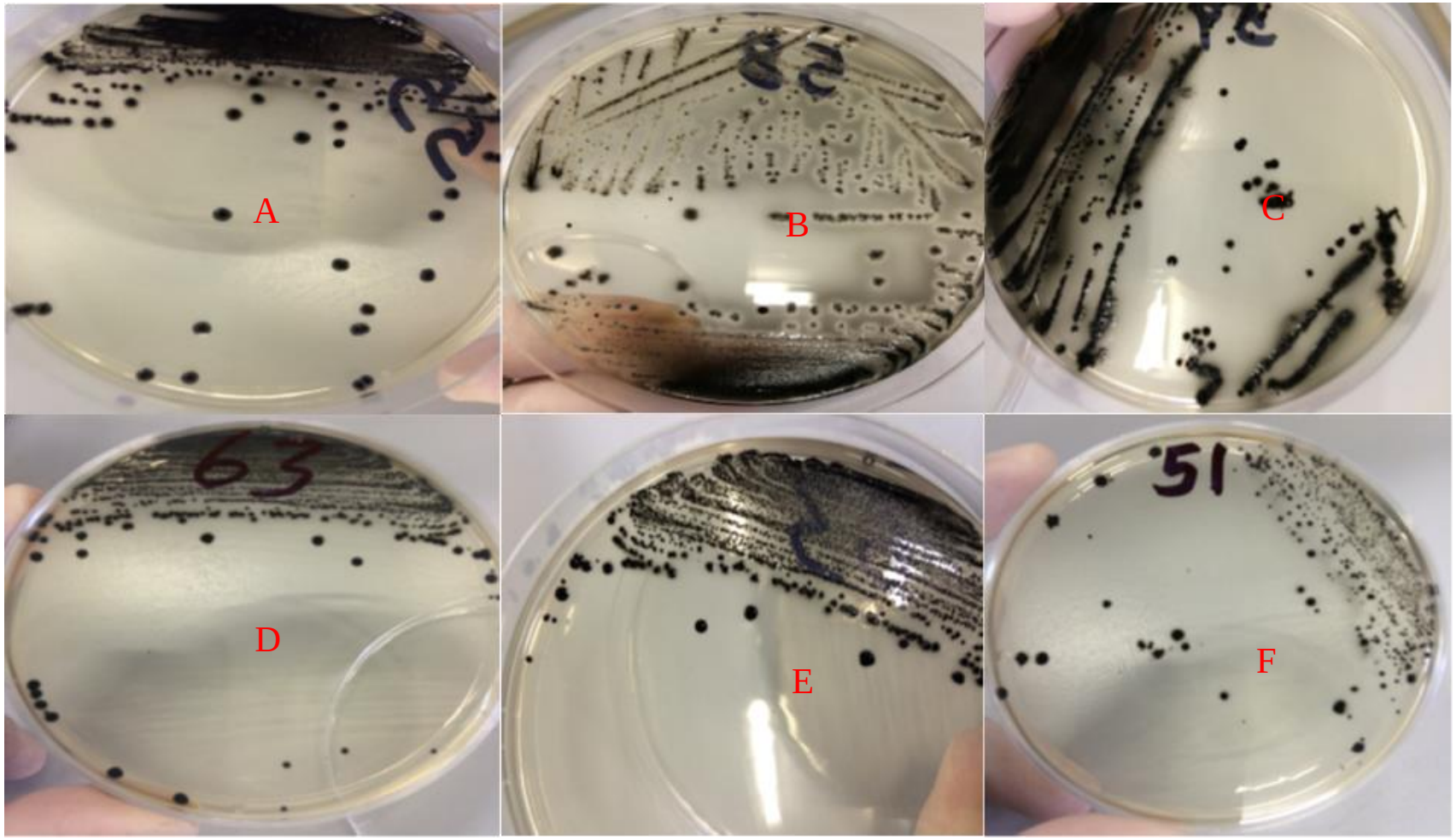


Figure 14. *C. perfringens* colonies on TSC agar. The colonies are circular with entire or undulated margins and have an elevated (convex) centre. The plates A, B, C, D, E, and F belong to *C. perfringens* strains number 55, 58, 59, 63, 3, and 51 respectively.

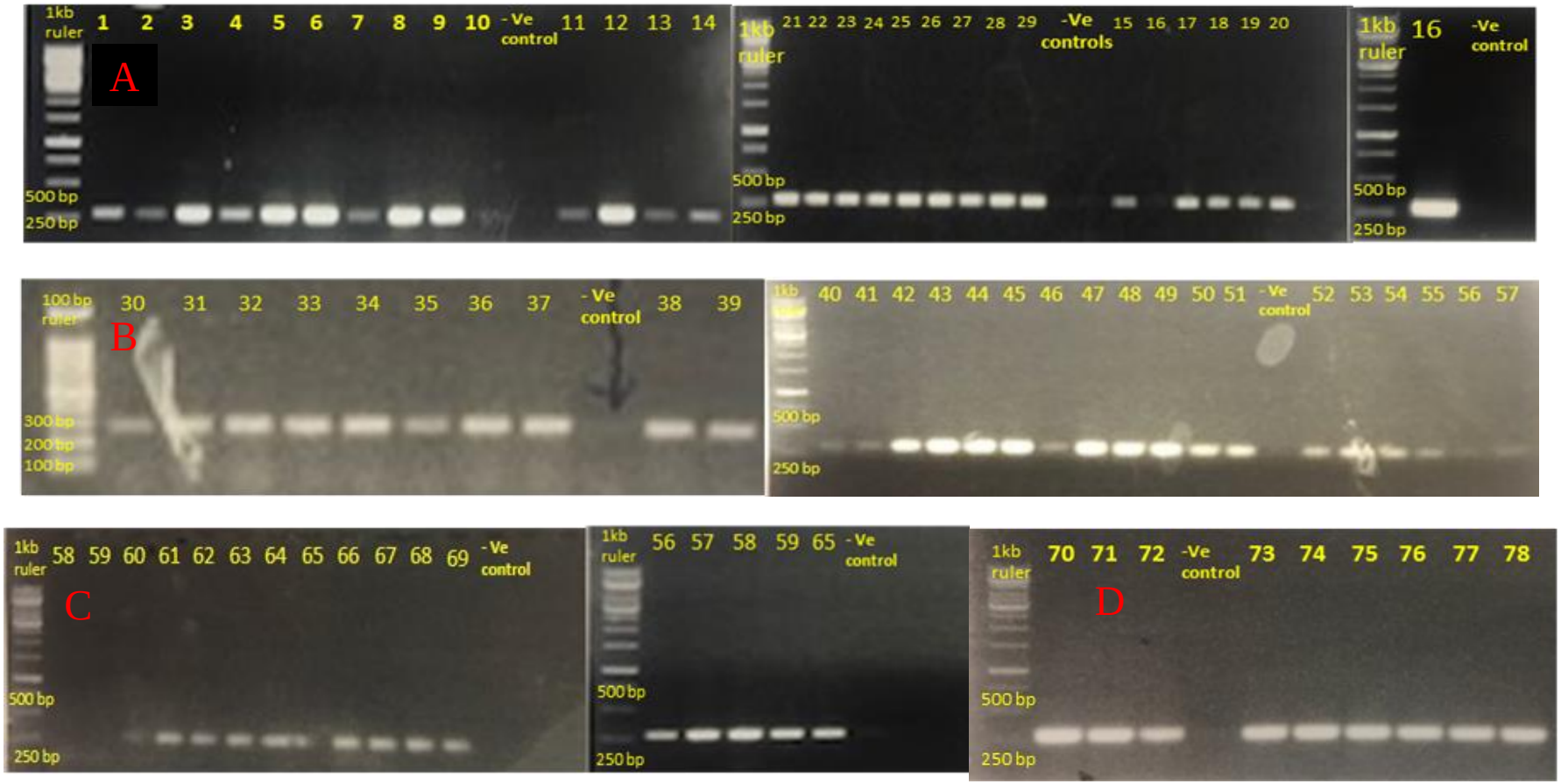


Figure 15. Gel electrophoresis images showing *C. perfringens* specific 16s rDNA amplification. A PCR product of 270bp can be seen with each successful amplification. The DNA ladders used are either the Bioline GeneRuler 1 kb (image A, right part of image B, images C and D) or the New England BioLab 100bp DNA ladder (left part of image B). Samples correspond to the following strains: (A) *C. perfringens* strains 1-29. (B) *C. perfringens* strains 30-57. (C) *C. perfringens* strains 56-69. (D) *C. perfringens* strains 70-78.

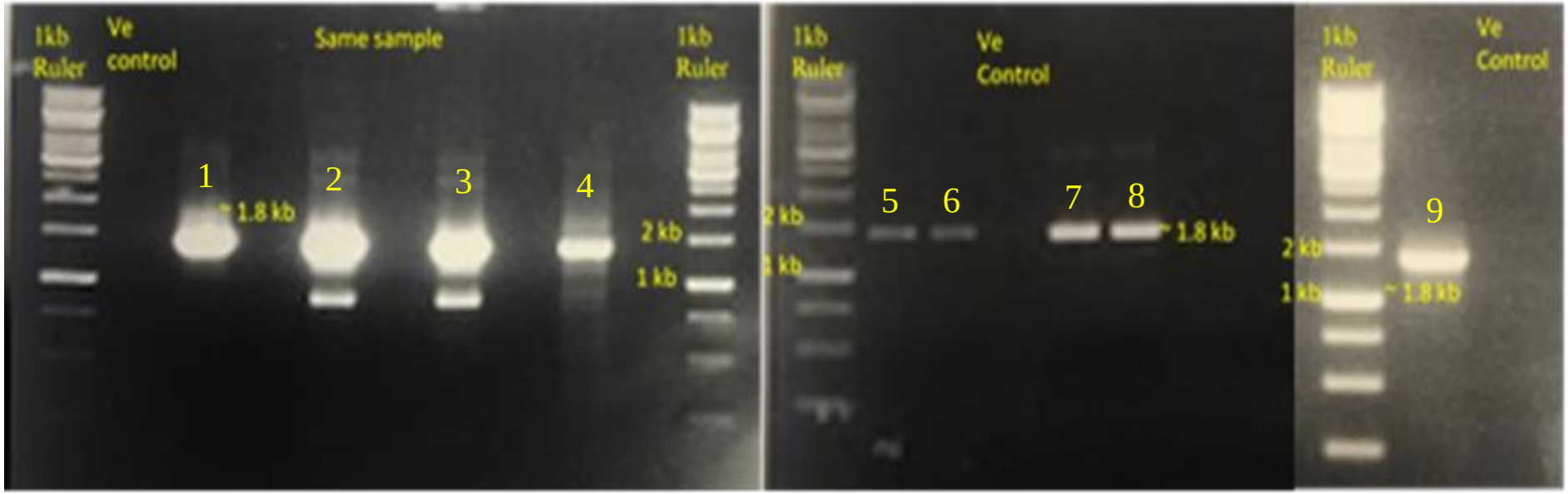


Figure 16. Amplification of bacterial 16s rDNA region. This amplification was of unknown black colonies that were repeatedly negative for *C. perfringens* specific16s rDNA amplification. The DNA ladder used was a Bioline GeneRuler 1 kb. The PCR products of ~1800 bp were sequenced and the blast of sequences revealed that these colonies belong to: *Enterococcus* (1), *Pseudomonas* (2, 3, 4 and 9), *Bacillus* (5 and 6), and *Proteus* (7 and 8) species.

4.4.2 *C. perfringens* toxin typing

78 *C. perfringens* strains (55 strains were isolated from samples collected from Leicestershire (Figure, 17), and 23 from samples provided by colleagues) were tested for the presence of the main toxin genes with multiplex PCR developed by van Asten *et al.* (2009) (Figure, 18). Additional individual PCRs were carried out to detect the presence of β, ε, and ι toxins, as all the isolates were negative for these toxin genes by multiplex PCR (Figure, 19).

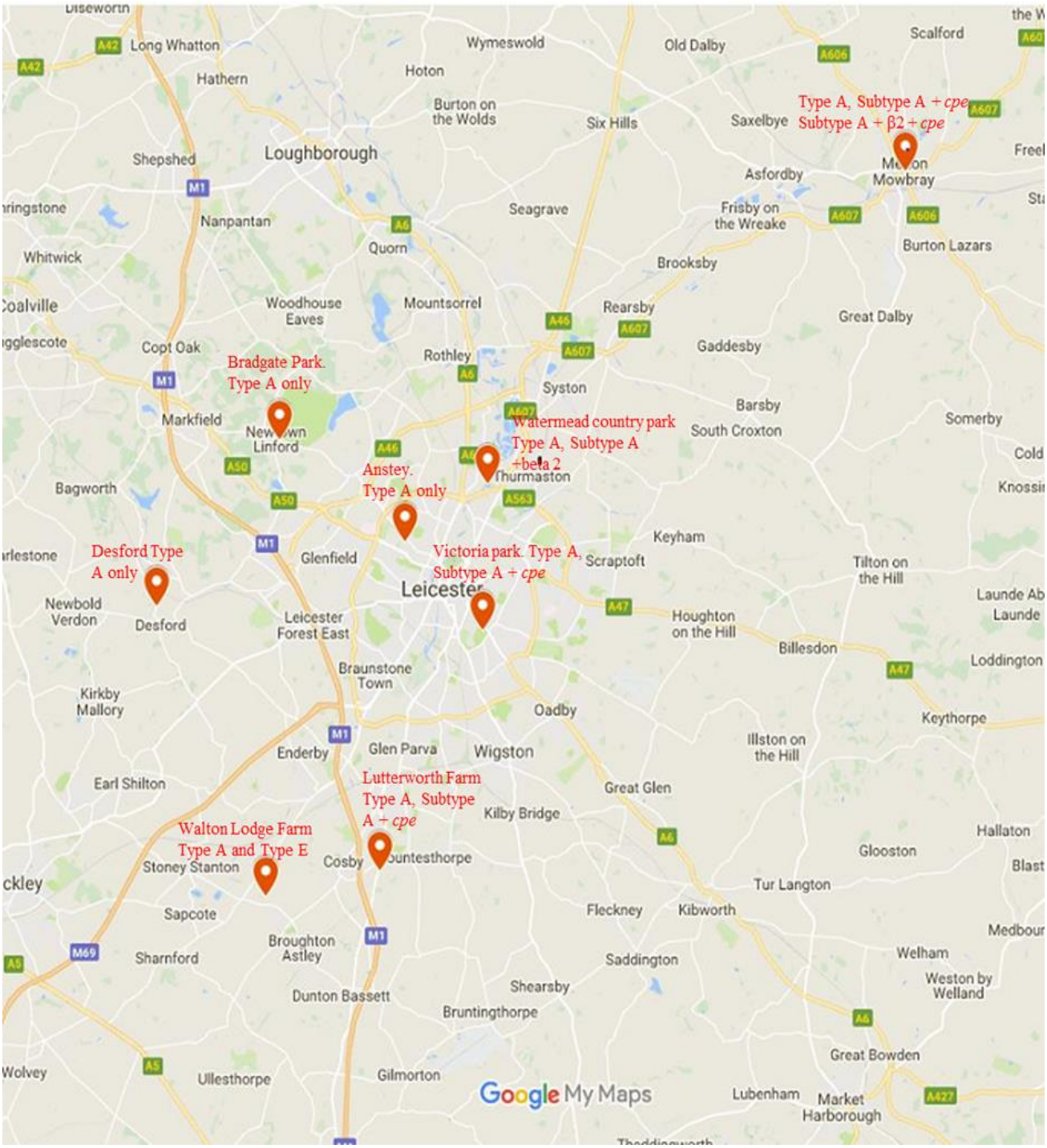


Figure 17. Sampling locations and the toxin genotypes of *C. perfringens* strains isolated in these locations. Faecal and soil samples were collected from a limited number of locations. Type A *C. perfringens* is dominant in and around Leicester. Only one strain of type E *C. perfringens* was found; it was isolated in south Leicester.

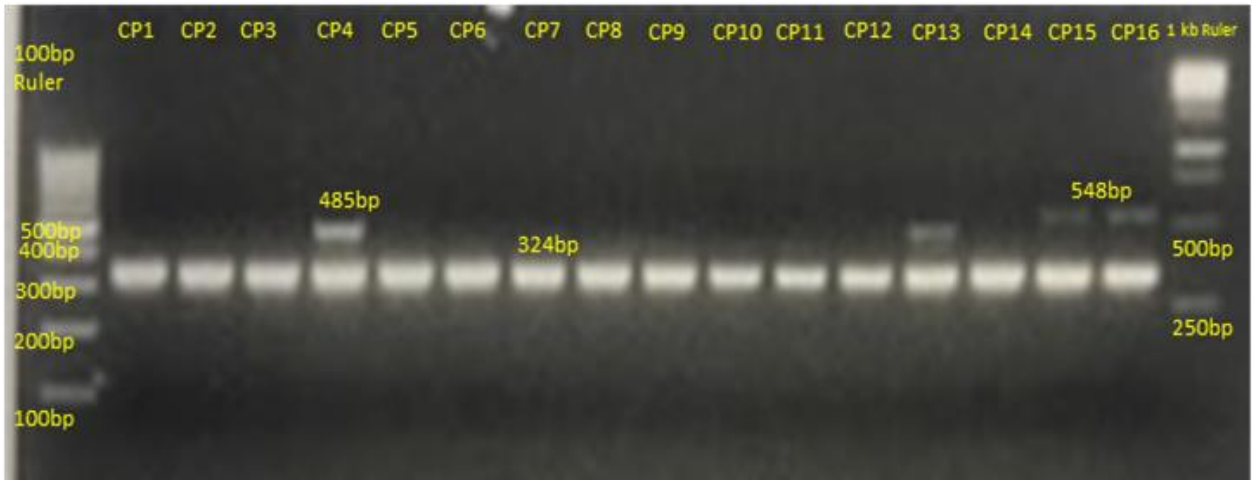


Figure 18. A representative electrophoresis image of multiplex PCR products of different *C. perfringens* toxin types. The New England BioLab 100bp DNA gene ruler and the 1kb Bioline GeneRuler can be seen in the left- and right-most lanes respectively. Each lane displays the results of the amplification of 3 genes, these being the Alpha toxin gene (α) that is 324bp, the enterotoxin (cpe) gene 485bp, and the beta 2 (β2) toxin gene 584bp. Type A *C. perfringens* has the α toxin gene only (e.g. CP1). *C. perfringens* type A variants possess cpe and β2 toxins in addition to the main α toxin. For example, CP4 and CP13 have the cpe toxin, while CP15 and CP16 have the β2 toxin. CP35 (not shown here) has both cpe and β2 toxins in addition to the α toxin. No toxin β, iota (ι), and epsilon (ε) genes were detected by the multiplex PCR.

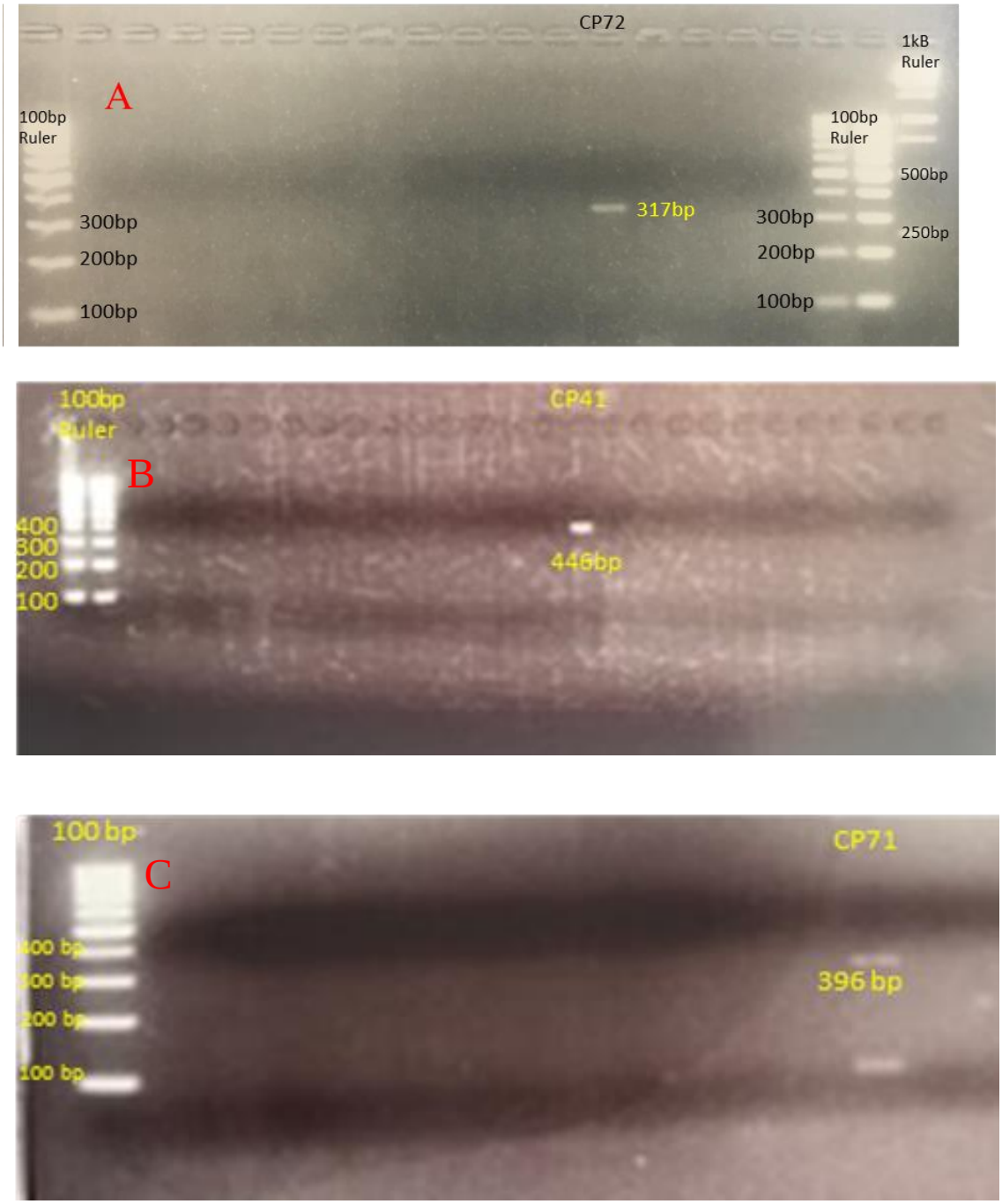


Figure 19. Electrophoresis of individual PCR products to detect *C. perfringens* β, ι, and ε toxins. The New England BioLab 100bp DNA gene ruler was used to determine the size of PCR products amplified from iota and epsilon toxins. Additionally, the 1kb Bioline GeneRuler was used with the beta toxin. A: 317bp sized PCR product amplified from the beta toxin gene which was only detected in *C. perfringens* strain 72. B: 446bp product amplified from the iota toxin gene; only *C. perfringens* strain 41 had this gene. C: 396bp product amplified from epsilon toxin gene, found only in *C. perfringens* strain 71.

The results revealed that type A is dominant among our collection of *C. perfringens* environmental isolates, with 96.1% percentage (64 out of 78), most of which only include the major *C. perfringens* toxin, the alpha toxin. Only one strain was assigned for each of the C (strain 72), D (strain 71), and E (strain 41) types. No type B strains were isolated (Figure, 20). The toxin type of each strain is shown in Appendix 5.

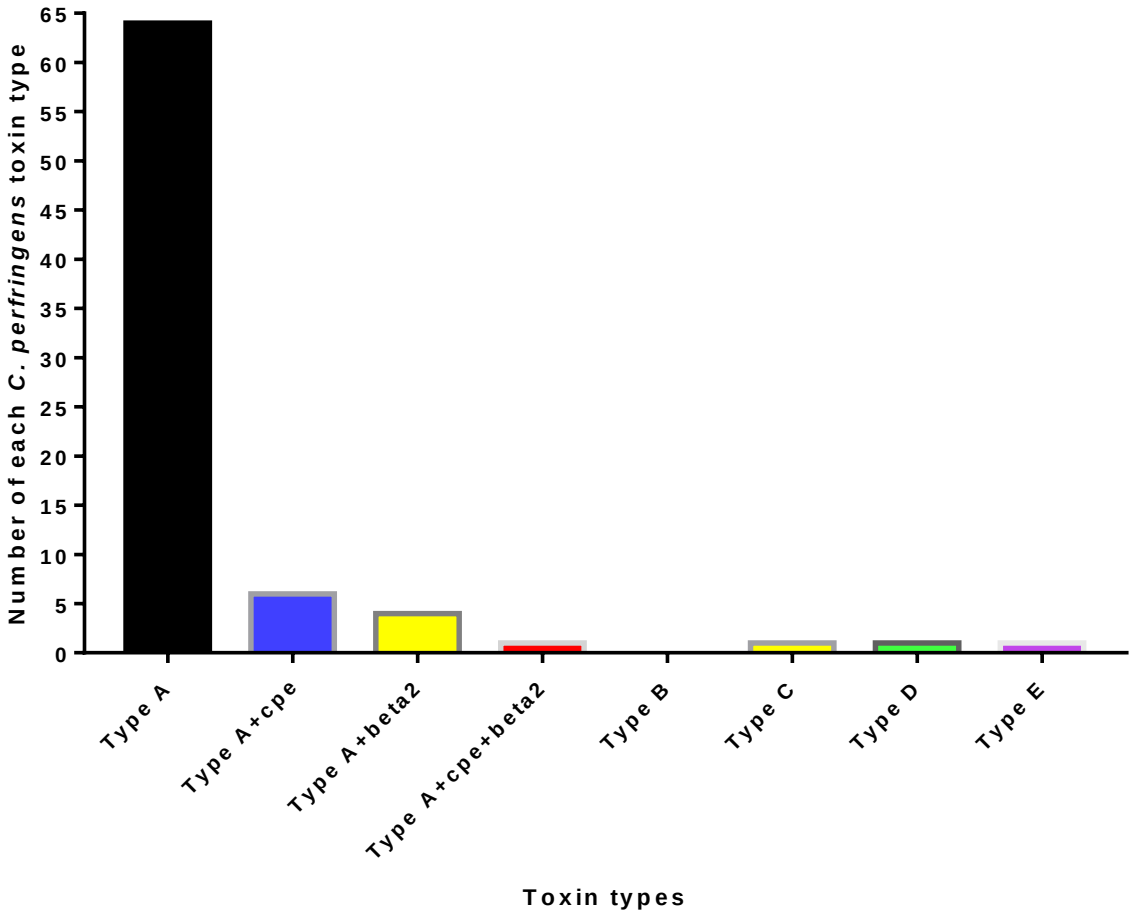


Figure 20. The number of each different toxin genotype of 78 environmental *C. perfringens* strains (69 were isolated in this study). Type A was dominant among the isolated environmental *C. perfringens* strains. Subtype A that has the enterotoxin gene (cpe), the beta2 toxin gene, or both enterotoxin and beta2 toxin genes were also isolated. No type B *C. perfringens* strain was isolated. Only one strain was assigned for each of the types C, D, and E.

The distribution of toxin types within *C. perfringens* isolates shown in Figure 17 can help to predict the diseases that can be caused by these types in each area based on our isolated collection. The north-western quarter of Leicestershire only has type A, which can solely produce the α toxin; in other words only gas gangrene, septicaemia, and chicken necrotic enteritis can arise from an infection from *C. perfringens* in this area. The other areas have a combination of A-subtypes, C, D, and E. Consequently, many types of diseases can arise as a result of a *C. perfringens* infection (Table, 7). These diseases were reviewed in section 2.1.2.

***C. perfringens* toxin genotype**	**Toxin produced**	**%**	**No.**	**Source**	**Potential diseases**	
					Human	Animals
Type A (comprises 96.1% of all the isolated strains)	α	82	64	Chicken, cow, deer, dog, duck, horse, lamb, sediment, sheep, soil, swan, turkey, and unknown	Gas gangrene Haemolysis	Gas gangrene and haemolysis
	α + *cpe*	7.7	6	Dog, duck, and soil	Food poisoning (Enteritis)	Enteritis, Haemorrhagic Diarrhoea
	α + β2	5.1	4	Dog, duck, swan, unknown	Enteritis	Necrotizing enteritis
	α + β2 + cpe	1.3	1	Sheep	Enteritis	Enteritis
Type B	α + β + ε	0	0	----------	Enteritis, Enterotoxemia Multiple sclerosis	Enteritis Neurological disorder
Type C	α + β	1.3	1	soil	Human enteritis Necroticans	Necrotizing enteritis
Type D	α + ε	1.3	1	soil	Not reported	Enterotoxemia
Type E	α + ι	1.3	1	soil	Not reported	Enteritis

Table 7. The percentage, number, source, toxin produced, and potential of each of the isolated *C. perfringens* toxin type to cause diseases

4.4.3 Isolation of temperate *C. perfringens* phages (induction of temperate phages)

Both mitomycin C (M) and norofloxacin (N) were able to induce prophages from *C. perfringens* at a concentration of 3µg/ml. Zones of lysis were observed in several cases, but no individual plaques were obtained after scraping the lysis zone, re-suspending it and carrying out a second round of both spot tests and plaque assay. There is a high level of prophage carriage in the *C. perfringens* strains examined, as all the induced strains gave lysis when tested on different *C. perfringens* strains from our collection (Table, 8).

	Indicator strain																	
Phage induced from	CP1		CP2		CP3		CP4		CP5		CP6		CP7		CP8		CP9	
	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N
CP1	x	x	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	Y	Y
CP2	-ve	-ve	X	x	lysis	lysis	-ve	-ve	-ve	-ve	lysis	lysis	lysis	lysis	lysis	lysis	-ve	-ve
CP3	-ve	-ve	-ve	-ve	x	x	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP4	-ve	-ve	-ve	-ve	-ve	-ve	x	x	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP5	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	x	x	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP6	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	x	x	-ve	-ve	-ve	-ve	-ve	-ve
CP7	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	x	x	-ve	-ve	-ve	-ve
CP8	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	x	x	-ve	-ve
CP9	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	x	x
CP10	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP11	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis
CP12	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP13	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP14	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP15	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis
CP16	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve
CP17	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
	Indicator strain																	
Phage induced from	CP10		CP11		CP12		CP13		CP14		CP15		CP16		CP17		CP18	
	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N
CP1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis
CP3	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis
CP4	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP5	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis
CP6	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP7	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP8	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP9	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP10	x	x	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP11	lysis	lysis	X	x	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP12	-ve	-ve	-ve	-ve	x	x	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis

CP13	-ve	-ve	-ve	-ve	-ve	-ve	x	x	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP14	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	x	x	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP15	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	x	x	-ve	-ve	-ve	-ve	-ve	-ve
CP16	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	x	x	-ve	-ve	-ve	-ve
CP17	-ve	-ve	-ve	-ve	lysis	lysis	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	x	x	-ve	-ve
Indicator strain																		
Phage induced from	CP19		CP20		CP21		CP22		CP23		CP24		CP25		CP26		CP27	
	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N
CP1	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP3	-ve	-ve	Lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP4	lysis	lysis	Lysis	lysis	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP5	-ve	-ve	Lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis
CP6	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve
CP7	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve
CP8	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	lysis	lysis	lysis	lysis	-ve	-ve	-ve	-ve
CP9	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP10	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve
CP11	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis
CP12	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve
CP13	-ve	-ve	Lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP14	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP15	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP16	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP17	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve

Table 8. Lysis caused by the induction lysate of 17 environmental *C. perfringens* strains on 27 environmental *C. perfringens* strains, all of which are alpha and alpha variant toxin types. Each induction lysate was able to produce lysis on at least one indicator strain.

Interestingly, the induction lysate of *C. perfringens* number 11 (CP11) has produced lysis on 7 strains of our *C. perfringens* collection out of 27 (Figure, 21). No lysis was produced on CP11 by the other 16 induction lysates. This may be due to encoding a large number of prophages that can cause lysis to many *C. perfringens* strains as well as protect the 11 lysogen from co-infection with other phages. Although there were another 8 strains which did not show lysis, the induction lysates of these strains caused lysis for only a few lawns of *C. perfringens* (2-5 strains) (Figure, 22).

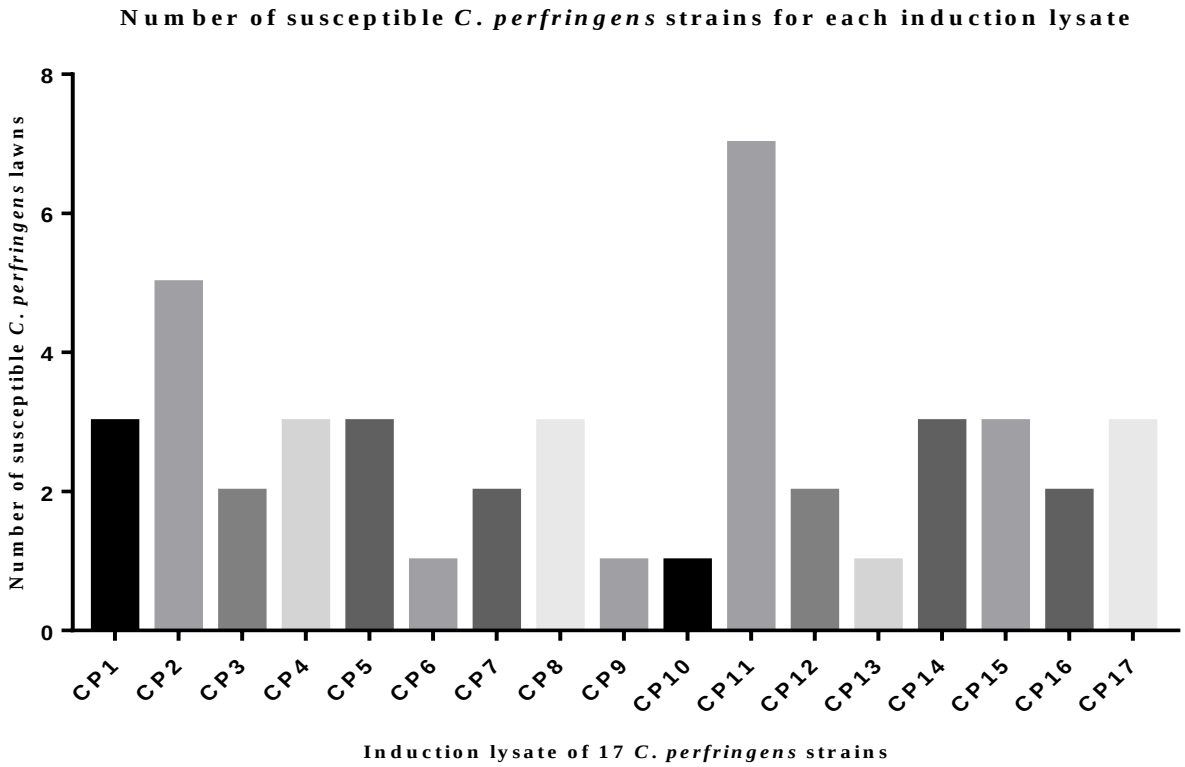


Figure 21. Number of susceptible *C. perfringens* strains to each induction lysate. As identified by the presence of lysis. The induction lysate of *C. perfringens* strain 11 was able to produce lysis on seven indicator strains.

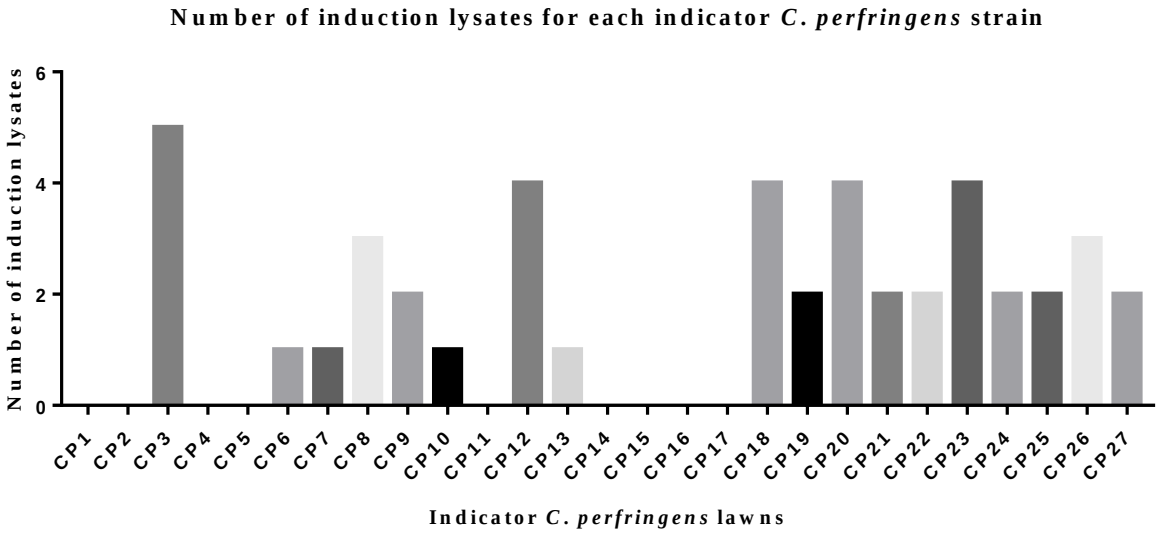


Figure 22. Number of induced lysates that each *C. perfringens* strain is sensitive to. Five induction lysates produced lysis on *C. perfringens* strain 3.

4.4.4 Isolation of free *C. perfringens* phage by enrichment culture

Five phages were isolated from faecal samples by enrichment culture; they were named CPAP1, CPAP2, CPAS1, CPAS2, and CPAS3 (Figure, 23). The podovirus CPAP1 produced medium clear plaques on a TY double layer, while the other podovirus, CPAP2, produced medium sized hazy plaques. The isolated siphoviruses CPA1, CPAS2, and CPAS3 produced medium hazy plaques (Figure, 24). The phages were purified and clonal stocks were prepared for them and stored at -80ºC, as described in section 3.7.

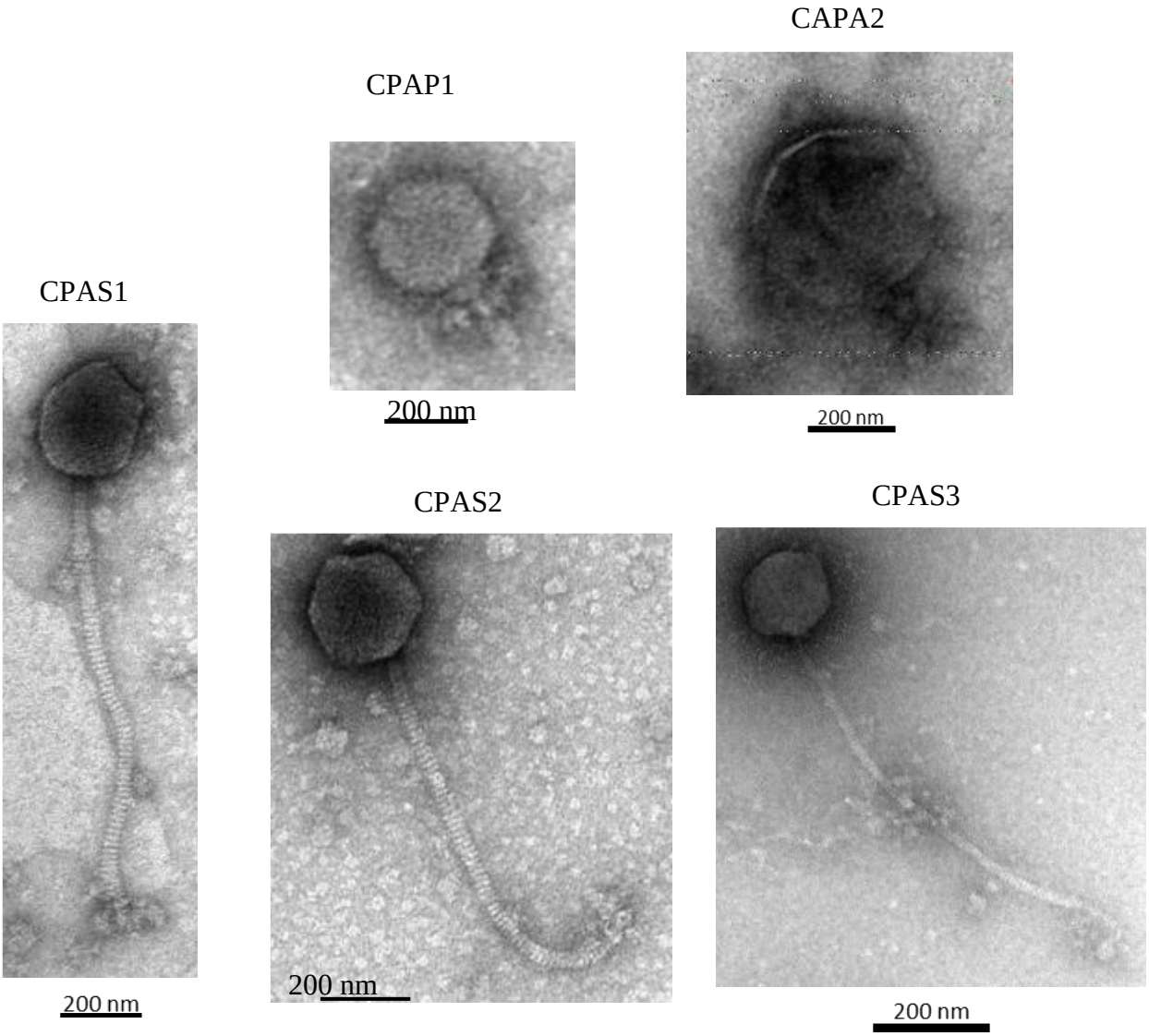


Figure 23. Morphology of five phages isolated from environmental faecal samples as shown by transmission electron microscopy. The scale bar represents 200nm. All the isolated phages belong to the *Caudovirales* order. The upper two images show the isolated *Podoviridae* CPAP1 and CPAP2. The other three images show the isolated *Siphoviridae* CPAS1, CPAS2, and CPAS3.

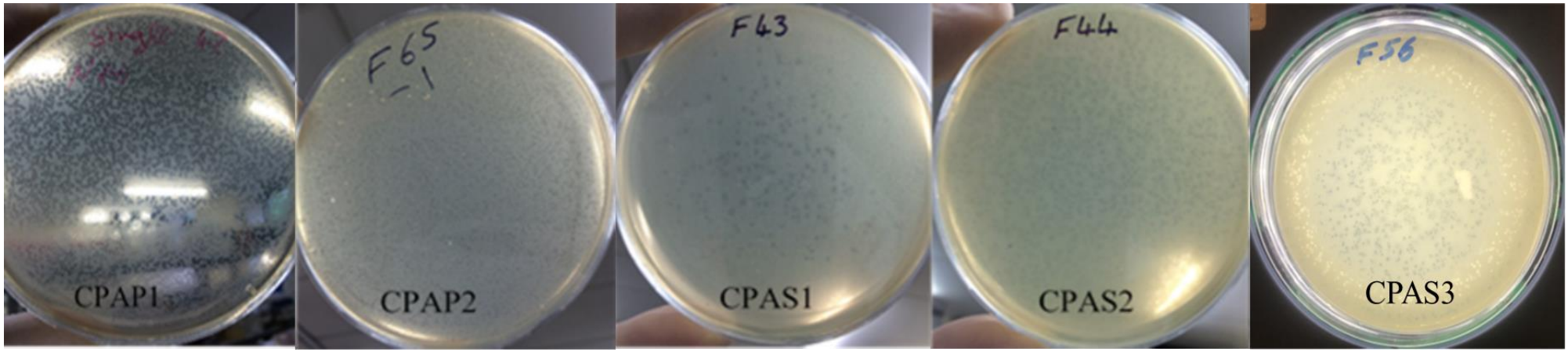
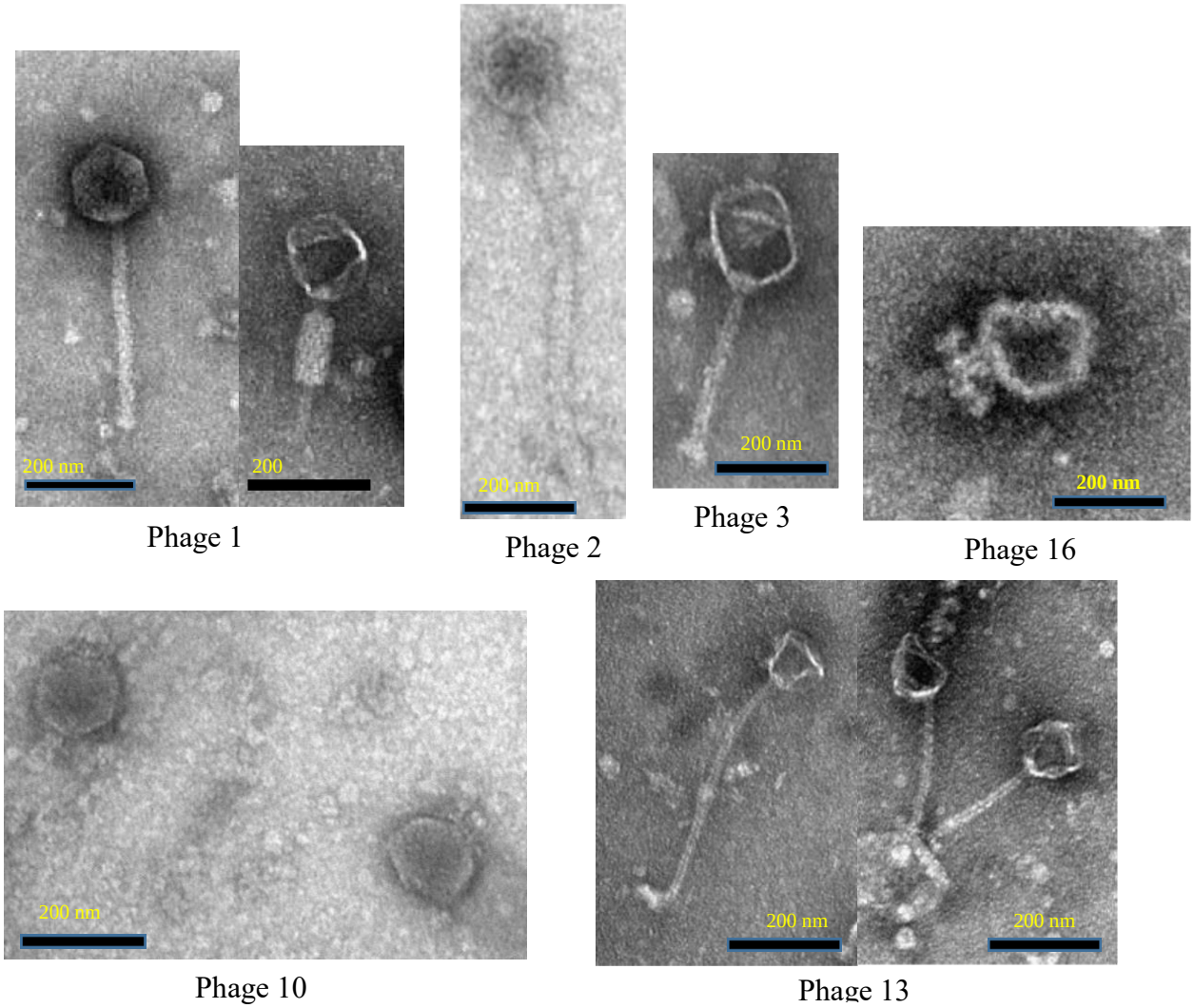


Figure 24. Plaque morphology of the isolated stable phages on a TY double layer medium. CPAP1 produces medium clear plaques. CPAP2, CPA1, CPAS2, and CPAS3 produce medium sized hazy plaques.

The double layer media can alter the plaques' morphology. The CPAP1 phage was able to produce two plaque morphologies on TS double layers, medium plaques and medium with a surrounding faint circle. The second plaques' morphology was dominant on TS media. Further purification rounds were carried out separately for each plaque, indicating that the sample has two different phages or a satellite phage. However, both morphologies were observed during the purification of each plaque. The TEM images revealed the presence of podovirus in both plaques, and the DNA sequencing was decisive as it confirmed that the DNA preparations of both plaques belong to the same phage. The CPAP1 plaque morphology on TY double layer was uniform, and were clear, medium-sized plaques (Figure, 25).

Figure 25. CPAP1 plaques morphology on both TS and TY double layer. The two plates on the left show plaques with a double lysis zone on TS agar. The other two plates on the right show plaques with one lysis zone on TY agar.

Another 6 *C. perfringens* phages were isolated (Figure, 26). Unfortunately, the purification rounds for each could not be completed. Interestingly, phage number 16 was able to enter lysogenic state (Figure, 27) and stay inside CP16.



Phage 1 Phage 2 Phage 3 Phage 16

Phage 10 Phage 13

Figure 26. Morphology of six phages isolated from environmental faecal and soil samples, as shown by transmission electron microscopy. The number under each image refers to the isolated *C. perfringens* strain. TEM analysis revealed that all the isolated unstable phages are also tailed phages belonging to the *Caudovirales* order. Two phages could be isolated on CP1. Phage 10 and phage 16 belong to the *Podoviridae* family, while the other three phages (phages 2, 3, and 13 belong to the *Siphoviridae* family. On strain CP1, one short myovirus belonging to the *Myoviridae* family and one siphovirus were isolated.

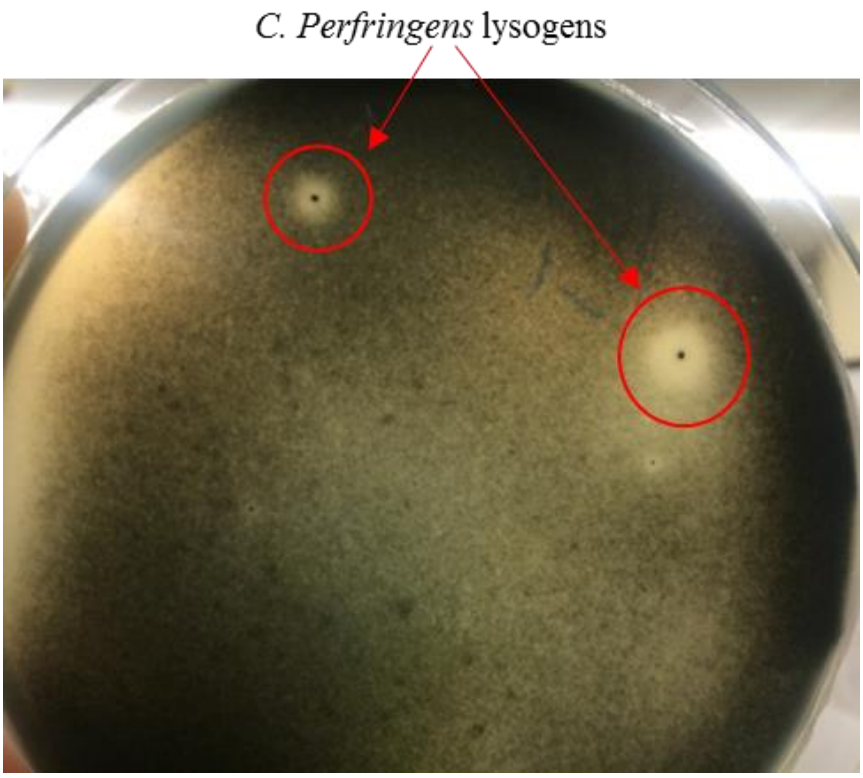


Figure 27. The *C. perfringens* strain 16 lysogen. Phage 16 was able to switch to lysogenic growth and integrate itself into CP16.

The other five phages could not be passaged for more than three passages (two plaque purification rounds and an initial isolation round). These phages were able to grow and produce plaques to a high titre on a *C. perfringens* lawn (Figure, 28). Thereafter, the phage progeny from the third passage lost their infectivity and could not be passed any more.

The lysate of the original isolation and of the second passage (first plaque purification round) were tested alongside with the progeny of the third passage. The first and second passages lysates were still able to grow and produce plagues on the *C. perfringens* indicator strain, whereas the third passage lysate were not. This may be due to the development of phage resistance by *C. perfringens*, or to mutations which may have occurred in these phages.

There was no relationship between the type and the location of environmental samples, and the type of isolated phages. Both podoviruses and siphoviruses were present in soil and faecal samples taken from different locations.

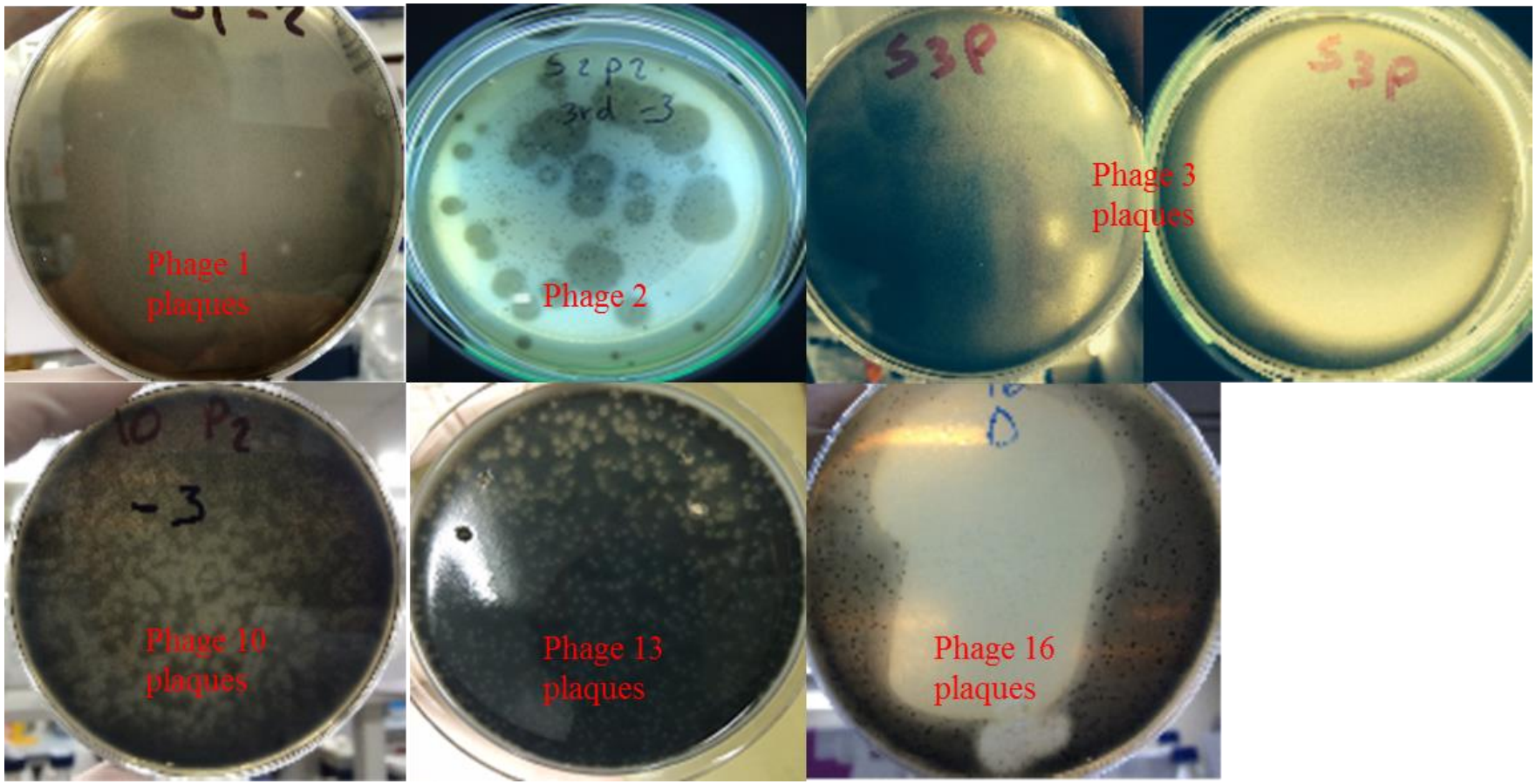


Figure 28. Plaque morphology of the isolated unstable free phages using a TS double layer. The upper four plates show the plaque morphology of the unstable phages isolated on *C. perfringens* strains 1, 2, and 3. The three other plates show the plaque morphology of the unstable phages isolated on *C. perfringens* strains 10 and 16. The phages were numbered same as their indicator strains.

4.4.5 Morphology of the isolated phages (TEM analysis)

TEM analysis revealed that all the isolated stable phages are tailed phages belonging to the *Caudovirales* order (Figure, 23). Two of them belong to the *Podoviridae* family (CPAP1 and CPAP2), and the other three phages (CPAS1, CPAS2, and CPAS3) belong to the *Siphoviridae* family. The morphological difference between the two families is in the length of the non-contractile tail (Fokine *et al.*, 2014).

The two Podoviruses varied in size and tail length. CPAP1 has a head diameter of ~51 nm and ~35 nm tail length, while CPAP2 has a head diameter of ~129 nm and ~58 nm tail length. The three siphophages also varied in head size and tail length; they differ slightly in the head diameter (between 85-90 nm). The tail length varied largely with a range of 306-445 nm (Table, 9).

Phage	Head diameter nm	Tail length nm
CPAP1	~51 nm	~35 nm
CPAP2	~129 nm	~58 nm
CPAS1	~90 nm	~306 nm
CPAS2	~85 nm	~339 nm
CPAS3	~88 nm	~445 nm

Table 9. Key dimensions of the five *C. perfringens* phages isolated in this study. The values in the table represent the mean of 5 measurements.

For sequencing, the type of phage helped in anticipating whether there was any contamination with DNA from another phage type, and also helped in blasting the new sequence against the same family database.

4.4.6 Optimization of TY medium to be used in spot tests and in the isolation of new phages

The use of a TY double layer has overcome the difficulties of distinguishing plaques incurred for the black colour produced by *C. perfringens* on a TS double layer demonstrated in Figure 29.

The use of the TY double layer has allowed for a confluent *C. perfringens* growth and very clear plaques when there was a phage in the tested enrichment samples. The titration of phages by spot test has also become available, as it was previously performed by plaque assay (Figures 29 and 30).

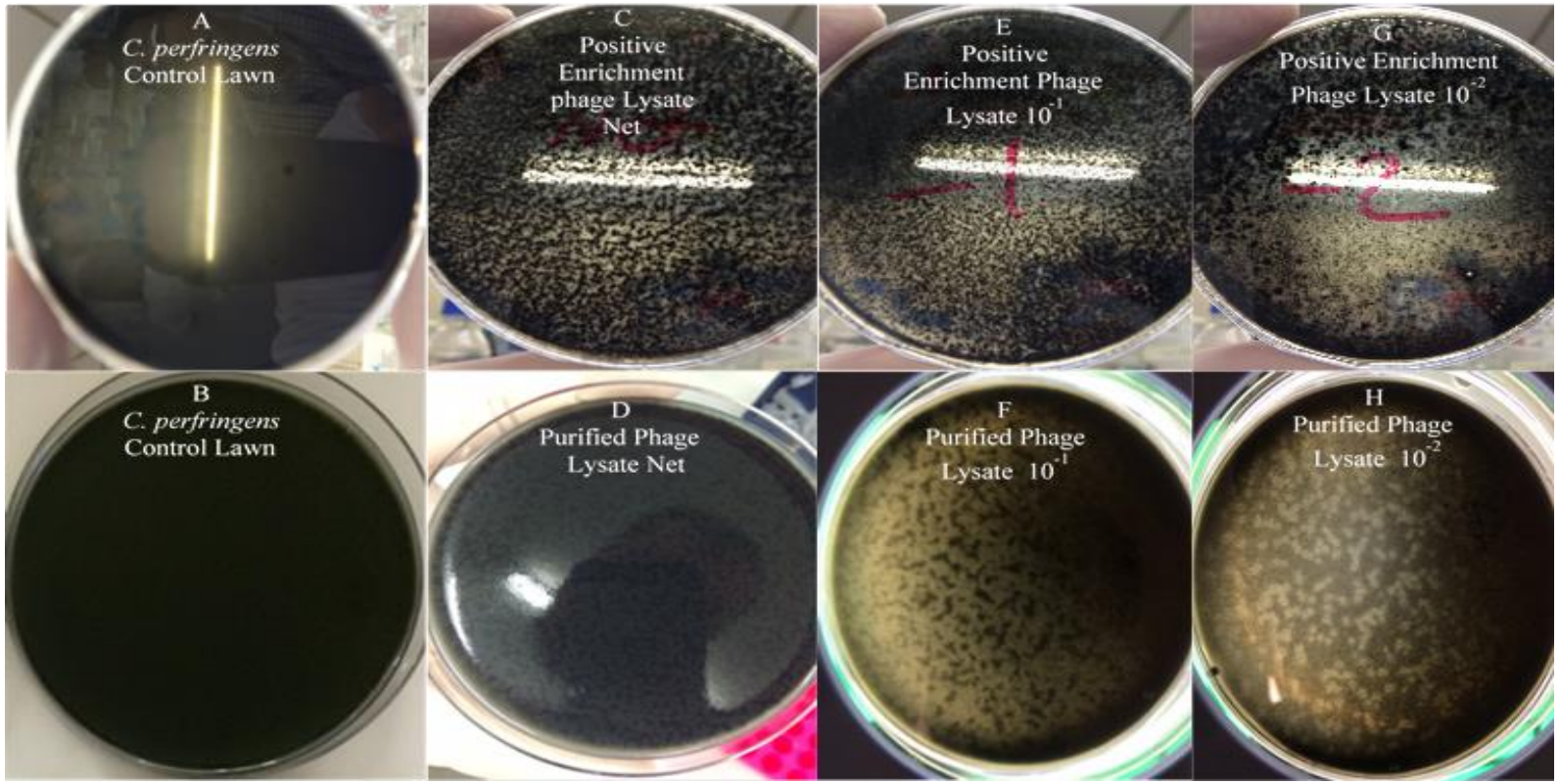


Figure 29. Morphological differences between plaques arise from the initial enrichment lysate and after five rounds of purification of the same phage on the TS double layer. The plaques produced by the initial enrichment lysate of CPAP1 are visualized on the upper four plates. On the left is the control plate which has confluent *C. perfringens* growth. The other three plates represent the net, one fold, and two fold dilutions of the initial lysate. The enigmatic appearance of the latter plates results from the presence of different bacterial metabolic products in the initial enrichment lysate. This made it difficult to determine whether a phage was present in the tested faecal sample or not. The lower four plates show the plaques morphology of CPAP1 after 5 rounds of purification.

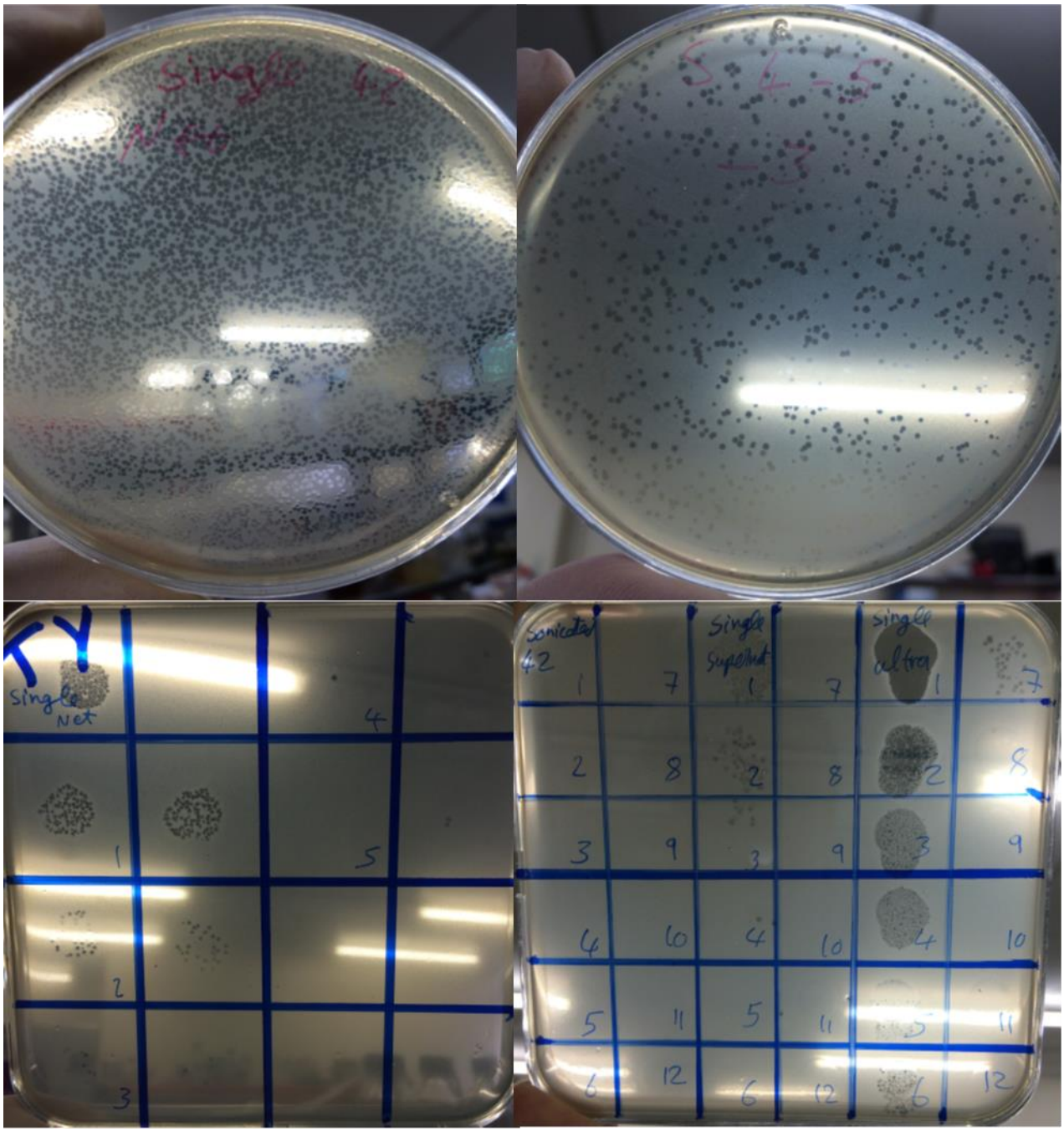


Figure 30. Plaque morphology on TY double layer. The plaques can easily be seen on the TY double layer plates.

4.4.7 Pulsed-field gel-electrophoresis

The approximate size of the phage CPAP1 genome is around 18.5 kb, as shown by PFGE (Figure, 31). Phages CPAS1 and CPAS2 were sent for sequencing right after their isolation, therefore the PFGE step was not necessary. The other two phages, CPAS2 and CPAP3, were unable to show bands because of their low titre; they cannot be propagated

to a titre higher than 10^6. CPAS2 and CPAS3 were sent for sequencing, but the genome preparations failed the quality control.

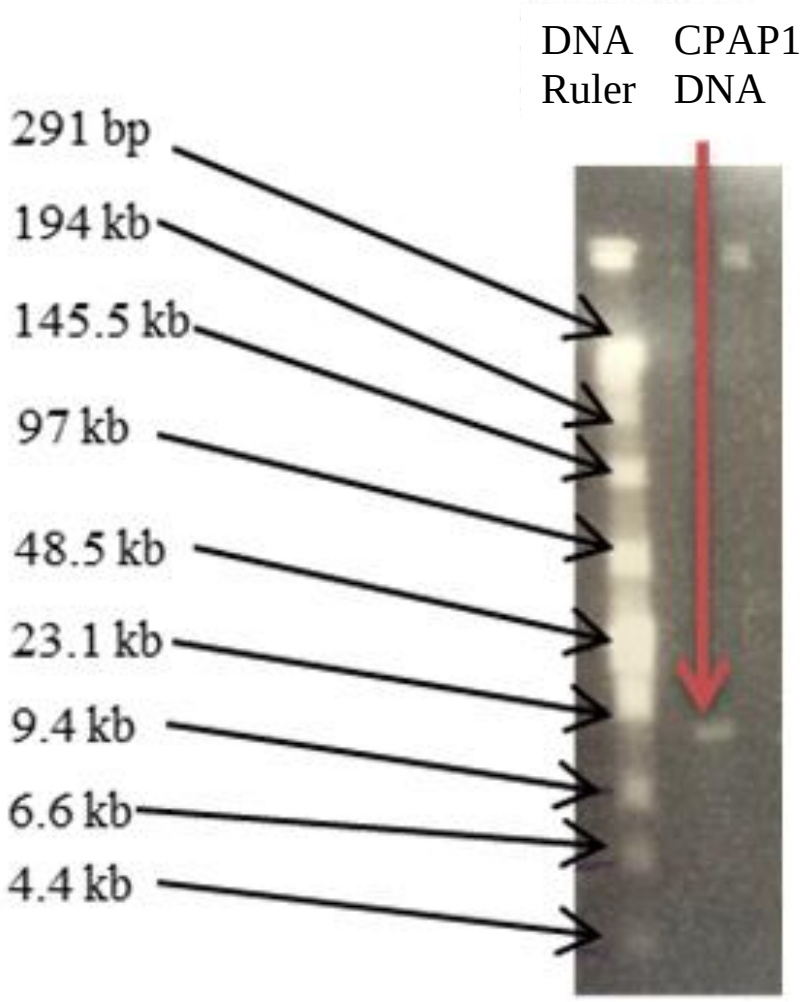


Figure 31. Pulsed-field gel-electrophoresis of phage CPAP1's DNA. It shows that the size of the phage DNA is ~18.5 kb.

4.4.8 Host range

The isolated phages were tested against 42 environmental *C. perfringens* strains using spot tests to determine their host range (Figure, 32). These strains included two strains of toxin type A which has the α toxin and enterotoxin (CP4, and CP13), three strains of toxin type A which have α and β2 toxins (CP15, CP16, and CP25), as well as strain CP35 of toxin type A which has α, enterotoxin, and β2 toxins. The other 36 *C. perfringens* strains are type A with the main α toxin only. The results showed that Podoviruses CPAP1 and

CPAP2 are able to infect and lyse *C. perfringens* strains 6 and 2 respectively, out of the tested 42 strains. Unfortunately, the Siphoviruses are unable to infect and lyse any of the tested *C. perfringens* strains, apart from their isolation strain (Table, 10).

Both CPAP1 and CPAP2 are able to cause lysis from without to *C. perfringens* strains 11 and 15 respectively. Lysis from without is a term used to describe bacterial lysis caused by the attachment of a high number of phages to a bacterial cell resulting in lysis without the production of phage progeny (Abedon, 2011). Both the virulent phages CPAP1 and the CPAP2 can attach to environmental *C. perfringens* strains 11 and 15 respectively (isolated from different areas and representing toxin type A and three variants of type A that possess toxins β2, cpe, or both, in addition to the main α toxin. In contrast to the podoviruses, the siphovirus CPAS1was unable to cause lysis from without to any of the tested strains. CPAS2 and CPAS3 caused lysis to strains 1 and 4 respectively (Table, 11). The ability of each phage to infect and lyse or to cause lysis from without is demonstrated in Figure 32. In total CPAP1, CPAP2, CPAS1, CPAS2, and CPAS3 can attach to ~40.5%, ~40.5%, ~2.4%, ~4.8%, and ~9.5% of the tested *C. perfringens* strains (Figure,32).

	Phages				
Sensitive CP strain	**CPAP1**	**CPAP2**	**CPAS1**	**CPAS2**	**CPAS3**
CP21		+			
CP23	+				
CP26	+				
CP27	+				
CP28	+				
CP40	+		+	+	
CP42	+				
CP56					+
CP65		+			

Table 10. Host range of the isolated phages on 42 environmental strains of *C. perfringens*. CPAP1 is able to produce plaques on 6 *C. perfringens* strains, while CPAP2 was able to produce plaques on two *C. perfringens* strains. In contrast, the *Siphoviruses* were only able to grow on their isolation strain. The experiment was repeated twice; each time, three replicates of each dilution were spotted on the bacterial lawn.

	Phages				
CP strain	**CPAP1**	**CPAP2**	**CPAS1**	**CPAS2**	**CPAS3**
CP 1	+	+			+
CP 2	+	+			+
CP 3	+	+			
CP 5	+	+		+	
CP 6		+			
CP 7		+			
CP 8		+			
CP 9	+	+			
CP 13	+	+			
CP 16	+	+			
CP 21		+			+
CP 22	+				+
CP 23		+			
CP 24		+			
CP 25	+	+			
CP 26	+	+			
CP 27					
CP 28					
CP 34	+				
CP 40			**Indicator strain**	**Indicator strain**	
CP 42	**Indicator strain**				
CP 56					**Indicator strain**
CP 65		**Indicator strain**			

Table 11. Ability of each phage to cause lysis from without on 42 *C. perfringens* environmental strains. The experiment was repeated twice; each time, three replicates of each dilution were spotted on the bacterial lawn. CPAP1 and CPAP2 can bind to a total of 11 and 15 *C. perfringens* strains respectively out of 42 strains.

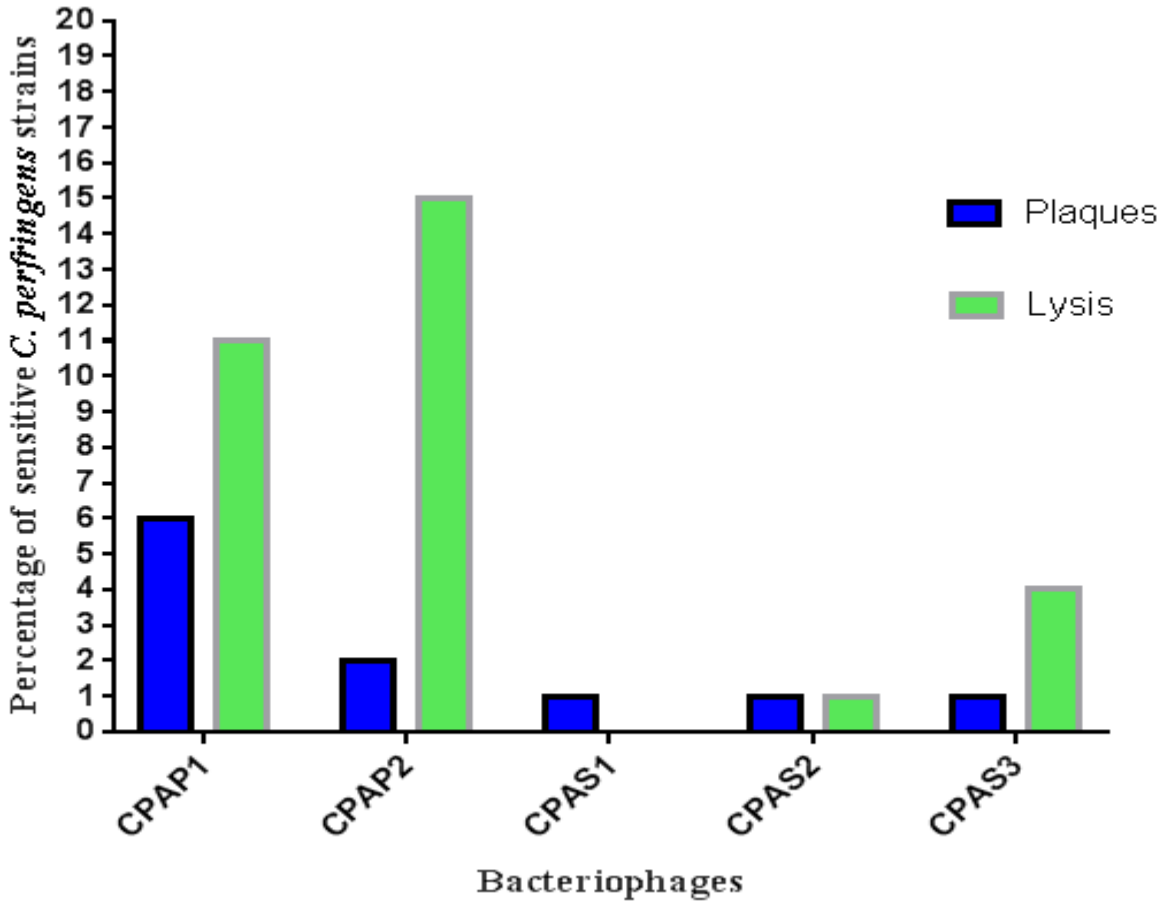


Figure 32. Phage host range against environmental *C. perfringens* strains. The figure illustrates the percentage of each phage's ability to grow (produce plaques) or to cause lysis from without on 42 *C. Perfringens* environmental strains.

4.4.9 *C. perfringens* growth curve

The growth curve of *C. perfringens* strain 42 was carried out in order to determine the starting and ending time points of the exponential growth phase. *C. perfringens* grows faster in TS broth (Figure, 33) than in TY broth when inoculum from TS agar plate is used. Both the optical density and colony forming unit/ml (CFU/ml) at which the exponential growth phase occurs were taken for TY broth culture. The OD cannot be taken for TS broth because of the black colour produced as a result for sulphur reduction. These values are required in order to calculate the MOI for further experiments. This curves shows that the log phase started between the 1st and 2nd hour post inoculation at OD550 nm of ~0.2 and CFU/ml of about 2 X 10^7, and ended after 5.5 hours post inoculation at OD550 nm of 1.445 and CFU/ml of 7 X 10^9. The CFU dropped sharply

after the end of the log phase to reach 4 X 10^8, while the stationary phase started at the 6^{th} hour post inoculation and lasted for another 13 hours (Figures 34 and 35).

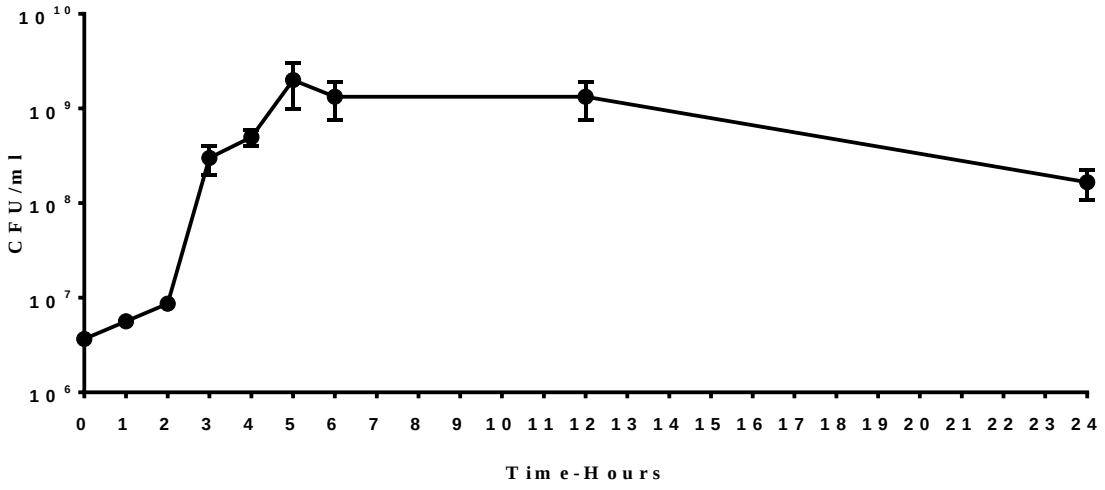


Figure 33. The growth curve of *C. perfringens* strain 42 in TS broth as measured by CFU testing. The log phase started at the second hour post inoculation. Initial inoculum was taken from TS agar plate.

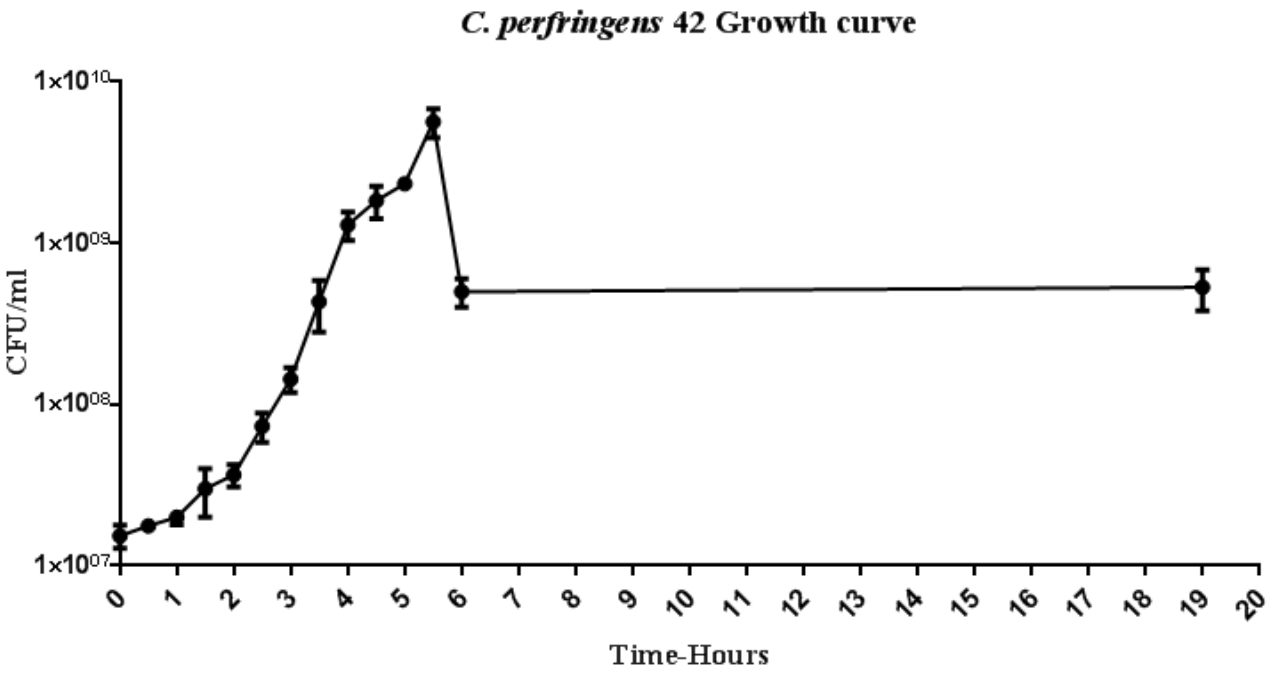


Figure 34. The growth curve of *C. perfringens* strain 42 in TY broth as measured by CFU testing.

The log phase started between the second hour post inoculation at CFU/ml of 2 X 10^7, and ended at 5.5 hours post inoculation at CFU of 7 X 10^9. Initial inoculum was taken from TS broth culture.

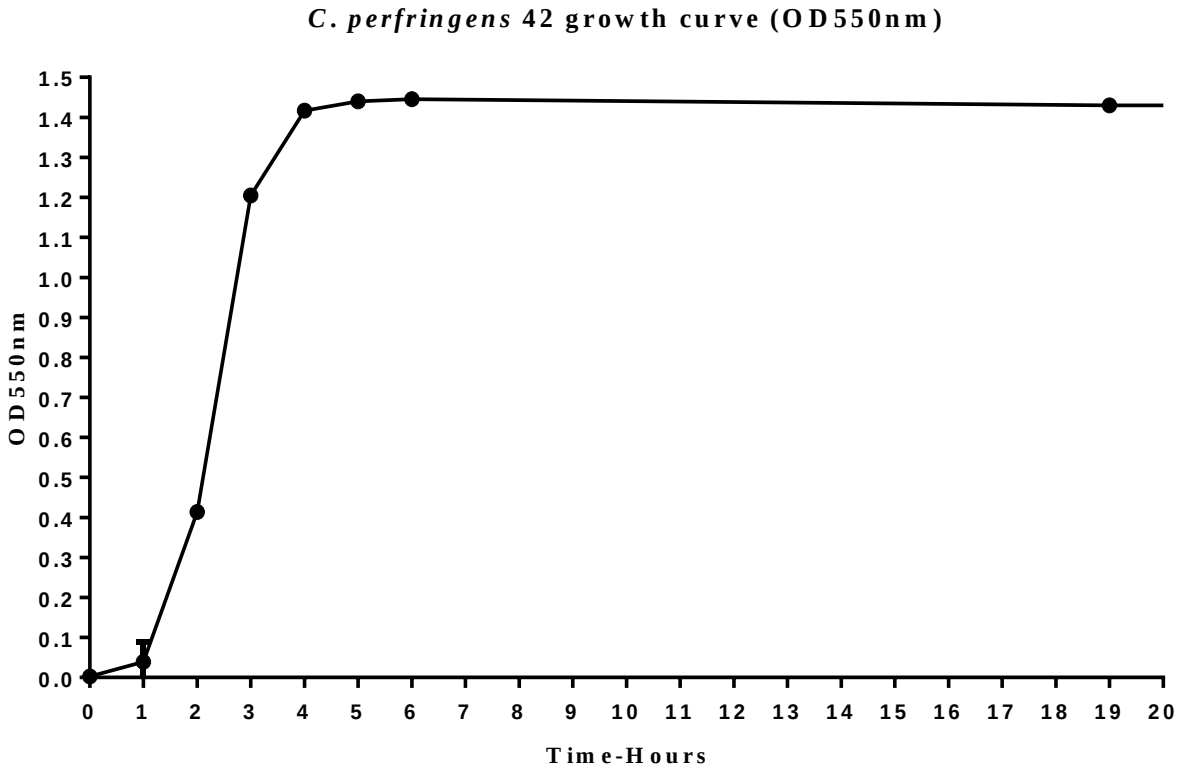


Figure 35. The growth curve of *C. perfringens* strain 42 as measured by optical density. The log phase started between the first and second hour post inoculation at OD of ~0.2, and ended at 5.5 hours post inoculation at OD of 1.445.

4.4.10 CPAP1 one-step growth curve

In order to obtain CPAP1 growth parameters that aid in assessing the time required for growth and the average number of progeny from each lytic lifecycle (in-situ amplification ability), a one-step growth curve was carried out. The data collected from this experiment also helped in the determination of CPAP1's ability to control *C. perfringens* strain 42 growth as reviewed in section 4.1.3.

The CPAP1 one-step growth curve of revealed that the overall time of one cycle is 100minutes. Both the adsorption time and the latent period are 20minutes, and the burst size is 136.585 virions per cell (Figure, 36). This size was also calculated by determining

the relative concentration of the phage at each time point, which was found to be ~137 (Figure, 37).

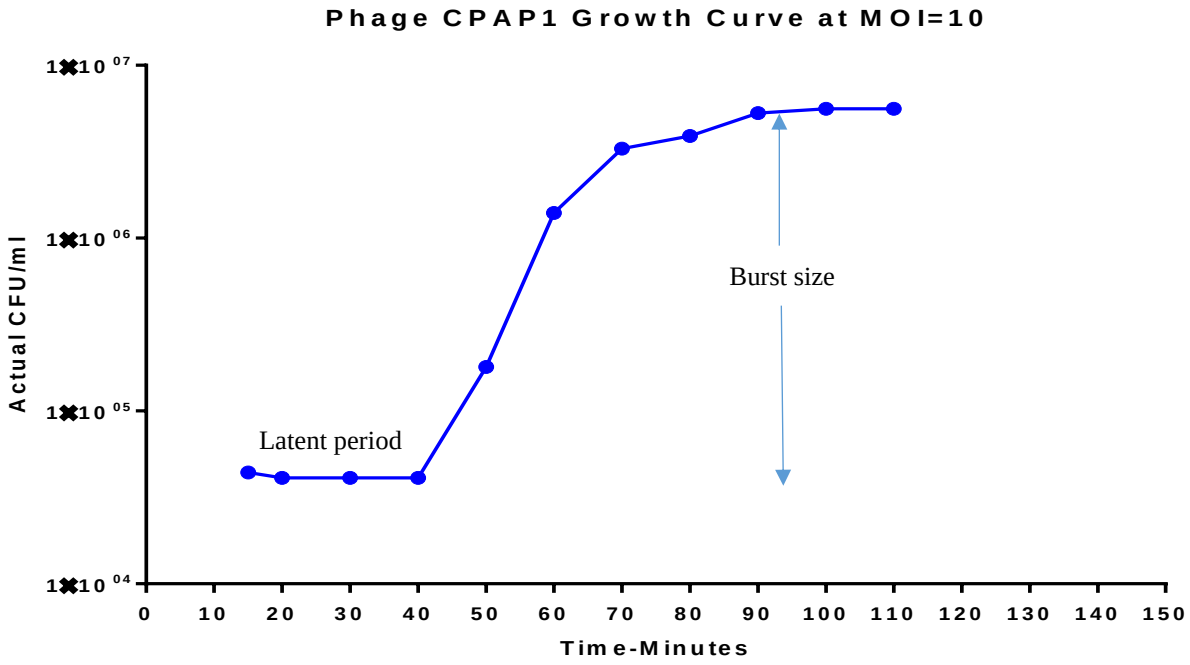


Figure 36. CPAP1 growth curve at MOI of 10. The adsorption time and the latent period are each 20 minutes. The whole cycle took 100 minutes. The burst size is about 137 (136.585) phages per infected bacterial cell.

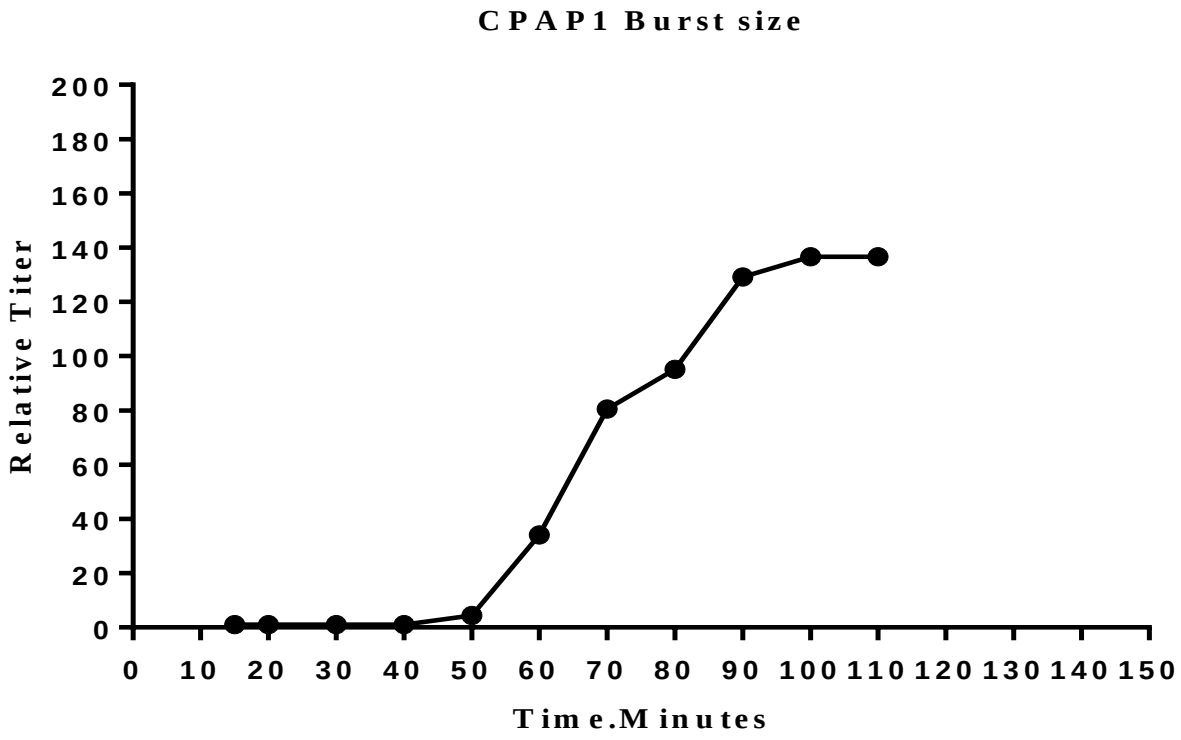


Figure 37. This illustrates the burst size of the CPAP1 phage (phages released from each infected bacterial cell) to be about 137 phages. The average of the actual PFU/ml of three time points from the latent period were taken as the baseline, then the relative phage concentration for the following time points were calculated by dividing the actual PFU/ml for that time point by the calculated baseline PFU/ml.

4.4.11 Genome sequencing and annotation of *C. perfringens* phages

The CPAP1 and CPAS1 genomes were successfully sequenced (Appendix, 7). The result of the CPAS2 sequence revealed the presence of two phages in the sample. Further work is required to separate these two phages and sequence them along with CPAP2 and CPAS3.

4.4.11.1 Phage CPAP1

CPAP1 is a virulent phage, it has a double stranded linear DNA which is 18.390 kb in size and has a G+C content of 28.6 %. 22 ORFs were identified, 19 on the positive strand (Figure, 38) and 3 on the negative strand. All the predicted ORFs functions are listed in Table 12. Both PROKKA and RAST predicted the same exact number and location of ORFs, except for 3; 11, 17, and 21 were at the same location but differed in size. The blast of these 3 ORFs prediction by both PROKKA and RAST agreed with the function

of ORF 11 and 21 as a hypothetical protein, and disagreed on ORF17. RAST predicted ORF 17 to start 21 nucleotides earlier and stop at the exact nucleotide predicted by PROKKA. According to DNA BLAST, RAST predicted ORF17 as a guanine nucleotide exchange factor (100% identity to *Poecilia formosa* with 0.001 E-value) while PROKKA predicted ORF 17 as a hypothetical protein (84% identity to *C. perfringens* phage phi24R with 2e-17). However, the blast of amino acids of ORF17 gene product predicted by both PROKKA and RAST gave 53% identity, uniquely for the phi24R phage.

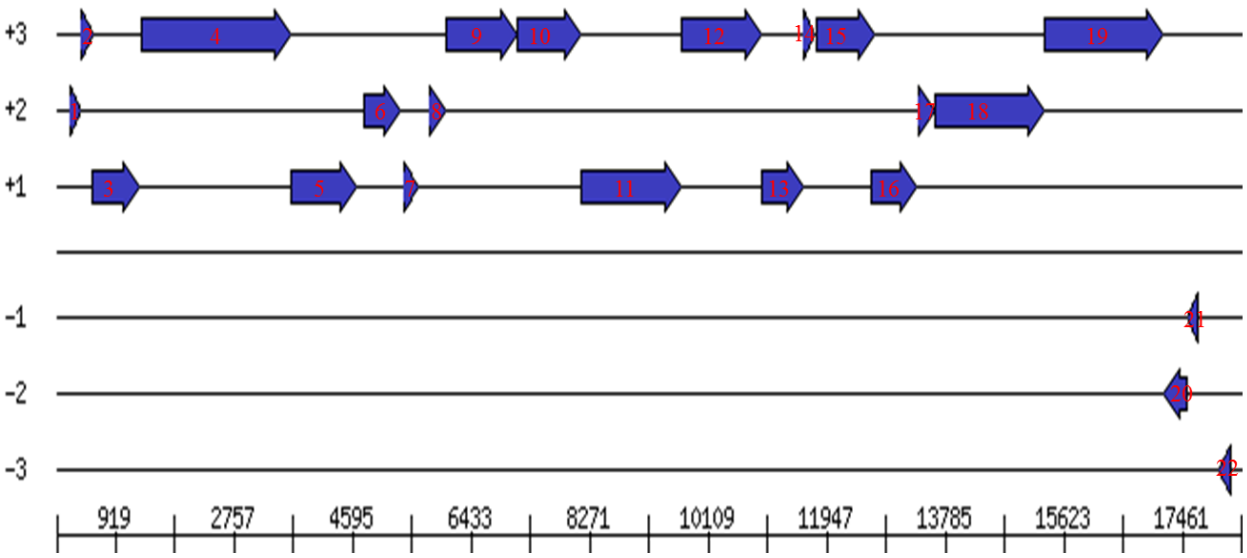


Figure 38. This shows the position of phage CPAP1 ORFs. The direction of the arrows indicates the transcription direction. Nineteen genes are on the positive strand, while the last three are on the negative strand.

ORF	Start	Stop	Gene (bp)	Strand	Product name	Group	Top blast	% identity
1	215	370	51	+	Hypothetical protein		Phi24R	96
2	378	587	69	+	Hypothetical protein		Phi24R	99
3	556	1296	246	+	Hypothetical protein		Phi24R	98
4	1317	3626	769	+	DNA polymerase	Phage replication	Phi24R	100
5	3628	4650	340	+	DNA encapsidation protein	Packaging protein	Phi24R	99
6	4757	5338	193	+	Hypothetical protein		Phi24R	97
7	5392	5628	78	+	Hypothetical protein		Phi24R	97
8	5774	6028	84	+	Hypothetical protein		Phi24R	98
9	6033	7133	366	+	Major head protein		Phi24R	93
10	7146	8138	330	+	Hypothetical protein		Phi24R	95
11	8131	9690	482	+	Lysozyme peptidase		Phi24R	97
12	9687	10934	415	+	Tail protein		Phi24R	99
13	10936	11574	212	+	N-acetylmuramoyl-L-alanine amidase		Phi24R	97
14	11574	11747	57	+	Hypothetical protein		Phi24R	100
15	11778	12680	300	+	Connector		Phi24R	99
16	12640	13344	234	+	Lower collar protein		Phi24R	93
17	13334	13615	93	+	Hypothetical protein		Phi24R	53
18	13616	15334	572	+	Hypothetical protein		Phi24R	63
19	15327	17156	609	+	Pre-neck appendage		Phi24R	96
20	17524	17171	117	-	Hypothetical protein		Phi24R	99
21	17697	17539	32	-	Hypothetical protein			Non
22	18209	18003	68	-	Hypothetical protein		*Candidatus woesebacteria*	47

Table 12. The predicted function of CPAP1 ORFs by the top BLAST hits. Five ORFs encode structural proteins while four ORFs encode enzymes. Thirteen ORFs encode proteins with unknown functions.

Nineteen of the predicted ORFs have a significant amino acid similarity (93-100 %) with zero E-values with phage phi24R isolated by Morales *et al.* (2012), nine of them with a known function. One ORF has a 53 % similarity with an E-value of 7e-22, while only two ORFs have no similarity with *C. perfringens* phi24R. The alignment of the two genomes using the Geneious software revealed that there are two areas of mismatch between them (Figure, 39).

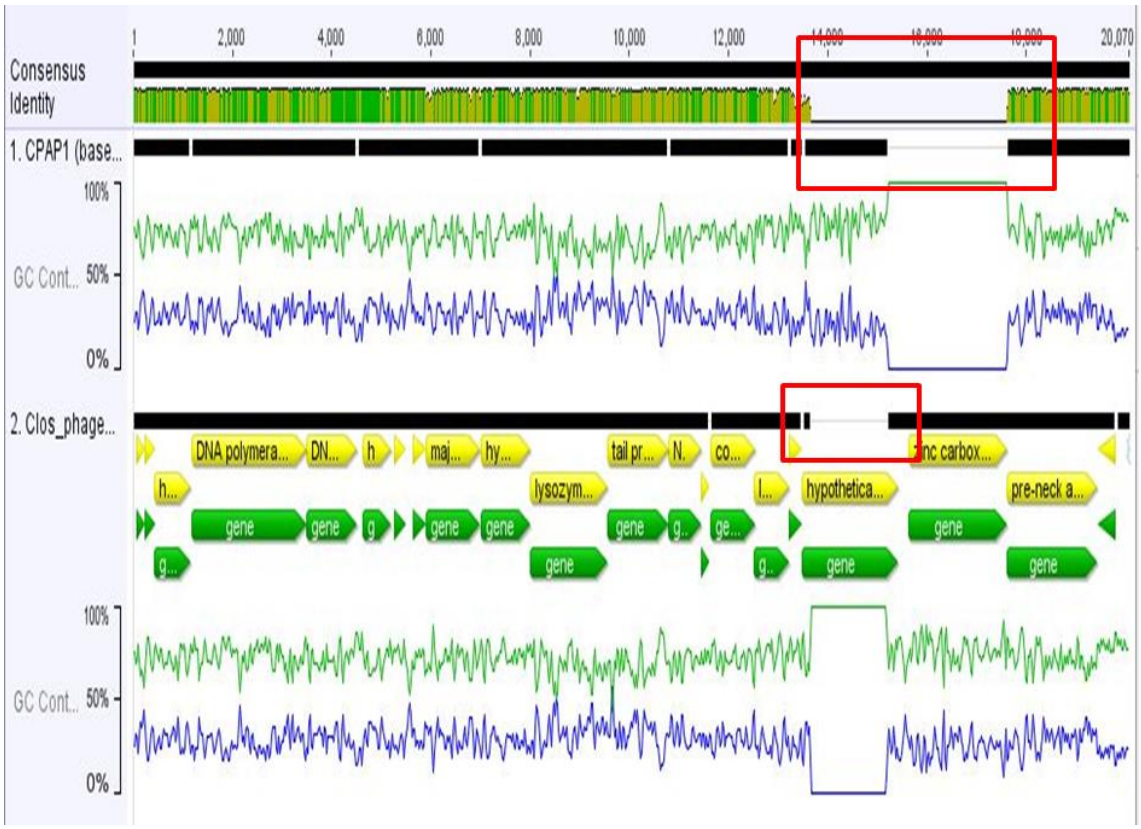


Figure 39. The results of the alignment of the CPAP1 genome and that of a previously identified *C. perfringens* phage, phi24R. The top image shows the CPAP1 genome while the bottom one represents the phi24R genome. Although there is a high level of similarity across each genome, there are also major differences between the two, as represented by the red squares highlighting regions of the genomes. It can be seen that at ~13700bp into the genomes of CPAP1 contains a large insertion distinct from the region in phi24R. In Phi24R, this region contains a putative zinc carboxidase gene not found within CPAP1.

The alignment of amino acid sequences of the CPAP1 endolysin with the phi24R and CPS1 endolysins shows a difference in their primary structures (Figure, 40). Despite these, the three endolysins have similar catalytic and cell wall-binding domains (CBD). Both domains of each enzymes begin and end at the same amino acid number, as demonstrated by the BLAST against the conserved protein domains (Figure, 41).

```
CPS1  mkigirdghspnckgaialrdeqacmrilcrevievlekhghevvycgsnartengelse 60
CPAP1 MKIGIRDGHSPNCKGAIGLRDEQSCMRVLCKEVIEILEKHGHEVVYCGSNASTQNGELSE 60
Φ24R  mkigirdghspnckgaiglrdeqscmrvlckevieilekhghevvycgsdastqnselse 60
      *****************.*****:***:**:****:*************:* *:*.****

CPS1  ginkansnnvdifislhmdvskehkangtcsfvakearksirdiaqrlvdnfetlglqnr 120
CPAP1 GVRKANNSNVDIFISLHMNSFN-GQAQGTEALVTVGARNSIKEIASRLCKNFASLGLVNR 119
Φ24R  gvrkannsnvdifislhmnsfn-gqaqgtesliavgarnsikeiasrlcknfaslglvnr 119
      *:.***..**********:  :  :*:** ::::  **:**::**.** .** :*** **

CPS1  gvresnyremrevnapniifetmfcdnlhdinevwsptpyekmallianaidptikenel 180
CPAP1 GVKEVNLYEMKNVKAPNIIFETMFCDNPHDINEVWSPTPYEKMALLIANAIDPTIKENEL 179
Φ24R  gvkevnlyemknvkapniifetmfcdnehdinevwsptpyekmallianaidptikenel 179
      **:* *  **::*:************* ******************************

CPS1  yrvvvqyfnskedaencqqeiakrwycfvegcn  213
CPAP1 YRVVVQYFNSKEDAENCQQEIAKRWYCFVEECN  212
Φ24R  yrvvvqyfnskkdaencqqeiakrwycfveecn  212
      ***********:****************** **
```

Figure 40. Alignment of CPAP1 amino acids sequence with phi24R and CPS1 endolysins sequences. There is a difference in the primary structure of the endolysins of the three phages. There are seven amino acids out of 212 that are different between CPAP1 and phi24R, and 44 amino acids out of 213 are different between CPAP1 and CPS1.

Five ORFs producing structural proteins were identified. ORF9 encodes the major head protein, ORF 12 encodes the tail protein, ORF 15 encodes the connector protein, ORF 16 encodes the lower collar protein, and ORF 19 encodes the pre-neck appendage protein. Four ORFs producing enzymes were identified as well; ORF 4 encodes DNA polymerase, ORF 5 encodes DNA the encapsidation enzyme, ORF 11 encodes lysozyme peptidase, and ORF 13 encodes N-acetylmuramoyl-L-alanine amidase (endolysin). Thirteen ORFs encode proteins with unknown functions. Figure 42 shows a circular map of the CPAP1 genome.

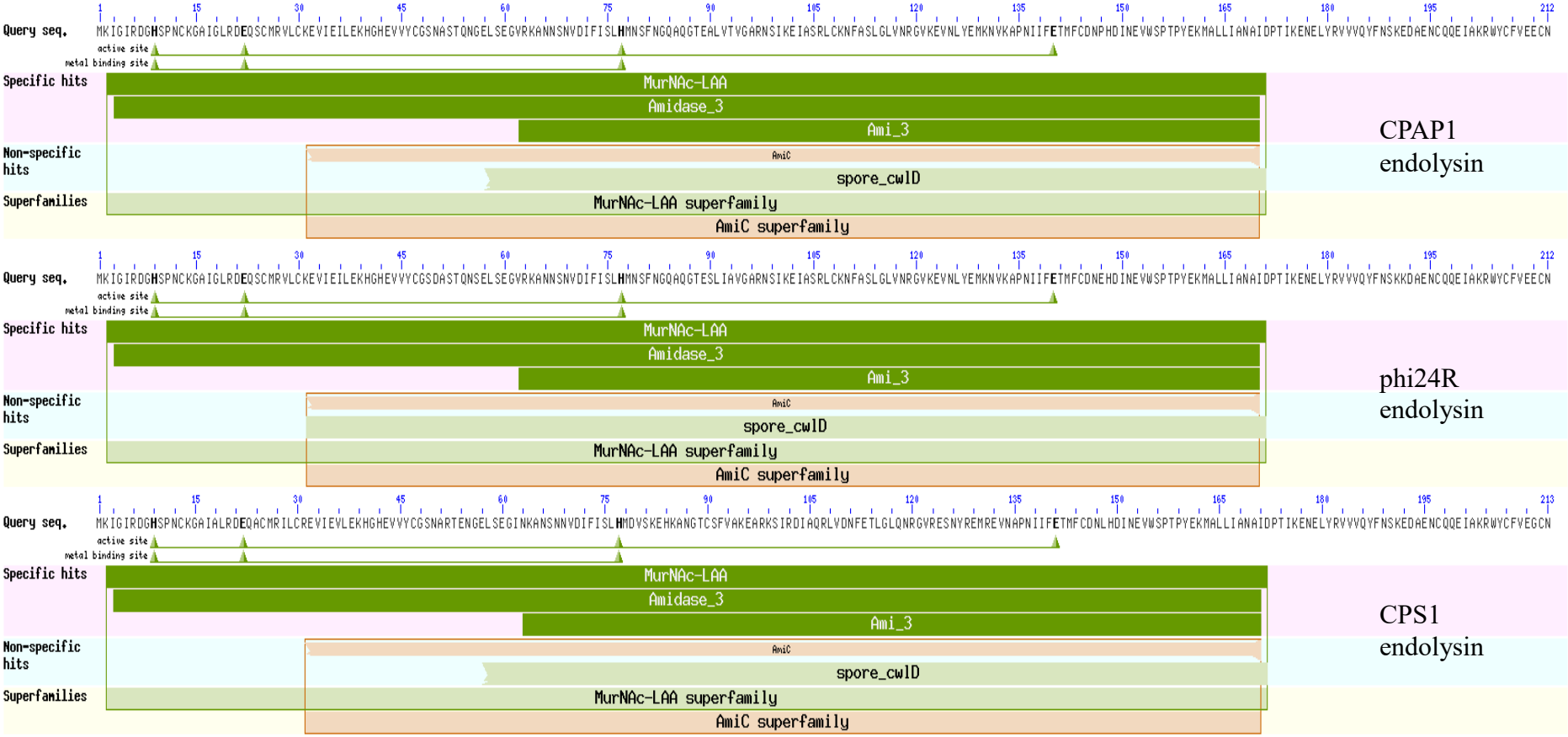


Figure 41. Comparison of two *C. perfringens* phages (phi24R and CPS1) endolysins with CPAP1 endolysin. The BLAST of endolysin amino acid sequence against the protein conserved domains showed that despite the differences in these enzymes primary structure, they have a similar catalytic and cell binding domains.

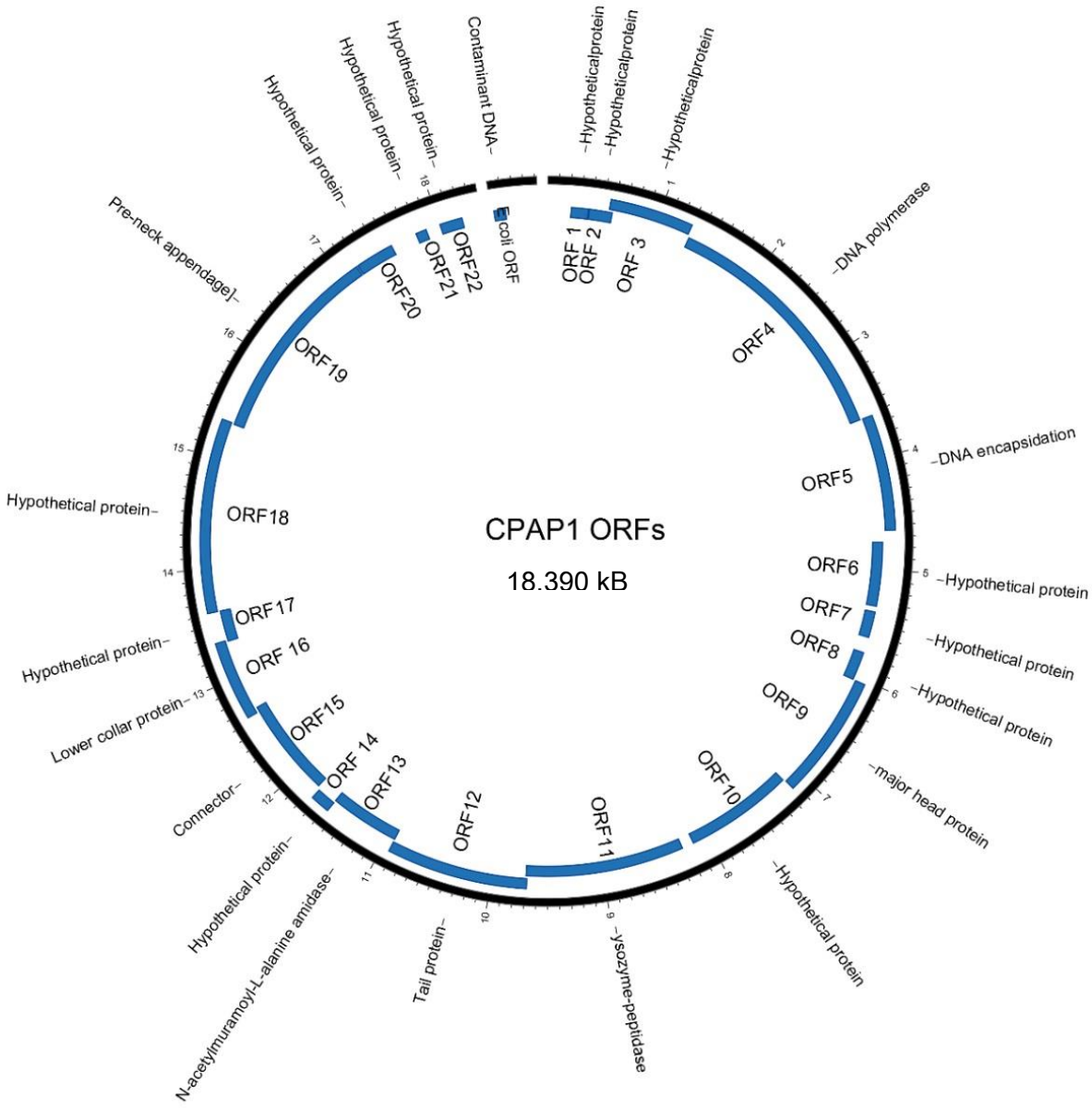


Figure 42. A circular genome map showing the putative function of each of the ORFs of the Podovirus phage CPAP1 (18.390 kB). The black outer circle represents the nucleotides' size (1= 1kb). The blue markers represent the location of ORFs within the genome, labelled with their function.

4.4.11.2 Phage CPAS1

CPAS1 is a temperate phage which has a double stranded linear DNA and is 152.340 kB in length with a G+C content of 33.24%. 226 ORFs were identified, and many of their functions were predicted. However, the phage has genes with no similarity to any of the known genes. The blast of the FASTA sequence revealed that there is no similarity between CPAS1 and any of the sequenced *C. perfringens* phages. Figure 43 shows the circular map of the phage CPAS1.

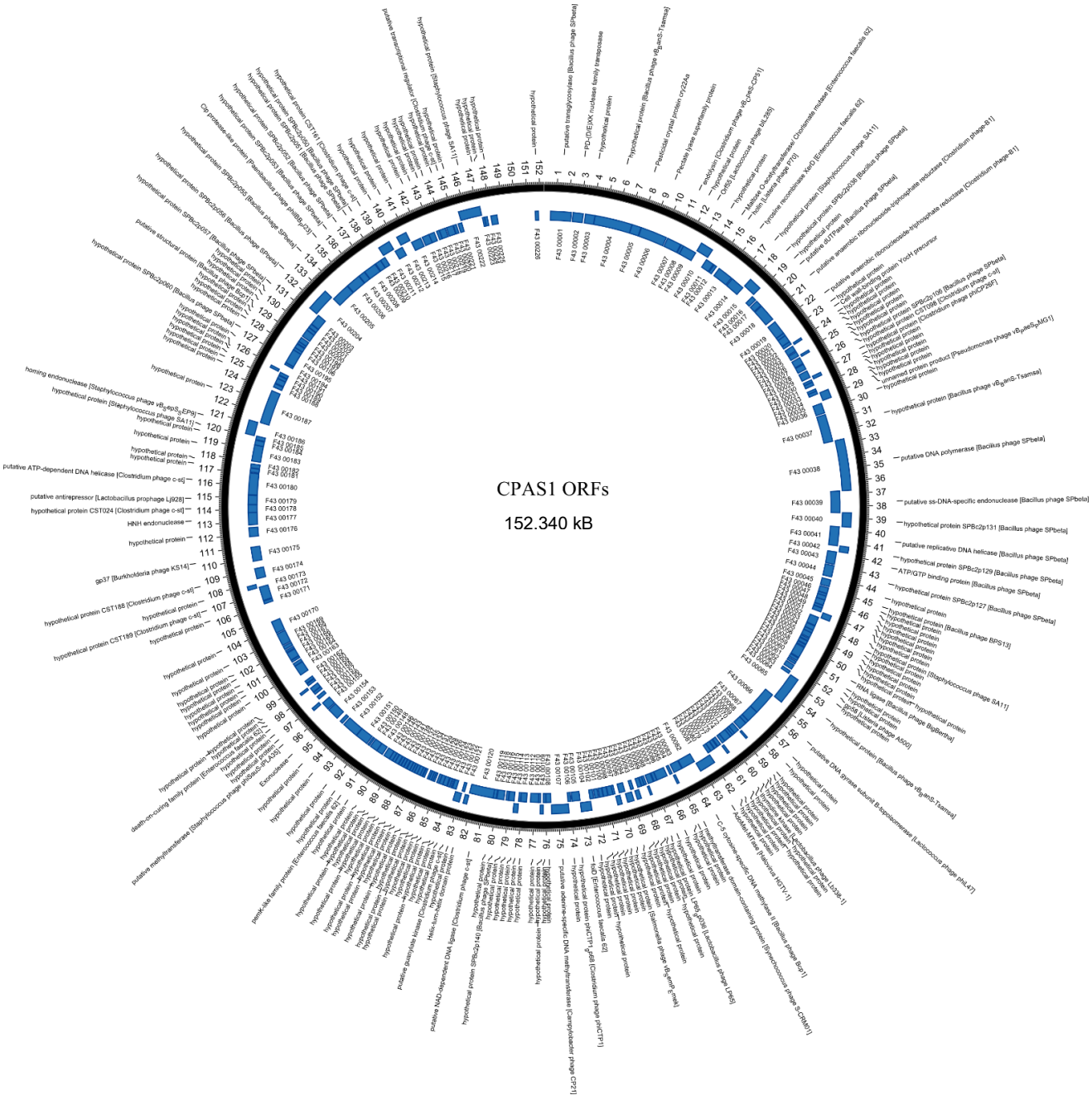


Figure 43. A circular genome map showing the putative function of each of the ORFs of the siphovirus phage CPAS1 (152.340 kB). The black outer circle represents the nucleotide size (1= 1kb). The blue markers represent the location of ORFs within the genome, labelled with their function.

4.5 Discussion

4.5.1 Toxin typing

In the areas where *C. perfringens* strains were isolated (limited number of areas in Leicestershire), the gas gangrene, food poisoning, and broiler chicken enteritis are the main diseases that can be caused by *C. perfringens*, as type A is dominant in my collection. A rare enteritis caused by types C, D, and E may also arise in these areas. The absence of types B *C. perfringens* from my collection potentially excludes the involvement of *C. perfringens* in cases of neurological disorders, multiple sclerosis, and enteritis that may occur in these areas.

4.5.2 Isolation of temperate *C. perfringens* phages (induction of temperate phages)

No temperate phage was isolated by induction. However, prophages could be induced from *C. perfringens* strains by both mitomycin C and norofloxacin. The induction results showed a high level of prophage carriage in my *C. perfringens* collection. This is one of the factors enabling *C. perfringens*' resistance to phage infection. Superinfection exclusion is the prevention of secondary infection with another (closely related) phage provided by an already established infection (Beperet *et al.*, 2014).

4.5.3 Isolation of *C. perfringens* free phages by enrichment culture

Five phages were isolated directly from faecal samples. CPAP1 is a virulent (strictly lytic) phage while CPAS1 is a temperate phage. The other three phages were not sequenced. Lytic phages are preferred over temperate phages as antimicrobial agents as the former cannot insert their genome in the bacterial chromosome, and will always lyse their bacterial host cell and kill it (Guenther *et al.*, 2009). Temperate phages are not suitable for therapeutic purposes as a result of their integration ability (Sahota, 2016).

These five phages are an addition to the already globally isolated *C. perfringens* phages. They can be tested on more *C. perfringens* strains to establish the possibility of using them to prepare a phage cocktail (containing phages isolated in different studies). Further study into each of these phage endolysins will also increase knowledge regarding said endolysins structure and efficiency. An endolysin cocktail composed of a combination of structurally different proteins (in terms of both binding and catalytic domains) may have the ability to lyse all known *C. perfringens* strains. It can also allow for the exploration of the use of their binding domains for diagnostic purposes.

C. perfringens strains 1, 2, 3, 10, and 13 exhibited a distinct pattern of resistance to phage infection; it was possible to pass the environmental phages three times only, afterwards, the phages from the third passage lost their infectivity. This pattern occurred with five phages. This resistance may be a result of phage mutations, change in the bacterial receptors (phage attachment site), or *C. perfringens* possibly has the ability to tag the phage DNA. Subsequently, the naive bacterial cells which have never been exposed to the phage may become able to recognize and destroy phage nucleic acids upon entry. Similar results were found with *C. difficile* temperate phages which were able to produce plaques only once on *C. difficile* 630 and could not be passed any further (Nale, 2013).

C. perfringens strain 16 showed some lysogens after the third passage (Figure 26). Some phages possess the integrase gene which enables them to integrate their genome into bacterial DNA and enter the lysogenic lifecycle. The phage DNA might be integrated into the *C. perfringens* genome, as has been documented for *C. perfringens* previously (Zimmer *et al.*, 2002a).

4.5.4 Optimization of TY medium

The TY double layer was optimized to be used in a spot test and for the isolation of new phages. The new media enables the accurate visualization of plaques during enrichment for phage isolation. The TY double layer represents an improved media system for the isolation of *C. perfringens* phages.

The inconsistent *C. perfringens* lawn (obtained from testing faecal enrichment lysate) was a result of the presence of different bacterial metabolic products, such as bacteriocins and lysozymes. These products originated from the microbial communities present in the faecal sample used for the enrichment culture. In bacterial communities, different bacterial species compete using a variety of mechanisms. The growth of a given species can be impaired as a result (Hibbing *et al.*, 2010).

4.5.5 Morphology of the isolated phages

All the isolated phages belong to two families, *Podoviridae* and *Siphoviridae*. Only one short tail myovirus was induced from the *C. perfringens* lysogen. It was unable to produce plaques on any *C. perfringens* strains and could not be purified.

There is a significant difference between the diameter and tail length of the two podoviruses, as shown by the TEM. While the three siphophages were very similar in head size, there was a large difference in tail length.

4.5.6 Plaque morphology

Although the podovirus produced small and medium clear plaques and the siphovirus produced medium sized hazy plaques, the low number of isolated phages cannot be used to establish a relation between the phage particle morphology and the plaques produced.

CPAP1 is able to produce two different plaque morphologies on two different mediums. This indicates that the type of nutrients provided for bacterial growth dictates the bacterial growth pattern, and subsequently the bacteria's ability to support phage growth. Moreover, this indicates that the phage may behave differently *in-vivo*.

4.5.7 Pulsed-field gel-electrophoresis (PFGE)

PFGE helps in the sequencing process as it provides a preliminary assessment of a phage's genome size. The genome size of the CPAP1 phage was assessed to be about 18.5 kB before it was sequenced.

4.5.8 Host ranges

The results of host range analysis revealed that the podovirus CPAP1 has a wider host range than the other isolated phages, which makes it a good candidate as a therapeutic agent, especially if used in a phage cocktail. Each of the podoviruses CPAP1 and CPAP2 can bind to the surface of approximately 40.4% of the *C. perfringens* environmental strains isolated in this study, which reflects the presence of a common receptor. Further study of these phages' tail fibre proteins which are presumably involved in the attachment of phages to *C. perfringens*' surface may be useful for diagnostic purpose.

Unfortunately, the three siphoviruses have a very narrow host range, and cannot infect and lyse any of the tested *C. perfringens* strain apart from their isolation strain. However, phages with a narrow range often infect bacterial strains that other phages cannot (Hargreaves, 2013). Thus, CPAS3 may be useful in a phage cocktail as it can only infect *C. perfringens* strain 65, which has not been infected by any other phage.

4.5.9 Phage one-step growth curve

The time required for a phage lifecycle to take place and the number of phages produced from each cycle are important parameters to estimate the magnitude of the in-

situ amplification capacity of the phage. The result of the CPAP1 one-step growth curve revealed that it has a high burst size, resulting in the production of a large number of phage progeny at the site of infection. This can be an advantage if the phage is used to treat infections at physiologic sites where the immune system has limited accessibility, such as the cerebrospinal fluid or intestines. However, in the case of systemic infections, this can be a disadvantage. The high number of phages produced in a relatively short time (110 minutes) will result in rapid cell lysis, consequently triggering an adverse immune response (severe hypersensitivity reaction).

4.5.10 Genome sequencing and annotation

The annotation of the CPAP1 genome revealed that it is a virulent phage as it does not appear to encode a lysogeny control or integrase gene. In contrast, the annotation of the CPAS1 genome revealed that it is a temperate phage. CPAP2, CPAS2, and CPAS3 are still to be sequenced.

Fortunately, CPAP1's genome does not have any bacterial toxin genes, antibiotic resistance genes, and transduction genes, meaning that it is a safe therapeutic agent. Given that phages can participate in the dissemination of bacterial toxin genes, and antibiotic resistance through phage-mediated transduction (Colavecchio *et al.*, 2017, Rossmann *et al.*, 2015), CPAP1 is a good candidate for phage therapy.

CPAS1 is not favourable as a therapeutic agent. Temperate phages have a 10 – 100 times larger ability of transduction in comparison with virulent phages (Saye *et al.*, 1990, Saye *et al.*, 1987). This is another reason why strictly lytic phages should be used for therapeutic purposes.

4.6 Conclusion

Among the *C. perfringens* strains collected from a limited number of locations in Leicestershire, toxin type A and its' variant (with cpe, beta or both toxins in addition to the main alpha toxin) is the dominant toxin type in our collection. All *C. perfringens* strains in this collection have a high level of prophage carriage, as all the induced strains could cause lysis on at least one of the indicator strains. They also have the ability to resist phage infection. This is explained by both their ability to develop resistance against phages which could grow and produce plaques three times on them, and the low number of isolated phages (5 phages out of 81 environmental samples).

Five phages were isolated; the *C. perfringens* Podoviruses have a wider host range than the Siphoviruses, along with a higher number of receptors on the surface of *C. perfringens*, as it was able to cause lysis from without for multiple *C. perfringens* strains.

Only one strictly lytic phage was isolated. The genome characteristics, host range, and biological parameter of this phage signify that it is a good candidate for phage therapy. The genome sequence of this phage also revealed that it is closely related to the phi24R lytic phage, as they have 95% similarity.

4.7 Future work

1. Isolation of more *C. perfringens* phages and determine their potential as a therapeutic agents for the treatment of *C. perfringens* infections.
2. Assessing the potential of using antibiotic with phage cocktails in the control of *C. perfringens* growth.

Chapter 5

Cloning, Expression, Purification and Assessment of Phage Endolysin as a Potential Antimicrobial for the Treatment of *C. perfringens* Infections

Chapter 5: Cloning, Expression, Purification and Assessment of Phage Endolysin as a Potential Antimicrobial for the Treatment of *C. perfringens* Infection

5.1 Introduction

The progressive development of antibiotic resistance as a result of its excessive and irresponsible use has become a global public healthcare problem. In Europe, about 25000 people die every year due to infection from multi-antibiotic resistant bacteria. Moreover, the situation is worse in the developing countries. At this rate of resistance development, it is estimated that by 2050, around 10 million people worldwide will die yearly because of infections from multi-antibiotic resistant bacteria (Rai *et al.*, 2017). Recently, politicians, physicians, and researchers have been urged to look for novel antimicrobials options (Dufour *et al.*, 2017).

5.1.1 Novel antimicrobials

Recently, researchers have been focusing on the rehabilitation of old compounds that are known to be therapeutically safe. This achieved by improving said compounds to have a new mechanism of action. The analogue of the old robenidine, an anti-coccidial drug, is an example (Ogunniyi *et al.*, 2017).

New chemotherapeutics belonging to the old oxazolidinone class of chemical antibacterials have recently been described. These new compounds represent an effective choice for the treatment of serious Gram-positive infections (Karpiuk *et al.*, 2017).

Moreover, the application of nanotechnology to develop an effective antimicrobial is a promising discipline. These new nano-antimicrobials could revolutionise the health sector; the safety and possibility of using them alone or in combination with antibiotics is currently under study (Rai *et al.*, 2017).

Phage therapy is yet another promising approach. Recent studies have shown that phages can be used in biocontrol, the development of new molecules that have clinical applications, and in nanomedicine utilizing phage display technology (El-Shibiny *et al.*, 2017, Dufour *et al.*, 2017).

5.1.2 Phages endolysins

Phage endolysins are effective novel antibacterial agents. They have been successfully used to treat a variety of bacterial infections, as reviewed in section 2.2.6. Moreover, their ability to successfully control or clear pathogenic bacteria from different anatomic sites of empirically infected laboratory animals has been shown. Endolysins also have other uses such as bio-preservation, elimination of biofilm and diagnosis purposes (Gunathilaka *et al.*, 2017).

There are many advantages of using endolysins as antibacterial agents. These include their rapid mode of action, wide host range within the bacterial species, a very low concentration is required to lyse high bacterial concentration regardless of their antibiotic sensitivity profile, novel mode of action, and the low possibility of developing resistance, as to date, no resistance against endolysins has been reported, and it is not expected to occur. This fact is due to the extracellular use of endolysin where there are a limited number for resistance mechanisms. Most of the antimicrobials' resistance mechanisms act inside the cell. More recently, engineered phage endolysin has been described to have successfully treated bacterial infection such as skin infection with *Staphylococcus aureus* (Totte *et al.*, 2017, Oliveira *et al.*, 2015, Borysowski *et al.*, 2006, Donovan *et al.*, 2009, Spratt, 1994).

However, there are disadvantages associated with endolysin therapy. Non-clinical studies have shown these to include the immunogenicity and the liberation of pro-inflammatory substances as a result of the rapid bacterial lysis. However, all data regarding safety and effectiveness obtained from non-clinical studies are still to be confirmed by clinical trials (Borysowski *et al.*, 2006). Another disadvantage is that the mass production and purification of endolysins is usually hindered by their low solubility, and many display lower lytic activity in comparison with their activity during phage infection (Fernandes *et al.*, 2012). Moreover, the exogenous endolysin activity can be blocked by the bacterial outer membranes such as the lipopolysaccharide (LPS) surrounding the Gram-negative bacteria cell wall, which prevents the access of exogenously added endolysin to the target murine (Gerstmans *et al.*, 2016, Briers *et al.*, 2014). Furthermore, the rapid release of pyogenes and lipopolysaccharides as a result of the fast endolysin action can lead to adverse immune responses and toxin shock (Shen *et al.*, 2013, Górski *et al.*, 2012, Hagens *et al.*, 2004, Rosenfeld *et al.*, 2006, Borysowski *et al.*, 2006).

The development of a chimeric endolysin by fusing the catalytic domain of the *Streptococcus* phage endolysin with new cell binding domains to extend the host range has been shown. This chimeric endolysin is able to lyse *Streptococcus* and *Staphylococcus* bacterial strains (Dong *et al.*, 2015). The lipopolysaccharide (LPS) surrounding the Gram-negative bacteria cell wall is a physical barrier that prevents the access of exogenously added endolysin to the target murine. As a consequence, Artilysins was developed in order to overcome this major hurdle and use endolysin as an antibacterial to treat Gram-negative bacterial infection. This is an engineered endolysin able to penetrate the LPS and lyse many pathogens, including *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (Briers *et al.*, 2014, Gerstmans *et al.*, 2016).

The native endolysin of *Streptococcus pyogenes* (*S. pyogenes*) phage PlyC is able to control intracellular infections caused by *S. pyogenes*. It can cross the epithelial cells' cytoplasmic membrane (CM) in a dose-dependent manner and lyse the intracellular *S. pyogenes.* PlyC's ability to traverse CM potentiates its engineering for other functionalities (Shen *et al.*, 2016). With further investigation, endolysins can truly be engineered to treat infections caused by Gram-negative bacteria and intracellular bacterial infections (O'Sullivan *et al.*, 2016).

5.1.3 *C. perfringens* phages endolysins

Although strictly lytic phages have been isolated for *C. perfringens*, there are some limitations that accompany their use to control or eliminate *C. perfringens* infection in comparison with the use of endolysins. These include resistance to phage infection, the presence of bacterial toxins within the phage lysate, the transduction ability of phage and the subsequent dispersion of bacterial virulent genes (Gunathilaka *et al.*, 2017, Wang *et al.*, 2016, Gupta *et al.*, 2011).

Endolysins encoded by *C. perfringens* phage phiSM101 employ two modular domains, a cell wall binding domain at the C-terminal (tandem repeated SH3_3 domains, SH3N and SH3C) and a catalytic domain at the N-terminal (Figure, 44). These endolysins have the ability to lyse most *C. perfringens* strains (Tamai *et al.*, 2014a). There is a variation in the lysis potential of different *C. perfringens* endolysins, as demonstrated by a few studies. Some were able to lyse all the tested strains while others lysed most of them (Gervasi *et al.*, 2014a, Zimmer *et al.*, 2002a, Gervasi *et al.*, 2014b). This variation may be due to differences in the enzyme cell wall binding domains, at the central SH3-3

domain of the C-terminal which is responsible for the enzyme specificity. However, further investigation in this area is required (Fischetti, 2010, Schmelcher *et al.*, 2012, Gervasi *et al.*, 2014a).

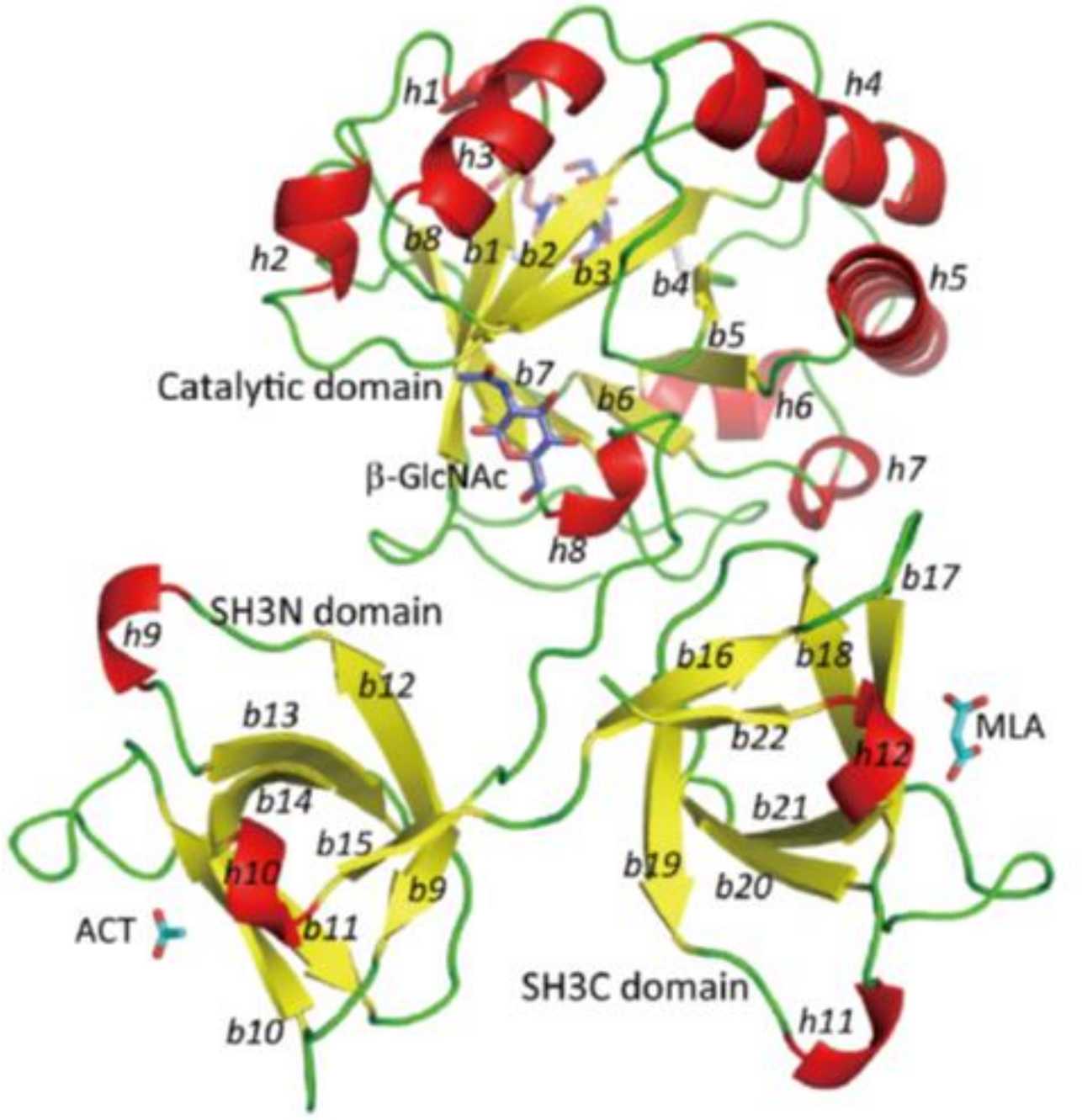


Figure 44. The overall structure of endolysin Psm encoded by *C. perfringens* phage SM101. The Psm endolysin consists of two domains, the N-terminal catalytic domain (upper part of the image) and the C-terminal cell wall binding domain (CBD) SH3_3, which consists of SH3N and SH3C (lower part of the image). The catalytic domain is linked to the CBD by 7 amino acids residue (Tamai et al., 2014a).

5.1.4 Endolysin mode of action

The endolysin enzyme is expressed by double-stranded bacteriophages at the end stage of the lifecycle. It can reach, bind, and cleave the peptidoglycan in a site-specific manner through the pores in the cytoplasmic membrane made by the holin enzyme. Subsequently, the host cell is lysed and progeny virions are released (Borysowski *et al.*, 2006). Holins are small proteins that accumulate at the cytoplasmic membrane, resulting in membrane permeability to the folded endolysin. The holin activity is regulated by anti-holin protein inhibitors (Wang *et al.*, 2000). Holins have been studied for their potential as antimicrobial agents (Veiga-Crespo *et al.*, 2007). For instance, the lambda phage holin has the potential for cancer treatment as it can disrupt the eukaryotic membrane integrity, as demonstrated in a mouse model (Agu *et al.*, 2006).

Interestingly, purified recombinant endolysins can cause rapid cell lysis when applied externally. Moreover, the binding domain at the C-terminal is not always necessary for the endolysin activity, as has been shown with a number of endolysins. Moreover, species specificity and the mode of action of endolysins make them an interesting therapeutic agent (Borysowski *et al.*, 2006, Mayer *et al.*, 2011).

5.2 Aims and objectives

In order to determine the potential of phage's endolysin to control or prevent the growth of *C. perfringens,* the following aims were set:

1. Clone and express the predicted endolysin gene of phage CPAP1, and purify the expressed protein.
2. Confirm the peptidoglycan amidase activity of the purified protein by zymography.
3. Determine the ability of the purified endolysin to lyse *C. perfringens* strains by lysis assay.

5.3 Material and methods

5.3.1 Bioinformatics analysis of the phage CPAP1 endolysin gene

The annotation of CPAP1 predicted ORF13 as a N--acetylmuramoyl-L-alanine amidase gene. The nucleotides sequence of ORF13 was translated to an amino acids sequence online using ExPASy available at http://web.expasy.org/translate/, and the molecular weight (MW) of CPAP1 Endolysin was also determined by ExPASy available at http://www.sciencegateway.org/tools/proteinmw.htm (Gasteiger *et al.*, 2003). The predicted amino acids sequence was identified as an endolysin via a comparison with the available proteins sequences online, using BLASTp against the non-redundant protein database (Altschul *et al.*, 1990). The functional domain of the predicted protein was searched against the Pfam database (Bateman *et al.*, 2004) available online at http://pfam.xfam.org/. The Clustal omega (multiple sequence alignment) (Sievers *et al.*, 2011) available at https://www.ebi.ac.uk/Tools/msa/clustalo/ was used to compare CPAP1 with other endolysins belonging to *C. perfringens* phages.

5.3.2 Cloning of endolysin

CPAP1 endolysin was cloned in to the pLEICS-02 plasmid which has a glutathione S-transferase (GST) tag (Figure, 45). The GST-tag facilitates the purification of the expressed endolysin by the column method. The cloning was carried out by the University of Leicester PROTEX service, department of molecular and cell biology which is available at http://www2.le.ac.uk/departments/molcellbiol/facilities/protex. The ORF13 of the CPAP1 phage was amplified with a pair of primers (Appendix, 3) with an adapter sequence (homologous sequence) compatible with the plasmid pLEICS-02 cloning site. The primers were designed according to the PROTEX instructions available online at http://www2.le.ac.uk/colleges/medbiopsych/facilities-and-services/cbs/protex/primers. The PCR was carried out using the following conditions: A 30second initial denaturation step at 98°C, 34 cycles of 10seconds denaturation at 98°C, 30seconds annealing at 57°C, 25seconds extension at 72°C, and a 10minute final extension at 72°C. The PCR reaction mix's volume was 50µl and contained: DNA template, 0.5µM of each primer, 200µM of dNTPs, 1X buffer, and 1 unit/50µl of phusion DNA polymerase (New England biolabs, UK). The PCR product was visualized using 1% agarose gel (BioLine, UK). The product was cleaned and sent to the cloning service.

After receiving the construct, it was sequenced using plasmid-based primers pGEX 3' and pGEX 5' (Appendix, 3). Then, the sequence was searched for any possible mutation or incorrect insertion before starting the expression.

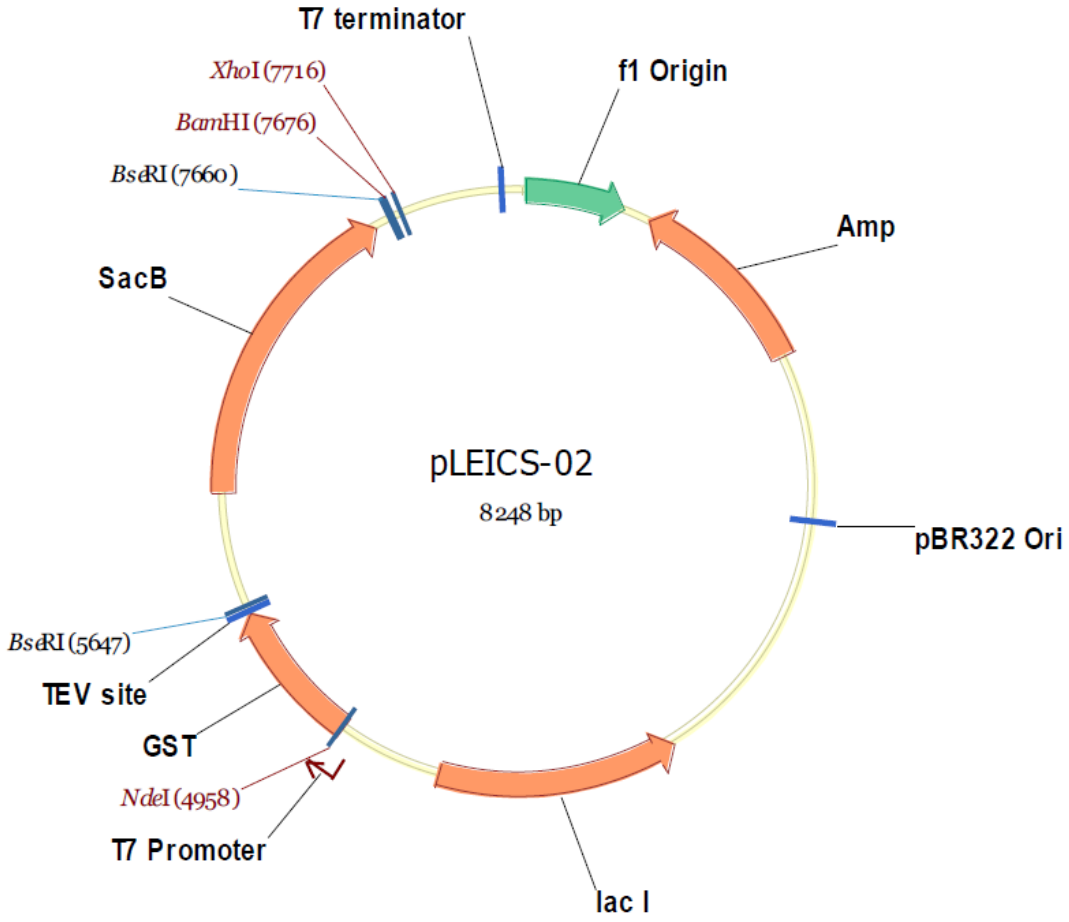


Figure 45. The structure of the pLEICS-02 plasmid. The plasmid has a GST-tag and an ampicillin/carbenicillin resistance gene as a selection marker.

5.3.3 Preparation of competent *E. coli* cells

Competent *E. coli* BL21 cells were prepared by the rubidium chloride method as described by (Green *et al.*, 2013), with modifications. *E. coli* BL21 was streaked onto LB agar and incubated aerobically overnight at 37°C. The following day, one colony was inoculated in 5ml of LB broth inside a 50ml falcon tube with a loosened cap. The tube was incubated overnight at 37°C with agitation at 220rpm using an Innova® 44 (New Brunswick scientific, USA) shaker incubator. After that, 2ml of the prepared culture was inoculated in 200ml of fresh LB broth in a 500ml flask and incubated aerobically at 37ºC with agitation at 220rpm. When the OD_{600} of the culture reached 0.3-0.4, it was chilled on ice for 15minutes. Then, the culture was transferred into a 50ml sterile polypropylene tube and centrifuged at 4500xg for 15minutes at 4°C. The supernatant was discarded and the tube was placed on ice. Following this, the pellet was carefully re-suspended in 0.4volume of the culture (20ml for each 50ml of cells) of pre-chilled transformation buffer 1 (TFB1). The TFB1 buffer consisted of 30mM potassium acetate (Sigma-Aldrich, USA), 100mM $RbCl_2$ (Sigma, Germany), 10mM of aqueous $CaCl_2$ (Fisher Scientific, UK), 50mM $MnCl_2$ (Sigma-Aldrich, USA), and 15% v/v glycerol (VWR Chemicals, Belgium). The pH was adjusted to 5.8 using diluted acetic acid (Fisher Scientific, UK).

The suspended cells were incubated on ice for an hour, then were centrifuged at 4500xg for 15minutes at 4°C using an Avanti J-E centrifuge (Beckman Coulter, USA). After that, the supernatant was discarded and the tube was placed on ice. The pellet was carefully re-suspended in 0.02 volume of the culture (1ml for each 50ml of cells) of a pre-chilled transformation buffer 2 (TFB2). The TFB2 buffer consisted of 10mM 3-N-morpholino propane-sulfonic acid (MOPS) (Sigma, USA), 75mM $CaCl_2$ (Fisher scientific, UK), 10mM $RbCl_2$ (Sigma, Germany), and 15% v/v of glycerol (VWR Chemicals, Belgium). The pH of the buffer was adjusted to 6.5 with the use of diluted NaOH (Fisher scientific, UK). The cell suspension was then incubated on ice for 15minutes. After that, aliquots of 100µl in 2ml sterile conical bottomed, screw capped tubes were prepared (Sarstedt, Germany), snap frozen in liquid Nitrogen and stored at -80ºC.

5.3.4 Transformation of *E. coli* BL21 with the recombinant plasmid

The PCR product of endolysin gene amplification was cleaned and sent for cloning as described in section 3.11.1. The construct that had the recombinant endolysin gene was amplified inside *E. coli* DH5α and extracted as described in section 3.11.2.

The success of the aforementioned steps thus far was confirmed by sending the construct to the PNACL service (The Protein Nucleic Acid Chemistry Laboratory (College of Medicine, Biological Sciences and Psychology) available at http://www2.le.ac.uk/colleges/medbiopsych/facilities-and-services/cbs/PNACL/dna-sequencing, to eliminate any probability of mutation that may have happened in the pLEICS-02 plasmid. The pGEX'5 and pGEX'3 primers (Appendix, 2) were used by the PNACL unit.

Thereafter, the recombinant endolysin construct was transformed into competent *E. coli* BL21 (DE3) to express the endolysin protein. Briefly, 100µl aliquot of the competent *E. coli* BL21 (DE3) was taken from -80^{0}C and placed on ice. 1µl of the extracted plasmid was added to the cells and left on ice for one hour. Then, a 42°C heat shock was performed for one minute, after which the tube was immediately placed on ice for another minute. 300µl of LB broth was added and it was incubated for 1 hour at 37°C with agitation at 220rpm, to enhance the recovery of bacterial cells.

After the incubation was over, 100µl of the mixture (bacteria + plasmid) was plated on LB agar supplemented with 150µg/ml of carbenicillin (Melford biolaboratories, UK) to select the transformant cells. Serial dilutions up to 10^{-2} were prepared and 100µl of each was plated on LB broth. The plates were incubated aerobically overnight at 37°C. The next day, transformant colonies containing the recombinant plasmid were ready.

5.3.5 Expression of recombinant endolysin

5ml of LB broth was poured on to plates that had moderate growth of transformant *E. coli* BL21, and the colonies were mixed and suspended in the LB brother using a spreader loop. The cell suspension was then added to 500ml of LB broth supplemented with 150µg/ml carbenicillin (Melford Biolaboratories, UK) in a 1litre flask. The expression incubation settings were carried out as described by (Oliveira *et al.*, 2015), with modifications. The flask was incubated at 37°C with agitation at 120rpm until the OD_{620} reached 0.5 (0.485 was used). The Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Melford biolaboratoris, UK) inducer was then added to a final concentration of 0.5mM to start the expression of the endolysin. The flask was further incubated at 16°C overnight (12-15 hours) with agitation at 150rpm. The next day, the culture was centrifuged at 5000xg for 20minutes at 4°C using an Avanti J-E centrifuge (Beckman Coulter, USA).

After the expression was completed, the pelleted cells were suspended with 50ml of suspension-lysis buffer that contained 25mM Tris-HCL (CemCruz, USA), 150mM NaCl (Fisher scientific, UK), pH 7.4. Then the following were added to the cell suspension, DNase (Invitrogen, UK) to a concentration of 50µg/ml, $MgCl_2$ 5mM (Acros organics, USA) and one tablet of protease inhibitor cocktail (Roche, Germany). The bacterial suspension was placed on ice and sonication was carried out. This sonication was carried out using a 9.5mm diameter probe at 60% of the maximum power of the sonicator (Sanyo, UK) for 20seconds for each pulse, for 7 pulses (cycles) with a minute rest (cooling intervals) between them to prevent the denaturation of the expressed protein as a result of the high temperature. After sonication, the lysate was centrifuged at 15000xg for 20minutes at 4°C using an Avanti J-E centrifuge (Beckman Coulter, USA) to remove debris. The supernatant was filtered with 0.22µM filter to further remove the debris.

5.3.6 Purification of recombinant endolysin

Glutathione sepharose resin (GE Healthcare, Sweden) was used to purify the recombinant endolysin by column affinity chromatography. 1ml of the resin was added to the purification column. After a moment, the column was calibrated with 30ml of binding buffer (25mM Tris-HCL (ChemCruz, USA) and 1M NaCl (Fisher scientific, UK), pH 7.4.

The clear lysate from the expression step was diluted 1:1 with the binding buffer and passed through the column. This step was repeated two more times to allow all the GST tagged endolysin to bind. After the sample was passed three more times through the column, the column was washed with 30ml of the binding buffer to get rid of the unwanted proteins.

The recombinant protein was eluted with 5ml of elution buffer. This buffer consisted of 50mM Tris-HCL (ChemCruz, USA), 10mM reduced L-Glutathione (Sigma-Aldrich, UK), and 5mM dithiothreitol (D.T.T) (Biomedicals, USA) pH 8. All the buffers and samples were allowed to pass through the purification column via gravity flow. The purified protein was further cleaned by an-ion exchange using an AKTA purifier (GE healthcare, Sweden) so as to remove both the elution buffer and any unwanted proteins. The purified protein concentration was then measured by using the Nano-Drop 1000 (Thermo-scientific, USA) machine.

5.3.7 Removing the GST tag

The purified recombinant protein was cleaved to remove the GST-tag using the Tobacco Etch Virus (TEV) protease (Sigma-Aldrich, UK). The TEV protease is an enzyme that strictly cleaves at a 7 amino acids recognition sequence. This recognition sequence is incorporated at the end of a tag's sequence to enable the removal of the tag from the recombinant proteins (Figure, 46) (Miladi *et al.*, 2012, Stols *et al.*, 2002). The cleavage was carried out according to the manufacturer's instructions. Briefly, 5ml of protein fractions were dialysed overnight at 4ºC in 2litres of Tris buffer (25mM Tris-HCl (ChemCruz, USA), 150mM NaCl (Fisher scientific, UK), and 14mM 2-mercaptoethanol (Sigma-Aldrich, UK) pH 8.0. The dialysis was carried out to replace the buffer with TEV buffer and to remove the reduced L-glutathione which will affect the subsequent binding of the cleaved GST tag in the purification column.

The next day, the dialysed 5ml protein was diluted 1:1 with 50mM Tris-HCl (final concentration of 25mM) and 300mM NaCl (final concentration of 150mM). 75µl of TEV protease added to the diluted protein and the mixture was incubated overnight at 4ºC. The digested protein was then passed through a newly prepared affinity purification column (1ml of Glutathione sepharose resin was added). The GST tag was bound to the column and the endolysin passed through. The purified tag-free protein was further cleaned by an-ion exchange using an AKTA purifier (GE healthcare, Sweden) to clear out and remove both the TEV nuclease and any unwanted proteins left in the sample. The flow through was used for the zymography and lysis experiment described in sections 5.3.8 and 5.3.9.

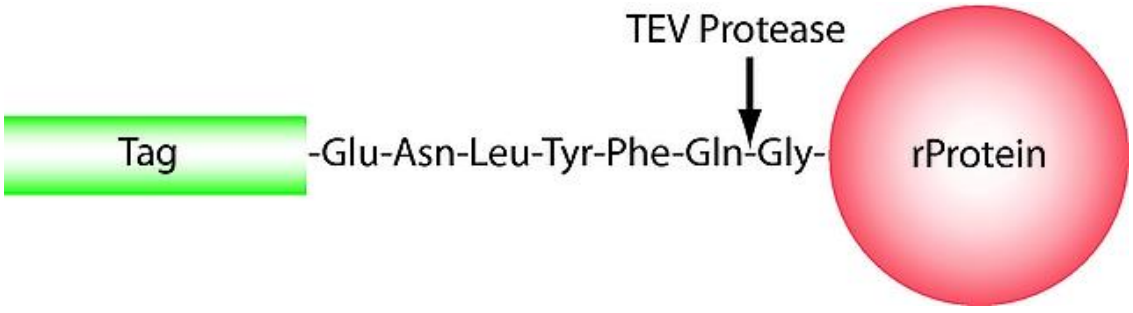


Figure 46. Recognition amino acids sequence and the cleavage site of TEV protease. This figure shows the 7 amino acids sequence is incorporated at the end of the tag, this is the site at which the TEV protease specifically acts. Adapted from http://www.sigmaaldrich.com/catalog/product/sigma/t4455?lang=en&region=GB

5.3.8 Zymography

In order to prove that the purified recombinant protein has muralytic activity, zymography was carried out as described by (Mukamolova *et al.*, 2006), with modifications. SDS-PAGE was carried out using a 12% polyacrylamide gel supplemented with 0.2% w/v of lyophilized *Micrococcus lysodeikticus* ATCC No. 4698 (Sigma-Aldrich, USA) as a substrate for the enzyme. 20mg of the substrate was added to 9.7ml of the SDS-PAGE gel (3.4ml distilled water, 2.5ml of 1.5M Tris–HCl (ChemCruz, USA) pH 8.8, 4ml of 30% (W/V) Acrylamide/Bis-acrylamide Protogel (National Diagnostics, England), and 100µl 0f 10% SDS (Fisher Scientific, USA), without the addition of ammonium persulfate (APS) solution and the N, N, N', N'-Tetramethylethylenediamine (TEMED). A nylon Pasteur pipette was used to homogenise the mixture. After this, 50µl of 10% APS (BioRad, USA), and 10µl of TEMED (Sigma, China) were added to the gel. The stacking gel was prepared as described in section 3.11.3.1 and was overlaid on the running gel. A comb of suitable size was used.

Endolysin aliquots with different concentrations (8.216, 8.907, 10.790, and 11.832 mg/ml), along with a pre-stained protein standard and a lysozyme solution from chicken egg white (Sigma-Aldrich, USA) as a positive control were loaded in the gel, as described in 3.11.3.2. The sample buffer used to prepare the endolysin for electrophoresis consisted of 0.313M Tris-HCl pH 6.8, 10%SDS, 0.01% bromophenol blue, 50% glycerol and 100mM DTT. The lysozyme control was prepared prior to use by dissolving 10mg in 1ml of 10mM Tris-HCL pH 8. Electrophoresis was performed at 150volt for 1hour using a PowerPac basic (BioRad, USA).

After running the samples using a 1X running buffer described in 3.11.3.3, the gels were rinsed and washed briefly (20minutes with slow shaking at room temperature on a rocker) with distilled water. Then, the gels were washed at room temperature for 3hours with a renaturation buffer consisting of 20mM KH_2PO_4 (Fisher scientific, UK), and 50mM NaCL (Fisher scientific, UK) pH 7.2, supplemented with 2% triton X100 (Fisher scientific, USA), to remove the SDS and allow for renaturation. This buffer was changed every hour. Thereafter, the gels were incubated overnight at 37ºC using the same renaturation buffer but without triton X100.

The following day, the gels were stained for 2hours with slow agitation at room temperature with methylene blue 0.1w/v (Sigma-Aldrich, USA) prepared in 0.01M

potassium hydroxide (FSA laboratory supplies, England). Then, it was de-stained with distilled water and slowly agitated at room temperature until the clearance became visible.

5.3.9 Lysis assay

A lysis assay was carried out in order to determine the ability of endolysin to lyse *C. perfringens* strains. The experiment was conducted as described by (Zimmer *et al.*, 2002b) with modifications. Briefly, *C. perfringens* was grown in TY broth until it reached log phase (OD_{550} ~ 0.2), it was then aliquoted in 1.8ml aliquots into 2ml micro-centrifuge tubes. After that, *C. perfringens* cells were harvested by centrifugation at 10000xg for 10minutes at 4ºC (Thermo-Scientific, Germany). The pelleted cells were washed twice with 1.8ml of pre-reduced phosphate buffered saline (PBS) (Oxoid, England) pH 7.5 and re-suspended with 1.6ml of pre-reduced TY broth, inside the anaerobic chamber. CFU was performed for each tested *C. perfringens* strain, after which the culture was distributed across a 96 microtiter plate (290µl per well). A diluted endolysin stock was mixed with the cell suspension to a 300µl total reaction volume in the microtiter plate. A final concentration of 2, 1, and 0.5µg/ml of endolysin was used with three repeats for each concentration.

TY broth was used as a negative control to exclude any contamination. An untreated culture of each tested *C. perfringens* strain was also set to monitor the normal growth and ensure that the reduction in the CFU in the treated wells is the result of endolysin treatment. The CFU of the treated strains was taken after one hour of anaerobic incubation at 37ºC. 10µl of each culture was directly spotted on the TY agar, while another 10µl was suspended in 90µl PBS that was kept at room temperature to prevent further cell growth. A serial dilution was made and 10µl of each dilution was spotted on TY agar.

5.4 Results

5.4.1 Bioinformatics analysis of CPAP1

The amino acid sequence of CPAP1 endolysin has 97% similarity with the endolysin of phage phi24R and 80% similarity with phage CPS1 (Figure 41 in 4.4.11). It also has between 54-57% similarities with endolysin genes of another 6 *C. perfringens* phages endolysin (Figure, 47).

```
ΦCP340      mkialrgghspnckganvlrdeqscmwaladevekvltshghtvvrcettlsneredvrq    60
Φ39-O       mkialrgghspnckganvlrdeqscmwaladevekvltshghtvvrcettlsneredvrq    60
CPS1        mkigirdghspnckgaialrdeqacmrilcrevievlekhghevvycgsnartengelse    60
CPAP1       MKIGIRDGHSPNCKGAIGLRDEQSCMRVLCKEVIEILEKHGHEVVYCGSNASTQNGELSE    60
Φ24R        mkigirdghspnckgaiglrdeqscmrvlckevieilekhghevvycgsdastqnselse    60
CPV1        miigsrtghskkclgavslrnewecmnilerevnkilkahghtiidcnssastengelse    60
ΦCP26F      miigsryghsencrgakglrdevdamkplhfefkkimeqyghtiidccsnantqngelse    60
            * *. * *** :* **  **:*  .*  *  *. :::  :** :: * :   .:. :: :

ΦCP340      gakkg--yncdmfislhmnasd-grgngteawvarsarssikeiasrlcknyatlglqnr   117
Φ39-O       gakkg--yncdmfislhmnasd-grgngteawvarsarssikeiasrlcknyatlglqnr   117
CPS1        ginkansnnvdifislhmdvskehkangtcsfvakearksirdiaqrlvdnfetlglqnr   120
CPAP1       GVRKANNSNVDIFISLHMNSFN-GQAQGTEALVTVGARNSIKEIASRLCKNFASLGLVNR   119
Φ24R        gvrkannsnvdifislhmnsfn-gqaqgtesliavgarnsikeiasrlcknfaslglvnr   119
CPV1        gcrkanaqhidiflsyhmnaskdhkghgtecwvhpnarasckeiasrissnlsklgfynr   120
ΦCP26F      garkanaqildlfiswhgnk---gggqgceawiannsra--kpyaermcknfsslgfknr   115
            * .*.     *:*:* * :      .:*  . :   :*   :  *.*: .*  .**: **

ΦCP340      gvke-knywemtdtncpniifetmfcddkhdi-diwastswdklarlianaidpniplek   175
Φ39-O       gvke-knywemtdtncpniifetmfcddkhdi-diwastswdklarlianaidpniplek   175
CPS1        gvre-snyremrevnapniifetmfcdnlhdinevwsptpyekmallianaidpti---k   176
CPAP1       GVKE-VNLYEMKNVKAPNIIFETMFCDNPHDINEVWSPTPYEKMALLIANAIDPTI---K   175
Φ24R        gvke-vnlyemknvkapniifetmfcdnehdinevwsptpyekmallianaidpti---k   175
CPV1        gvkt-gllyemknvnapniiietcfcdnekdi-giwsptpyevmarhianaldpsipiee   178
ΦCP26F      gvkysdkyyemrninapniifetlfldsekdi-siwspipyevmarylanaidpniplek   174
            **:      ** : :.****:** * *. :**  :*:   :: :*  :***:**.*   :

ΦCP340      eqdyyrvavqrfkskedaekakqrisselgyycfteki-        213
Φ39-O       eqdyyrvcvqrftnkedaekaqqrisnelgyycfaeki-        213
CPS1        enelyrvvvqyfnskedaencqqeiak--rwycfvegcn        213
CPAP1       ENELYRVVVQYFNSKEDAENCQQEIAK--RWYCFVEECN        212
Φ24R        enelyrvvvqyfnskkdaencqqeiak--rwycfveecn        212
CPV1        kpkrwqvrvyaftskeeaqkysdritkelkaynvveei-        216
ΦCP26F      eqdyyrvcvqrftnkedaekaqqrisnelgyycfaeki-        212
            : . ::* *  *..*::*:: .:.*:.    * ..*
```

Figure 47. Alignment of the CPAP1 endolysin amino acids sequence with endolysin sequences of six *C. perfringens* phages. The CPAP1 endolysin has different percentages of similarities with other *C. perfringens* phages endolysins; 97% with Φ24R, 80% with CPS1 phage, 57% with ΦCP340, 57% with ΦCP39-O, 55% with ΦCP26F, and 54% with CPV1.

The structural analysis of CPAP1 endolysin using Pfam domain analysis showed that it belongs to the amidase-3 (*N*-acetylmuramoyl-L-alanine amidase) family. It is composed of 212 amino acids. Moreover, the molecular weight of this endolysin is 23.884kD, as determined by ExPASy online and shown on SDS-PAGE.

5.4.2 Cloning, expression, and purification of recombinant endolysin

The CPAP1 endolysin was cloned as described in 5.3.2. A gradient PCR was carried out to amplify the coding sequence (Figure, 48); annealing temperatures ranging between 56-58ºC annealing temperatures gave a PCR product. The analysis of the recombinant plasmid sequence revealed that no mutation occurred in the insert.

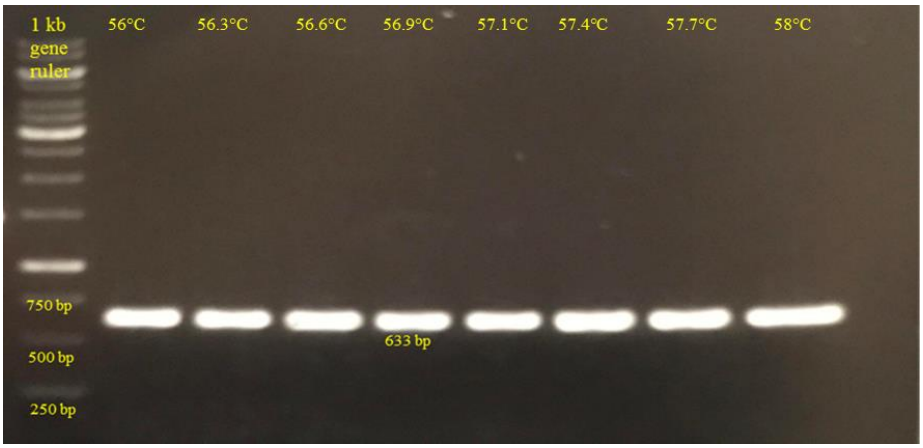


Figure 48. Gel electrophoresis image of a gradient PCR showing a successful amplification of endolysin gene and the adapter sequence. (633bp) compatible with pLEICS-02 plasmid. A range between 56-58°C annealing temperatures gave a PCR product, which was used for endolysin cloning by the PROTEX service.

After the expression and purification of the recombinant endolysin, it was analysed by SDS-PAGE. The GST-tagged endolysin bands were approximately 50kB in size (Figure, 49). Although the enzyme was successfully expressed and purified, further optimization for both the expression and purification processes is required so as to produce more pure endolysin with higher final yields.

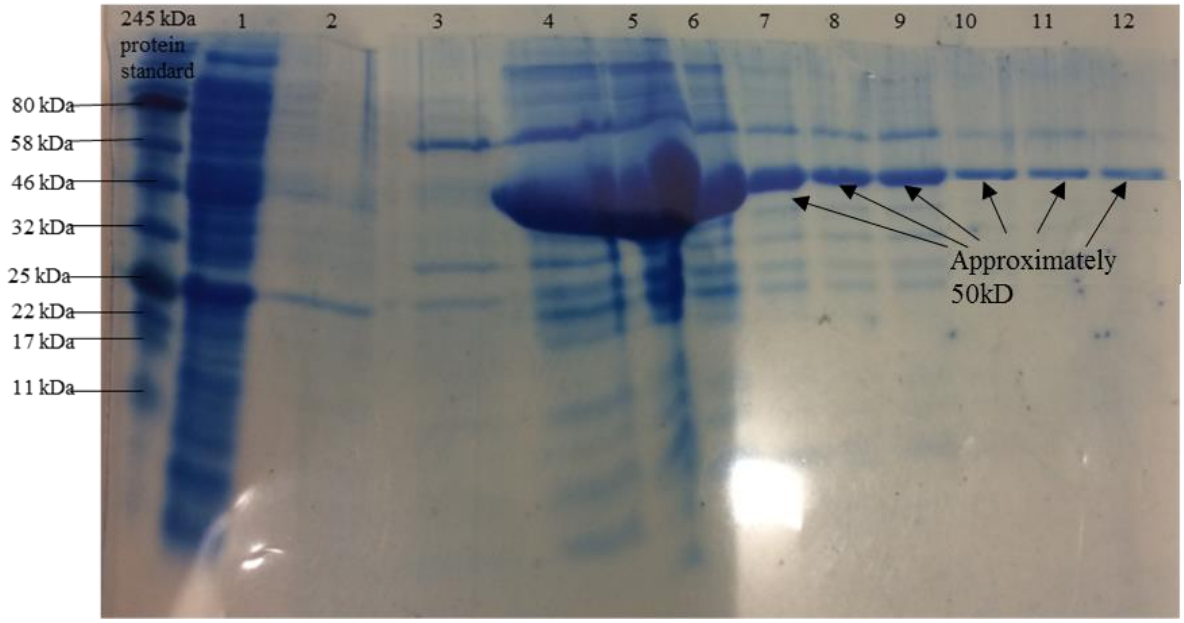


Figure 49. SDS-PAGE image shows the purified recombinant endolysin after it was cleaned by anionic exchange. The first lane on the left is a wide range protein standard (New England Biolabs, UK). (1) eluted protein before cleaning. (2) control of un-induced E. coli culture. (3-12) are the 50kB (approximately) GST-tagged recombinant endolysin obtained after cleaning with the AKTA purifier. This purification yielded 0.5ml fractions.

5.4.3 Removing the GST tag

The endolysin enzyme has an N-terminal catalytic domain, as reviewed in 2.2.7. The GST tag was removed as described in section 5.3.7 to free the catalytic domain of the enzyme. After the cleavage of the tag, the solution was column purified again using a binding buffer without reduced L-glutathione to keep the tag bound to the column while the endolysin passed and accumulated in the flow through.

The result revealed that the recombinant protein was partially cleaved as two bands were obtained; one had a molecular weight of approximately 24kDa, which is the endolysin, and the other weighed approximately 50kDa, which is the tagged endolysin (Figure, 50). It seems that the high concentration of protein resulted in a partial cleavage of the tag and in the passage of the un-cleaved protein through the column. This result suggests that the cleavage conditions need further optimization.

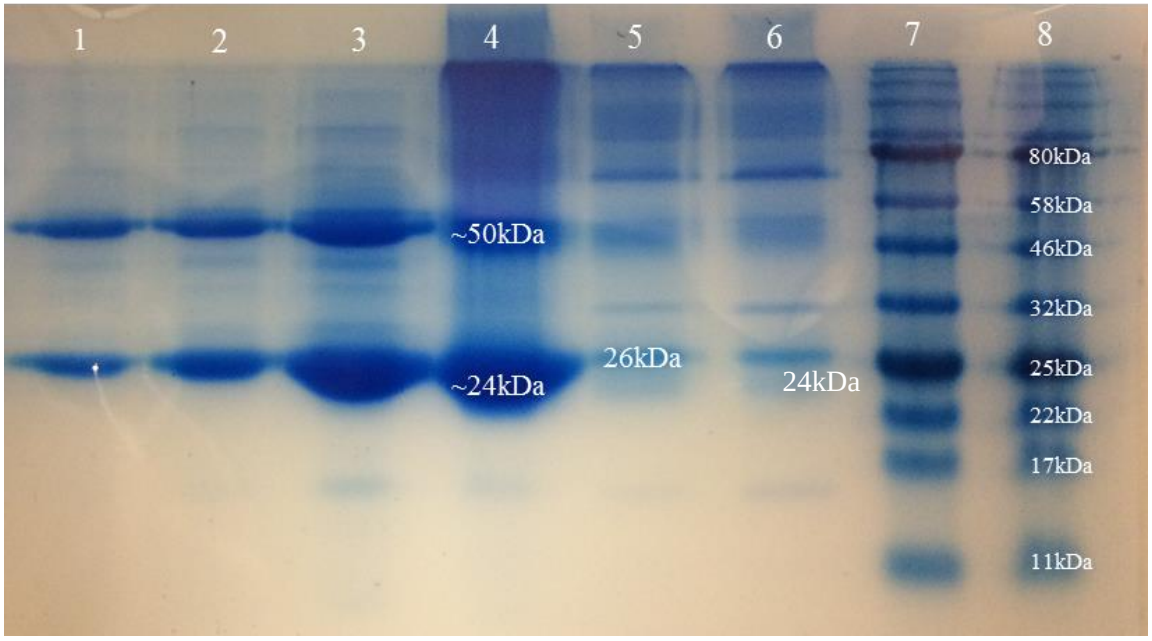


Figure 50. SDS-PAGE image shows the recombinant endolysin after it was cleaved by the TEV enzyme and cleaned by an AKTA purifier. (1-6) show two bands, a ~24kDa of the endolysin only and ~50kDa of the GST-tagged endolysin. Lanes (5 & 6) show three bands, a 26 kDa band of the GST tag only, ~24kDa band of the endolysin, and ~50kDa of the GST-tagged endolysin. (7 & 8) are wide range protein standard (New England Biolabs).

5.4.4 Zymography

After cleaving the GST tag and cleaning the recombinant protein with an AKTA purifier, the solution was filter sterilized and used for zymography. The result confirmed that the recombinant endolysin of the *C. perfringens* phage has muralytic activity, as it was able to hydrolase the cell wall substrate of *Micrococcus lysodeikticus* supplemented in the polyacrylamide gel. The activity was confirmed as a clearance band occurred when using a renaturation buffer of 7.2 pH (Figure, 51).

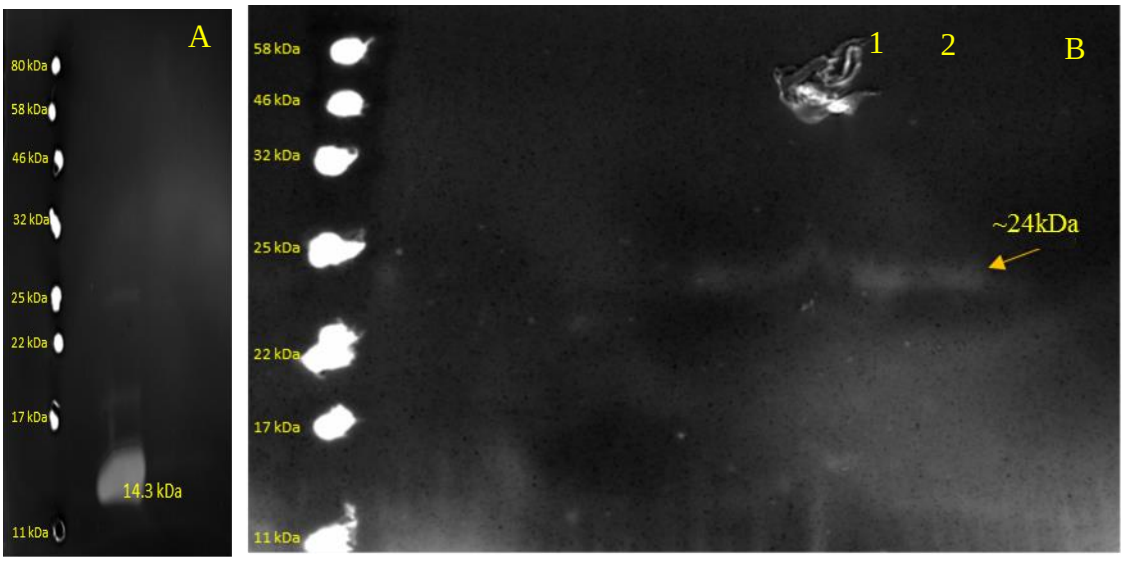


Figure 51. The muralytic activity of recombinant endolysin in a zymogram. (A) The clearance caused by the lysozymes (positive control), protein standard on the left and a clearance band at ~14.3 kDa. Part B shows the clearance caused by the purified, cleaned recombinant endolysin. Protein standard on the left of the image and clearance bands on lanes 1 and 2 (highlighted with an arrow on the right hand side of the image) at ~24 kDa,. The recombinant protein has renatured in a buffer that had 20 mM KH_2PO_4, and 50 mM NaCL pH 7.2.

5.4.5 Lysis assay

The ability of the recombinant endolysin to lyse *C. perfringens* strains (CP7, CP14, CP28, CP41, CP53, CP65, and CP73) was assessed using a lysis assay. Seven strains were treated with the enzyme, and the results revealed that it was able to stop the growth of six *C. perfringens* strains and completely lyse them. Only one strain was not completely terminated (Figure, 52).

The endolysin has a wider host range in comparison with the parent phage. While the endolysin parent phage could propagate on six *C. perfringens* strains, the derived endolysin could lyse another seven *C. perfringens* strains that the parent phage could not propagate on or cause lysis from without.

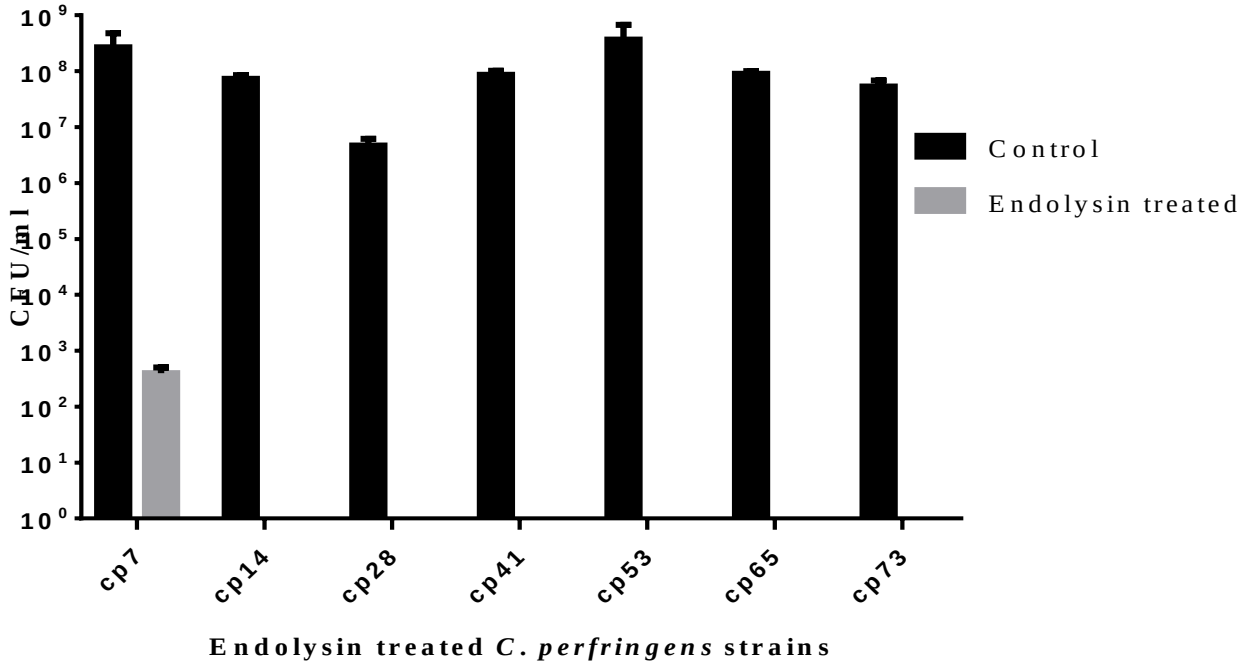


Figure 52. The lysis of seven strains treated with the phage endolysin. All the strains were completely lysed except for the *C. perfringens* number 7 strain (the first two columns on the left), which was partially lysed. An untreated *C. perfringens* culture of each strain was used as a control (black columns).

5.5 Discussion

In this chapter, we identified and proved that the ORF13 of the CPAP1 phage encodes N-acetylmuramoyl-L-alanine amidase. This enzyme is expressed by *C. perfringens* phage CPAP1. The recombinant CPAP1 endolysin has the potent ability to completely lyse most of the tested *C. perfringens* strains, making it a candidate medication for *C. perfringens* treatment.

The ability of *C. perfringens* strain number 7 to partially resist the lysis caused by endolysin reinforces the need of using an endolysin cocktail. Such a cocktail with the ability to completely lyse known *C. perfringens* strains would definitely provide a strong case for the prophylactic use against gas gangrene if administered early after traumatic injury (via a spray or ointment). It may also be able to treat the systemic infection (upon early diagnosis), gas gangrene, and all other types of intestinal infections caused by *C. perfringens*, as it lyses *C. perfringens* strains regardless of which toxin-type they are.

In order to confirm endolysin's suitability to treat these infections, a clinical trial should be carried out. The laboratory settings can determine the outcome of the

interactions between the enzyme and the bacteria under optimum conditions for both. However, the infection environment has many factors which may possibly affect the bacteria and the enzyme positively or negatively. For example, the different bacterial metabolic products which are produced by other microorganisms at the site of infection, gut microbiota (and other wound contaminants) may inhibit, block or lyse the enzyme, as well as provoke or inhibit the growth of *C. perfringens.* The immune response against the enzyme as an antigen is another example.

The conditions of the expression and purification of the *C. perfringens* phage endolysin was optimized as described in 5.2.5 and 5.2.6. These conditions can possibly be used to express and purify other *C. perfringens* phage endolysins. The lytic activity of the recombinant endolysin was confirmed with a zymogram.

The study of interactions between endolysin and growing *C. perfringens* cells in TY broth using direct CFU/ml can uncover any ability of these strains to resist lysis caused by endolysin, if there is any resistance found. It can also uncover the ability of *C. perfringens* strains grown in the presence of this enzyme by forming a biofilm to physically hide the bacteria away and keep it out of the enzyme's reach. While the turbidity reduction test can show the time required for an endolysin to lyse *C. perfringens* strains in a suspension buffer, the bacteria will not have any metabolic activity inside the buffer used in turbidity test due to the presence of oxygen and the absence of essential nutrients.

5.6 Conclusion

The findings of this chapter show that the recombinant endolysin can be used for the treatment of local and intestinal infection caused by *C. perfringens.* However, more characterisation is required, as explained in the discussion. It can also be used as a prophylactic agent to prevent gas gangrene by covering the burns and traumatically injured areas of the body with the enzyme.

The endolysin has a wider host range than the parent phage; this highlights the potential of endolysin as a novel antimicrobial agent. It can act at a pH and temperature similar to that of blood and can thus be used to treat the systemic infections caused by *C. perfringens* upon early diagnosis. However, further studies are needed to determine the time required by the immune system to clear the endolysin from the circulation.

5.7 Future work

1. Clone, express, and purify the endolysin of more *C. perfringens* phages.
2. Determine the host range (lytic spectrum) of these endolysins on a large number of *C. perfringens* strains.
3. Determine the ability of endolysins to prevent spore germination.
4. Determine the minimum inhibitory concentration of endolysins.
5. Determine the specificity of these endolysins by testing their lytic ability on different bacterial species. This is particularly important when treating intestinal infections.
6. Test the lytic activity of endolysins in different media, pH and temperature so as to determine their lytic activity in different conditions that mimic the infection environment (intestine, skin, and blood stream).
7. Assess the stability of these endolysins in different suspension liquids after long storage at room temperature and at 4ºC.
8. Determine the *in-vivo* activity of the endolysins using animal models for both local and systemic infections.
9. Test the ability of endolysin cocktails to lyse all the isolated *C. perfringens* strains.

Chapter 6

Optimization of electro-transformation parameters for *C. difficile*

Chapter 6. Optimization of electro-transformation parameters for *C. difficile*

6.1 Introduction

In the first part of this chapter, we test the hypothesis stating that by using a wide range of both transmembrane voltage and pulse durations (Ut), it is possible to transform *C. difficile* by electroporation. Using our understanding of electroporation mechanism and considering the successful electro-transformation of *Clostridium* species other than *C. difficile*, we applied variable parameters. Changes in each parameter value in every experiment setting were decided depending on the effect (role) of that particular parameter. These parameters were changed in the context of gradually increasing the damage caused by Ut (the thermal electric effect) to the *C. difficile* physical barriers, in order to determine an interval at which this damage remains reversible and the DNA can pass through into the cell. After applying many settings, it became obvious that the reasons for the unsuccessful transformation is due to the complex structure of the *C. difficile* physical barriers (thick peptidoglycan and surface layer proteins) which restrict the passage of DNA into *C. difficile*, and the biofilm production which prevents physical contact between the DNA and *C. difficile* cells.

In the second part of this chapter, we test the hypothesis that by preventing biofilm formation and diminishing the thickness of *C. difficile* physical barriers, the cells may become susceptible for transformation. Considering our understanding of the processes of biofilm formation, surface layer proteins formation, and the peptidoglycan bonds, we used different treatments and growth conditions to prepare *C. difficile* for electroporation.

C. difficile grows in a biofilm which enables it to resist a variety of environmental stresses such as oxygen stress, antibiotics, and immune defence systems (Dapa *et al.*, 2013). Biofilm prevents direct physical contact between bacterial cells and the environmental stress factors by providing an enclosed environment (Beloin *et al.*, 2008, Watnick *et al.*, 2001). Similarly, the presence of *C. difficile* cells in a biofilm prevents the contact of these cells with the DNA that is used to transform these cells. *C. difficile* was grown with agitation to disperse cells and prevent biofilm formation.

C. difficile has a thick protein coat mainly consisting of two proteins, the high molecular weight and the low molecular weight proteins. These two proteins result from the cleavage of the precursor protein SlpA inside the cell. Consequently, these two proteins reassemble on the cell surface to form a proteinaceous lattice layer on the cell surface (Calabi *et al.*, 2002, Fagan *et al.*, 2009, Dang *et al.*, 2010). A cysteine protease inhibitor (reviewed in detail in section 6.1.5) was used to prevent the cleavage of the surface layer protein precursor SlpA into high and low molecular weight proteins, and thus to diminish the outer protein layer so as to facilitate the passage of DNA by electroporation.

Lysostaphin (reviewed in detail in 6.1.6) was used to increase the diameter of the normally existing tiny pores in the peptidoglycan layer. The diameter of pores in *E.coli* range between 16-24Å (Bonardi *et al.*, 2011).

6.1.1 *C. difficile* infection and phage therapy

The CDI number has largely grown in the last decade with increased incidence of multi-antibiotic resistance *C. difficile* strains in Europe. The development of this resistance can be attributed to the sub-inhibitory doses of the antibiotic in the lumen, the ability of *C. difficile* to grow in a biofilm, the heterogenicity of metronidazole resistance, the presence of proteins in the intestine which helps the bacterial DNA repair, and the mutation in the vancomycin binding site (Mirecka, 2017). A new antimicrobial agent that does not affect the gut microbiota or participate in the dissemination of antibiotic resistance is urgently required (Kirk *et al.*, 2017b). Consequently, interest in phage therapy has been renewed (Garneau *et al.*, 2017). The use of *C. difficile* phage combinations has demonstrated potential to treat CDI, as it was able to terminate *C. difficile* culture *in-vitro* and delayed the onset of symptoms *in-vivo* using an animal model. However, few studies have focused on the use of phage therapy to treat CDI, mainly because all the isolated *C. difficile* phages are temperate (Nale *et al.*, 2016b). Yet, there is no record of a strictly lytic *C. difficile* phage in the literature.

6.1.2 Genetic manipulation of *C. difficile*

Currently, *C. difficile* genetic manipulation is dependent on conjugation as the only available method to transform *C. difficile* with plasmid DNA. The transformation efficiency varies among *C. difficile* strains, with *C. difficile* ribotype 20291 being refractory to transformation (Kirk *et al.*, 2016).

The optimization of the electroporation parameters for microorganisms which are difficult and laborious to transform by conventional methods provides an additional tool to genetically manipulate these microorganisms. Electroporation has a higher efficiency in comparison with chemical transformation (Deng *et al.*, 2017).

Since highly effective transformation protocols have been described for *E. coli* and *Pseudomonas aeruginosa*, phages that infect these species are almost the only engineered viruses, as the *in-vitro* modified phage nucleic acid needs to be delivered back into bacterial host cells so as to obtain a viable phage progeny (Pires *et al.*, 2016). Electroporation has been successfully used to deliver whole phage DNA to *E. coli* (Planelles *et al.*, 1999, Inoue *et al.*, 2016).

C. difficile phages can be genetically improved to be used for CDI treatment. It is worth investigating the effects of the deletion of the phage integrase gene, how integrative the phage remains and how the lytic activity is affected. The properties of phages can also be improved by the insertion of genes that can disrupt *C. difficile* toxin genes. In order to accomplish this, a potent *C. difficile* genetic transformation system is required to deliver the engineered genetic material back to the bacterium.

6.1.3 Electro-transformation of *C. difficile*

There is only one report for the transformation of *C. difficile* by electroporation by Ackerman et al. (2001). The study states that the most important factor for the successful electro-transformation of *C. difficile* is strain characteristic. Another important factor is the type of nucleic acid used. This conclusion was reached given the outcome of the experiments, as only one strain out of nine was transformed in that study, and the transformation efficiency varied (using the same settings) with different plasmid types.

6.1.4 *C. difficile* cell wall

The *C. difficile* cell wall (Figure, 53) consists of a thick de-acetylated peptide-glycan (PG) layer that has unique cell wall glycol-polymers (CWGs) bound to a surface layer (S-layer) of protein lattice surrounding the whole PG. The S-layer has an unusual structure; it is composed of two distinct proteins, a high molecular weight (42–50 kDa) protein and a low molecular weight (22–38 kDa) protein, and an additional 28 other proteins (Brüggemann *et al.*, 2009, Kirk *et al.*, 2017a, Cerquetti *et al.*, 2000). These proteins are expressed at a very high level and have the ability to form two dimension arrays. There is a variability in the molecular weight of these two proteins between

different *C. difficile* strains. Consequently, they present on SDS-PAGE between 22kDa and 55kDa (Dawson *et al.*, 2009). The above-mentioned cell wall complexity and thickness represents a physical barrier that inhibits and limits the passage of DNA by electroporation.

The *Bacillus spp*. S-layer protein differs from the *C. difficile* S-layer; it is composed of two similar thick protein layers consisting of a high molecular weight protein. The S-layer of *Bacillus spp*. does not have a low molecular weight protein (Leduc *et al.*, 1977).

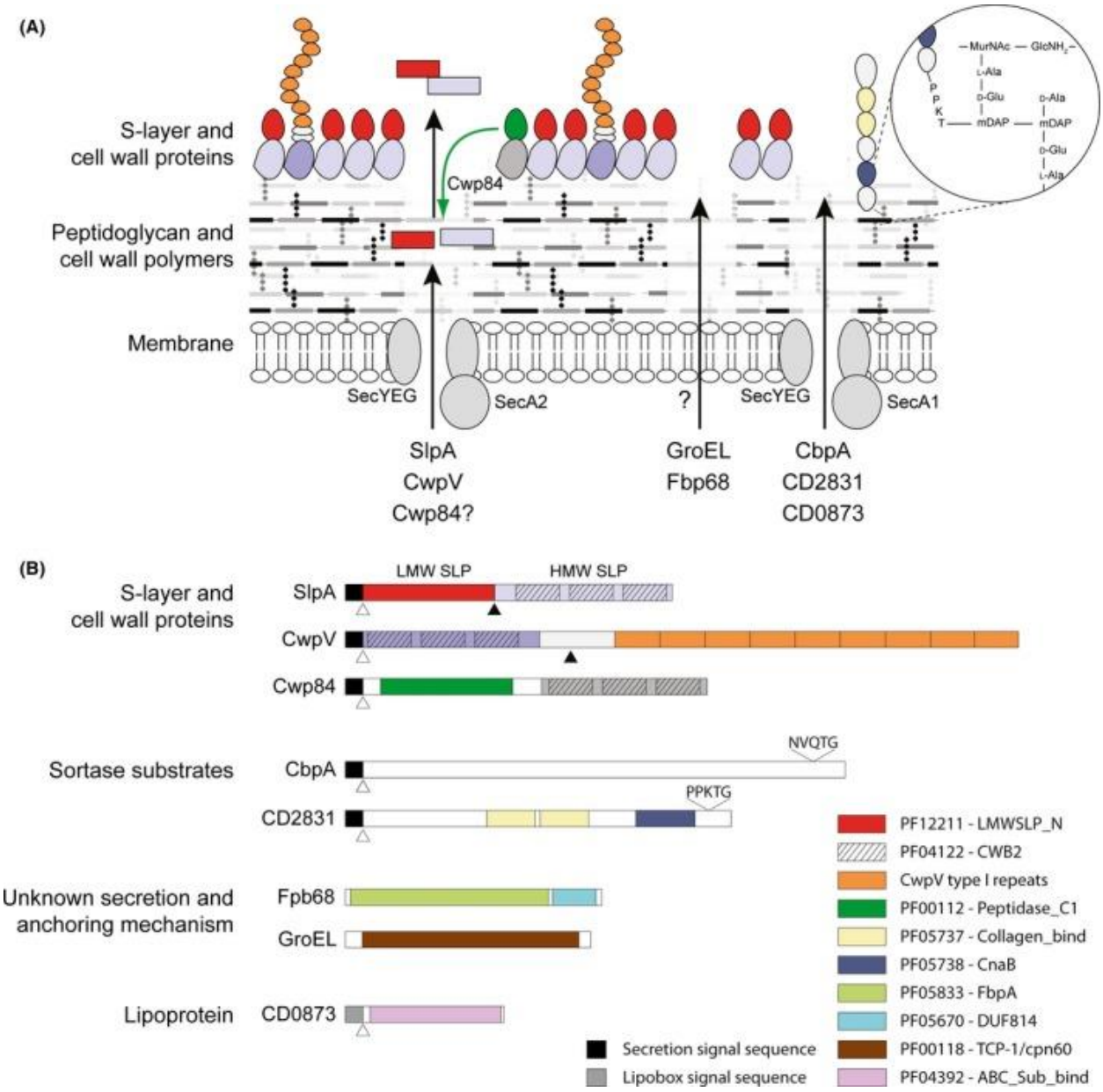
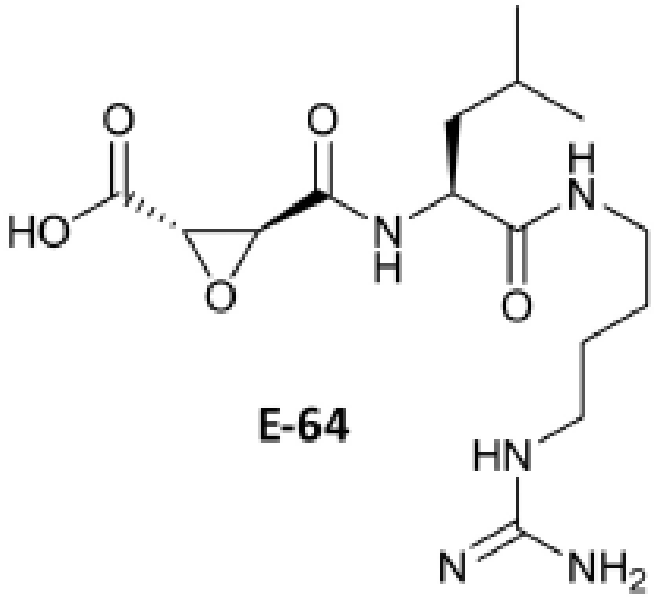


Figure 53. Complexity and thickness of *C. difficile* cell envelope.

A) The *C. difficile* cell envelope. The outer layer is decorated by the cell wall proteins family. SlpA, S-layer, and cell wall binding proteins are shown at the top. In the middle, the peptidoglycan and the glycopolymers are shown. The plasma membrane is shown beneath the peptidoglycan layer. This complicated structure can explain why *C. difficile* is refractory to transformation. B) Organization of the protein domains are shown. Black boxes refer to the secretion signals of N-terminal. Black arrows refer to the post-secretion cleavage sites of the SlpA and CwpV. The sorting motifs location and sequence are shown above CD2831 and CbpA (Kirk *et al.*, 2017a).

6.1.5 Cysteine protease inhibitors

Proteases play an important role in the regulation of biological processes through the degradation of enzymes and protein molecules that are involved in these processes (Domsalla *et al.*, 2008, Vicik *et al.*, 2006). The HMW and LMW proteins of the *C. difficile* cell wall are generated by the cleavage of the surface layer protein precursor SlpA by a cysteine protease Cwp84. This process can be inhibited using a cysteine protease inhibitor E-64 (Figure, 54). Thus, the SlpA precursor will be produced and trapped inside the bacterial cell. Consequently, the thick surface protein layer will become thinner (de la Riva *et al.*, 2011, Dang *et al.*, 2010).



E-64

Figure 54. Structure of the synthetic cysteine protease inhibitor E-64. (Dang et al., 2010). Above is a schematic of the chemical structure of E-64.

6.1.6 Lysostaphin

Lysostaphin is an antimicrobial enzyme that is able to cleave the penta-glycine bridge found in the peptidoglycan of *Staphylococci.* It was previously thought to be a bacteriocine that helps *Staphylococci* to compete and survive in the presence of other bacterial species in the environment (Bastos *et al.*, 2010). However, it was recently found that lysostaphin plays an important role during *Staphylococcus aureus* division. It allows the cell to divide through the cleavage of the cell wall peptidoglycan (Raulinaitis *et al.*, 2017).

Lysostaphin has been used to optimize electroporation parameters for *Staphylococcus carnosus* and *C. perfringens* because of the penta-glycine peptidase activity that it possesses (Gao *et al.*, 2014, Lanckriet *et al.*, 2009). It may also help in the optimization of electroporation parameters for *C. difficile*.

6.1.7 Aims and objectives

In this chapter, we will test the hypothesis that an electroporation protocol can be optimized and used for the genetic manipulation of *C. difficile* phages in order to improve and manipulate their properties as therapeutic agents. The following objectives are set:

1. Empirically optimize electroporation parameters to transform *C. difficile* ribotypes R076 with phage DNA, and R220 with plasmid DNA. This can be achieved by:
    a. Using varying transmembrane pulses.
    b. Reducing the biofilm formation by culturing *C. difficile* with agitation.
    c. Diminishing the physical barriers (surface layer protein and peptide glycan) by the treatment of bacteria with cysteine protease inhibitor (E-64) and lysostaphin.

6.2 Materials and methods

6.2.1 Phage DNA preparation

Phage phiCDHM1 was ultra-centrifuged at 30,900RPM for 3 hours at 4^0C using an ultra centrifuge (Sorvall-Discovery SE/Kendro laboratory products, Hitachi, Japan). Phage DNA was extracted by the phenol/chloroform/isoamyl alcohol method as described in 3.10.1. The extracted DNA was concentrated using a SpeedVac savant (Thermo-Scientific, UK) vacum drier and DNA concentration was determined. The DNA was stored at -20^0C to be used in the optimization of electroporation of *C. difficile* R076, as it is the propagation strain of phiCDHM1.

6.2.2 Plasmid DNA preparation

E. coli strains that harbor the plasmids pMTL82151 and pMTL84151 (Heap *et al.*, 2009b) were grown on Luria Bertani broth, (Fischer scientific, U.K) supplemented with 30µg/ml chloramphenicol (Sigma, Germany), aerobically overnight at 37ºC in a shaker incubator using the Innova® 44 (New Brunswick scientific, USA) incubator. The plasmid was extracted using a mini-prep kit (Sigma Alderich, USA) as described in 3.11.2. The extracted plasmid DNA was concentrated using a vacuum drier and the DNA concentration was determined. The DNA was stored at -20^0C to be used in the optimization of electroporation of *C. difficile* R220, as it harbours phiCDHM1 as a prophage.

6.2.3 Electroporation of *C. difficile* using different transmembrane pulses

6.2.3.1 Preparation of C. difficile cells for electroporation

C. difficile R076 was chosen as it is susceptible to infection with 10 of the isolated phages (phiCDHM1, phiCDHM2, phiCDHM3, phiCDHM5, phiCDHM6, phiCDHM10, phiCDHM11, phiCDHM13, phiCDHM15, and phiCDHM16) available from Professor Clokie's laboratory. The success of the optimization of R076's electroporation would make the genetic manipulation of all these 10 phages possible.

R220 was chosen because it harbours the CDHM1 and CDHM3 bacteriophages as prophages; these phages can be engineered by homologous recombination using the semi-suicide plasmids pMTL82151. The engineered prophage can be induced from R220 as described by (Hargreaves, 2013).

Electroporation was conducted as described by (Ackermann *et al.*, 2001) with modifications. *C. difficile* was grown on blood agar as described in section 3.2.1, but 100µl of FA culture was inoculated in to 1ml of pre-reduced and pre-warmed BHI broth. The tube was incubated anaerobically for 12hours at 37ºC. It was then centrifuged at 3000xg for 20minutes at 4ºC. The pellet was washed once with 10ml of a cooled pre-reduced electroporation buffer (sodium magnesium phosphate [SMP] buffer consisting of aqueous magnesium chloride, 0.2g, sucrose 92.4g, 70ml of 100mM tri sodium phosphate in 1litre, pH 7.4). The pellet was re-suspended gently in the SMP buffer without vortexing and centrifuged again at 3000xg for 20minutes at 4ºC. Finally, the pellet was re-suspended in 5ml of cooled pre-reduced SMP buffer incubated in ice for half an hour. Volumes of 0.8ml or 0.4ml of cell suspension was used for the electroporation experiment. The setting was repeated but at room temperature without cooling the SMP buffer on incubation in ice.

6.2.3.2 Setting the transmembrane pulse (Ut)

A BIORAD *E. coli* Pulser (USA) was used to transform *C. difficile* by electroporation. A wide range of pulse durations were used (1-2.5kV/cm and 5-50.1mSec.). The field intensity (kV/cm) and the time constant of the pulses (U_t) were calculated using the following equation, as instructed by the BioRad guide available online at http://www.bio-rad.com/webroot/web/pdf/lsr/literature/4006174B.pdf

$$\text{Field intensity kV/cm} = \frac{\text{Voltage (kV)}}{\text{Cuvatte gap (cm)}}$$

$$Pulse\ duration\ (msec.) = Capacitance\ (\mu F) \times \text{Resistance } (\Omega)$$

Two different cuvettes were used; the first was with a 2mm gap and 1cm^2 electrode, and the second was a cuvette with a 4mm gap and 2cm^2 electrode (Gene Flow, UK).

6.2.3.3 Electroporation

0.8ml or 0.4ml of *C. difficile* cells were transported into the electroporation cuvette and the DNA was added at different concentrations (1-10µg/ml). The experiment was carried out with pre-pulse incubation at room temperature and with incubation on ice. After electroporation was performed the cuvette was incubated for 10minutes on ice. This

step was excluded when the experiment was carried out at room temperature. Thereafter, 4.8ml of a pre-reduced Trypticase glucose-yeast extract (TGY) broth (Appendix, 1) maintained at room temperature was inoculated with the cell suspension and incubated anaerobically for 3hours at 37ºC to recover the electroporated cells, as described by (Ackermann *et al.,* 2001). After the incubation was completed:

1. For phage DNA, 500µl of bacterial suspension was mixed with 8ml of BHI soft agar and poured onto brain heart infusion agar square plates. 10 plates were prepared for each 4.8ml electroporated bacterial suspension. 5µl of the extracted phage DNA used for the electroporation experiment was directly spotted on a bacterial lawn as a control to make sure that it does not contain any intact virion. The plates were incubated anaerobically for 24hours at 37ºC. Post incubation, the plates were then examined for the presence of plaques.

2. For plasmid DNA, bacterial suspension was poured onto 4 circular plates (1.2ml per plate); two Brucella agar plates and 2plates of TGY agar circular plates (Appendix, 1) supplemented with 15µg/ml thiamphenicol (Appendix, 1). These were incubated anaerobically for 48-72 hours at 37ºC.

The retrieved colonies on TGY and Brucella agars were sub-cultured twice onto TGY and Brucella agars respectively before the extraction of DNA and the running of a PCR to establish the presence of plasmid. An un-shocked bacterial suspension was used as a control.

6.2.4 Culturing of *C. difficile* outside the anaerobic chamber and weakening its biofilm

Both *C. difficile* ribotypes 076 and 220 grow in a biofilm. This is confirmed by the presence of visual viscous cloudy clumps of bacterial aggregations in FA broth surrounded by a clear broth.

Different broths were used for the culturing of *C. difficile* outside the anaerobic chamber. BHI-glucose Na-thioglycollate (BHIS-Na) (3.7% BHI (Oxoid, UK), 0.5% yeast extract (Oxoid, UK), 1.5% glucose (Fisher scientific, UK) and 0.1% sodium thioglycollate (Sigma-Aldrich, UK) pH7.4. The BHI, yeast extract and Na-thioglycollate were autoclaved in 480ml of distilled water. Then, the glucose was dissolved in 20ml of distilled water and filter sterilized onto the autoclaved BHIS-NA.

Fluid thioglycollate (FTG) broth was also used to culture *C. difficile* outside the anaerobic chamber (1.5% tryptone (Oxoid, UK), 0.5% yeast extract (Oxoid, UK), 0.55% glucose (Fisher Scientific, USA), 0.05% sodium thioglycollate, 0.25% sodium chloride (Fisher Scientific, USA), 0.0001% resazurin (Sigma-Aldrich, USA), 0.05% L-cysteine (Sigma-Aldrich, USA) pH7.2, available online at http://www.oxoid.com/uk/blue/prod_detail/prod_detail.asp?pr=cm0173&cat=&sec=1&c=uk&lang=en.

C. difficile was grown with agitation at 120rpm outside the anaerobic chamber at 37ºC for 1hour. A control culture was grown inside the anaerobic chamber and the CFU of both were compared. Two 15ml tubes of both FTG and BHIS-Na broths were pre-reduced overnight inside the anaerobic chamber. Starting from the FA culture, 150µl of the *C. difficile* overnight culture (cells at stationary phase) was inoculated in all tubes. One tube of FTG and one of BHIS-Na broth were left in the anaerobic chamber at 37ºC for 1hour as a control. The other FTG and BHIS-Na broths were sealed with para-film and incubated outside the anaerobic chamber at 37ºC with agitation at 120rpm. After 1hour of incubation, CFU/ml was carried out for the all the tubes as described in section 3.4.

6.2.5 Treatment of *C. difficile* with cysteine-protease E-64 inhibitor and lysostaphin

In order to diminish the surface layer protein of *C. difficile* and make pores in the peptidoglycan, the culture was treated with cysteine protease inhibitor E-64 (Sigma-Aldrich, UK) and lysostaphin (Sigma-Aldrich, UK). A stock solution of 5mg/ml (13.9899mM) of E-64 was prepared and used to a final concentration of 100mM, 250mM, and 500mM. After the treatment with cysteine inhibitor, lysostaphin was used to help make pores in the peptidoglycan. 1mg/ml stock solution of lysostaphin was prepared and added to the E-64 treated *C. difficile* cells at a final concentration of 10µg/ml.

C. difficile was grown as described in section 6.2.4, 150µl of FA culture was inoculated in 15ml of BHIS-Na (a control tube was also prepared) and the E-64 was added to the test tube only at the same time of inoculation. The tubes were sealed with para-film and transported to a shaker incubator. After 1hour of incubation at 120rpm and 37ºC, lysostaphin was added to the test tube only. The mixture was mixed by inversion and washed twice with a pre-reduced SMP buffer by centrifugation at 3500xg for 10 minutes. The pellet of the final washing step was re-suspended in pre-reduced SMP buffer and was then ready for electroporation.

6.2.6 Extraction of *C. difficile* surface layer protein

The extraction of the surface layer protein was conducted as described by (Fagan *et al.*, 2010). Briefly, 5ml of each of the E-64 treated *C. difficile* R076 culture and of the control (untreated) culture in the BHIs-Na broth were transported into 15ml tubes. Then, the tubes were centrifuged at 3500xg for 15minutes at room temperature. The pellet was re-suspended in 500µl of PBS and washed two more times. After the last washing step, the PBS was completely drained from the tube. The pellet was re-suspended in 50µl of low pH glycine-HCL (Acros Organics, USA) pH 2.2, and incubated at room temperature for 20minutes with gentle interval agitation. The suspension was then transported into eppendorf tubes and centrifuged at a speed of 21,000xg for 10minutes at 4ºC. The supernatant containing the cell wall surface layer proteins was carefully transferred to new tubes and neutralized with 2µl of 2M Tris-base (Fisher scientific, UK). Thereafter, the protein concentration was analyzed by SDS-PAGE as described in 3.5.3.

6.2.7 Electroporation of enzymatically treated *C. difficile*

No ice incubation was used throughout the experiment. The *C. difficile* culture was used for electroporation right after the enzymatic treatment. CFU/ml was taken before starting electroporation. Thereafter, 800µl of the treated cells were transferred into the electroporation cuvette and 5µg of plasmid DNA was added. Cells were mixed with the DNA by inversion a few times. The electroporation was carried out using voltage magnitudes 0.625, 1.250, 2 and 2.5kV/cm as the successful electroporation of other *Clostridium* species was carried out using the last two voltages. The 0.625 and 1.25 kV/cm were used to allow achieve a very long pulse duration without having an "Arch". Variable pulse duration was used for each of these voltages. A 2mm gap cuvette with a $1cm^2$ electrode area was used.

The electroporated cells were instantly inoculated in to 5ml of a pre-reduced BHIS-Na broth maintained at room temperature and 10µl was used to perform the CFU count. After 2hours of incubation, thiamphenicol was added to the final concentration of 15µg/ml and the tubes were further incubated for an hour. The purpose of the addition of antibiotic was to select for the transformed cells and kill any untransformed cell. Thereafter, one ml of the culture was poured on a Brucella agar plate supplemented with 15µg/ml thiamphenicol, hemin and vitamin K1. The electroporated cells were distributed on the agar surface using a plastic spreader (5 plates for each tube). During the 48-72 hours of anaerobic incubation at 37ºC, the plates were monitored for the appearance of

any transformant colonies. Colony PCR using M13 primers was performed to confirm the presence of the plasmid.

6.3 Results

6.3.1 Empirical optimization of *C. difficile* electroporation parameters

In order to optimize electroporation conditions, one should consider changing the main parameters that are crucial for the success of electro-transformation. These parameters include the composition of buffer used, electric field strength, pulse duration, DNA type and concentration, and the treatment of cells prior to electroporation (Deng *et al.*, 2017, Oh *et al.*, 2015, Rols *et al.*, 1998).

The electroporation parameters were set to deliver increasing field strength and pulse duration (Appendix, 6). *C. difficile* was refractory to transformation despite all the changes in the experiment settings shown in appendix 6. The viability of cells after electroporation was very high, as shown in the following figures, revealing the ability of *C. difficile* to survive the electrical and thermal stresses caused by the transmembrane pulse. Figures 55 and 56 shows the viability of *C. difficile* R076 after electroporation using different voltages and pulse durations. Phage CDHM1 DNA was used in these attempts.

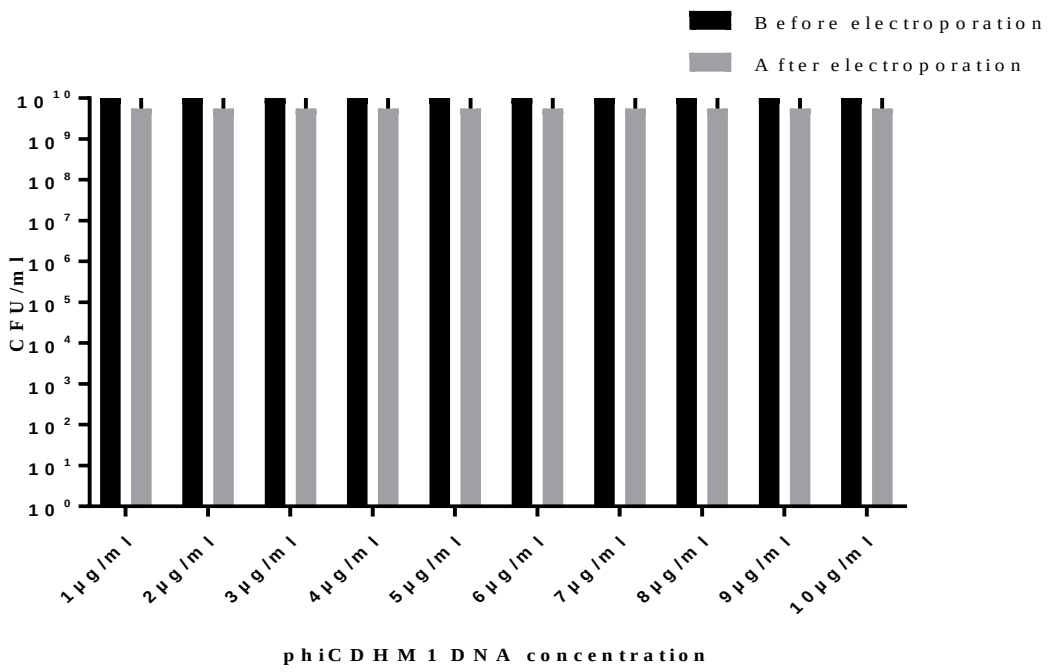


Figure 55. The viability of *C. difficile* ribotype 076 with varying amounts of phiCDHM1 DNA concentrations. Survival of the ribotype 076 cells using Ut of 2.5kV for 5.2 milliseconds (exponential decay pulse) and DNA concentrations between 1-10µg/ml. The experiment was performed twice; once with pre-pulse incubation on ice and the other at room temperature. No significant difference found between the conditions.

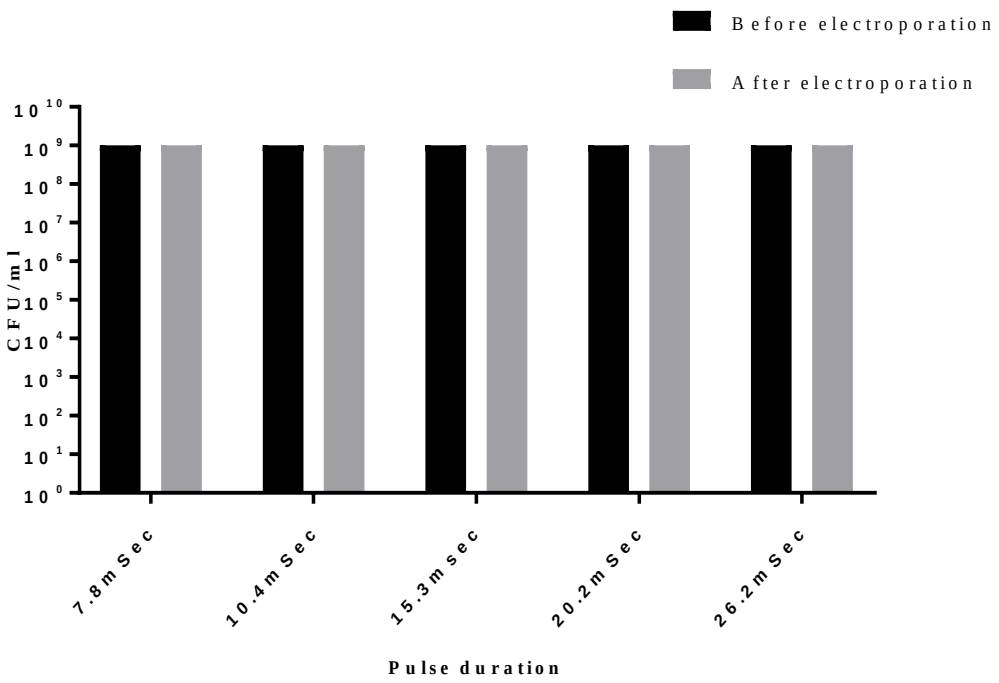


Figure 56. The viability of *C. difficile* ribotype 076 with ranging pulse durations. The survival rate of *C. difficile* ribotype 076 cells using 2.5kV, pulse durations between 7.8-26.2 millisecond (exponential decay pulse), with a phiCDHM1 DNA concentration of 5µg/ml. The experiment was performed once with pre-pulse incubation on ice. No significant difference found between the conditions.

The large genome size of phiCDHM1 (54,279bp) is an important factor that affects the success of *C. difficile* ribotype 076 transformation by electroporation. It was decided to use a plasmid DNA as it is much smaller. Plasmids pMTL82151 (5254bp) and pMTL84151 (6297bp) were used. Different experimental settings were applied including different voltages, pulse durations, DNA concentrations, and pre-pulse incubations. The rate of *C. difficile* ribotypes 076 and 220 cells survival after electroporation was very high. Figures 57, 58, 59, 60, 61, 62, and 63 show the viability of *C. difficile* ribotype 076 after electroporation, while figures 64, 65, 66, 67, 68, 69, and 70 show the viability of *C. difficile* ribotype 220 after electroporation.

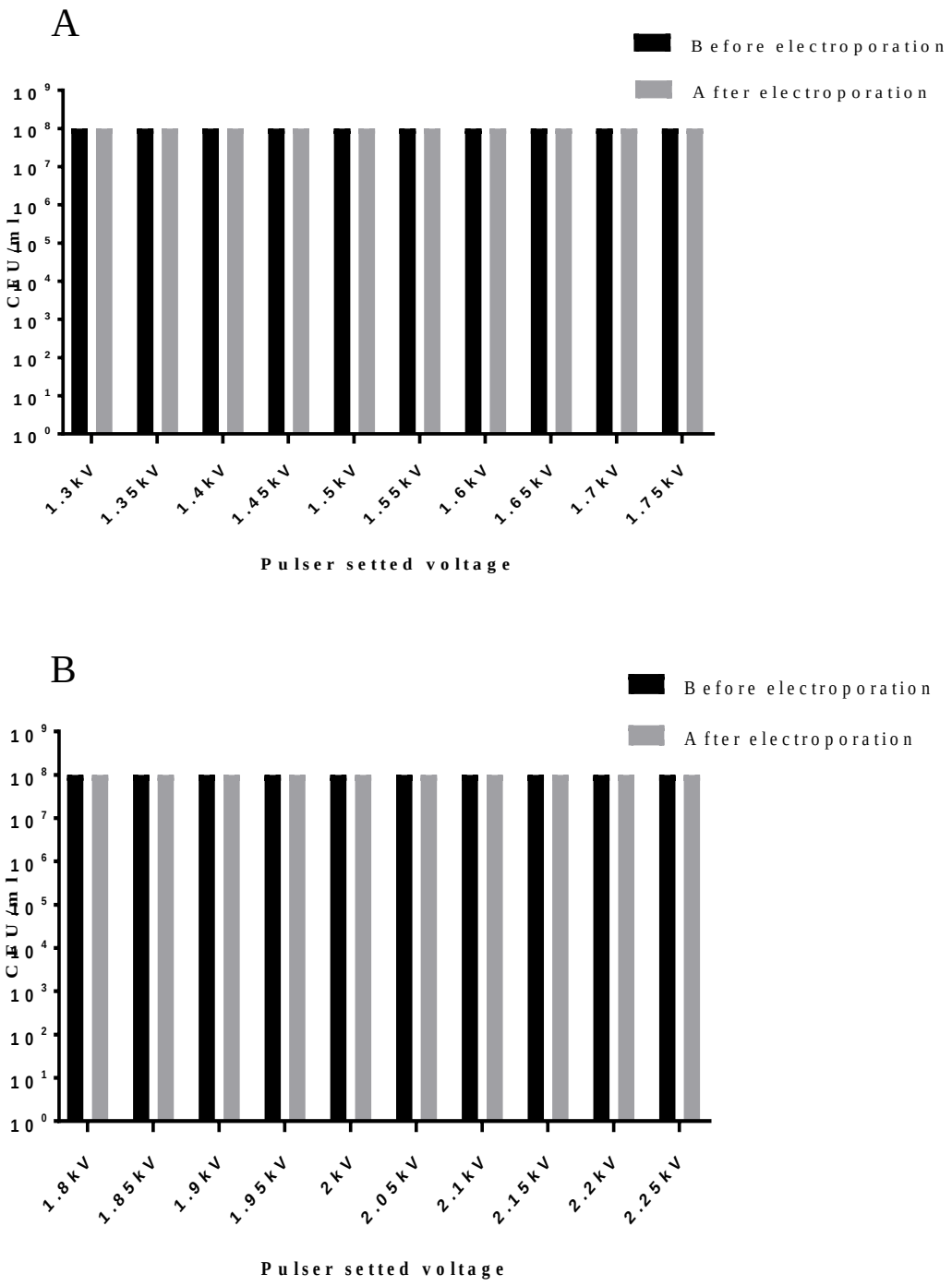


Figure 57. Viability of *C. difficile* ribotype 076 cells with differing pulser voltages. Shown are the survival of *C. difficile* cells when challenged with pulse duration of 5.2±0.1 milliseconds (exponential decay pulse), 5µg/ml plasmid pMTL82151 DNA, pre-pulse incubation on ice, and different voltages (1.3-2.25kV). A) Shows cells' survival rate when 1.3-1.75kV was used. B) Shows cells' survival rate when 1.8-2.25kV was used. No significant difference found in any of the conditions.

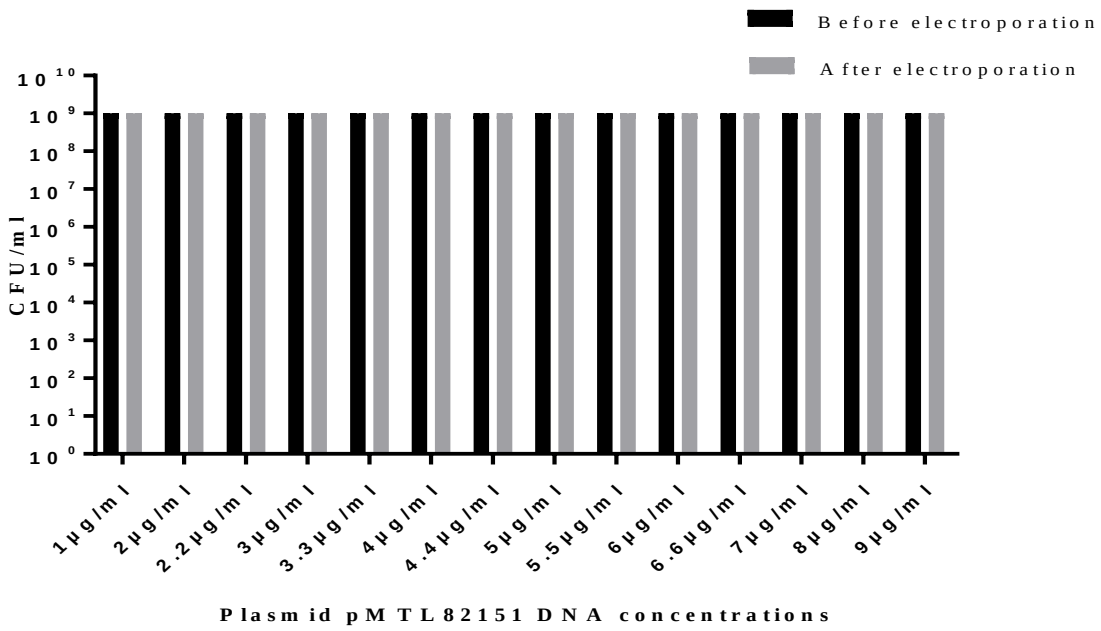


Figure 58. Viability of *C. difficile* ribotype 076 cells with varying plasmid DNA concentrations & electroporation parameters. This electroporation was performed using 2.5kV, pulse duration of 5.2±0.1 milliseconds (exponential decay pulse), pre-pulse incubation on ice, and different DNA concentrations of plasmid pMTL82151. No significant difference was found with any changes in DNA concentrations.

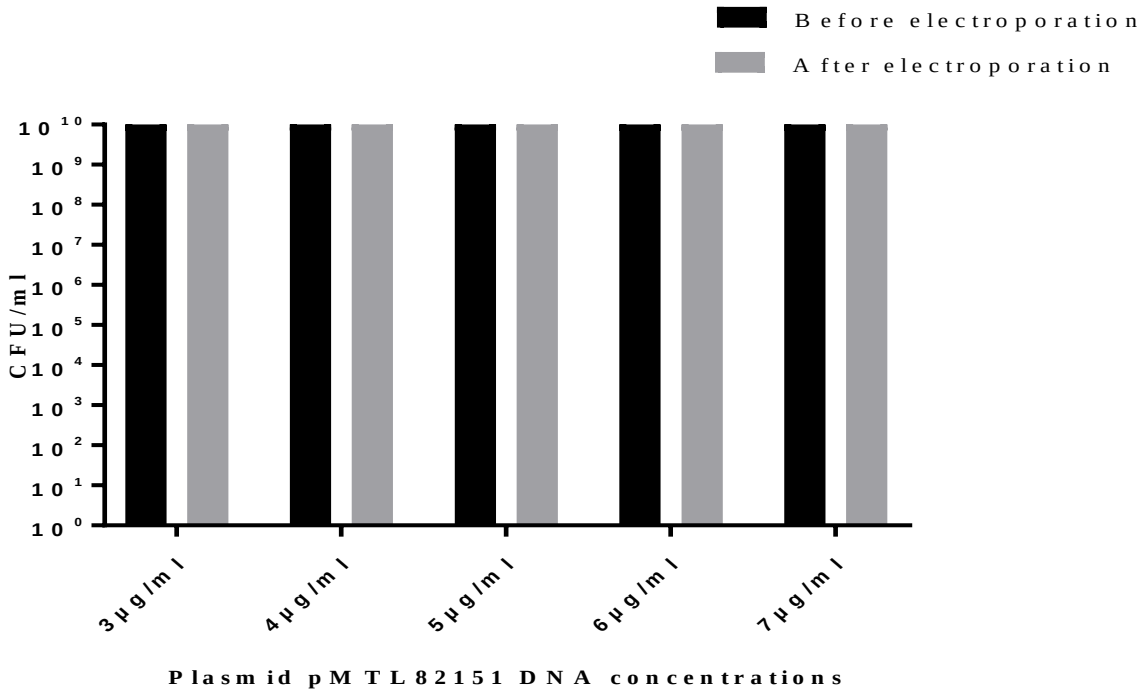


Figure 59. Viability of *C. difficile* ribotype 076 cells with varying plasmid DNA concentrations & electroporation parameters. The electroporation was performed using 2.5kV, a pulse duration of 10.3 millisecond (exponential decay pulse), pre-pulse incubation on ice, and varying concentrations of plasmid pMTL82151 DNA. No significant difference found in the CFU values in any of the conditions shown.

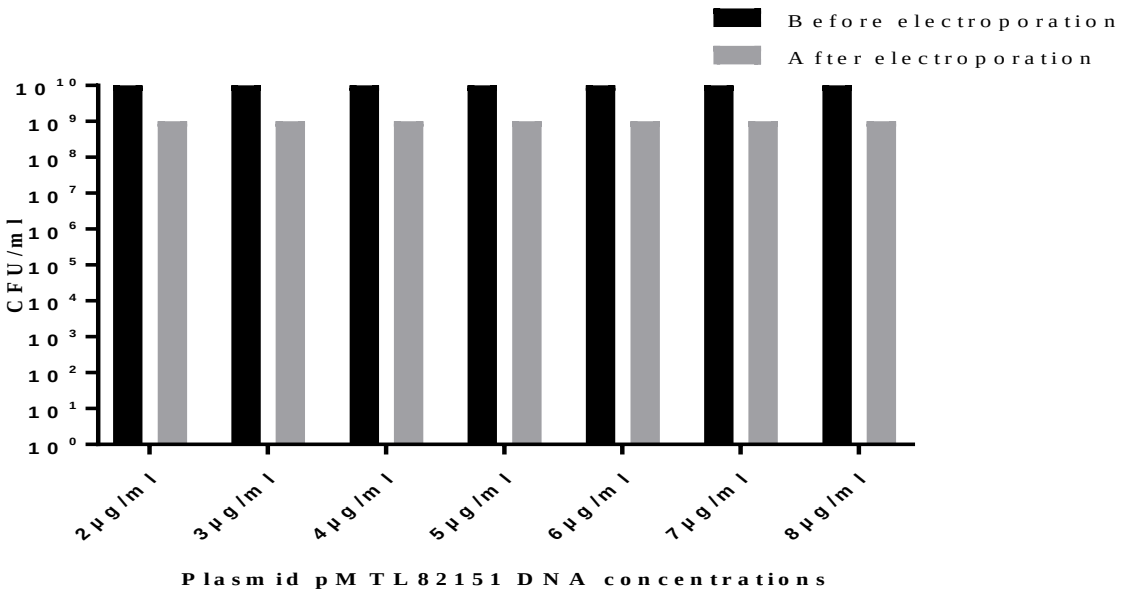


Figure 60. Viability of *C. difficile* ribotype 076 cells with varying plasmid DNA concentrations & electroporation parameters. This electroporation was carried out using 3kV for 5.3 milliseconds (exponential decay pulse), pre-pulse incubation on ice, and different concentrations of plasmid pMTL82151 DNA. No significant difference found between the different conditions used, though a one log drop in CFU/ml can be seen.

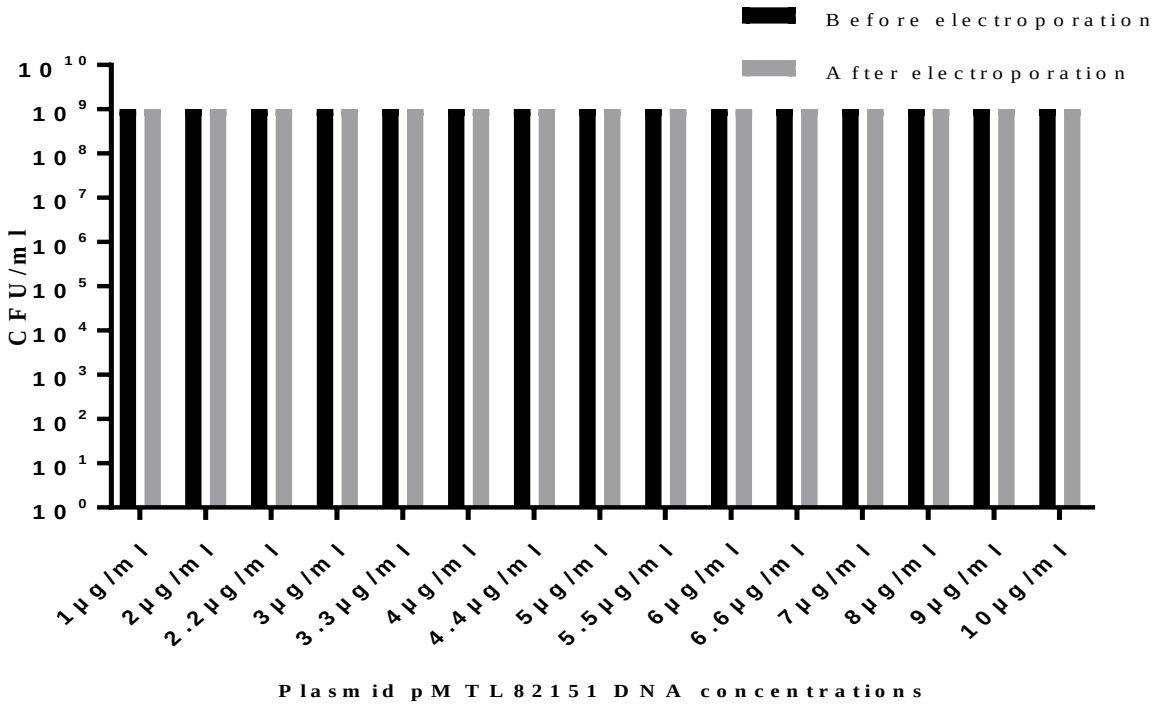


Figure 61. Viability of *C. difficile* ribotype 076 cells with varying plasmid DNA concentrations & electroporation parameters. This electroporation was carried out using 2.5kV for 5.2±0.1 milliseconds (exponential decay pulse), pre-pulse incubation at room temperature, and different concentrations of plasmid pMTL82151 DNA. No significant difference found in the CFU/ml values in any of the conditions shown.

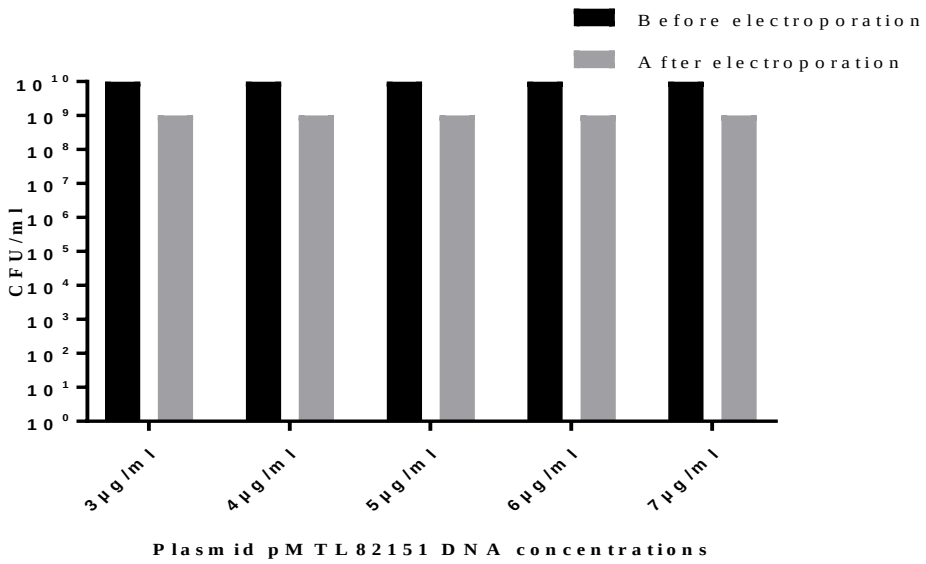


Figure 62. *C. difficile* ribotype 076 survival rates cells with varying plasmid DNA concentrations & electroporation parameters. This electroporation was carried out using 2.5kV for 10.3 milliseconds (exponential decay pulse), pre-pulse incubation at room temperature, and different concentrations of plasmid pMTL82151 DNA. Approximately a one log drop in CFU can be seen.

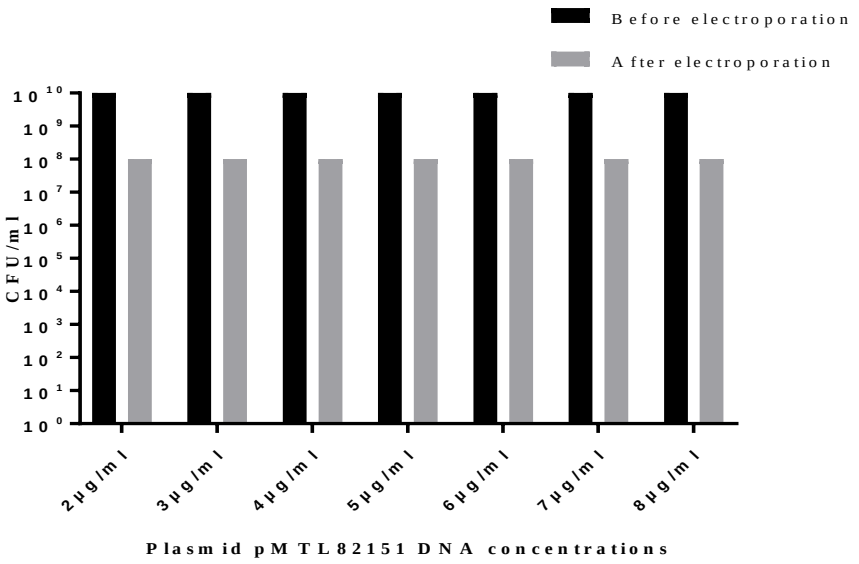


Figure 63. *C. difficile* ribotype 076 survival rates cells with varying plasmid DNA concentrations & electroporation parameters. This electroporation was carried out using 3kV for 5.3 milliseconds (exponential decay pulse), pre-pulse incubation at room temperature, and different concentrations of plasmid pMTL82151 DNA. Approximately a two log drop in CFU/ml can be seen.

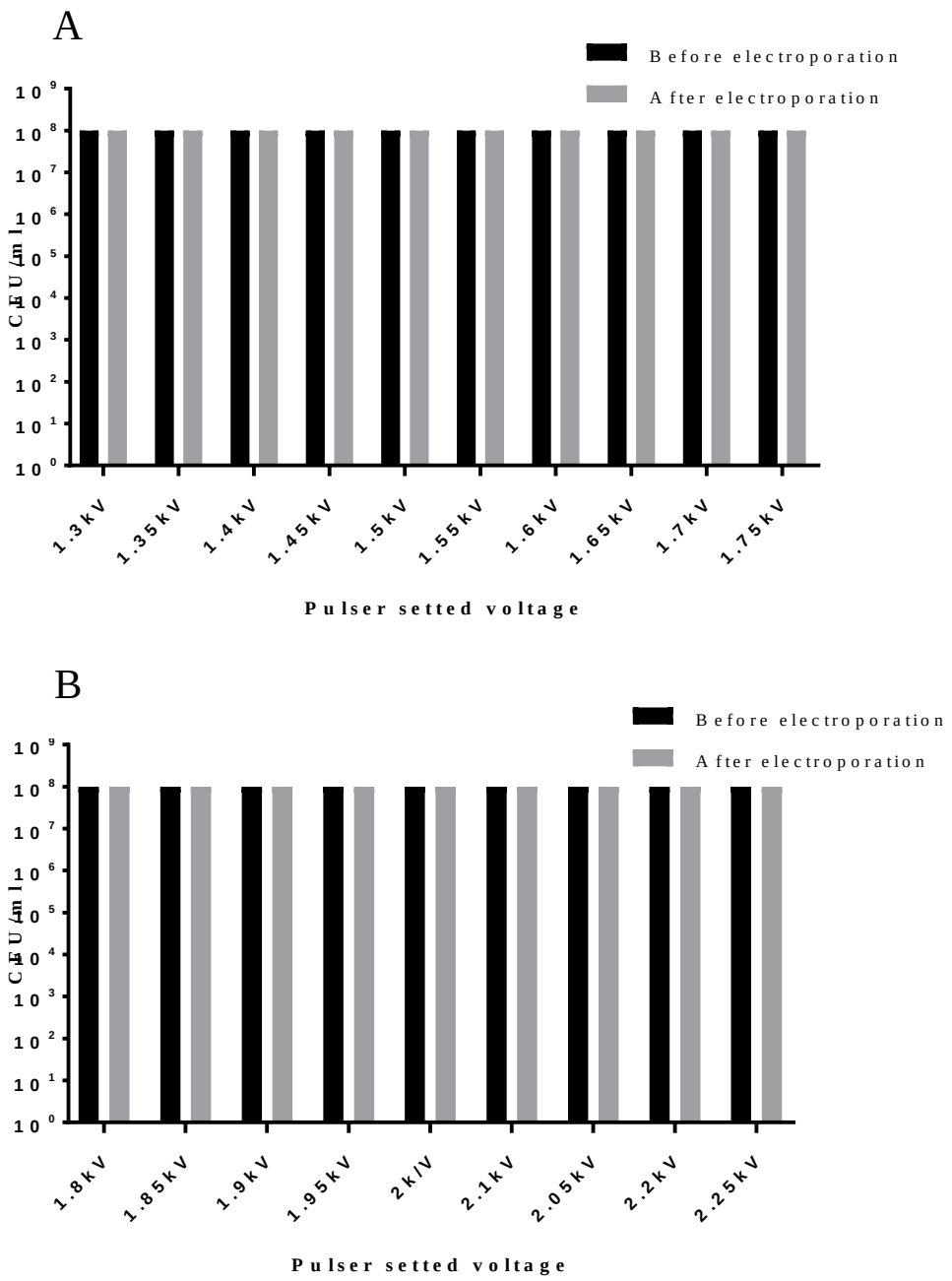


Figure 64. Viability of *C. difficile* ribotype 220 cells with varying voltage settings & electroporation parameters. This electroporation was carried out using a pulse duration of 5.3±0.1 milliseconds (exponential decay pulse), 5µg/ml plasmid pMTL82151 DNA, pre-pulse incubation on ice, and different voltages (1.3-2.25kV). A) Shows cells' survival rate when 1.3-1.75kV was used. B) Shows cells' survival rate when 1.8-2.25kV was used. No significant difference found in the CFU/ml values in any of the conditions shown.

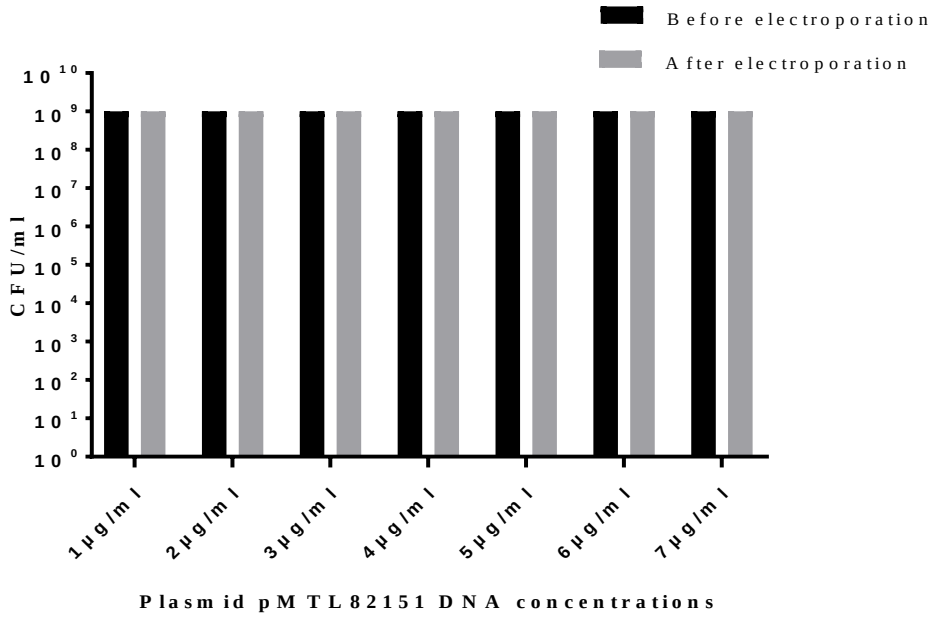


Figure 65. Viability of *C. difficile* ribotype 220 cells with varying plasmid DNA concentrations & electroporation parameters. This electroporation was carried out using 2.5kV, pulse duration of 5.3 milliseconds (exponential decay pulse), pre-pulse incubation on ice, and different concentrations of plasmid pMTL82151 DNA. No significant difference found in the CFU/ml values in any of the conditions shown.

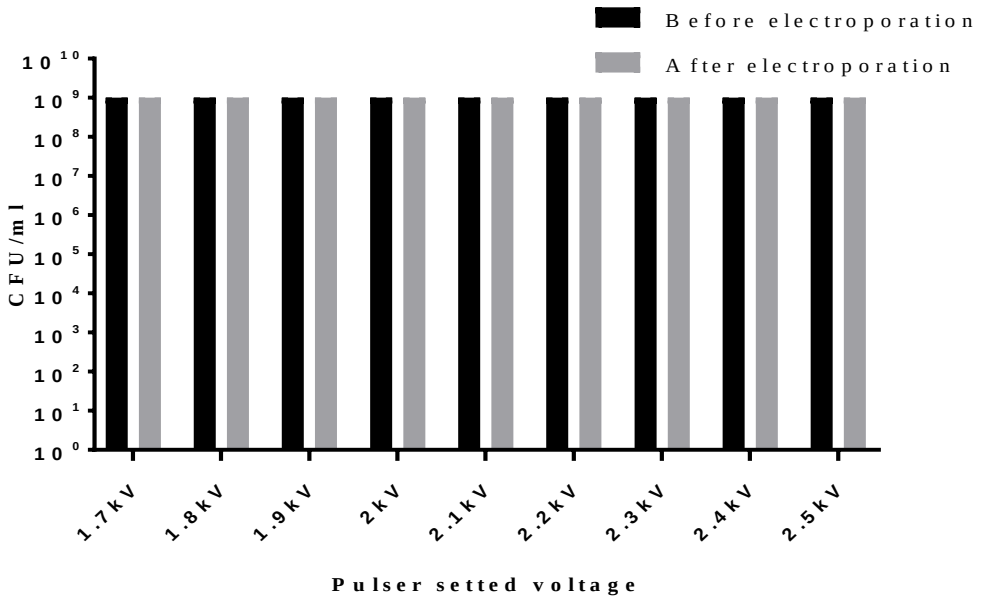


Figure 66. Viability of *C. difficile* ribotype 220 cells with varying voltage settings & electroporation parameters. This electroporation was carried out using a pulse duration of 5 milliseconds (square wave pulse) with 5 seconds rest between each pulse, 5µg/ml plasmid pMTL84151 DNA, pre-pulse incubation on ice, and different voltages (1.7-2.25kV). No significant difference found in the CFU/ml values in any of the conditions shown.

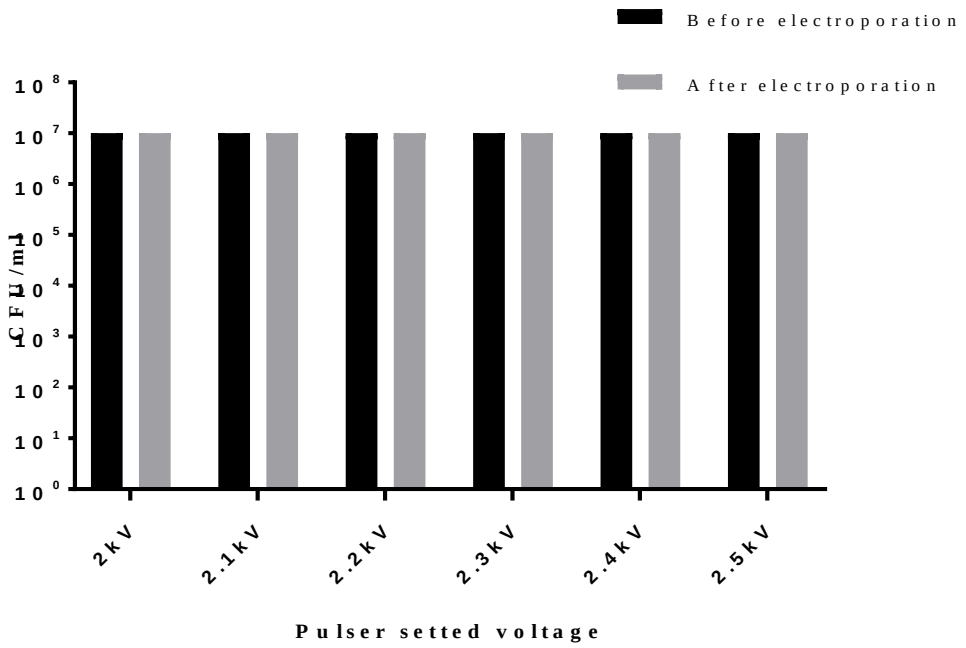


Figure 67. Viability of *C. difficile* ribotype 220 cells with varying voltage settings & electroporation parameters. This electroporation was carried out using a pulse duration of 4.1 milliseconds (square wave pulse) with 5 seconds rest between each pulse, 5µg/ml plasmid pMTL83151 DNA, pre-pulse incubation on ice, and different voltages (2-2.25kV). No significant difference found in the CFU values in any of the conditions shown.

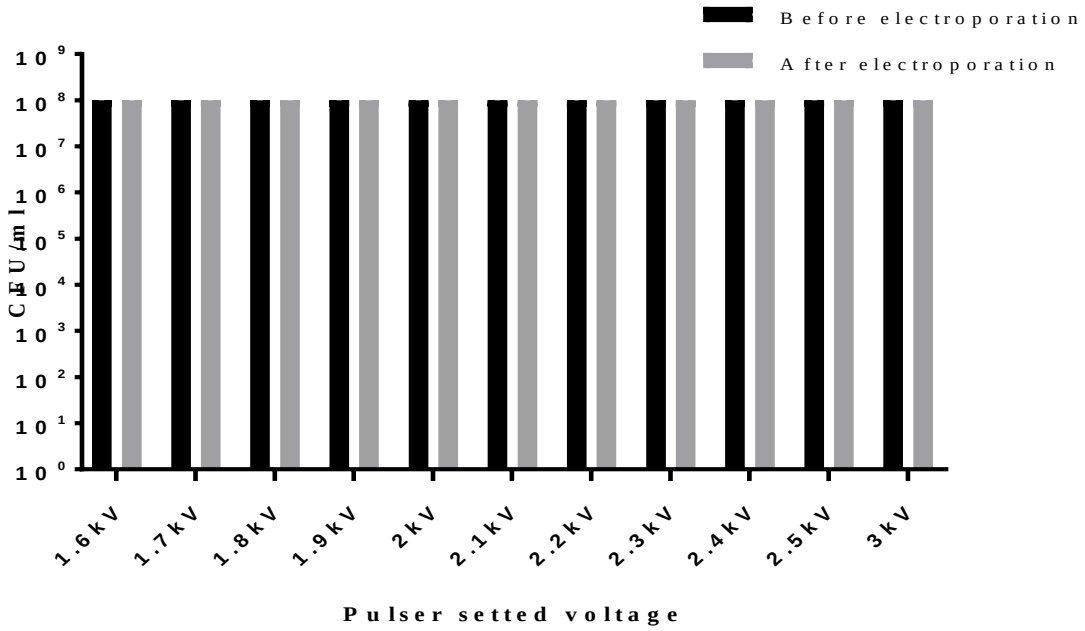


Figure 68. Viability of *C. difficile* ribotype 220 cells with varying voltage settings & electroporation parameters. This electroporation was carried out using a pulse duration of 5.1-5.2 milliseconds (time constant pulse), 5µg/ml plasmid pMTL82151 DNA, pre-pulse incubation on ice, and different voltages (1.6-3kV). No significant difference found in the CFU/ml values in any of the conditions shown.

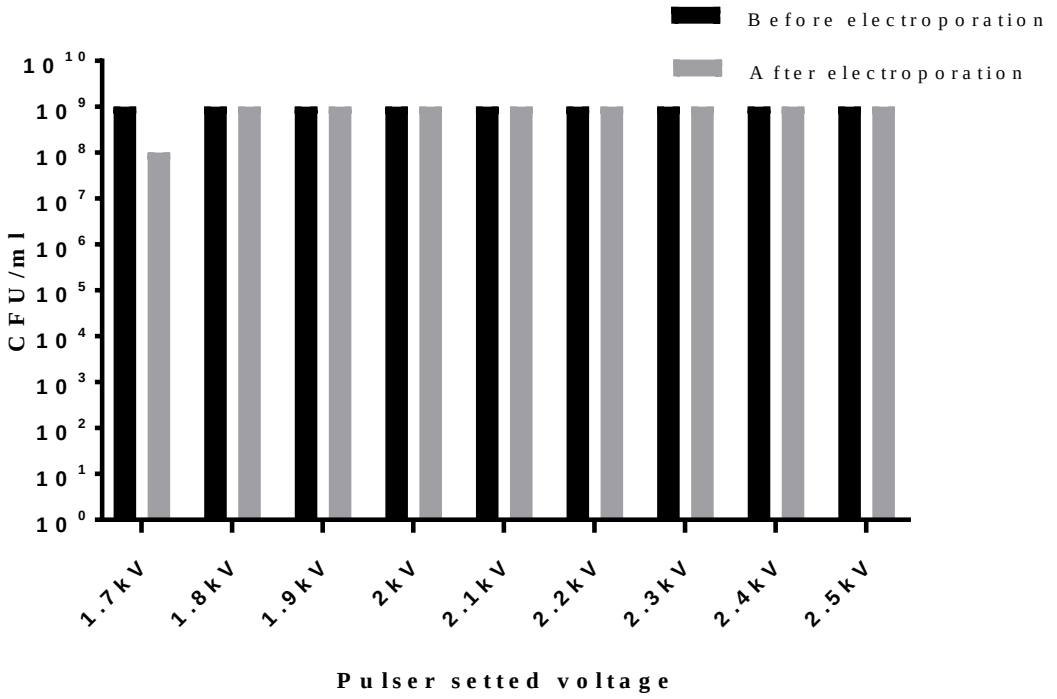


Figure 69. Viability of *C. difficile* ribotype 220 cells with varying voltage settings & electroporation parameters. This electroporation was carried out using a pulse duration between 5.3-7.3 milliseconds (time constant pulse), 5µg/ml plasmid pMTL82151 DNA, pre-pulse incubation on ice, and different voltages (1.7-2.5kV). No significant difference found in the CFU values in any of the conditions shown.

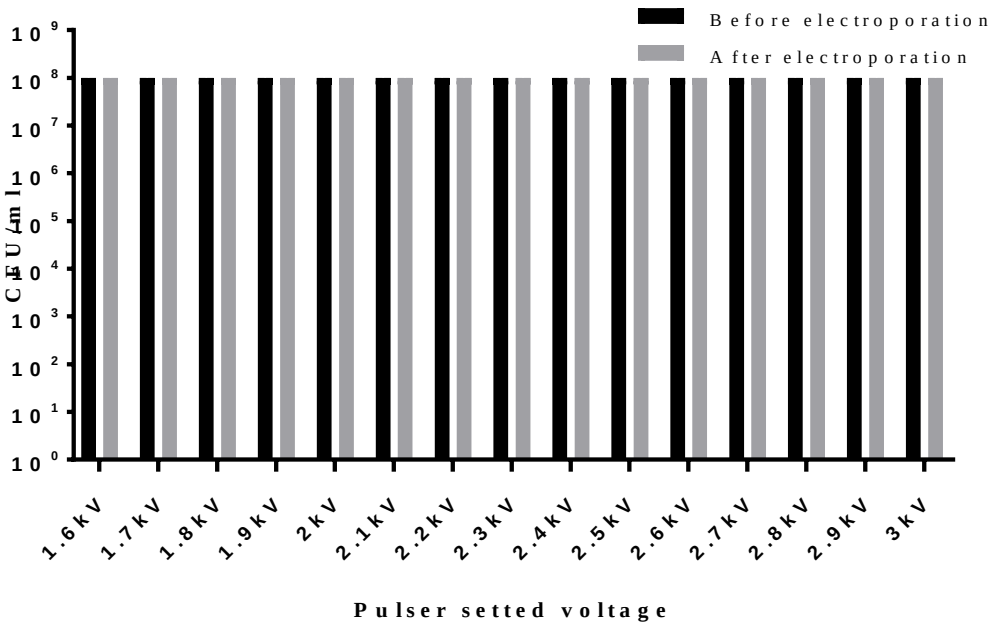


Figure 70. Viability of *C. difficile* ribotype 220 cells with varying voltage settings & electroporation parameters. This electroporation was carried out using a pulse duration between 5.5-6.8 milliseconds (time constant pulse), 5µg/ml plasmid pMTL84151 DNA, pre-pulse incubation on ice, and different voltages (1.6-3kV). No significant difference found in the CFU/ml values in any of the conditions shown.

Applying all these Uts has not affected the viability of *C. difficile* cells. Even when a Ut of 3kV and 5.3, a 26.2 milliseconds pulse duration was used, the viability of cells was only slightly reduced (Figure, 71). In order to determine the ability of *C. difficile* cells to survive the electrical stress caused by the transmembrane pulse, two consecutive pulses of 2.5kV for 5.2 milliseconds were applied. None of the cells survived.

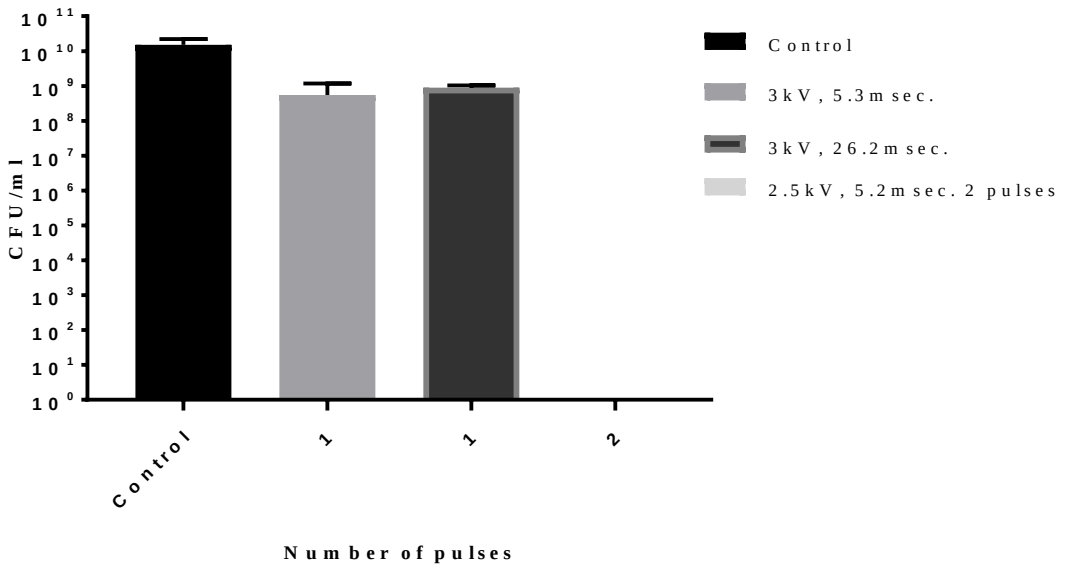


Figure 71. The tolerance of *C. difficile* ribotype 076 to transmembrane pulse. The black column represents the CFU/ml of a control *C. difficile* ribotype 076 which did not receive a pulse. 1. The grey column represents the CFU/ml of *C. difficile* ribotype 076 after one pulse using 3kV and a pulse duration of 5.3 milliseconds. 2. The dark grey column represents the CFU/ml of *C. difficile* ribotype 076 after one pulse using 3kV and a pulse duration of 26.2 milliseconds. 3. Represents the CFU/ml of *C. difficile* ribotype 076 after two pulses using 2.5kV and a pulse duration of 5.2 milliseconds. No bar is seen as the value of CFU/ml is zero indicating no survival.

6.3.2 Culturing *C. difficile* outside the anaerobic chamber

The *C. difficile* biofilm formation was highly reduced by culturing the cells with agitation. This was confirmed by the disappearance of viscosity when the cell pellet was re-suspended with 5ml of SMP buffer prior to electroporation. On the other hand, the cells suspension of *C. difficile* grown without agitation remained viscous. Two liquid media were tested to be used for culturing *C. difficile* outside the anaerobic chamber. *C. difficile* was unable to grow in the FTG broth outside the anaerobic chamber. However, the FTG broth could support the growth of *C. difficile* inside the chamber up to 10^4 cell/ml after being pre-reduced for 24 hours. The BHIS-Na broth was able to support the growth of *C. difficile* outside the anaerobic chamber. The CFU/ml was slightly different between the cultures inside and outside the anaerobic chamber when BHIS-Na was used (Figure, 72). However, BHIS-Na needed to be pre-reduced for 24 hours inside the anaerobic chamber before it became as efficient as shown.

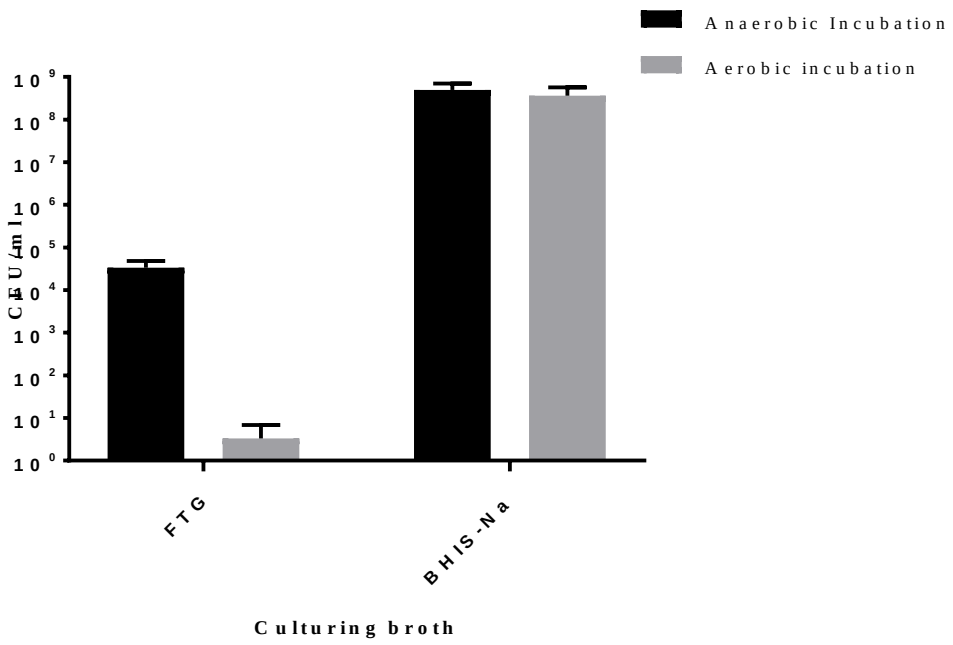


Figure 72. The ability of FTG and BHIS-Na broths to support *C. difficile* growth outside the anaerobic chamber. The black bars represent CFU/ml of C. difficile R076 when it is cultured inside the anaerobic chamber. The grey bars represent CFU/ml of *C. difficile* R076 when it is cultured outside the anaerobic chamber with agitation at 220 rpm.

6.3.3 Enzymatic treatment of *C. difficile*

6.3.3.1 Treatment of C. difficile with cysteine protease inhibitor E-64

The cysteine protease inhibitor was able to reduce the thickness of surface layer protein at the concentrations of 100, 250, and 500mM (Figure, 73). Although this treatment could not lead to a successful transformation, the survival of electroporated cells was much lower (three to four folds less) then it was before electroporation. This treatment does not affect the growth of *C. difficile*, as the CFU/ml was slightly different than the control (untreated) cell count (Figure, 74).

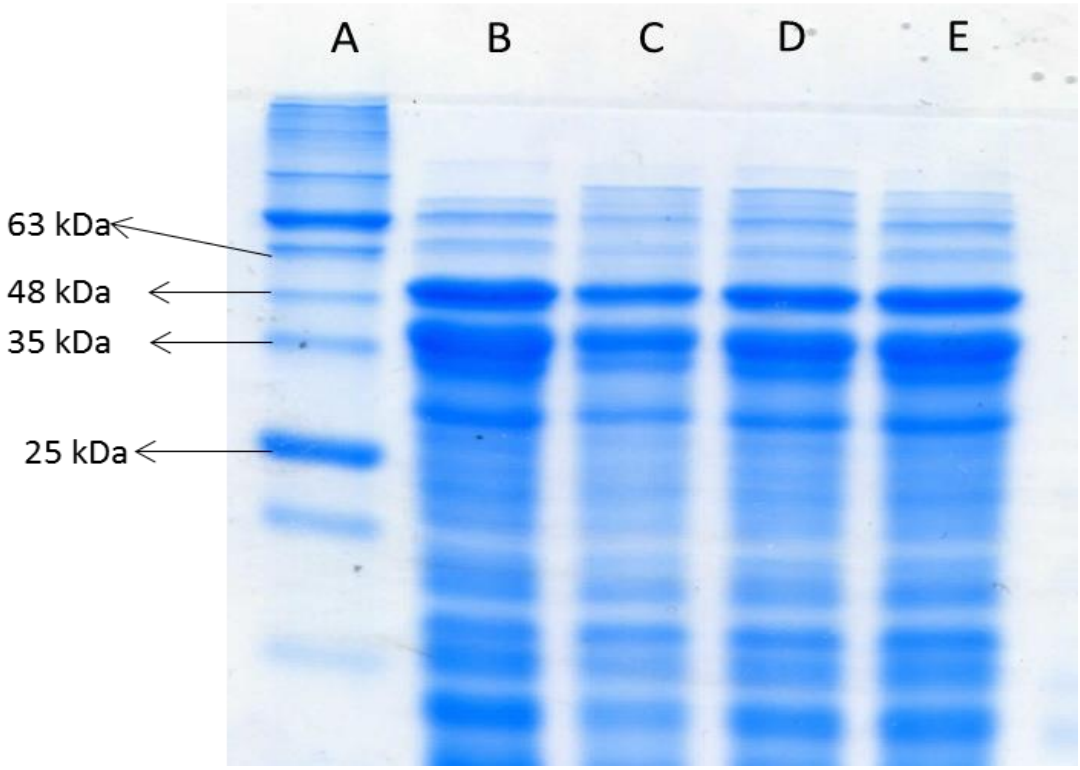


Figure 73. The effect of E-64 cysteine protease inhibitor treatment on the *C. difficile* R076 outer layer protein thickness. The figure shows the reduction of surface layer proteins of *C. difficile* R076 as a result of the E-64 treatment. Both the LMW (35 kDa) and the HMW (49kDa) protein concentration was reduced. A. Is a protein ladder (New England Biolabs). B. Shows the cell wall extract of untreated (control) *C. difficile* R076. C. Illustrates the bands of LMW and HMW of C. difficile R076 treated with 500mM, final concentration of E-64. D. Shows the bands after treatment with 250mM inhibitor. E. Shows the bands after treatment with 100mM inhibitor.

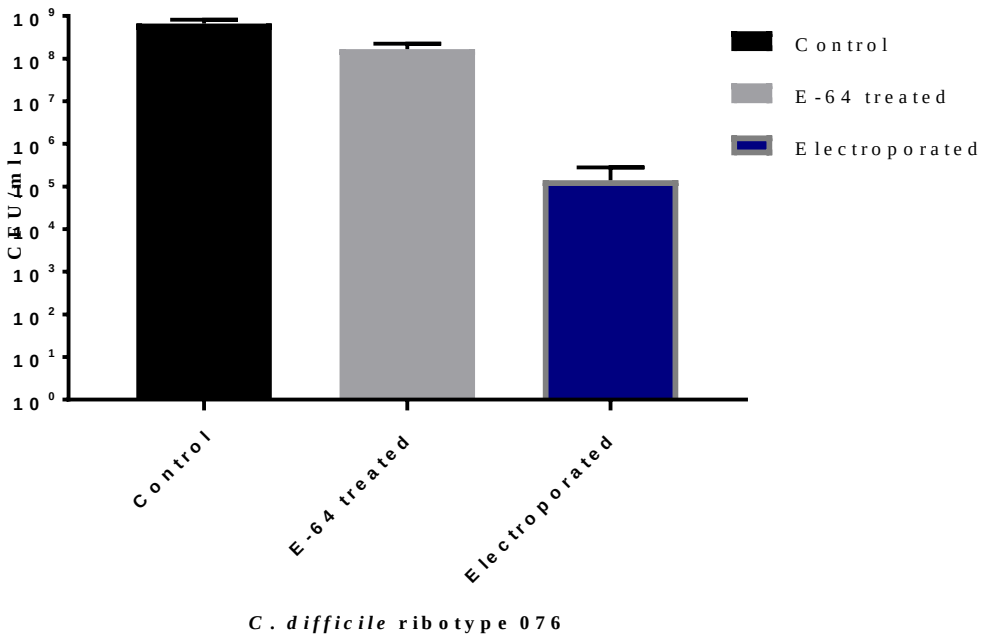


Figure 74. Treatment of *C. difficile* ribotype 076 with the E-64 protease inhibitor. The black column represents the CFU/ml of a control *C. difficile* ribotype 076 cultured inside the anaerobic chamber. The grey column represents the CFU/ml of E-64 treated *C. difficile* ribotype 076 cultured outside the anaerobic chamber with agitation at 220rpm. The blue column represents the mean of CFU/ml of three pulse durations. E-64 treated *C. difficile* ribotype 076 was electroporated using 2.5kV and pulse durations of 9, 12.8, 25.3 milliseconds, 5µg/ml plasmid pMTL82151 and with pre-pulse incubation at room temperature.

6.3.3.2 Treatment of *C. difficile* with lysostaphin

The lysostaphin has the ability to lyse the *C. difficile* R076 strain after incubation in an SMP buffer for 1hour, as the CFU/ml was reduced by about 50% after treatment (Figure, 75). Thus, the incubation of *C. difficile* R076 for an hour with 10µg/ml step was replaced with one washing step with the enzyme. The CFU/ml of E-64 treated *C. difficile* culture was slightly reduced when washed with 10µg/ml of lysostaphin. The CFU/ml of *C. difficile* culture treated with both enzymes was reduced by approximately 4 fold after electroporation (Figure, 76).

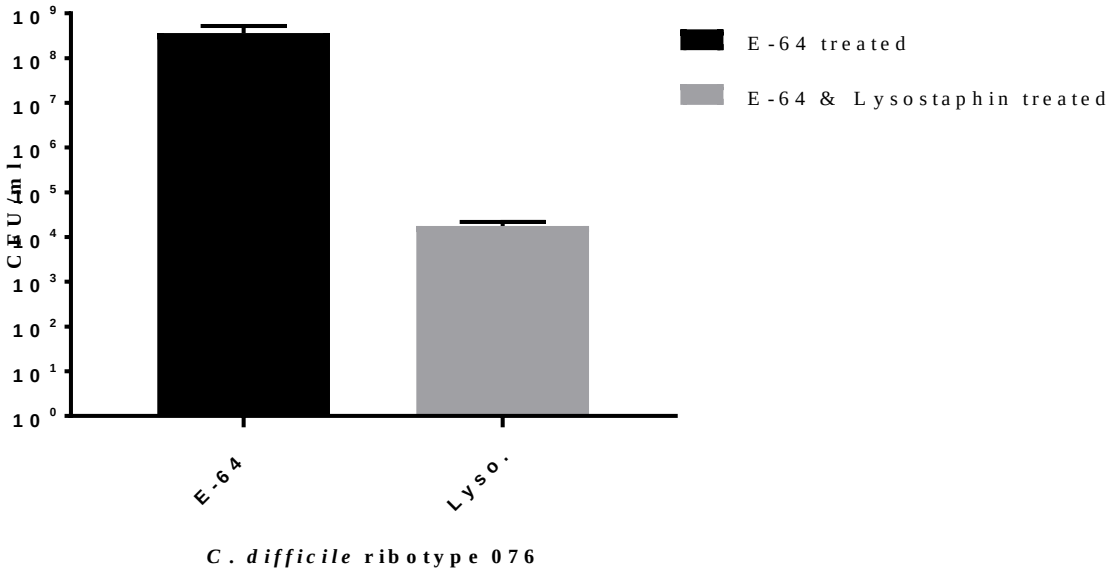


Figure 75. Treatment of *C. difficile* ribotype 076 with lysostaphin. The black column represents the CFU/ml of E-64 treated *C. difficile* ribotype 076. The grey column represents the CFU/ml of E-64 and lysostaphin treated *C. difficile* ribotype 076, after the treatment with E-64, the cells were incubated with lysostaphin in SMP buffer for an hour.

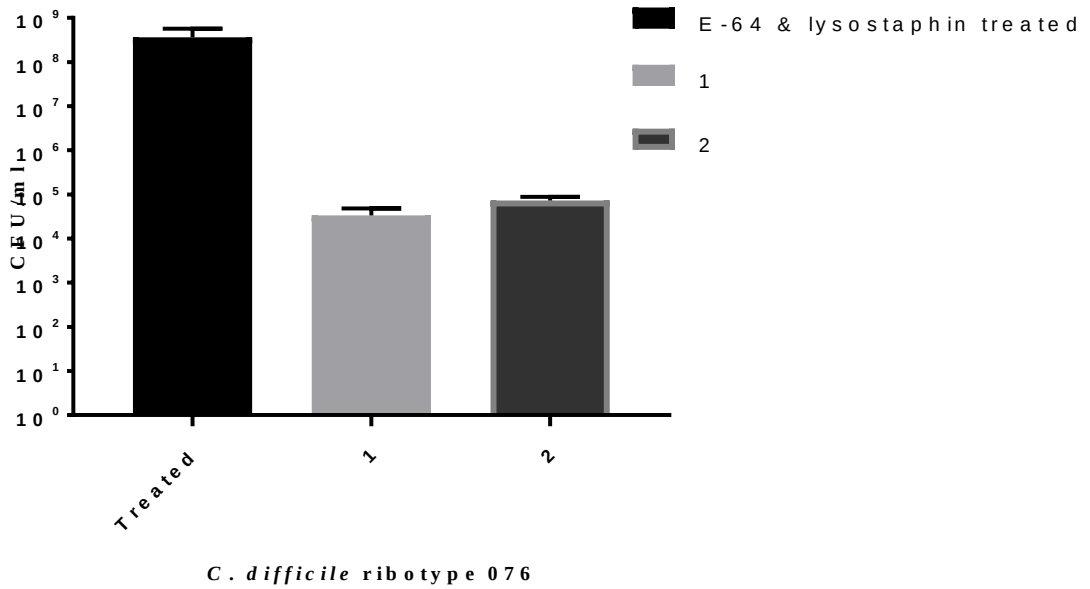


Figure 76. Electroporation of enzymatically treated *C. difficile* ribotype 076. The black column represents the CFU/ml of E-64 & lysostaphin treated *C. difficile* before electroporation. 1. The grey column represents the CFU/ml of the culture after electroporation using 2.5kV and a 25.4 milliseconds pulse duration. 2. The dark grey column represents the CFU/ml of the culture after electroporation using 1.25kV and a 49.6 milliseconds pulse duration.

6.4 Discussion

All the available specific *C. difficile* phages which have been isolated to date and characterised in order to develop therapeutic agents are temperate phages. No lytic phages have been isolated (Horgan *et al.*, 2010, Dei, 1989, Fortier *et al.*, 2007, Goh *et al.*, 2005, Govind *et al.*, 2006, Mahony *et al.*, 1985, Mayer *et al.*, 2008, Meessen-Pinard *et al.*, 2012, NAGY *et al.*, 1991, Nale, 2013, Sell *et al.*, 1983, Sekulovic *et al.*, 2016, Shan *et al.*, 2012) However, these types of phages are of limited value as therapeutic agents (Gill and Hyman, 2010; Meader *et al*., 2010), as reviewed in 2.2.4. Having said that, the genetic manipulation of phage genome has extended the therapeutic potential of phage therapy. For example, genetically modified temperate phages have been used to treat multiple antibiotic-resistant *E. coli.* A phage was engineered to deliver a functional CRISPR-cas to target the antibiotic resistance gene. Thus, the bacteria became sensitive to the antibiotic (Lin *et al.*, 2017, Yosef *et al.*, 2015). However, a sophisticated transformation method is required to accomplish phage engineering.

As mentioned above, in order to be able to improve the properties of *C. difficile* temperate phages, it is necessary to develop a sophisticated transformation method. Electroporation of engineered phage genome is a possible method, given that it is a spore forming organism, competent cells of *C. difficile* cannot be prepared. In this chapter, we attempted to empirically optimize a *C. difficile* electro-transformation protocol with the intention of using it for the genetic manipulation of *C. difficile* phages, so as to improve their properties.

Although the transformation of *C. difficile* was not accomplished, the experimental observations provides a baseline for further work. It has been confirmed that classical electroporation cannot transform *C. difficile*, at least for the ribotypes 076 and 220.

Changing the main factors (reviewed in 2.3.6.2) leading to a successful electroporation had no influence on the viability of *C. difficile* cells. For instance, applying a pulse (Ut) of 3kV for short (5.3 milliseconds) or long (26.2 milliseconds) pulse durations (Figure 71 in results) has only slightly decreased the viability of *C. difficile* cells, while similar conditions reduced the viability of *E. coli* cells by 25-30% (Dower *et al.*, 1988). Cell wall treatment is important for the success of electroporation.

The importance of enzymatic treatment can be explained by comparing the viability of an enzymatically treated culture to an untreated culture. The CFU/ml was almost

always the same after electroporation without enzymatic treatment, while it was reduced by 4 fold (around 50%) using the same pulser settings with an enzymatically treated culture. Moreover, applying an intensive trans-membrane pulse (Ut) at 2.5 kV/cm for a 5m.Sec pulse duration had no effect on the survival of the culture. In contrast, applying two consecutive Ut with the same settings resulted in the death of all culture cells. This observation suggested that the presence of the cell wall (peptidoglycan and surface protein layer) enables the cells to survive both the thermal and electrical stress caused until a threshold point, after which the cell will be broken down (Figure 71 in the result section 6.3.1).

The treatment of *C. difficile* with a cysteine protease inhibitor (up to 500mM) diminished the Slp, but did not affect the growth rate. This allows further treatment with higher concentrations of the enzyme to accomplish a thinner surface protein layer.

Ackerman *et al*. (2001) used two washing steps to highly pack the cells and eliminate biofilm. These washing steps were not sufficient to eliminate the *C. difficile* R076 and R220 biofilms (the biofilms of both ribotypes can be seen as cloudy viscous clumps surrounded by a clear broth). Despite the fact that the cells were highly packed and difficult to re-suspend, the final cells suspension was still viscous. This viscosity (clumping of *C. difficile* cells) represents an extra physical barrier and provide *C. difficile* cells with extra protection due to the sticking together of cells. Reducing the biofilm formation by culturing *C. difficile* with agitation will disperse the cells and expose them to the DNA, thereby increasing the possibility of electroporation success.

6.5 Conclusion

1. The nature of the *C. difficile* cell wall is the main reason for the failure of electroporation. The thick peptidoglycan layer and surface layer protein confront the passage of DNA into *C. difficile* cells.
2. This procedure is worthwhile in the context of improving phage characteristics. The delivery of *in-vitro* engineered phage DNA will save a lot of time, money, and effort. However, it can become laborious, time-consuming, and cost-ineffective if developed to transform *C. difficile* with plasmid DNA. The transformation of *C. difficile* by conjugation is cost-effective in comparison with the enzymatic treatment required for the preparation of *C. difficile* for electroporation.

3. Lysostaphin is able to partially lyse *C. difficile* R076 as noted during the optimization of *C. difficile* electroporation. It may therefore be potentially suitable for therapeutic purposes.

6.6 Future work

Further optimization is required, which may result in the successful electroporation of *C. difficile*. This optimization includes:

1. Treating *C. difficile* with a higher concentration of E-64 (more than 500mM). This treatment will reduce the surface layer protein thickness more than the 500mM has done.
2. Treating *C. difficile* with lower concentrations of lysostaphin. A lower concentration of lysostaphin (less than 10µg/ml) will reduce the lysis of *C. difficile* cells and weaken the peptidoglycan layer.

Hypothetically, the enzymatic treatment mentioned above will significantly weaken *C. difficile* cells. In order to assess whether cells have been weakened, a one fold decrease in CFU/ml caused by enzymatic treatment should be observed. The experiment must be carried out after a decrease of CFU/ml by one-fold or more will occur.

3. Using a new buffer that does not contain magnesium ions and replacing the sucrose with D-glucosamine. The absence of ions from the buffer will allow for the use of a higher field strength without having an Arch. D-glucosamine can increase the transformation efficiency by 30%, as it increases both the DNA uptake and the cytoplasmic membrane stability under pulse stresses (Igawa *et al.*, 2014). The presence of D-glucosamine in the electroporation buffer will help maintain the integrity of the cytoplasmic membrane. If electroporation is optimized for *C. difficile,* it can be used to deliver the engineered phage genome.
4. *In-vitro* engineering the phage DNA. This can be achieved by the amplification of the phage DNA in three or more parts with restriction sites enable the subsequent ligation. The amplification of the first part will stop at the beginning of the gene to be deleted, and the amplification of the second part will start at the end of gene to be deleted. Thus, the integrase gene and any undesired gene can be excluded during the amplification. Then, the amplified parts can be ligated.
5. Transforming *C. difficile* with the engineered phage DNA.
6. Characterizing the improved phage progeny properties to establish whether it has the potential as a therapy for CDI.

Chapter 7

General Discussion

Chapter 7. General Discussion

7.1 Introduction

C. perfringens is a Gram-positive, anaerobic, spore-forming bacterium. The spores are responsible for the environmental survival of *C. perfringens* and the spread of infections. These infections include facial myonecrosis and chicken enteritis. Considering the difficulties involving the treatment of human facial myonecrosis and the problems associated with the long term administration of antibiotics after the resection of the infected part of the body, along with the threat of economic loss as a result of chicken enteritis, which is treated by antibiotics; it is clear that there is a need for new medication to control these infections. Several non-antibiotic treatments have been used to treat chicken enteritis, such as vaccination and the administration of monoclonal antibodies to the intestine. Phage therapy is a good candidate for the treatment of *C. perfringens* infections. Although it has its own limitations it has been successfully used to control chicken enteritis. Moreover, it has many advantages in comparison with antibiotics.

It has been shown that phages inhabit the sites where their bacterial hosts are available (Hargreaves, 2013). *C. perfringens* is found in soil (Voidarou *et al.*, 2011), and in animal faecal samples (Goldstein *et al.*, 2012). *C. perfringens* phages have been isolated from environmental sources such as poultry faeces, chicken offal wash and intestinal contents (Seal *et al.*, 2011, Volozhantsev *et al.*, 2011).

In this study, environmental *C. perfringens* strains have been isolated by the enrichment culture method. A similar approach was used by (Goldstein *et al.*, 2012). The strain with the potential to cause diseases have been assessed by determining the toxin genes that they carry. Lytic and temperate phages that are able to grow in a lytic manner on *C. perfringens* were isolated using these strains. The new phages were characterised and investigated in terms of their potential as therapeutic agents. Moreover, the potential of using a recombinant endolysin derived from a *C. perfringens* lytic phage as a therapeutic agent for *C. perfringens* infection was explored.

Similar to *C. perfringens*, *C. difficile* is a Gram-positive spore-forming anaerobic bacterium that causes antibiotic-associated diarrhoea. *C. difficile* infection is spread by the ingestion of these spores. However, the diarrhoea starts after the disruption of gut

microbiota by antibiotics usually used to treat another illness. CDI is treated by antibiotics, which provokes further disruption of the gut microbiota. The emergence of hyper-virulent *C. difficile* strains with multiple-antibiotic resistance ability has resulted in the poor outcome or failure of antibiotic treatment. It has also highlighted the need for new medication. Although several non-antibiotic therapies have been used to treat CDI, such as faecal transplantation and probiotics, phage therapy is a good candidate for the treatment of CDI as it does not affect the microbiota and targets *C. difficile* in a specific manner. One limitation of *C. difficile* phage therapy is that all the isolated *C. difficile* phages to date are temperate phages which express the integrase gene. In order to improve the properties of *C. difficile* phages, an efficient transformation system is required.

In this study, we attempted to optimize an electroporation protocol in order to allow for the genetic manipulation of *C. difficile* phages to improve their properties. Unfortunately, all the attempts to transform *C. difficile* failed. Despite the failure of these attempts, the experiment observations provide baseline information for further work to optimize the protocol.

In the context of *Clostridium spp.* phage therapy, we isolated *C. perfringens* phages and attempted to improve the properties of already isolated *C. difficile* temperate phages by the optimization of the electroporation protocol for their genetic manipulation. Both of which contribute towards the development of phages for therapeutic purposes. Despite the failure of the empirical optimization of electroporation, further work in the future built on these findings may yield positive results.

7.2 *C. perfringens* phages and their endolysins as potential therapeutic agents

Many *C. perfringens* lytic and temperate phages have been isolated and characterized, and their potential as therapeutic agents to control *C. perfringens* infection has been examined (reviewed in 1.1.2 and 2.2.5). This is the case with the lytic phages ΦCP24R (Morales *et al.*, 2012), ΦCPV1 (Volozhantsev *et al.*, 2011), and ΦCPV4 & ΦZP2 (Volozhantsev *et al.*, 2012) and the temperate phages Φ3626 and Φ8533 (Zimmer *et al.*, 2002a), ΦCP39O and ΦCP26F (Seal *et al.*, 2011), ΦS9 and ΦS63 (Kim *et al.*, 2012), and phiSM101 (Nariya *et al.*, 2011). Few studies have examined the *C. perfringens* phage-derived endolysin as a therapeutic agent to overcome the complication that may accompany whole phage particles (Caly *et al.*, 2015), such as that seen with the study into the endolysin of Φ3626 (Zimmer *et al.*, 2002b).

In this study, the isolated *C. perfringens* phages contributed towards the current collection of phages by increasing the total number and the diversity of the collection. The new phages isolated in this study can be used with other isolated *C. perfringens* phages to prepare phage cocktails for the treatment of *C. perfringens* infections. They also provide a new source for endolysin genes which can be expressed and examined individually or in cocktails as therapeutic agents.

It has been shown that lytic phages are favourable for phage therapy (Sahota, 2016, Thanki, 2016). They have proved to be effective against *C. perfringens in-vivo* (Miller *et al.*, 2010, Caly *et al.*, 2015). In this study, we isolated a strictly lytic *C. perfringens* podovirus phage which is desirable for phage therapy. Both the relatively wide host range of this phage (~14.3%) and its burst size (~137 phage/cell) makes it a suitable candidate to be used in the preparation of a phage cocktail.

It has been shown that temperate *C. perfringens* phages can be induced using Ultraviolet irradiation (Zimmer *et al.*, 2002a, Kim *et al.*, 2012), and by spot testing (Seal *et al.*, 2011, Volozhantsev *et al.*, 2011). In this study, we attempted to induce bacteriophages from environmental *C. perfringens* strains using mitomycin-C and norfloxacin. Our results found a high level of prophage carriage, as all the induction lysates of *C. perfringens* strains gave lysis when investigated via spot tests. However we could not recover any of the induced phages. This was in part due to our inability to identify environmental *C. perfringens* strain susceptible to infection by these induced prophages thus no propogation could be performed. Furthermore, there it was possible that *C. perfringens* strains we worked with have the ability to resist phage infection via phage defence mechanisms. The prophage's super infection exclusion genes can protect the lysogen from infection with other phages (Canchaya *et al.*, 2003).

Additionally, the lytic activity of endolysins derived from *C. perfringens* phages has been assessed. Some endolysins were able to completely lyse all the tested *C. perfringens* strains, while others could only partially lyse them (Tamai *et al.*, 2014b, Zimmer *et al.*, 2002b, Gervasi *et al.*, 2014a).

In this study, we expressed and purified the endolysin of *C. perfringens* phage CPAP1 and assessed its lytic activity. Although one of tested *C. perfringens* strains was partially lysed, all the other strains were completely lysed. The partial lysis of one of the tested

strains may be due to a difference in the binding affinity of the enzyme binding domain or to a difference in the cell wall structure of this particular strain.

This endolysin shares the same modular structure with phiCP24R (Morales *et al.*, 2012), comprising of a single N-terminal catalytic domain and C-terminal binding domains. Both endolysins belong to the amidase-3 family. The majority of the endolysins of phages that infect Gram-positive bacteria have a modular structure comprising of a C-terminal binding domain and an N-terminal catalytic domain (Fischetti, 2008, Oechslin *et al.*, 2013, Dunne *et al.*, 2016).

7.3 Optimization of electroporation protocol for *C. difficile*

Electroporation is the process of introducing DNA into a cell by applying short and intensive electric pulses to the cell (Rubinsky, 2010, Bennett *et al.*, 2014). It has been successfully used to deliver an engineered phage genome to *E. coli* (Planelles *et al.*, 1999, Inoue *et al.*, 2016). In this study, we have attempted to empirically optimize an electro-transformation protocol for *C. difficile* in order to deliver engineered phage genome in the context of phage therapy.

It has been shown that successful molecular uptake by electroporation is affected by the presence of a cell wall. The pore formation which takes place in the cytoplasmic membrane as a result for the transmembrane pulse does not occur in the cell wall. Consequently, the molecular uptake by cells which have a cell wall is much lower in comparison to cells which do not possess a cell wall. For example, the uptake of external molecules by a *Chlamydomonas* strain lacking a cell wall is eight times higher than the *Chlamydomonas* strain with the cell wall (Azencott, 2003).

It has also been shown that the cell wall provides cells with structural strength that protects cell from the lethal effect of electroporation and increase cell viability. The successful delivery of molecules by electroporation is usually accompanied with a reduction in cell viability (Azencott, 2003).

The results obtained in this study suggest that the *C. difficile* cell wall is the main obstacle preventing the uptake of DNA and the success of *C. difficile* electroporation. Our empirical experiments' observations suggest that the different transmembrane pulses used to optimize an electroporation protocol are unable to disrupt *C. difficile* cell wall without enzymatic treatment. Further cell wall treatment is required to diminish both the

peptidoglycan and the surface protein layers. This may result in a successful electroporation as the cells will become more fragile, and the chance of large pore formation will increase. Subsequently, DNA uptake may occur.

Unfortunately, the effect of each electroporation parameter could not be determined as there was no transformation. Transformation efficiency is required in order to assess the role of each parameter such as field strength, pulse duration, type and number of pulses, DNA concentration, buffer composition, and pre-pulse incubation condition. However, the role of each parameter in increasing the transformation efficiency can be determined if the electroporation has been successfully performed.

7.4 Suggestion for future work

1. Isolation of more *C. perfringens* phages and determine their potential as a therapeutic agents in cocktails and in combination with antibiotic for the treatment of *C. perfringens* infections.

2. Cloning of more endolysins of *C. perfringens* phages, determining their minimum inhibitory concentrations, their ability to prevent spore germination, their stability in different suspension liquids after long storage at room temperature and at 4ºC, and the ability of endolysin cocktails to lyse all the isolated *C. perfringens* strains.

3. Examine the effect of further cell wall treatment on the viability of *C. difficile* cells in order to optimize electro-transformation parameters for *C. difficile.*

7.5 conclusion

Our attempts to optimize electro-transformation protocol which could provide a method of engineering *C. difficile* phages have failed. This failure is in part due to the complex structure of *C. difficile* cell wall, and to our inability to identify a *C. difficile* strain susceptible for electro-transformation.

Lytic *C. perfringens* phages can be isolated from environmental samples and they have a promising characteristics as a therapeutic agents to treat *C. perfringens* infections. Phage derived endolysins also have a potential to treat these infections as they possess a considerable lytic activity against *C. perfringens* strains.

Overall, further work is required to optimize *C. difficile* electro-transformation protocol. The development of such a protocol enables the improvement of *C. difficile*

phages in the context of CDI phage therapy. Increasing the current collection of *C. perfringens* phages and their endolysins enables the preparation of phages and endolysin cocktails for the treatment of *C. perfringens* infections.

8. Bibliography

Abedon, S. T., 2011. Lysis from without. *Bacteriophage,* **1**, 46-49.

Abedon, S. T., 2018. Phage Therapy: Various Perspectives on How to Improve the Art. *Methods Mol Biol,* **1734**, 113-127.

Abid, S., Azeem, T., Chaudhary, Z., Rehman, Z., Umar, S., 2016. Emeging Threat Of Necrotic Enteritis in Poultry and its Control Without Use of Antibiotics: A Review. *JAPS: Journal of Animal & Plant Sciences,* **26**.

Abidor, I., Arakelyan, V., Chernomordik, L., Chizmadzhev, Y. A., Pastushenko, V., Tarasevich, M., 1979. Electric breakdown of bilayer lipid membranes: I. The main experimental facts and their qualitative discussion. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry,* **104**, 37-52.

Abou Chakra, C. N., Pepin, J., Sirard, S., Valiquette, L., 2014. Risk Factors for Recurrence, Complications and Mortality in Clostridium difficile Infection: A Systematic Review. *PLoS One,* **9**, e98400.

Abouhmad, A., Mamo, G., Dishisha, T., Amin, M. A., Hatti-Kaul, R., 2016. T4 lysozyme fused with cellulose-binding module for antimicrobial cellulosic wound dressing materials. *J Appl Microbiol,* **121**, 115-125.

Ackermann, G., Tang, Y., Henderson, J., Rodloff, A., Silva Jr, J., Cohen, S., 2001. Electroporation of DNA sequences from the pathogenicity locus (PaLoc) of toxigenic< i> Clostridium difficile</i> into a non-toxigenic strain. *Molecular and cellular probes,* **15**, 301-306.

Ackermann, H.-W., 2006. Classification of bacteriophages. *The bacteriophages,* **635**, 8-16.

Afshari, A., Jamshidi, A., Razmyar, J., Rad, M., 2015. Genotyping of Clostridium perfringens isolated from broiler meat in northeastern of Iran. *Vet Res Forum,* **6**, 279-284.

Agu, C. A., Klein, R., Schwab, S., Konig-Schuster, M., Kodajova, P., Ausserlechner, M., Binishofer, B., Blasi, U., Salmons, B., Gunzburg, W. H., Hohenadl, C., 2006. The cytotoxic activity of the bacteriophage lambda-holin protein reduces tumour growth rates in mammary cancer cell xenograft models. *J Gene Med,* **8**, 229-241.

Alberts, B., Johnson, A., Lewis, J., Walter, P., Raff, M., Roberts, K. 2002. *Molecular Biology of the Cell 4th Edition: International Student Edition.* Routledge.

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., Lipman, D. J., 1990. Basic local alignment search tool. *J Mol Biol,* **215**, 403-410.

Alvarez-Perez, S., Blanco, J. L., Harmanus, C., Kuijper, E., Garcia, M. E., 2017. Subtyping and antimicrobial susceptibility of Clostridium difficile PCR ribotype 078/126 isolates of human and animal origin. *Vet Microbiol,* **199**, 15-22.

Ananthakrishnan, A. N., 2011. Clostridium difficile infection: epidemiology, risk factors and management. *Nat Rev Gastroenterol Hepatol,* **8**, 17-26.

Ando, H., Lemire, S., Pires, D. P., Lu, T. K., 2015. Engineering modular viral scaffolds for targeted bacterial population editing. *Cell systems,* **1**, 187-196.

Anfasa, F., Siegers, J. Y., van der Kroeg, M., Mumtaz, N., Stalin Raj, V., de Vrij, F. M. S., Widagdo, W., Gabriel, G., Salinas, S., Simonin, Y., Reusken, C., Kushner, S. A., Koopmans, M. P. G., Haagmans, B., Martina, B. E. E., van Riel, D., 2017. Phenotypic Differences between Asian and African Lineage Zika Viruses in Human Neural Progenitor Cells. *mSphere,* **2**.

Attai, H., Rimbey, J., Smith, G. P., Brown, P. J. B., 2017. Expression of a Peptidoglycan Hydrolase from Lytic Bacteriophages Atu_ph02 and Atu_ph03 Triggers Lysis of Agrobacterium tumefaciens. *Appl Environ Microbiol,* **83**.

Awad, M. M., Bryant, A. E., Stevens, D. L., Rood, J. I., 1995. Virulence studies on chromosomal α-toxin and Θ-toxin mutants constructed by allelic exchange provide genetic evidence for the essential role of α-toxin in Clostridium perfringens-mediated gas gangrene. *Molecular microbiology,* **15**, 191-202.

Azencott, H. R. 2003. *Influence of the cell wall on intracellular delivery by electroporation and acoustic cavitation.* Ph. D. Georgia Institute of Technology.

Aziminia, P., Pilehchian-Langroudi, R., Esmaeilnia, K., 2016. Cloning and expression of Clostridium perfringens type D vaccine strain epsilon toxin gene in E. coli as a recombinant vaccine candidate. *Iran J Microbiol,* **8**, 226-231.

Badagliacca, P., Di Provvido, A., Scattolini, S., Pompei, G., Di Giannatale, E., 2010. Toxin genotyping of Clostridium perfringens strains using a polymerase chain reaction protocol. *Vet Ital,* **46**, 113-118, 107-112.

Barkin, J. A., Sussman, D. A., Fifadara, N., Barkin, J. S., 2017. Clostridium difficile Infection and Patient-Specific Antimicrobial Resistance Testing Reveals a High Metronidazole Resistance Rate. *Dig Dis Sci,* **62**, 1035-1042.

Bartlett, J. G., Chang, T. W., Gurwith, M., Gorbach, S. L., Onderdonk, A. B., 1978. Antibiotic-associated pseudomembranous colitis due to toxin-producing clostridia. *New England Journal of Medicine,* **298**, 531-534.

Bastos, M. D., Coutinho, B. G., Coelho, M. L., 2010. Lysostaphin: A Staphylococcal Bacteriolysin with Potential Clinical Applications. *Pharmaceuticals (Basel),* **3**, 1139-1161.

Bateman, A., Coin, L., Durbin, R., Finn, R. D., Hollich, V., Griffiths-Jones, S., Khanna, A., Marshall, M., Moxon, S., Sonnhammer, E. L., 2004. The Pfam protein families database. *Nucleic acids research,* **32**, D138-D141.

Baums, C. G., Schotte, U., Amtsberg, G., Goethe, R., 2004a. Diagnostic multiplex PCR for toxin genotyping of Clostridium perfringens isolates. *Veterinary microbiology,* **100**, 11-16.

Baums, C. G., Schotte, U., Amtsberg, G., Goethe, R., 2004b. Diagnostic multiplex PCR for toxin genotyping of Clostridium perfringens isolates. *Veterinary microbiology,* **100**, 11-16.

Beloin, C., Roux, A., Ghigo, J.-M., 2008. Escherichia coli biofilms. *Bacterial Biofilms.* Springer 249-289.

Bengelsdorf, F. R., Poehlein, A., Linder, S., Erz, C., Hummel, T., Hoffmeister, S., Daniel, R., Durre, P., 2016. Industrial Acetogenic Biocatalysts: A Comparative Metabolic and Genomic Analysis. *Front Microbiol,* **7**, 1036.

Bennett, W. D. & Tieleman, D. P., 2014. The Importance of Membrane Defects ▪ Lessons from Simulations. *Accounts of Chemical Research*.

Beperet, I., Irons, S. L., Simón, O., King, L. A., Williams, T., Possee, R. D., López-Ferber, M., Caballero, P., 2014. Superinfection exclusion in alphabaculovirus infections is concomitant with actin reorganization. *J Virol,* **88**, 3548-3556.

Bernasconi, O. J., Dona, V., Tinguely, R., Endimiani, A., 2017. In vitro activity of three commercial bacteriophage cocktails against multidrug-resistant Escherichia coli and Proteus spp. strains of human and non-human origin. *J Glob Antimicrob Resist,* **8**, 179-185.

Bhatia, B., Solanki, A. K., Kaushik, H., Dixit, A., Garg, L. C., 2014. B-cell epitope of beta toxin of Clostridium perfringens genetically conjugated to a carrier protein: expression, purification and characterization of the chimeric protein. *Protein Expr Purif,* **102**, 38-44.

Bi, C., Jones, S. W., Hess, D. R., Tracy, B. P., Papoutsakis, E. T., 2011. SpoIIE is necessary for asymmetric division, sporulation, and expression of σF, σE, and σG but does not control solvent production in Clostridium acetobutylicum ATCC 824. *J Bacteriol,* **193**, 5130-5137.

Bibbo, S., Lopetuso, L. R., Ianiro, G., Di Rienzo, T., Gasbarrini, A., Cammarota, G., 2014. Role of microbiota and innate immunity in recurrent Clostridium difficile infection. *J Immunol Res,* **2014**, 462740.

Bikard, D., Euler, C. W., Jiang, W., Nussenzweig, P. M., Goldberg, G. W., Duportet, X., Fischetti, V. A., Marraffini, L. A., 2014. Exploiting CRISPR-Cas nucleases to produce sequence-specific antimicrobials. *Nat Biotechnol,* **32**, 1146-1150.

Billington, S. J., Wieckowski, E. U., Sarker, M. R., Bueschel, D., Songer, J. G., McClane, B. A., 1998. Clostridium perfringens type E animal enteritis isolates with highly conserved, silent enterotoxin gene sequences. *Infection and immunity,* **66**, 4531-4536.

Birge, E. A., 2006. *Bacterial and bacteriophage genetics.* Springer.

Bonardi, F., Halza, E., Walko, M., Du Plessis, F., Nouwen, N., Feringa, B. L., Driessen, A. J., 2011. Probing the SecYEG translocation pore size with preproteins conjugated with sizable rigid spherical molecules. *Proceedings of the National Academy of Sciences,* **108**, 7775-7780.

Borysowski, J., Weber-Dabrowska, B., Gorski, A., 2006. Bacteriophage endolysins as a novel class of antibacterial agents. *Exp Biol Med (Maywood),* **231**, 366-377.

Briers, Y., Volckaert, G., Cornelissen, A., Lagaert, S., Michiels, C. W., Hertveldt, K., Lavigne, R., 2007. Muralytic activity and modular structure of the endolysins of Pseudomonas aeruginosa bacteriophages φKZ and EL. *Molecular microbiology,* **65**, 1334-1344.

Briers, Y., Walmagh, M., Van Puyenbroeck, V., Cornelissen, A., Cenens, W., Aertsen, A., Oliveira, H., Azeredo, J., Verween, G., Pirnay, J.-P., 2014. Engineered endolysin-based "Artilysins" to combat multidrug-resistant gram-negative pathogens. *MBio,* **5**, e01379-01314.

Broom, L., 2017. Necrotic enteritis; current knowledge and diet-related mitigation. *World's Poultry Science Journal*, **73**, 281-292.

Brown, N. F., Wickham, M. E., Coombes, B. K., Finlay, B. B., 2006. Crossing the line: selection and evolution of virulence traits. *PLoS pathogens*, **2**, e42.

Brüggemann, H. & Gottschalk, G., 2009. *Clostridia: molecular biology in the post-genomic era.* Horizon Scientific Press.

Brumbaugh, D. E., De Zoeten, E. F., Pyo-Twist, A., Fidanza, S., Hughes, S., Dolan, S. A., Child, J., Dominguez, S. R., 2017. An Intragastric Fecal Microbiota Transplantation Program for Treatment of Recurrent Clostridium difficile in Children is Efficacious, Safe, and Inexpensive. *J Pediatr*.

Brüssow, H., Canchaya, C., Hardt, W.-D., 2004. Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiology and Molecular Biology Reviews*, **68**, 560-602.

Brzychczy-Wloch, M., Ochonska, D., Piotrowska, A., Bulanda, M., 2017. Gas Gangrene of Different Origin Associated with Clostridium perfringens Type A in Three Patients Simultaneously Hospitalized in a Single Department of Orthopedics and Traumatology in Poland. *Pol J Microbiol*, **65**, 399-406.

Buzby, J. C. & Roberts, T., 1997. Economic costs and trade impacts of microbial foodborne illness. *World Health Stat Q*, **50**, 57-66.

Calabi, E., Calabi, F., Phillips, A. D., Fairweather, N. F., 2002. Binding of Clostridium difficile surface layer proteins to gastrointestinal tissues. *Infection and immunity*, **70**, 5770-5778.

Caly, D. L., D'Inca, R., Auclair, E., Drider, D., 2015. Alternatives to Antibiotics to Prevent Necrotic Enteritis in Broiler Chickens: A Microbiologist's Perspective. *Frontiers in Microbiology*, **6**.

Campbell, A., 2003. The future of bacteriophage biology. *Nature Reviews Genetics*, **4**, 471-477.

Canchaya, C., Proux, C., Fournous, G., Bruttin, A., Brüssow, H., 2003. Prophage genomics. *Microbiology and Molecular Biology Reviews*, **67**, 238-276.

Carding, S., Verbeke, K., Vipond, D. T., Corfe, B. M., Owen, L. J., 2015. Dysbiosis of the gut microbiota in disease. *Microbial ecology in health and disease*, **26**, 26191.

Carnazza, S. & Guglielmino, S., 2010. PHAGE DISPLAY AS A TOOL FOR SYNTHETIC BIOLOGY. 79.

Carretero, R. G., Brugera, M. R., Vazquez-Gomez, O., Rebollo-Aparicio, N., 2016. Massive haemolysis, gas-forming liver abscess and sepsis due to Clostridium perfringens bacteraemia. *BMJ Case Reports*, **2016**, bcr2016218014.

Carter, G. P., Larcombe, S., Li, L., Jayawardena, D., Awad, M. M., Songer, J. G., Lyras, D., 2014. Expression of the large clostridial toxins is controlled by conserved regulatory mechanisms. *Int J Med Microbiol*.

Carter, P., 1986. Site-directed mutagenesis. *Biochemical Journal*, **237**, 1.

Carvalho, C., Costa, A. R., Silva, F., Oliveira, A., 2017. Bacteriophages and their derivatives for the treatment and control of food-producing animal infections. *Crit Rev Microbiol*, 1-19.

Caspers, P., Locher, H. H., Pfaff, P., Diggelmann, S., Rueedi, G., Bur, D., Ritz, D., 2017. Different Resistance Mechanisms for Cadazolid and Linezolid in C. difficile found by Whole Genome Sequencing Analysis. *Antimicrob Agents Chemother*.

Cassir, N., Fahsi, N., Durand, G., Lagier, J. C., Raoult, D., Fournier, P. E., 2017. Emergence of Clostridium difficile tcdC variant 078 in Marseille, France. *Eur J Clin Microbiol Infect Dis*.

Cerquetti, M., Molinari, A., Sebastianelli, A., Diociaiuti, M., Petruzzelli, R., Capo, C., Mastrantonio, P., 2000. Characterization of surface layer proteins from different Clostridium difficile clinical isolates. *Microbial pathogenesis*, **28**, 363-372.

Chan, L. Y., Kosuri, S., Endy, D., 2005. Refactoring bacteriophage T7. *Molecular systems biology*, **1**.

Chandrasekaran, R. & Lacy, D. B., 2017. The role of toxins in Clostridium difficile infection. *FEMS Microbiol Rev*, **41**, 723-750.

Chang, D. C., Chassy, B. M., Saunders, J. A., Sowers, A. E., 1992. *Guide to electroporation and electrofusion.* Academic Press.

Chanishvili, N., 2012. Phage therapy—history from Twort and d'Herelle through Soviet experience to current approaches. *Bacteriophages*, **83**, 1.

Chellapandi, P. & Prisilla, A., 2016. Structure, Function and Evolution of Clostridium botulinum C2 and C3 Toxins: Insight to Poultry and Veterinary Vaccines. *Curr Protein Pept Sci*.

Cheng, W., Chen, H., Yan, S., Su, J., 2014. Illumina sequencing-based analyses of bacterial communities during short-chain fatty-acid production from food waste and sewage sludge fermentation at different pH values. *World J Microbiol Biotechnol*.

Cheng, X., Zhang, X., Pflugrath, J. W., Studier, F. W., 1994. The structure of bacteriophage T7 lysozyme, a zinc amidase and an inhibitor of T7 RNA polymerase. *Proceedings of the National Academy of Sciences*, **91**, 4034-4038.

Cherenfant, J., Nikfarjam, M., Mathew, A., Kimchi, E. T., Staveley-O'Carroll, K. F., 2009. Completion Pancreatectomy for Treatment of a Clostridium perfringens Pancreatic Infection. *Archives of Surgery,* **144**, 368-370.

Chizmadzhev, Y. A., Arakelyan, V., Pastushenko, V., 1979. 248-Electric breakdown of bilayer lipid membranes III. Analysis of possible mechanisms of defect origination. *Bioelectrochemistry and Bioenergetics,* **6**, 63-70.

Choi, I. Y., Lee, J. H., Kim, H. J., Park, M. K., 2017. Isolation and Characterization of a Novel Broad-host-range Bacteriophage Infecting Salmonella enterica subsp. enterica for Biocontrol and Rapid Detection. *J Microbiol Biotechnol,* **27**, 2151-2155.

Chukwu, E. E., Nwaokorie, F. O., Coker, A. O., Avila-Campos, M. J., Solis, R. L., Llanco, L. A., Ogunsola, F. T., 2016. Detection of toxigenic Clostridium perfringens and Clostridium botulinum from food sold in Lagos, Nigeria. *Anaerobe,* **42**, 176-181.

Cisek, A. A., Dabrowska, I., Gregorczyk, K. P., Wyzewski, Z., 2017. Phage Therapy in Bacterial Infections Treatment: One Hundred Years After the Discovery of Bacteriophages. *Curr Microbiol,* **74**, 277-283.

Cochrane, J., Bland, L., Noble, M., 2015. Intravascular hemolysis and septicemia due to Clostridium perfringens emphysematous cholecystitis and hepatic abscesses. *Case reports in medicine,* **2015**.

Colavecchio, A., Cadieux, B., Lo, A., Goodridge, L. D., 2017. Bacteriophages Contribute to the Spread of Antibiotic Resistance Genes among Foodborne Pathogens of the Enterobacteriaceae Family - A Review. *Front Microbiol,* **8**, 1108.

Coletti, L., Battaglia, V., De Simone, P., Turturici, L., Bartolozzi, C., Filipponi, F., 2017. Safety and feasibility of electrochemotherapy in patients with unresectable colorectal liver metastases: A pilot study. *Int J Surg*.

Collery, M. M., Kuehne, S. A., McBride, S. M., Kelly, M. L., Monot, M., Cockayne, A., Dupuy, B., Minton, N. P., 2016. What's a SNP between friends: The influence of single nucleotide polymorphisms on virulence and phenotypes of Clostridium difficile strain 630 and derivatives. *Virulence*, 1-15.

Colombet, J. & Sime-Ngando, T., 2012. Use of PEG, Polyethylene glycol, to characterize the diversity of environmental viruses. *Microbial Ecology*, **58**, 728-736.

Cooksley, C. M., Zhang, Y., Wang, H., Redl, S., Winzer, K., Minton, N. P., 2012. Targeted mutagenesis of the< i> Clostridium acetobutylicum</i> acetone–butanol–ethanol fermentation pathway. *Metabolic engineering*, **14**, 630-641.

Cooper, K. K., Bueschel, D. M., Songer, J. G., 2013. Presence of Clostridium perfringens in retail chicken livers. *Anaerobe*, **21**, 67-68.

Costa, M., Walbott, H., Monachello, D., Westhof, E., Michel, F., 2016. Crystal structures of a group II intron lariat primed for reverse splicing. *Science*, **354**.

Cukkemane, N., Bikker, F. J., Nazmi, K., Brand, H. S., Veerman, E. C., 2014. Identification and characterization of a salivary-pellicle-binding peptide by phage display. *Arch Oral Biol*, **59**, 448-454.

Dang, T. T., Riva, L. d. l., Fagan, R. P., Storck, E. M., Heal, W. P., Janoir, C., Fairweather, N. F., Tate, E. W., 2010. Chemical probes of surface layer biogenesis in Clostridium difficile. *ACS chemical biology*, **5**, 279-285.

Dapa, T. & Unnikrishnan, M., 2013. Biofilm formation by Clostridium difficile. *Gut Microbes*, **4**, 397-402.

Dave, G. A., 2017. A rapid qualitative assay for detection of Clostridium perfringens in canned food products. *Acta Biochim Pol*, **64**, 207-213.

Dawson, L. F., Stabler, R. A., Wren, B. W., 2009. Comparative genomics of Clostridium difficile. *Clostridia: Molecular Biology in the Post-genomic Era*, 143.

de la Riva, L., Willing, S. E., Tate, E. W., Fairweather, N. F., 2011. Roles of cysteine proteases Cwp84 and Cwp13 in biogenesis of the cell wall of Clostridium difficile. *J Bacteriol*, **193**, 3276-3285.

Dei, R., 1989. Observations on phage-typing of Clostridium difficile: preliminary evaluation of a phage panel. *European journal of epidemiology,* **5**, 351-354.

Dembek, M., Barquist, L., Boinett, C. J., Cain, A. K., Mayho, M., Lawley, T. D., Fairweather, N. F., Fagan, R. P., 2015. High-throughput analysis of gene essentiality and sporulation in Clostridium difficile. *MBio,* **6**, e02383-02314.

Deng, Y., Zhang, X., Zhang, X., 2017. Recent advances in genetic modification systems for Actinobacteria. *Appl Microbiol Biotechnol,* **101**, 2217-2226.

Diez-Martinez, R., De Paz, H. D., Garcia-Fernandez, E., Bustamante, N., Euler, C. W., Fischetti, V. A., Menendez, M., Garcia, P., 2015. A novel chimeric phage lysin with high in vitro and in vivo bactericidal activity against Streptococcus pneumoniae. *J Antimicrob Chemother,* **70**, 1763-1773.

Domsalla, A. & Melzig, M. F., 2008. Occurrence and properties of proteases in plant latices. *Planta medica,* **74**, 699-711.

Dong, Q., Wang, J., Yang, H., Wei, C., Yu, J., Zhang, Y., Huang, Y., Zhang, X. E., Wei, H., 2015. Construction of a chimeric lysin Ply187N-V12C with extended lytic activity against staphylococci and streptococci. *Microb Biotechnol,* **8**, 210-220.

Dong, X., Stothard, P., Forsythe, I. J., Wishart, D. S., 2004. PlasMapper: a web server for drawing and auto-annotating plasmid maps. *Nucleic acids research,* **32**, W660-W664.

Donovan, D., Becker, S., Dong, S., Baker, J., Foster-Frey, J., Pritchard, D., 2009. Peptidoglycan hydrolase enzyme fusions for treating multi-drug resistant pathogens. *Biotech International,* **21**, 6.

Dower, W. J., Miller, J. F., Ragsdale, C. W., 1988. High efficiency transformation of E. coli by high voltage electroporation. *Nucleic acids research,* **16**, 6127-6145.

Dubberke, E. R. & Burnham, C.-A. D., 2011. Clostridium difficile: Methods and Protocols. *Clinical Infectious Diseases,* **52**, 964-965.

Dufour, N. & Debarbieux, L., 2017. [Phage therapy: a realistic weapon against multidrug resistant bacteria]. *Med Sci (Paris),* **33**, 410-416.

Dunne, M., Leicht, S., Krichel, B., Mertens, H. D., Thompson, A., Krijgsveld, J., Svergun, D. I., Gomez-Torres, N., Garde, S., Uetrecht, C., Narbad, A., Mayer, M. J., Meijers, R., 2016. Crystal Structure of the CTP1L Endolysin Reveals How Its Activity Is Regulated by a Secondary Translation Product. *J Biol Chem,* **291**, 4882-4893.

Dunne, M., Mertens, H. D., Garefalaki, V., Jeffries, C. M., Thompson, A., Lemke, E. A., Svergun, D. I., Mayer, M. J., Narbad, A., Meijers, R., 2014. The CD27L and CTP1L endolysins targeting Clostridia contain a built-in trigger and release factor. *PLoS Pathog,* **10**, e1004228.

Durovic, A., Widmer, A. F., Frei, R., Tschudin-Sutter, S., 2017. Distinguishing Clostridium difficile Recurrence From Reinfection: Independent Validation of Current Recommendations. *Infect Control Hosp Epidemiol*, 1-6.

Eisgruber, H., Wiedmann, M., Stolle, A., 1996. Plasmid profiling for strain differentiation and characterization of Clostridium perfringens isolates. *Journal of Veterinary Medicine, Series B,* **43**, 137-146.

El-Shibiny, A. & El-Sahhar, S., 2017. Bacteriophages: The possible solution to treat pathogenic bacteria. *Can J Microbiol*.

Ellis, J. T., Sims, R. C., Miller, C. D., 2014. Microbial bioproducts from cheese whey through fermentation with wastewater sludge Clostridium isolates. *Can J Microbiol*, 1-5.

Erickson, J. E. & Deibel, R. H., 1978. New medium for rapid screening and enumeration of Clostridium perfringens in foods. *Appl Environ Microbiol,* **36**, 567-571.

Fagan, R. & Fairweather, N., 2010. Dissecting the cell surface. *Clostridium difficile: Methods and Protocols*, 117-134.

Fagan, R. P., Albesa-Jové, D., Qazi, O., Svergun, D. I., Brown, K. A., Fairweather, N. F., 2009. Structural insights into the molecular organization of the S-layer from Clostridium difficile. *Molecular microbiology,* **71**, 1308-1322.

Faulds-Pain, A. & Wren, B. W., 2013. Improved bacterial mutagenesis by high-frequency allele exchange, demonstrated in Clostridium difficile and Streptococcus suis. *Applied and environmental microbiology,* **79**, 4768-4771.

Fehér, T., Karcagi, I., Blattner, F. R., Pósfai, G., 2012. Bacteriophage recombineering in the lytic state using the lambda red recombinases. *Microbial biotechnology*, **5**, 466-476.

Fernandes, S., Proenca, D., Cantante, C., Silva, F. A., Leandro, C., Lourenco, S., Milheirico, C., de Lencastre, H., Cavaco-Silva, P., Pimentel, M., Sao-Jose, C., 2012. Novel chimerical endolysins with broad antimicrobial activity against methicillin-resistant Staphylococcus aureus. *Microb Drug Resist,* **18**, 333-343.

Ferreira, M. R., Moreira, G. M., Cunha, C. E., Mendonca, M., Salvarani, F. M., Moreira, A. N., Conceicao, F. R., 2016. Recombinant Alpha, Beta, and Epsilon Toxins of Clostridium perfringens: Production Strategies and Applications as Veterinary Vaccines. *Toxins (Basel),* **8**.

Finegold, S. M., Summanen, P. H., Downes, J., Corbett, K., Komoriya, T., 2017. Detection of Clostridium perfringens toxin genes in the gut microbiota of autistic children. *Anaerobe*.

Finsterer, J. & Hess, B., 2007. Neuromuscular and Central Nervous System Manifestations of Clostridium perfringens Infections. *Infection,* **35**, 396-405.

Fischetti, V. A., 2008. Bacteriophage lysins as effective antibacterials. *Current opinion in microbiology,* **11**, 393-400.

Fischetti, V. A., 2010. Bacteriophage endolysins: a novel anti-infective to control Gram-positive pathogens. *International Journal of Medical Microbiology,* **300**, 357-362.

Fokine, A. & Rossmann, M. G., 2014. Molecular architecture of tailed double-stranded DNA phages. *Bacteriophage,* **4**, e28281.

Fortier, L.-C. & Moineau, S., 2007. Morphological and genetic diversity of temperate phages in Clostridium difficile. *Applied and environmental microbiology,* **73**, 7358-7366.

Galtier, M., De Sordi, L., Sivignon, A., de Vallee, A., Maura, D., Neut, C., Rahmouni, O., Wannerberger, K., Darfeuille-Michaud, A., Desreumaux, P., Barnich, N., Debarbieux, L., 2017. Bacteriophages targeting adherent invasive Escherichia coli strains as a promising new treatment for Crohn's disease. *J Crohns Colitis*.

Gandon, S., 2016. Why Be Temperate: Lessons from Bacteriophage lambda. *Trends Microbiol,* **24**, 356-365.

Gao, J., Wang, Y., Liu, Z., Wang, Z., 2010. Phage display and its application in vaccine design. *Annals of microbiology,* **60**, 13-19.

2014. *Proceedings of the 2012 International Conference on Applied Biotechnology (ICAB 2012).* Springer.

Garcia-Jimenez, A., Prim, N., Crusi, X., Benito, N., 2016. Septic arthritis due to Clostridium ramosum. *Semin Arthritis Rheum,* **45**, 617-620.

García-Lechuz, J., Hernangomez, S., Juan, R. S., Pelaez, T., Alcala, L., Bouza, E., 2001. Extra-intestinal infections caused by Clostridium difficile. *Clinical Microbiology and Infection,* **7**, 453-457.

García, S. & Heredia, N., 2011. Clostridium perfringens: A dynamic foodborne pathogen. *Food and Bioprocess Technology,* **4**, 624-630.

Garneau, J. R., Depardieu, F., Fortier, L. C., Bikard, D., Monot, M., 2017. PhageTerm: a tool for fast and accurate determination of phage termini and packaging mechanism using next-generation sequencing data. *Sci Rep,* **7**, 8292.

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R. D., Bairoch, A., 2003. ExPASy: the proteomics server for in-depth protein knowledge and analysis. *Nucleic acids research,* **31**, 3784-3788.

Gaucher, M. L., Perron, G. G., Arsenault, J., Letellier, A., Boulianne, M., Quessy, S., 2017. Recurring Necrotic Enteritis Outbreaks in Commercial Broiler Chicken Flocks Strongly Influence Toxin Gene Carriage and Species Richness in the Resident Clostridium perfringens Population. *Front Microbiol,* **8**, 881.

Gerding, D. N., Johnson, S., Rupnik, M., Aktories, K., 2014. Clostridium difficile binary toxin CDT: mechanism, epidemiology, and potential clinical importance. *Gut microbes,* **5**, 15-27.

Gerstmans, H., Rodriguez-Rubio, L., Lavigne, R., Briers, Y., 2016. From endolysins to Artilysin(R)s: novel enzyme-based approaches to kill drug-resistant bacteria. *Biochem Soc Trans,* **44**, 123-128.

Gervasi, T., Horn, N., Wegmann, U., Dugo, G., Narbad, A., Mayer, M. J., 2014a. Expression and delivery of an endolysin to combat Clostridium perfringens. *Appl Microbiol Biotechnol,* **98**, 2495-2505.

Gervasi, T., Lo Curto, R., Minniti, E., Narbad, A., Mayer, M. J., 2014b. Application of Lactobacillus johnsonii expressing phage endolysin for control of Clostridium perfringens. *Lett Appl Microbiol,* **59**, 355-361.

Gharaibeh, S., Al Rifai, R., Al-Majali, A., 2010. Molecular typing and antimicrobial susceptibility of Clostridium perfringens from broiler chickens. *Anaerobe,* **16**, 586-589.

Gillis, H. D., Lang, A. L. S., ElSherif, M., Martin, I., Hatchette, T. F., McNeil, S. A., LeBlanc, J. J., 2017. Assessing the diagnostic accuracy of PCR-based detection of Streptococcus pneumoniae from nasopharyngeal swabs collected for viral studies in Canadian adults hospitalised with community-acquired pneumonia: a

Serious Outcomes Surveillance (SOS) Network of the Canadian Immunization Research (CIRN) study. *BMJ Open,* **7**, e015008.

Goh, S., Riley, T. V., Chang, B. J., 2005. Isolation and characterization of temperate bacteriophages of Clostridium difficile. *Applied and environmental microbiology,* **71**, 1079-1083.

Gohari, I. M., Kropinski, A. M., Weese, S. J., Whitehead, A. E., Parreira, V. R., Boerlin, P., Prescott, J. F., 2017. NetF-producing Clostridium perfringens: Clonality and plasmid pathogenicity loci analysis. *Infection, Genetics and Evolution*.

Goldenberg, J. Z., Yap, C., Lytvyn, L., Lo, C. K., Beardsley, J., Mertz, D., Johnston, B. C., 2017. Probiotics for the prevention of Clostridium difficile-associated diarrhea in adults and children. *Cochrane Database Syst Rev,* **12**, CD006095.

Goldstein, M. R., Kruth, S. A., Bersenas, A. M., Holowaychuk, M. K., Weese, J. S., 2012. Detection and characterization of Clostridium perfringens in the feces of healthy and diarrheic dogs. *Can J Vet Res,* **76**, 161-165.

Górski, A., Miedzybrodzki, R., Borysowski, J., Dabrowska, K., Wierzbicki, P., Ohams, M., Korczak-Kowalska, G., Olszowska-Zaremba, N., Lusiak-Szelachowska, M., Klak, M., 2012. 2 Phage as a Modulator of Immune Responses: Practical Implications for Phage Therapy. *Advances in virus research,* **83**, 41.

Gould, E. N., Giannone, R., Kania, S. A., Tolbert, M. K., 2017. Cysteine protease 30 (CP30) contributes to adhesion and cytopathogenicity in feline Tritrichomonas foetus. *Vet Parasitol,* **244**, 114-122.

Govind, R., Fralick, J. A., Rolfe, R. D., 2006. Genomic organization and molecular characterization of Clostridium difficile bacteriophage ΦCD119. *J Bacteriol,* **188**, 2568-2577.

Govind, R., Vediyappan, G., Rolfe, R. D., Dupuy, B., Fralick, J. A., 2009. Bacteriophage-mediated toxin gene regulation in Clostridium difficile. *J Virol,* **83**, 12037-12045.

Green, E. M., Boynton, Z. L., Harris, L. M., Rudolph, F. B., Papoutsakis, E. T., Bennett, G. N., 1996. Genetic manipulation of acid formation pathways by gene inactivation in Clostridium acetobutylicum ATCC 824. *Microbiology,* **142**, 2079-2086.

Green, R. & Rogers, E. J., 2013. Chemical transformation of E. coli. *Methods in enzymology,* **529**, 329.

Griffiths, A. J., 2002. *Modern genetic analysis: integrating genes and genomes.* Macmillan.

Guenther, S., Huwyler, D., Richard, S., Loessner, M. J., 2009. Virulent bacteriophage for efficient biocontrol of Listeria monocytogenes in ready-to-eat foods. *Applied and environmental microbiology*, **75**, 93-100.

Gunathilaka, G. U., Tahlan, V., Mafiz, A., Polur, M., Zhang, Y., 2017. Phage in urban wastewater have the potential to disseminate antibiotic resistance. *Int J Antimicrob Agents*.

Gupta, R. & Prasad, Y., 2011. P-27/HP endolysin as antibacterial agent for antibiotic resistant Staphylococcus aureus of human infections. *Current microbiology*, **63**, 39.

Gurjar, A. A., Hegde, N. V., Love, B. C., Jayarao, B. M., 2008. Real-time multiplex PCR assay for rapid detection and toxintyping of Clostridium perfringens toxin producing strains in feces of dairy cattle. *Mol Cell Probes*, **22**, 90-95.

Hagens, S., Habel, A., Von Ahsen, U., Von Gabain, A., Bläsi, U., 2004. Therapy of experimental Pseudomonas infections with a nonreplicating genetically modified phage. *Antimicrobial Agents and Chemotherapy*, **48**, 3817-3822.

Hahnke, S., Wibberg, D., Tomazetto, G., Puhler, A., Klocke, M., Schluter, A., 2014. Whole genome sequence of Clostridium sp. strain M2/40 isolated from a lab-scale mesophilic two-phase biogas reactor digesting maize silage and wheat straw. *J Biotechnol*.

Hall, I. C. & O'TOOLE, E., 1935. Intestinal flora in new-born infants: with a description of a new pathogenic anaerobe, Bacillus difficilis. *American journal of diseases of children,* **49**, 390-402.

Hall, M. P., Unch, J., Binkowski, B. F., Valley, M. P., Butler, B. L., Wood, M. G., Otto, P., Zimmerman, K., Vidugiris, G., Machleidt, T., Robers, M. B., Benink, H. A., Eggers, C. T., Slater, M. R., Meisenheimer, P. L., Klaubert, D. H., Fan, F., Encell, L. P., Wood, K. V., 2012. Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. *ACS Chem Biol*, **7**, 1848-1857.

Han, D., Byun, S. H., Kim, J., Kwon, M., Pleasure, S. J., Ahn, J. H., Yoon, K., 2017. Human cytomegalovirus IE2 protein disturbs brain development by dysregulating neural stem cell maintenance and the polarization of migrating neurons. *J Virol*.

Hargreaves, K. R. 2013. *Isolation and Characterisation of Bacteriophages Infecting Environmental Strains of Clostridium difficile.* Ph. D. Department of Infection, Immunity and Inflammation.

Hargreaves, K. R. & Clokie, M. R. J., 2014. Clostridium difficile phages: still difficult? *Frontiers in Microbiology,* **5**.

Harmsen, A. M., van Tol, E., Giannakopoulos, G. F., de Brauw, L. M., 2016. Clostridial Gas Gangrene of the Abdominal Wall After Laparoscopic Cholecystectomy: A Case Report and Review. *Surg Laparosc Endosc Percutan Tech,* **26**, 278-281.

Harper, P. S., 2010. *Practical Genetic Counselling 7th Edition.* CRC Press.

Heap, J. T., Cartman, S. T., Pennington, O. J., Cooksley, C. M., Scott, J. C., Blount, B., Burns, D. A., Minton, N. P., 2009a. Development of genetic knock-out systems for clostridia. *Clostridia: molecular biology in the post-genomic era. Caister Academic Press, Norfolk, United Kingdom*, 179-198.

Heap, J. T., Kuehne, S. A., Ehsaan, M., Cartman, S. T., Cooksley, C. M., Scott, J. C., Minton, N. P., 2010. The ClosTron: Mutagenesis in Clostridium refined and streamlined. *J Microbiol Methods,* **80**, 49-55.

Heap, J. T., Pennington, O. J., Cartman, S. T., Carter, G. P., Minton, N. P., 2007. The ClosTron: a universal gene knock-out system for the genus Clostridium. *J Microbiol Methods,* **70**, 452-464.

Heap, J. T., Pennington, O. J., Cartman, S. T., Minton, N. P., 2009b. A modular system for< i> Clostridium</i> shuttle plasmids. *J Microbiol Methods,* **78**, 79-85.

Hegarty, J. P., Sangster, W., Ashley, R. E., Myers, R., Hafenstein, S., Stewart, D. B., Sr., 2016. Induction and Purification of C. difficile Phage Tail-Like Particles. *Methods Mol Biol,* **1476**, 167-175.

Henry, M., Lavigne, R., Debarbieux, L., 2013. Predicting In Vivo Efficacy of Therapeutic Bacteriophages Used To Treat Pulmonary Infections. *Antimicrobial Agents and Chemotherapy,* **57**, 5961-5968.

Hensgens, M., Keessen, E., Squire, M., Riley, T., Koene, M., de Boer, E., Lipman, L., Kuijper, E., 2012. Clostridium difficile infection in the community: a zoonotic disease? *Clinical microbiology and infection,* **18**, 635-645.

Herman, N. A., Li, J., Bedi, R., Turchi, B., Liu, X., Miller, M. J., Zhang, W., 2017. Development of a High-Efficiency Transformation Method and Implementation of Rational Metabolic Engineering for the Industrial Butanol Hyperproducer

Clostridium saccharoperbutylacetonicum Strain N1-4. *Appl Environ Microbiol,* **83**.

Hernandez, L. D., Kroh, H. K., Hsieh, E., Yang, X., Beaumont, M., Sheth, P. R., DiNunzio, E., Rutherford, S. A., Ohi, M. D., Ermakov, G., Xiao, L., Secore, S., Karczewski, J., Racine, F., Mayhood, T., Fischer, P., Sher, X., Gupta, P., Lacy, D. B., Therien, A. G., 2017. Epitopes and Mechanism of Action of the Clostridium difficile Toxin A-Neutralizing Antibody Actoxumab. *J Mol Biol,* **429**, 1030-1044.

Hibbing, M. E., Fuqua, C., Parsek, M. R., Peterson, S. B., 2010. Bacterial competition: surviving and thriving in the microbial jungle. *Nature reviews. Microbiology,* **8**, 15.

Hifumi, T., Koido, Y., Takahashi, M., Yamamoto, A., 2013. Antitoxin treatment for liver abscess caused by Clostridium perfringens. *Clinical and molecular hepatology,* **19**, 97-98.

Hifumi, T., Nakano, D., Chiba, J., Takahashi, M., Yamamoto, A., Fujisawa, Y., Kawakita, K., Kuroda, Y., Nishiyama, A., 2018. Combined therapy with gas gangrene antitoxin and recombinant human soluble thrombomodulin for Clostridium perfringens sepsis in a rat model. *Toxicon,* **141**, 112-117.

Holmes, R. & Jobling, M., 1996. Genetics: conjugation. *Barons medical microbiology,* **4**.

Horgan, M., O'Sullivan, O., Coffey, A., Fitzgerald, G. F., van Sinderen, D., McAuliffe, O., Ross, R. P., 2010. Genome analysis of the Clostridium difficile phage ΦCD6356, a temperate phage of the Siphoviridae family. *Gene,* **462**, 34-43.

Hu, B., Margolin, W., Molineux, I. J., Liu, J., 2015. Structural remodeling of bacteriophage T4 and host membranes during infection initiation. *Proc Natl Acad Sci U S A,* **112**, E4919-4928.

Hu, Y., Yang, H., Wang, J., Zhang, Y., Yu, J., Wei, H., 2016. Comparison between a chimeric lysin ClyH and other enzymes for extracting DNA to detect methicillin resistant Staphylococcus aureus by quantitative PCR. *World J Microbiol Biotechnol,* **32**, 1.

Huber, C. A., Foster, N. F., Riley, T. V., Paterson, D. L., 2013. Challenges for standardization of Clostridium difficile typing methods. *Journal of clinical microbiology,* **51**, 2810-2814.

Hugelshofer, M., Achermann, Y., Kovari, H., Dent, W., Hombach, M., Bloemberg, G., 2012. Meningoencephalitis with subdural empyema caused by toxigenic Clostridium perfringens type A. *Journal of clinical microbiology,* **50**, 3409-3411.

Hutton, M. L., Cunningham, B. A., Mackin, K. E., Lyon, S. A., James, M. L., Rood, J. I., Lyras, D., 2017. Bovine antibodies targeting primary and recurrent Clostridium difficile disease are a potent antibiotic alternative. *Sci Rep*, **7**, 3665.

Hwang, K.-E., Hwang, Y.-R., Seol, C.-H., Park, C., Park, S.-H., Yoon, K.-H., Park, D.-S., Lee, M.-K., Jeong, E.-T., Kim, H.-R., 2012. Clostridium difficile Infection in Lung Cancer Patients. *Japanese journal of infectious diseases,* **66**, 379-382.

Hyman, P. & Abedon, S. T., 2009. Practical methods for determining phage growth parameters. *Methods Mol Biol,* **501**, 175-202.

Hyman, P. & Abedon, S. T., 2010a. Bacteriophage host range and bacterial resistance. *Advances in applied microbiology,* **70**, 217-248.

Hyman, P. & Abedon, S. T., 2010b. Bacteriophage host range and bacterial resistance. *Adv Appl Microbiol,* **70**, 217-248.

Iakobachvili, N. 2015. *Mycobacterial resuscitation promoting factors: roles and mechanisms in infected macrophages.* Ph. D. Department of Infection, Immunity and Inflammation.

Igawa, K., Ohara, N., Kawakubo, A., Sugimoto, K., Yanagiguchi, K., Ikeda, T., Yamada, S., Hayashi, Y., 2014. D-Glucosamine Promotes Transfection Efficiency during Electroporation. *BioMed Research International,* **2014**.

Inoue, I., Ishikawa, Y., Uraoka, Y., Yamashita, I., Yasueda, H., 2016. Selection of a novel peptide aptamer with high affinity for TiO2-nanoparticle through a direct electroporation with TiO2-binding phage complexes. *J Biosci Bioeng*, **122**, 528-532.

Ivanova, K., Ramon, E., Hoyo, J., Tzanov, T., 2017. Innovative Approaches for Controlling Clinically Relevant Biofilms: Current Trends and Future Prospects. *Curr Top Med Chem*.

Ivarsson, M. E., Leroux, J.-C., Castagner, B., 2015. Investigational new treatments for Clostridium difficile infection. *Drug discovery today,* **20**, 602-608.

Jaeger, D., Hubner, W., Huser, T., Mussgnug, J. H., Kruse, O., 2017. Nuclear transformation and functional gene expression in the oleaginous microalga Monoraphidium neglectum. *J Biotechnol,* **249**, 10-15.

Jain, P., Hartman, T. E., Eisenberg, N., O'Donnell, M. R., Kriakov, J., Govender, K., Makume, M., Thaler, D. S., Hatfull, G. F., Sturm, A. W., Larsen, M. H., Moodley,

P., Jacobs, W. R., Jr., 2012. phi(2)GFP10, a high-intensity fluorophage, enables detection and rapid drug susceptibility testing of Mycobacterium tuberculosis directly from sputum samples. *J Clin Microbiol,* **50**, 1362-1369.

Javed, M. A., Poshtiban, S., Arutyunov, D., Evoy, S., Szymanski, C. M., 2013. Bacteriophage receptor binding protein based assays for the simultaneous detection of Campylobacter jejuni and Campylobacter coli. *PLoS One,* **8**, e69770.

Jayaraman, S., Das, P. P., Saini, P. C., Roy, B., Chatterjee, P. N., 2017. Use of Bacillus Subtilis PB6 as a potential antibiotic growth promoter replacement in improving performance of broiler birds. *Poult Sci*, pex079.

Jirásková, A., Vítek, L., Fevery, J., Ruml, T., Branny, P., 2005. Rapid protocol for electroporation of< i> Clostridium perfringens</i>. *J Microbiol Methods,* **62**, 125-127.

Johansson, A. 2006. *Clostridium perfringens the causal agent of necrotic enteritis in poultry. Faculty of Veterinary Medicine and Animal Science, Department of Biomedical Sciences and Veterinary Public Health, Uppsala.* Ph. D. Doctoral thesis, Swedish University of Agricultural Sciences, Uppsala.

Jones, S. W., Tracy, B. P., Gaida, S. M., Papoutsakis, E. T., 2011. Inactivation of σF in Clostridium acetobutylicum ATCC 824 blocks sporulation prior to asymmetric division and abolishes σE and σG protein expression but does not block solvent formation. *J Bacteriol,* **193**, 2429-2440.

Jordan, E. T., Collins, M., Terefe, J., Ugozzoli, L., Rubio, T., 2008. Optimizing electroporation conditions in primary and other difficult-to-transfect cells. *Journal of biomolecular techniques: JBT,* **19**, 328.

Joshi, L. T., Welsch, A., Hawkins, J., Baillie, L., 2017. The effect of hospital biocide sodium dichloroisocyanurate on the viability and properties of Clostridium difficile spores. *Lett Appl Microbiol*.

Just, I. & Gerhard, R., 2005. Large clostridial cytotoxins. *Reviews of physiology, biochemistry and pharmacology.* Springer 23-47.

Kadra, B., Guillou, J., Popoff, M., Bourlioux, P., 1999. Typing of sheep clinical isolates and identification of enterotoxigenic Clostridium perfringens strains by classical methods and by polymerase chain reaction (PCR) 1. *FEMS Immunology & Medical Microbiology,* **24**, 259-266.

Kaistha, S. D. & Umrao, P. D., 2016. Bacteriophage for Mitigation of Multiple Drug Resistant Biofilm Forming Pathogens. *Recent Pat Biotechnol,* **10**, 184-194.

Kaliniene, L., Simoliunas, E., Truncaite, L., Zajanckauskaite, A., Nainys, J., Kaupinis, A., Valius, M., Meskys, R., 2017. Molecular analysis of Arthrobacter myovirus vB_ArtM-ArV1: we blame it on the tail. *J Virol*.

Kannuchamy, S., Mukund, N., Saleena, L. M., 2016. Genetic engineering of Clostridium thermocellum DSM1313 for enhanced ethanol production. *BMC biotechnology*, **16**, 34.

Karpiuk, I. & Tyski, S., 2017. Looking for the new preparations for antibacterial therapy. V. New antimicrobial agents from the oxazolidinones groups in clinical trials. *Przegl Epidemiol*, **71**, 207-219.

Keessen, E. C., Hensgens, M. P., Spigaglia, P., Barbanti, F., Sanders, I. M., Kuijper, E. J., Lipman, L. J., 2013. Antimicrobial susceptibility profiles of human and piglet Clostridium difficile PCR-ribotype 078. *Antimicrobial resistance and infection control*, **2**, 14.

Khalili, S., Jahangiri, A., Hashemi, Z. S., Khalesi, B., Mard-Soltani, M., Amani, J., 2017. Structural pierce into molecular mechanism underlying Clostridium perfringens Epsilon toxin function. *Toxicon*, **127**, 90-99.

Khan, M., Nazir, J., Anjum, A. A., Nawaz, M., Shabbir, M. Z., 2014. Toxinotyping and antimicrobial susceptibility of enterotoxigenic Clostridium perfringens isolates from mutton, beef and chicken meat. *Journal of Food Science and Technology*, 1-6.

Khanna, S., Shin, A., Kelly, C. P., 2017. Management of Clostridium difficile Infection in Inflammatory Bowel Disease: Expert Review from the Clinical Practice Updates Committee of the AGA Institute. *Clin Gastroenterol Hepatol*, **15**, 166-174.

Kim, K.-P., Born, Y., Lurz, R., Eichenseher, F., Zimmer, M., Loessner, M. J., Klumpp, J., 2012. Inducible Clostridium perfringens bacteriophages WS9 and WS63.

Kirk, J. A., Banerji, O., Fagan, R. P., 2017a. Characteristics of the Clostridium difficile cell envelope and its importance in therapeutics. *Microbial biotechnology*, **10**, 76-90.

Kirk, J. A. & Fagan, R. P., 2016. Heat shock increases conjugation efficiency in Clostridium difficile. *Anaerobe*, **42**, 1-5.

Kirk, J. A., Gebhart, D., Buckley, A. M., Lok, S., Scholl, D., Douce, G. R., Govoni, G. R., Fagan, R. P., 2017b. New class of precision antimicrobials redefines role of Clostridium difficile S-layer in virulence and viability. *Sci Transl Med,* **9**.

Kiro, R., Shitrit, D., Qimron, U., 2014. Efficient engineering of a bacteriophage genome using the type IE CRISPR-Cas system. *RNA biology,* **11**, 42-44.

Kittler, S., Fischer, S., Abdulmawjood, A., Glünder, G., Klein, G., 2014. Colonisation of a Phage Susceptible Campylobacter jejuni Population in Two Phage Positive Broiler Flocks. *PLoS One,* **9**, e94782.

Kolek, J., Sedlar, K., Provaznik, I., Patakova, P., 2016. Dam and Dcm methylations prevent gene transfer into Clostridium pasteurianum NRRL B-598: development of methods for electrotransformation, conjugation, and sonoporation. *Biotechnology for biofuels,* **9**, 14.

Korndörfer, I. P., Danzer, J., Schmelcher, M., Zimmer, M., Skerra, A., Loessner, M. J., 2006. The Crystal Structure of the Bacteriophage PSA Endolysin Reveals a Unique Fold Responsible for Specific Recognition of Listeria Cell Walls. *J Mol Biol,* **364**, 678-689.

Kosloske, A. M., Ball, W. S., Umland, E., Skipper, B., 1985. Clostridial necrotizing enterocolitis. *Journal of pediatric surgery,* **20**, 155-159.

Kotnik, T. & Miklavčič, D., 2006. Theoretical evaluation of voltage inducement on internal membranes of biological cells exposed to electric fields. *Biophysical journal,* **90**, 480-491.

Kropinski, A. M., Mazzocco, A., Waddell, T. E., Lingohr, E., Johnson, R. P., 2009. Enumeration of bacteriophages by double agar overlay plaque assay. *Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization, and Interactions*, 69-76.

Krylov, V., 2001. Phage therapy in terms of bacteriophage genetics: hopes, prospects, safety, limitations. *Russian Journal of Genetics,* **37**, 715-730.

Kuehne, S. A. & Minton, N. P., 2012. ClosTron-mediated engineering of Clostridium. *Bioengineered,* **3**, 247-254.

Kumar, R. B. & Alam, S. I., 2017. Effect of continuous sub-culturing on infectivity of Clostridium perfringens ATCC13124 in mouse gas gangrene model. *Folia Microbiol (Praha),* **62**, 343-353.

Lacey, J. A., Johanesen, P. A., Lyras, D., Moore, R. J., 2016. Genomic diversity of necrotic enteritis-associated strains of Clostridium perfringens: a review. *Avian Pathol,* **45**, 302-307.

Lanckriet, A., Timbermont, L., Happonen, L. J., Pajunen, M. I., Pasmans, F., Haesebrouck, F., Ducatelle, R., Savilahti, H., Van Immerseel, F., 2009. Generation of single-copy transposon insertions in Clostridium perfringens by electroporation of phage Mu DNA transposition complexes. *Applied and environmental microbiology,* **75**, 2638-2642.

Landi, G., Gualtieri, G., Bello, I. S., Kirsch, D., 2017. A case of fatal Clostridium perfringens bacteremia and sepsis following CT-guided liver biopsy of a rare neuroendocrine hepatic tumor. *Forensic Sci Med Pathol*.

Latka, A., Maciejewska, B., Majkowska-Skrobek, G., Briers, Y., Drulis-Kawa, Z., 2017. Bacteriophage-encoded virion-associated enzymes to overcome the carbohydrate barriers during the infection process. *Appl Microbiol Biotechnol,* **101**, 3103-3119.

Leang, C., Ueki, T., Nevin, K. P., Lovley, D. R., 2013. A genetic system for Clostridium ljungdahlii: a chassis for autotrophic production of biocommodities and a model homoacetogen. *Appl Environ Microbiol,* **79**, 1102-1109.

Leduc, M., ROUSSEAU, M., HEIJENOORT, J., 1977. Structure of the Cell Wall of Bacillus Species CIP 76-111. *The FEBS Journal,* **80**, 153-163.

Lee, K. E., Lim, S. I., Shin, S. H., Kwon, Y. K., Kim, H. Y., Song, J. Y., An, D. J., 2014. Distribution of Clostridium perfringens isolates from piglets in South Korea. *J Vet Med Sci,* **76**, 745-749.

Lee, K. W., Lillehoj, H. S., Park, M. S., Jang, S. I., Ritter, G. D., Hong, Y. H., Jeong, W., Jeoung, H. Y., An, D. J., Lillehoj, E. P., 2012. Clostridium perfringens alpha-toxin and NetB toxin antibodies and their possible role in protection against necrotic enteritis and gangrenous dermatitis in broiler chickens. *Avian Dis,* **56**, 230-233.

Lee, P. S., 2008. Quantitation of microorganisms. *Practical Handbook of Microbiology,* 11-29.

Leffler, D. A. & Lamont, J. T., 2015. Clostridium difficile infection. *New England Journal of Medicine,* **372**, 1539-1548.

Lessa, F. C., Mu, Y., Bamberg, W. M., Beldavs, Z. G., Dumyati, G. K., Dunn, J. R., Farley, M. M., Holzbauer, S. M., Meek, J. I., Phipps, E. C., 2015. Burden of Clostridium difficile infection in the United States. *New England Journal of Medicine,* **372**, 825-834.

Leverentz, B., Conway, W. S., Camp, M. J., Janisiewicz, W. J., Abuladze, T., Yang, M., Saftner, R., Sulakvelidze, A., 2003. Biocontrol of Listeria monocytogenes on fresh-cut produce by treatment with lytic bacteriophages and a bacteriocin. *Applied and environmental microbiology,* **69**, 4519-4526.

Li, J., Adams, V., Bannam, T. L., Miyamoto, K., Garcia, J. P., Uzal, F. A., Rood, J. I., McClane, B. A., 2013. Toxin plasmids of Clostridium perfringens. *Microbiology and Molecular Biology Reviews,* **77**, 208-233.

Li, J., Freedman, J. C., Evans, D. R., McClane, B. A., 2017. CodY Promotes Sporulation and Enterotoxin Production by Clostridium perfringens Type A Strain SM101. *Infection and immunity,* IAI. 00855-00816.

Li, S., 2008. *Electroporation protocols: preclinical and clinical gene medicine.* Humana Press.

Li, X. & Heyer, W.-D., 2008. Homologous recombination in DNA repair and DNA damage tolerance. *Cell research,* **18**, 99-113.

Liew, F., Henstra, A. M., Kpke, M., Winzer, K., Simpson, S. D., Minton, N. P., 2017. Metabolic engineering of Clostridium autoethanogenum for selective alcohol production. *Metab Eng*.

Lin, D. M., Koskella, B., Lin, H. C., 2017. Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. *World journal of gastrointestinal pharmacology and therapeutics,* **8**, 162.

Litster, J., 1975. Stability of lipid bilayers and red blood cell membranes. *Physics Letters A,* **53**, 193-194.

Liu, X., Jiang, S., Fang, C., Li, H., Zhang, X., Zhang, F., June, C. H., Zhao, Y., 2017. Novel T cells with improved in vivo anti-tumor activity generated by RNA electroporation. *Protein Cell*.

Liu, Y. J., Zhang, J., Cui, G. Z., Cui, Q., 2015. Current progress of targetron technology: development, improvement and application in metabolic engineering. *Biotechnol J,* **10**, 855-865.

Liubakka, A. & Vaughn, B. P., 2016. Clostridium difficile Infection and Fecal Microbiota Transplant. *AACN Adv Crit Care,* **27**, 324-337.

Loc-Carrillo, C. & Abedon, S. T., 2011. Pros and cons of phage therapy. *Bacteriophage,* **1**, 111-114.

Loessner, M. J., Rees, C. E., Stewart, G. S., Scherer, S., 1996. Construction of luciferase reporter bacteriophage A511::luxAB for rapid and sensitive detection of viable Listeria cells. *Appl Environ Microbiol,* **62**, 1133-1140.

Löfmark, S., Edlund, C., Nord, C. E., 2010. Metronidazole is still the drug of choice for treatment of anaerobic infections. *Clinical Infectious Diseases,* **50**, S16-S23.

Lu, T. K. & Collins, J. J., 2007. Dispersing biofilms with engineered enzymatic bacteriophage. *Proceedings of the National Academy of Sciences,* **104**, 11197-11202.

Lu, T. K. T., Koeris, M. S., Chevalier, B. S., Holder, J. W., McKenzie, G. J., Brownell, D. R. 2012. *Recombinant phage and methods.* Google Patents.

Maciejewska, B., Roszniowski, B., Espaillat, A., Kesik-Szeloch, A., Majkowska-Skrobek, G., Kropinski, A. M., Briers, Y., Cava, F., Lavigne, R., Drulis-Kawa, Z., 2017. Klebsiella phages representing a novel clade of viruses with an unknown DNA modification and biotechnologically interesting enzymes. *Appl Microbiol Biotechnol,* **101**, 673-684.

Mahony, D., 1974. Bacteriocin susceptibility of Clostridium perfringens: a provisional typing schema. *Applied microbiology,* **28**, 172-176.

Mahony, D., 1977. Induction of bacteriocins from Clostridium perfringens by treatment with mitomycin C. *Antimicrobial Agents and Chemotherapy,* **11**, 1067-1068.

Mahony, D., Bell, P., Easterbrook, K., 1985. Two bacteriophages of Clostridium difficile. *Journal of clinical microbiology,* **21**, 251-254.

Manni, M. M., Valero, J. G., Perez-Cormenzana, M., Cano, A., Alonso, C., Goni, F. M., 2017. Lipidomic profile of GM95 cell death induced by Clostridium perfringens alpha-toxin. *Chem Phys Lipids*.

Marinelli, L. J., Piuri, M., Swigoňová, Z., Balachandran, A., Oldfield, L. M., van Kessel, J. C., Hatfull, G. F., 2008. BRED: a simple and powerful tool for constructing mutant and recombinant bacteriophage genomes. *PLoS One,* **3**, e3957.

Mattila, E., Arkkila, P., Mattila, P. S., Tarkka, E., Tissari, P., Anttila, V.-J., 2013. Extra-intestinal Clostridium difficile infections. *Clinical Infectious Diseases*.

Mayer, M. J., Garefalaki, V., Spoerl, R., Narbad, A., Meijers, R., 2011. Structure-based modification of a Clostridium difficile-targeting endolysin affects activity and host range. *J Bacteriol,* **193**, 5477-5486.

Mayer, M. J., Narbad, A., Gasson, M. J., 2008. Molecular characterization of a Clostridium difficile bacteriophage and its cloned biologically active endolysin. *J Bacteriol*, **190**, 6734-6740.

Mazaheri Nezhad Fard, R., Barton, M. D., Heuzenroeder, M. W., 2011. Bacteriophage-mediated transduction of antibiotic resistance in enterococci. *Lett Appl Microbiol*, **52**, 559-564.

Mazzocco, A., Waddell, T. E., Lingohr, E., Johnson, R. P., 2009. Enumeration of bacteriophages using the small drop plaque assay system. *Bacteriophages: Methods and Protocols, Volume 1: Isolation, Characterization, and Interactions*, 81-85.

McDonel, J. L., 1980. Clostridium perfringens toxins (type A, B, C, D, E). *Pharmacology & Therapeutics*, **10**, 617-655.

Meader, E., Mayer, M. J., Gasson, M. J., Steverding, D., Carding, S. R., Narbad, A., 2010. Bacteriophage treatment significantly reduces viable< i> Clostridium difficile</i> and prevents toxin production in an< i> in vitro</i> model system. *Anaerobe*, **16**, 549-554.

Meader, E., Mayer, M. J., Steverding, D., Carding, S. R., Narbad, A., 2013. Evaluation of bacteriophage therapy to control Clostridium difficile and toxin production in an in vitro human colon model system. *Anaerobe*, **22**, 25-30.

Meer, R. & Songer, J. G., 1997. Multiplex polymerase chain reaction assay for genotyping Clostridium perfringens. *American journal of veterinary research*, **58**, 702-705.

Meessen-Pinard, M., Sekulovic, O., Fortier, L.-C., 2012. Evidence of in vivo prophage induction during Clostridium difficile infection. *Applied and environmental microbiology*, **78**, 7662-7670.

Messick, C. A., Hammel, J. P., Hull, T., 2017. Risk Factors that Predict Recurrent Clostridium difficile Infections in Surgical Patients. *Am Surg*, **83**, 653-659.

Miladi, B., El Marjou, A., Boeuf, G., Bouallagui, H., Dufour, F., Di Martino, P., Elm'selmi, A., 2012. Oriented immobilization of the tobacco etch virus protease for the cleavage of fusion proteins. *J Biotechnol*, **158**, 97-103.

Miller, R. W., Skinner, J., Sulakvelidze, A., Mathis, G. F., Hofacre, C. L., 2010. Bacteriophage therapy for control of necrotic enteritis of broiler chickens experimentally infected with Clostridium perfringens. *Avian diseases*, **54**, 33-40.

Milton, A. A. P., Agarwal, R. K., Bhuvana Priya, G., Saminathan, M., Aravind, M., Reddy, A., Athira, C. K., Ramees, T., Sharma, A. K., Kumar, A., 2017. Prevalence and molecular typing of Clostridium perfringens in captive wildlife in India. *Anaerobe,* **44**, 55-57.

Minton, N. P., Ehsaan, M., Humphreys, C. M., Little, G. T., Baker, J., Henstra, A. M., Liew, F., Kelly, M. L., Sheng, L., Schwarz, K., Zhang, Y., 2016. A roadmap for gene system development in Clostridium. *Anaerobe,* **41**, 104-112.

Mirecka, A., 2017. Clostridium difficile infection a current therapeutic and epidemiological problem. *Przegl Epidemiol,* **71**, 155-164.

Mishra, N. & Smyth, J. A., 2017. Oral vaccination of broiler chickens against necrotic enteritis using a non-virulent NetB positive strain of Clostridium perfringens type A. *Vaccine,* **35**, 6858-6865.

Mohanta, T. K., Bashir, T., Hashem, A., Abd Allah, E. F., Bae, H., 2017. Genome Editing Tools in Plants. *Genes (Basel),* **8**.

Moradpour, Z. & Ghasemian, A., 2011. Modified phages: novel antimicrobial agents to combat infectious diseases. *Biotechnology advances,* **29**, 732-738.

Morales, C. A., Oakley, B. B., Garrish, J. K., Siragusa, G. R., Ard, M. B., Seal, B. S., 2012. Complete genome sequence of the podoviral bacteriophage PhiCP24R, which is virulent for Clostridium perfringens. *Arch Virol,* **157**, 769-772.

Mukamolova, G. V., Murzin, A. G., Salina, E. G., Demina, G. R., Kell, D. B., Kaprelyants, A. S., Young, M., 2006. Muralytic activity of Micrococcus luteus Rpf and its relationship to physiological activity in promoting bacterial growth and resuscitation. *Molecular microbiology,* **59**, 84-98.

Mullany, P., Williams, R., Langridge, G. C., Turner, D. J., Whalan, R., Clayton, C., Lawley, T., Hussain, H., McCurrie, K., Morden, N., Allan, E., Roberts, A. P., 2012. Behavior and target site selection of conjugative transposon Tn916 in two different strains of toxigenic Clostridium difficile. *Appl Environ Microbiol,* **78**, 2147-2153.

Nagahama, M., 2013. Vaccines against Clostridium perfringens alpha-toxin. *Curr Pharm Biotechnol,* **14**, 913-917.

NAGY, E. & Földes, J., 1991. Electron microscopic investigation of lysogeny of Clostridium difficile strains isolated from antibiotic-associated diarrhea cases and from healthy carriers. *Apmis,* **99**, 321-326.

Nakamura, I., Yamaguchi, T., Tsukimori, A., Sato, A., Fukushima, S., Mizuno, Y., Matsumoto, T., 2014. Fulminant colitis from Clostridium difficile infection, the epidemic strain ribotype 027, in Japan. *J Infect Chemother,* **20**, 380-383.

Nakonieczna, A., Cooper, C. J., Gryko, R., 2015. Bacteriophages and bacteriophage-derived endolysins as potential therapeutics to combat Gram-positive spore forming bacteria. *J Appl Microbiol,* **119**, 620-631.

Nale, J. Y. 2013. *Isolation and Characterisation of Temperate Bacteriophages of the Hypervirulent Clostridium difficile 027 Strains.* Ph. D. University of Leicester.

Nale, J. Y., Chutia, M., Carr, P., Hickenbotham, P. T., Clokie, M. R., 2016a. 'Get in Early'; Biofilm and Wax Moth (Galleria mellonella) Models Reveal New Insights into the Therapeutic Potential of Clostridium difficile Bacteriophages. *Front Microbiol,* **7**, 1383.

Nale, J. Y., Shan, J., Hickenbotham, P. T., Fawley, W. N., Wilcox, M. H., Clokie, M. R., 2012. Diverse temperate bacteriophage carriage in Clostridium difficile 027 strains. *PLoS One,* **7**, e37263.

Nale, J. Y., Spencer, J., Hargreaves, K. R., Buckley, A. M., Trzepinski, P., Douce, G. R., Clokie, M. R., 2016b. Bacteriophage Combinations Significantly Reduce Clostridium difficile Growth In Vitro and Proliferation In Vivo. *Antimicrob Agents Chemother,* **60**, 968-981.

Nanda, A. M., Thormann, K., Frunzke, J., 2015. Impact of spontaneous prophage induction on the fitness of bacterial populations and host-microbe interactions. *J Bacteriol,* **197**, 410-419.

Napotnik, T. B., Reberšek, M., Kotnik, T., Lebrasseur, E., Cabodevila, G., Miklavčič, D., 2010. Electropermeabilization of endocytotic vesicles in B16 F1 mouse melanoma cells. *Medical & biological engineering & computing,* **48**, 407-413.

Nariya, H., Miyata, S., Tamai, E., Sekiya, H., Maki, J., Okabe, A., 2011. Identification and characterization of a putative endolysin encoded by episomal phage phiSM101 of Clostridium perfringens. *Appl Microbiol Biotechnol,* **90**, 1973-1979.

Nayak, S. U., Griffiss, J. M., Blumer, J., O'Riordan, M. A., Gray, W., McKenzie, R., Jurao, R. A., An, A. T., Le, M., Bell, S. J., Ochsner, U. A., Jarvis, T. C., Janjic, N., Zenilman, J. M., 2017. Safety, tolerability, systemic exposure and metabolism of CRS3123, a methionyl-tRNA synthetase inhibitor developed for treatment of Clostridium difficile infections, in a Phase I study. *Antimicrob Agents Chemother*.

Neely, M. N. & Friedman, D. I., 1998. Arrangement and functional identification of genes in the regulatory region of lambdoid phage H-19B, a carrier of a Shiga-like toxin. *Gene,* **223**, 105-113.

Ng, Y. K., Ehsaan, M., Philip, S., Collery, M. M., Janoir, C., Collignon, A., Cartman, S. T., Minton, N. P., 2013. Expanding the repertoire of gene tools for precise manipulation of the Clostridium difficile genome: allelic exchange using pyrE alleles. *PLoS One,* **8**, e56051.

Nickoloff, J. A., 1995. *Animal cell electroporation and electrofusion protocols.* Springer.

Nilsson, A. S., 2014. Phage therapy-constraints and possibilities. *Ups J Med Sci,* **119**, 192-198.

Nyati, K. K., Prasad, K. N., Agrawal, V., Husain, N., 2017. Matrix metalloproteinases-2 and -9 in Campylobacter jejuni-induced paralytic neuropathy resembling Guillain-Barre syndrome in chickens. *Microb Pathog*.

O'connor, J. R., Lyras, D., Farrow, K. A., Adams, V., Powell, D. R., Hinds, J., Cheung, J. K., Rood, J. I., 2006. Construction and analysis of chromosomal Clostridium difficile mutants. *Molecular microbiology,* **61**, 1335-1351.

O'Sullivan, L., Buttimer, C., McAuliffe, O., Bolton, D., Coffey, A., 2016. Bacteriophage-based tools: recent advances and novel applications. *F1000Res,* **5**, 2782.

Oakley, C. L. & Warrack, G. H., 1953. Routine typing of Clostridium welchii. *The Journal of Hygiene,* **51**, 102-107.

Oechslin, F., Daraspe, J., Giddey, M., Moreillon, P., Resch, G., 2013. In vitro characterization of PlySK1249, a novel phage lysin, and assessment of its antibacterial activity in a mouse model of Streptococcus agalactiae bacteremia. *Antimicrobial Agents and Chemotherapy,* **57**, 6276-6283.

Ogunniyi, A. D., Khazandi, M., Stevens, A. J., Sims, S. K., Page, S. W., Garg, S., Venter, H., Powell, A., White, K., Petrovski, K. R., Laven-Law, G., Totoli, E. G., Salgado, H. R., Pi, H., Coombs, G. W., Shinabarger, D. L., Turnidge, J. D., Paton, J. C., McCluskey, A., Trott, D. J., 2017. Evaluation of robenidine analog NCL195 as a novel broad-spectrum antibacterial agent. *PLoS One,* **12**, e0183457.

Oh, Y. H., Eom, G. T., Kang, K. H., Choi, J. W., Song, B. K., Lee, S. H., Park, S. J., 2015. Optimized Transformation of Newly Constructed Escherichia coli-Clostridia Shuttle Vectors into Clostridium beijerinckii. *Appl Biochem Biotechnol,* **177**, 226-236.

Oliveira, A., Leite, M., Kluskens, L. D., Santos, S. B., Melo, L. D., Azeredo, J., 2015. The First Paenibacillus larvae Bacteriophage Endolysin (PlyPl23) with High Potential to Control American Foulbrood. *PLoS One,* **10**, e0132095.

Oppenheim, A. B. & Adhya, S. L., 2007. A new look at bacteriophage λ genetic networks. *J Bacteriol,* **189**, 298-304.

Oppenheim, A. B., Rattray, A. J., Bubunenko, M., Thomason, L. C., Court, D. L., 2004. In vivo recombineering of bacteriophage λ by PCR fragments and single-strand oligonucleotides. *Virology,* **319**, 185-189.

Orlova, E., 2012. *Bacteriophages and their structural organisation.* InTech.

Orrell, K. E., Zhang, Z., Sugiman-Marangos, S. N., Melnyk, R. A., 2017. Clostridium difficile toxins A and B: Receptors, pores, and translocation into cells. *Crit Rev Biochem Mol Biol,* 1-13.

Osman, K. & Elhariri, M., 2013. Antibiotic resistance of Clostridium perfringens isolates from broiler chickens in Egypt. *Rev Sci Tech Off Int Epiz,* **32**, 841-850.

Ozkan, I., Akturk, E., Yeshenkulov, N., Atmaca, S., Rahmanov, N., Atabay, H. I., 2016. Lytic Activity of Various Phage Cocktails on Multidrug-Resistant Bacteria. *Clin Invest Med,* **39**, 27504.

Papatheodorou, P. & Aktories, K., 2016. Receptor-Binding and Uptake of Binary Actin-ADP-Ribosylating Toxins. *Curr Top Microbiol Immunol*.

Park, J. Y., Kim, S., Oh, J. Y., Kim, H. R., Jang, I., Lee, H. S., Kwon, Y. K., 2015. Characterization of Clostridium perfringens isolates obtained from 2010 to 2012 from chickens with necrotic enteritis in Korea. *Poult Sci,* **94**, 1158-1164.

Park, Y., Lim, J. A., Kong, M., Ryu, S., Rhee, S., 2014. Structure of bacteriophage SPN1S endolysin reveals an unusual two-module fold for the peptidoglycan lytic and binding activity. *Mol Microbiol,* **92**, 316-325.

Payandeh, Z., Rasooli, I., Mousavi Gargari, S. L., Rajabi Bazl, M., Ebrahimizadeh, W., 2014. Immunoreaction of a recombinant nanobody from camelid single domain antibody fragment with Acinetobacter baumannii. *Trans R Soc Trop Med Hyg,* **108**, 92-98.

Perelle, S., Gibert, M., Boquet, P., Popoff, M. R., 1993. Characterization of Clostridium perfringens iota-toxin genes and expression in Escherichia coli. *Infection and immunity,* **61**, 5147-5156.

Peterson, E. C. & Daugulis, A. J., 2014. The use of high pressure CO -facilitated pH swings to enhance in situ product recovery of butyric acid in a two-phase partitioning bioreactor. *Biotechnol Bioeng*.

Petit, L., Gibert, M., Popoff, M. R., 1999. Clostridium perfringens: toxinotype and genotype. *Trends Microbiol,* **7**, 104-110.

Petridis, M., Bagdasarian, M., Waldor, M. K., Walker, E., 2006. Horizontal transfer of Shiga toxin and antibiotic resistance genes among Escherichia coli strains in house fly (Diptera: Muscidae) gut. *J Med Entomol,* **43**, 288-295.

Pires, D. P., Cleto, S., Sillankorva, S., Azeredo, J., Lu, T. K., 2016. Genetically engineered phages: a review of advances over the last decade. *Microbiology and Molecular Biology Reviews,* **80**, 523-543.

Pitt, T. L. & Gaston, M. A., 1995. Bacteriocin Typing. In Howard, J. & Whitcombe, D. M. (eds.) *Diagnostic Bacteriology Protocols.* Totowa, NJ: Humana Press 5-14.

Planelles, L., Marañón, C., Requena, J. M. a., López, M. C., 1999. Phage recovery by electroporation of naked DNA into host cells avoids the use of packaging extracts. *Analytical biochemistry,* **267**, 234-235.

Popoff, M. R., 2011. Epsilon toxin: a fascinating pore-forming toxin. *Febs Journal*, **278**, 4602-4615.

Porter, R. J. & Fogg, C., 2015. Faecal microbiota transplantation for Clostridium difficile infection in the United Kingdom. *Clinical Microbiology and Infection*, **21**, 578-582.

Postma, N., Kiers, D., Pickkers, P., 2015. The challenge of Clostridium difficile infection: overview of clinical manifestations, diagnostic tools and therapeutic options. *International Journal of Antimicrobial Agents,* **46**, S47-S50.

Powell, I. B., Achen, M. G., Hillier, A. J., Davidson, B. E., 1988. A simple and rapid method for genetic transformation of lactic streptococci by electroporation. *Applied and environmental microbiology,* **54**, 655-660.

Prokhorov, N. S., Riccio, C., Zdorovenko, E. L., Shneider, M. M., Browning, C., Knirel, Y. A., Leiman, P. G., Letarov, A. V., 2017. Function of bacteriophage G7C esterase tailspike in host cell adsorption. *Mol Microbiol*.

Prpar Mihevc, S., Pavlin, M., Darovic, S., Zivin, M., Podbregar, M., Rogelj, B., Mars, T., 2017. Modelling FUS Mislocalisation in an In Vitro Model of Innervated Human Muscle. *J Mol Neurosci*.

Pruteanu, M. & Shanahan, F., 2013. Digestion of epithelial tight junction proteins by the commensal Clostridium perfringens. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, **305**, G740-G748.

Ptashne, M., 2004. *A genetic switch: phage lambda revisited.* Cold Spring Harbor Laboratory Press Cold Spring Harbor, NY:.

Purdy, D., O'Keeffe, T. A., Elmore, M., Herbert, M., McLeod, A., Bokori-Brown, M., Ostrowski, A., Minton, N. P., 2002. Conjugative transfer of clostridial shuttle vectors from Escherichia coli to Clostridium difficile through circumvention of the restriction barrier. *Molecular microbiology,* **46**, 439-452.

Rai, M., Ingle, A. P., Pandit, R., Paralikar, P., Gupta, I., Chaud, M. V., Dos Santos, C. A., 2017. Broadening the spectrum of small-molecule antibacterials by metallic nanoparticles to overcome microbial resistance. *Int J Pharm*.

Rashid, S. J. 2016. *Understanding Clostridium Difficile and the Bacteriophages from the Environment.* Ph. D. Department of Infection, Immunity and Inflammation.

Raulinaitis, V., Tossavainen, H., Aitio, O., Juuti, J. T., Hiramatsu, K., Kontinen, V., Permi, P., 2017. Identification and structural characterization of LytU, a unique peptidoglycan endopeptidase from the lysostaphin family. *Sci Rep,* **7**, 6020.

Raya, R. l. R. & H'bert, E. M., 2008. Isolation of Phage via Induction of Lysogens. *Methods in molecular biology (Clifton, NJ),* **501**, 23-32.

Razmyar, J., Kalidari, G. A., Tolooe, A., Rad, M., Movassaghi, A. R., 2014. Genotyping of Clostridium perfringens isolated from healthy and diseased ostriches (Struthio camelus). *Iran J Microbiol,* **6**, 31-36.

Reberšek, M. & Miklavčič, D., 2011. Advantages and disadvantages of different concepts of electroporation pulse generation. *Automatika,* **52**, 12-19.

Reece-Hoyes, J. S. & Walhout, A. J. M., 2018. Gateway Recombinational Cloning. *Cold Spring Harb Protoc*, **2018**, pdb top094912.

Rodriguez-Rubio, L., Gerstmans, H., Thorpe, S., Mesnage, S., Lavigne, R., Briers, Y., 2016. DUF3380 Domain from a Salmonella Phage Endolysin Shows Potent N-Acetylmuramidase Activity. *Appl Environ Microbiol,* **82**, 4975-4981.

Rols, M.-P. & Teissié, J., 1998. Electropermeabilization of Mammalian Cells to Macromolecules: Control by Pulse Duration. *Biophysical journal,* **75**, 1415-1423.

Rosenfeld, Y. & Shai, Y., 2006. Lipopolysaccharide (Endotoxin)-host defense antibacterial peptides interactions: role in bacterial resistance and prevention of sepsis. *Biochimica et Biophysica Acta (BBA)-Biomembranes,* **1758**, 1513-1522.

Ross, A., Ward, S., Hyman, P., 2016. More is better: selecting for broad host range bacteriophages. *Frontiers in microbiology,* **7**.

Rossmann, F. S., Racek, T., Wobser, D., Puchalka, J., Rabener, E. M., Reiger, M., Hendrickx, A. P., Diederich, A. K., Jung, K., Klein, C., Huebner, J., 2015. Phage-mediated dispersal of biofilm and distribution of bacterial virulence genes is induced by quorum sensing. *PLoS Pathog,* **11**, e1004653.

Rubinsky, B., 2010. *Irreversible electroporation.* Springer.

Rumah, K. R., Linden, J., Fischetti, V. A., Vartanian, T., 2013. Isolation of Clostridium perfringens type B in an individual at first clinical presentation of multiple sclerosis provides clues for environmental triggers of the disease. *PLoS One,* **8**, e76359.

Sabouri, S., Sepehrizadeh, Z., Amirpour-Rostami, S., Skurnik, M., 2017. A minireview on the in vitro and in vivo experiments with anti-Escherichia coli O157:H7 phages as potential biocontrol and phage therapy agents. *Int J Food Microbiol,* **243**, 52-57.

Sabry, M., Abd El-Moein, K., Hamza, E., Abdel Kader, F., 2016. Occurrence of Clostridium perfringens Types A, E, and C in Fresh Fish and Its Public Health Significance. *J Food Prot,* **79**, 994-1000.

Sahota, J. S. 2016. *Investigations to underpin the development of bacteriophages as therapeutic agents to target pseudomonas aeruginosa infections in patients with cystic fibrosis.* Ph. D. Department of Infection, Immunity and Inflammation.

Sakurai, J., Nagahama, M., Oda, M., 2004. Clostridium perfringens alpha-toxin: characterization and mode of action. *Journal of biochemistry,* **136**, 569-574.

Saunders, J. R., Allison, H., James, C. E., McCarthy, A. J., Sharp, R., 2001. Phage-mediated transfer of virulence genes. *Journal of Chemical Technology and Biotechnology,* **76**, 662-666.

Sawires, Y. S. & Songer, J. G., 2005. Multiple-locus variable-number tandem repeat analysis for strain typing of Clostridium perfringens. *Anaerobe,* **11**, 262-272.

Saye, D. J., Ogunseitan, O., Sayler, G., Miller, R. V., 1987. Potential for transduction of plasmids in a natural freshwater environment: effect of plasmid donor concentration and a natural microbial community on transduction in Pseudomonas aeruginosa. *Applied and environmental microbiology,* **53**, 987-995.

Saye, D. J., Ogunseitan, O., Sayler, G., Miller, R. V., 1990. Transduction of linked chromosomal genes between Pseudomonas aeruginosa strains during incubation in situ in a freshwater habitat. *Applied and environmental microbiology,* **56**, 140-145.

Schalch, B., Bader, L., Schau, H.-P., Bergmann, R., Rometsch, A., Maydl, G., Keßler, S., 2003. Molecular typing of Clostridium perfringens from a food-borne disease outbreak in a nursing home: ribotyping versus pulsed-field gel electrophoresis. *Journal of clinical microbiology,* **41**, 892-895.

Schmelcher, M., Donovan, D. M., Loessner, M. J., 2012. Bacteriophage endolysins as novel antimicrobials. *Future microbiology,* **7**, 1147-1171.

Schmelcher, M., Powell, A. M., Camp, M. J., Pohl, C. S., Donovan, D. M., 2015. Synergistic streptococcal phage lambdaSA2 and B30 endolysins kill streptococci in cow milk and in a mouse model of mastitis. *Appl Microbiol Biotechnol,* **99**, 8475-8486.

Seal, B. S., 2013. Characterization of bacteriophages virulent for Clostridium perfringens and identification of phage lytic enzymes as alternatives to antibiotics for potential control of the bacterium. *Poult Sci,* **92**, 526-533.

Seal, B. S., Fouts, D. E., Simmons, M., Garrish, J. K., Kuntz, R. L., Woolsey, R., Schegg, K. M., Kropinski, A. M., Ackermann, H.-W., Siragusa, G. R., 2011. Clostridium perfringens bacteriophages ΦCP39O and ΦCP26F: genomic organization and proteomic analysis of the virions. *Archives of virology,* **156**, 25-35.

Segar, L., Easow, J. M., Srirangaraj, S., Hanifah, M., Joseph, N. M., Seetha, K. S., 2017. Prevalence of Clostridium difficile infection among the patients attending a tertiary care teaching hospital. *Indian J Pathol Microbiol,* **60**, 221-225.

Seike, S., Takehara, M., Kobayashi, K., Nagahama, M., 2016. Role of pannexin 1 in Clostridium perfringens beta-toxin-caused cell death. *Biochimica et Biophysica Acta (BBA)-Biomembranes,* **1858**, 3150-3156.

Seike, S., Takehara, M., Takagishi, T., Miyamoto, K., Kobayashi, K., Nagahama, M., 2017. Delta-toxin from Clostridium perfringens perturbs intestinal epithelial barrier function in Caco-2 cell monolayers. *Biochim Biophys Acta*.

Sekulovic, O. & Fortier, L. C., 2016. Characterization of Functional Prophages in Clostridium difficile. *Methods Mol Biol*, **1476**, 143-165.

Sell, T. L., Schaberg, D. R., Fekety, F. R., 1983. Bacteriophage and bacteriocin typing scheme for Clostridium difficile. *Journal of clinical microbiology*, **17**, 1148-1152.

Seller, M., Burghardt, R., Rolling, T., Hansen-Algenstaedt, N., Schaefer, C., 2016. Clostridium perfringens: a rare cause of spondylodiscitis case report and review of the literature. *British Journal of Neurosurgery*, 1-3.

Semler, D. D., Goudie, A. D., Finlay, W. H., Dennis, J. J., 2014. Aerosol phage therapy efficacy in Burkholderia cepacia complex respiratory infections. *Antimicrobial Agents and Chemotherapy*, AAC. 02388-02313.

Serroni, A., Magistrali, C. F., Pezzotti, G., Bano, L., Pellegrini, M., Severi, G., Pancrazio, C., Luciani, M., Tittarelli, M., Tofani, S., 2017. Expression of deleted, atoxic atypical recombinant beta2 toxin in a baculovirus system and production of polyclonal and monoclonal antibodies. *Microbial cell factories*, **16**, 94.

Shan, J., Patel, K. V., Hickenbotham, P. T., Nale, J. Y., Hargreaves, K. R., Clokie, M. R., 2012. Prophage carriage and diversity within clinically relevant strains of Clostridium difficile. *Applied and environmental microbiology*, **78**, 6027-6034.

Sharan, S. K., Thomason, L. C., Kuznetsov, S. G., 2009. Recombineering: a homologous recombination-based method of genetic engineering. *Nature protocols*, **4**, 206-223.

Shen, Y., Barros, M., Vennemann, T., Gallagher, D. T., Yin, Y., Linden, S. B., Heselpoth, R. D., Spencer, D. J., Donovan, D. M., Moult, J., 2016. A bacteriophage endolysin that eliminates intracellular streptococci. *Elife*, **5**, e13152.

Shen, Y., Koller, T., Kreikemeyer, B., Nelson, D. C., 2013. Rapid degradation of Streptococcus pyogenes biofilms by PlyC, a bacteriophage-encoded endolysin. *J Antimicrob Chemother*, **68**, 1818-1824.

Shin, H., Lee, J.-H., Yoon, H., Kang, D.-H., Ryu, S., 2014. Genomic investigation of lysogen formation and host lysis systems of the Salmonella temperate bacteriophage SPN9CC. *Applied and environmental microbiology*, **80**, 374-384.

Sievers, F., Wilm, A., Dineen, D., Gibson, T. J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular systems biology,* **7**, 539.

Silva, R. O., Almeida, L. R., Oliveira Junior, C. A., Lima, P. C., Soares, D. F., Pereira, P. L., Silva, I. J., Lobato, F. C., 2016. ISOLATION AND GENOTYPING OF CLOSTRIDIUM PERFRINGENS FROM FREE-LIVING SOUTH AMERICAN COATI (NASUA NASUA). *J Zoo Wildl Med,* **47**, 333-336.

Skånseng, B., Kaldhusdal, M., Rudi, K., 2006. Comparison of chicken gut colonisation by the pathogens< i> Campylobacter jejuni</i> and< i> Clostridium perfringens</i> by real-time quantitative PCR. *Molecular and cellular probes,* **20**, 269-279.

Skrivanova, E., Van Immerseel, F., Hovorkova, P., Kokoska, L., 2016. In Vitro Selective Growth-Inhibitory Effect of 8-Hydroxyquinoline on Clostridium perfringens versus Bifidobacteria in a Medium Containing Chicken Ileal Digesta. *PLoS One,* **11**, e0167638.

Smith, H. O., Hutchison, C. A., 3rd, Pfannkoch, C., Venter, J. C., 2003. Generating a synthetic genome by whole genome assembly: phiX174 bacteriophage from synthetic oligonucleotides. *Proc Natl Acad Sci U S A,* **100**, 15440-15445.

Smits, W. K., Lyras, D., Lacy, D. B., Wilcox, M. H., Kuijper, E. J., 2016. Clostridium difficile infection. *Nature Reviews Disease Primers,* **2**, 16020.

Songer, J. G., 1996. Genotyping of< i> Clostridium perfringens</i> by Polymerase Chain Reaction is a Useful Adjunct to Diagnosis of Clostridial Enteric Disease in Animals. *Anaerobe,* **2**, 197-203.

Spiceland, C. M., Khanna, S., Pardi, D. S., 2016. Outcomes With Fidaxomicin Therapy in Clostridium difficile Infection. *J Clin Gastroenterol*.

Spratt, B. G., 1994. Resistance to antibiotics mediated by target alterations. *Science-AAAS-Weekly Paper Edition-including Guide to Scientific Information,* **264**, 388-396.

Sridhara, V. & Joshi, R. P., 2014. Evaluations of a mechanistic hypothesis for the influence of extracellular ions on electroporation due to high-intensity, nanosecond pulsing. *Biochim Biophys Acta,* **1838**, 1793-1800.

Stanley, D., Wu, S. B., Rodgers, N., Swick, R. A., Moore, R. J., 2014. Differential responses of cecal microbiota to fishmeal, Eimeria and Clostridium perfringens in a necrotic enteritis challenge model in chickens. *PLoS One,* **9**, e104739.

Stiles, B. G., Barth, G., Barth, H., Popoff, M. R., 2013. Clostridium perfringens epsilon toxin: a malevolent molecule for animals and man? *Toxins (Basel),* **5**, 2138-2160.

Stiles, B. G., Pradhan, K., Fleming, J. M., Samy, R. P., Barth, H., Popoff, M. R., 2014. Clostridium and bacillus binary enterotoxins: bad for the bowels, and eukaryotic being. *Toxins (Basel),* **6**, 2626-2656.

Stoffels, L., Taunt, H. N., Charalambous, B., Purton, S., 2017. Synthesis of bacteriophage lytic proteins against Streptococcus pneumoniae in the chloroplast of Chlamydomonas reinhardtii. *Plant Biotechnol J.*

Stols, L., Gu, M., Dieckman, L., Raffen, R., Collart, F. R., Donnelly, M. I., 2002. A new vector for high-throughput, ligation-independent cloning encoding a tobacco etch virus protease cleavage site. *Protein Expr Purif,* **25**, 8-15.

Stringer, M., Turnbull, P., Gilbert, R., 1980. Application of serological typing to the investigation of outbreaks of Clostridium perfringens food poisoning, 1970–1978. *Journal of Hygiene,* **84**, 443-456.

Stuckle, C. A., Sonntag, C., Kulik, M., Nitschke, T., 2017. [Fulminant Course of a Clostridium Perfringens Infection with Pathognomonic CT finding]. *Dtsch Med Wochenschr,* **142**, 747-751.

Sudharsanan, S., Vijayakumar, C., Manish, K., Elamurugan, T. P., Manwar, A. S., 2017. Anal Canal Gas Gangrene in Aplastic Anaemia: Rare yet Lethal Entity - A Caveat. *Cureus,* **9**, e1469.

Sun, X. & Hirota, S. A., 2015. The roles of host and pathogen factors and the innate immune response in the pathogenesis of Clostridium difficile infection. *Mol Immunol,* **63**, 193-202.

Surawicz, C. M., Brandt, L. J., Binion, D. G., Ananthakrishnan, A. N., Curry, S. R., Gilligan, P. H., McFarland, L. V., Mellow, M., Zuckerbraun, B. S., 2013. Guidelines for diagnosis, treatment, and prevention of Clostridium difficile infections. *The American journal of gastroenterology,* **108**, 478-498.

Suttle, C. A., 2005. Viruses in the sea. *Nature,* **437**, 356-361.

Swift, S. M., Rowley, D. T., Young, C., Franks, A., Hyman, P., Donovan, D. M., 2016. The endolysin from the Enterococcus faecalis bacteriophage VD13 and conditions stimulating its lytic activity. *FEMS Microbiol Lett*.

Szafranski, S. P., Winkel, A., Stiesch, M., 2017. The use of bacteriophages to biocontrol oral biofilms. *J Biotechnol*.

Talukdar, P. K., Udompijitkul, P., Hossain, A., Sarker, M. R., 2017. Inactivation Strategies for Clostridium perfringens Spores and Vegetative Cells. *Appl Environ Microbiol*, **83**.

Tamai, E., Yoshida, H., Sekiya, H., Nariya, H., Miyata, S., Okabe, A., Kuwahara, T., Maki, J., Kamitori, S., 2014a. X-ray structure of a novel endolysin encoded by episomal phage phiSM101 of Clostridium perfringens. *Mol Microbiol*, **92**, 326-337.

Tamai, E., Yoshida, H., Sekiya, H., Nariya, H., Miyata, S., Okabe, A., Kuwahara, T., Maki, J., Kamitori, S., 2014b. X-ray structure of a novel endolysin encoded by episomal phage phiSM101 of Clostridium perfringens. *Molecular microbiology,* **92**, 326-337.

Tamariz, J. H., Lezameta, L., Guerra, H., 2014. Phagotherapy faced with Staphylococcus aureus methicilin resistant infections in mice. *Revista peruana de medicina experimental y salud publica,* **31**, 69-77.

Tang, V. H., Stewart, G. A., Chang, B. J., 2017. Dermatophagoides pteronyssinus lytFM encoding an NlpC/P60 endopeptidase is also present in mite-associated bacteria that express LytFM variants. *FEBS Open Bio,* **7**, 1267-1280.

Taupin, C., Dvolaitzky, M., Sauterey, C., 1975. Osmotic pressure-induced pores in phospholipid vesicles. *Biochemistry,* **14**, 4771-4775.

Tenover, F. C., Tickler, I. A., Persing, D. H., 2012. Antimicrobial-resistant strains of Clostridium difficile from North America. *Antimicrobial Agents and Chemotherapy,* **56**, 2929-2932.

Thandar, M., Lood, R., Winer, B. Y., Deutsch, D. R., Euler, C. W., Fischetti, V. A., 2016. Novel Engineered Peptides of a Phage Lysin as Effective Antimicrobials against Multidrug-Resistant Acinetobacter baumannii. *Antimicrob Agents Chemother,* **60**, 2671-2679.

Thanki, A. M. 2016. *Development of a phage-based diagnostic test for the identification of Clostridium difficile.* Ph. D. Loughborough University.

Totte, J., de Wit, J., Pardo, L., Schuren, F., van Doorn, M., Pasmans, S., 2017. Targeted anti-staphylococcal therapy with endolysins in atopic dermatitis and the effect on steroid use, disease severity and the microbiome: study protocol for a randomized controlled trial (MAAS trial). *Trials*, **18**, 404.

Tracy, B. P., Jones, S. W., Papoutsakis, E. T., 2011. Inactivation of σE and σG in Clostridium acetobutylicum illuminates their roles in clostridial-cell-form biogenesis, granulose synthesis, solventogenesis, and spore morphogenesis. *J Bacteriol*, **193**, 1414-1426.

Tsoneva, I., Tomov, T., Panova, I., Strahilov, D., 1990. Effective production by electrofusion of hybridomas secreting monoclonal antibodies against Hc-antigen of< i> Salmonella</i>. *Journal of Electroanalytical Chemistry and Interfacial Electrochemistry*, **299**, 41-49.

Turner, S., Pryer, K. M., Miao, V. P., Palmer, J. D., 1999. Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. *Journal of Eukaryotic Microbiology*, **46**, 327-338.

Uchiyama, J., Takemura, I., Hayashi, I., Matsuzaki, S., Satoh, M., Ujihara, T., Murakami, M., Imajoh, M., Sugai, M., Daibata, M., 2011. Characterization of lytic enzyme open reading frame 9 (ORF9) derived from Enterococcus faecalis bacteriophage phiEF24C. *Appl Environ Microbiol*, **77**, 580-585.

Vaishnavi, C., Singh, G., Singh, K., 2011. Cost-effective screening of pooled faecal specimens from patients with nosocomial diarrhoea for Clostridium perfringens enterotoxin. *Indian journal of medical microbiology*, **29**, 56.

van Asten, A. J., Allaart, J. G., Meeles, A. D., Gloudemans, P. W., Houwers, D. J., Grone, A., 2008. A new PCR followed by MboI digestion for the detection of all variants of the Clostridium perfringens cpb2 gene. *Vet Microbiol*, **127**, 412-416.

van Asten, A. J., van der Wiel, C. W., Nikolaou, G., Houwers, D. J., Gröne, A., 2009. A multiplex PCR for toxin typing of Clostridium perfringens isolates. *Veterinary microbiology*, **136**, 411-412.

Van Regenmortel, M. H., Fauquet, C. M., Bishop, D. H., Carstens, E., Estes, M., Lemon, S., Maniloff, J., Mayo, M., McGeoch, D., Pringle, C., 2000. *Virus taxonomy: classification and nomenclature of viruses. Seventh report of the International Committee on Taxonomy of Viruses.* Academic Press.

Van Tassell, M. L., Ibarra-Sanchez, L. A., Hoepker, G. P., Miller, M. J., 2017. Hot topic: Antilisterial activity by endolysin PlyP100 in fresh cheese. *J Dairy Sci*.

Veiga-Crespo, P., Barros-Velázquez, J., Villa, T., 2007. What can bacteriophages do for us. *Commun Curr Res Educ Top Trends Appl Microbiol Mendez-Vilas-Spain Formatex,* **2**, 885-893.

Ventura, M., Turroni, F., Lima-Mendez, G., Foroni, E., Zomer, A., Duranti, S., Giubellini, V., Bottacini, F., Horvath, P., Barrangou, R., 2009. Comparative analyses of prophage-like elements present in bifidobacterial genomes. *Applied and environmental microbiology,* **75**, 6929-6936.

Vernacchio, L., Vezina, R. M., Mitchell, A. A., Lesko, S. M., Plaut, A. G., Acheson, D. W., 2006. Diarrhea in American infants and young children in the community setting: incidence, clinical presentation and microbiology. *Pediatr Infect Dis J,* **25**, 2-7.

Vicik, R., Busemann, M., Baumann, K., Schirmeister, T., 2006. Inhibitors of cysteine proteases. *Curr Top Med Chem,* **6**, 331-353.

Voidarou, C., Bezirtzoglou, E., Alexopoulos, A., Plessas, S., Stefanis, C., Papadopoulos, I., Vavias, S., Stavropoulou, E., Fotou, K., Tzora, A., Skoufos, I., 2011. Occurrence of Clostridium perfringens from different cultivated soils. *Anaerobe,* **17**, 320-324.

Volozhantsev, N. V., Oakley, B. B., Morales, C. A., Verevkin, V. V., Bannov, V. A., Krasilnikova, V. M., Popova, A. V., Zhilenkov, E. L., Garrish, J. K., Schegg, K. M., 2012. Molecular characterization of podoviral bacteriophages virulent for Clostridium perfringens and their comparison with members of the Picovirinae. *PLoS One,* **7**, e38283.

Volozhantsev, N. V., Verevkin, V. V., Bannov, V. A., Krasilnikova, V. M., Myakinina, V. P., Zhilenkov, E. L., Svetoch, E. A., Stern, N. J., Oakley, B. B., Seal, B. S., 2011. The genome sequence and proteome of bacteriophage ΦCPV1 virulent for Clostridium perfringens. *Virus research,* **155**, 433-439.

Wagstaff, P. G., Buijs, M., van den Bos, W., de Bruin, D. M., Zondervan, P. J., de la Rosette, J. J., Laguna Pes, M. P., 2016. Irreversible electroporation: state of the art. *Onco Targets Ther,* **9**, 2437-2446.

Waldor, M. K. & Mekalanos, J. J., 1996. Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science,* **272**, 1910.

Wang, I.-N., Smith, D. L., Young, R., 2000. Holins: the protein clocks of bacteriophage infections. *Annual Reviews in Microbiology,* **54**, 799-825.

Wang, W., Li, M., Lin, H., Wang, J., Mao, X., 2016. The Vibrio parahaemolyticus-infecting bacteriophage qdvp001: genome sequence and endolysin with a modular structure. *Arch Virol,* **161**, 2645-2652.

Watnick, P. I., Lauriano, C. M., Klose, K. E., Croal, L., Kolter, R., 2001. The absence of a flagellum leads to altered colony morphology, biofilm development and virulence in Vibrio cholerae O139. *Molecular microbiology,* **39**, 223-235.

Watts, T. D., Johanesen, P. A., Lyras, D., Rood, J. I., Adams, V., 2017. Evidence that compatibility of closely related replicons in Clostridium perfringens depends on linkage to parMRC-like partitioning systems of different subfamilies. *Plasmid,* **91**, 68-75.

Weaver, J. C., 1993. Electroporation: a general phenomenon for manipulating cells and tissues. *Journal of cellular biochemistry,* **51**, 426-426.

Weaver, J. C. & Barnett, A., 1992. Progress toward a theoretical model for electroporation mechanism: membrane electrical behavior and molecular transport. *Guide to electroporation and electrofusion*, 91-117.

Weaver, J. C. & Chizmadzhev, Y. A., 1996. Theory of electroporation: a review. *Bioelectrochemistry and Bioenergetics,* **41**, 135-160.

Wei, C., Liu, J., Maina, A. N., Mwaura, F. B., Yu, J., Yan, C., Zhang, R., Wei, H., 2017. Developing a bacteriophage cocktail for biocontrol of potato bacterial wilt. *Virol Sin,* **32**, 476-484.

White, S., 1978. Formation of" solvent-free" black lipid bilayer membranes from glyceryl monooleate dispersed in squalene. *Biophysical journal,* **23**, 337-347.

Wilkesman, J. & Kurz, L., 2017. Zymography Principles. *Methods Mol Biol,* **1626**, 3-10.

Wilkinson, S. R. & Young, M., 1994. Targeted integration of genes into the Clostridium acetobutylicum chromosome. *Microbiology,* **140**, 89-95.

Winston, J. A., Thanissery, R., Montgomery, S. A., Theriot, C. M., 2016. Cefoperazone-treated Mouse Model of Clinically-relevant Clostridium difficile Strain R20291. *JoVE (Journal of Visualized Experiments),* e54850-e54850.

Wioland, L., Dupont, J. L., Doussau, F., Gaillard, S., Heid, F., Isope, P., Pauillac, S., Popoff, M. R., Bossu, J. L., Poulain, B., 2014. Epsilon toxin from Clostridium perfringens acts on oligodendrocytes without forming pores, and causes demyelination. *Cell Microbiol*.

Wormald, J. C., Dindyal, S., Mellor, F., Behar, N., 2016. Adult necrotising enterocolitis-pig-bel disease: a Pacific disease in London. *BMJ Case Rep,* **2016**.

Wu, X., Kwon, S. J., Kim, J., Kane, R. S., Dordick, J. S., 2017. Biocatalytic Nanocomposites for Combating Bacterial Pathogens. *Annu Rev Chem Biomol Eng,* **8**, 87-113.

Yan, W., 1989. Use of host modified bacteriophages in development of a phage typing scheme for Clostridium perfringens. *Medical laboratory sciences,* **46**, 186-193.

Yang, H., Wang, D. B., Dong, Q., Zhang, Z., Cui, Z., Deng, J., Yu, J., Zhang, X. E., Wei, H., 2012. Existence of separate domains in lysin PlyG for recognizing Bacillus anthracis spores and vegetative cells. *Antimicrob Agents Chemother,* **56**, 5031-5039.

Yang, W. C., Hsu, T. C., Cheng, K. C., Liu, J. R., 2017. Expression of the Clonostachys rosea lactonohydrolase gene by Lactobacillus reuteri to increase its zearalenone-removing ability. *Microb Cell Fact,* **16**, 69.

Yelland, T. S., Naylor, C. E., Bagoban, T., Savva, C. G., Moss, D. S., McClane, B. A., Blasig, I. E., Popoff, M., Basak, A. K., 2014. Structure of a C. perfringens enterotoxin mutant in complex with a modified Claudin-2 extracellular loop 2. *J Mol Biol,* **426**, 3134-3147.

Ying, Z., Zhang, M., Yan, S., Zhu, Z., 2013. Gas Gangrene in Orthopaedic Patients. *Case reports in orthopedics,* **2013**.

Yonogi, S., Kanki, M., Ohnishi, T., Shiono, M., Iida, T., Kumeda, Y., 2016. Development and application of a multiplex PCR assay for detection of the Clostridium perfringens enterotoxin-encoding genes cpe and becAB. *J Microbiol Methods,* **127**, 172-175.

Yoo, H. S., Lee, S. U., Park, K. Y., Park, Y. H., 1997. Molecular typing and epidemiological survey of prevalence of Clostridium perfringens types by multiplex PCR. *Journal of clinical microbiology,* **35**, 228-232.

Yosef, I., Manor, M., Kiro, R., Qimron, U., 2015. Temperate and lytic bacteriophages programmed to sensitize and kill antibiotic-resistant bacteria. *Proceedings of the National Academy of Sciences,* **112**, 7267-7272.

Young, V. B. & Hanna, P. C., 2014. Overlapping Roles for Toxins in Clostridium difficile Infection. *Journal of Infectious Diseases,* **209**, 9-11.

Zelasko, S., Gorski, A., Dabrowska, K., 2017. Delivering phage therapy per os: benefits and barriers. *Expert Rev Anti Infect Ther,* **15**, 167-179.

Zeng, L., Skinner, S. O., Zong, C., Sippy, J., Feiss, M., Golding, I., 2010. Decision Making at a Subcellular Level Determines the Outcome of Bacteriophage Infection. *Cell,* **141**, 682-691.

Zhang, D., Coronel-Aguilera, C. P., Romero, P. L., Perry, L., Minocha, U., Rosenfield, C., Gehring, A. G., Paoli, G. C., Bhunia, A. K., Applegate, B., 2016. The Use of a Novel NanoLuc -Based Reporter Phage for the Detection of Escherichia coli O157:H7. *Sci Rep,* **6**, 33235.

Zhu, L., Zhou, W., Wang, T., Xiang, H., Ji, X., Han, Y., Tian, Y., Sun, Y., Liu, J., Guo, X., 2017. Isolation of Clostridium perfringens type A from wild bharals (Pseudois nayaur) following sudden death in Tibet, China. *Anaerobe,* **44**, 20-22.

Zimmer, M., Scherer, S., Loessner, M. J., 2002a. Genomic analysis of Clostridium perfringens bacteriophage φ3626, which integrates into guaA and possibly affects sporulation. *J Bacteriol,* **184**, 4359-4368.

Zimmer, M., Vukov, N., Scherer, S., Loessner, M. J., 2002b. The murein hydrolase of the bacteriophage φ3626 dual lysis system is active against all tested Clostridium perfringens strains. *Applied and environmental microbiology,* **68**, 5311-5317.

Chapter 9

Appendices

9. Appendices

9.1 Appendix 1 - Media and antibiotics

Prestige Medical Autoclave, England was used to autoclave all the media and buffers under 121ºC, 15psi (1bar) for 15minutes. All the media in the following table were made up to 500ml using distilled water, except for the semi-solid Brain Heart Infusion and the salt.

Table 13. All the media and antibiotics used during the study are listed here.

Media	Composition in 500ml	Manufacturer	Purpose
Blood agar (Brain heart infusion (BHI) 3.7%, agar 5%, and 7% Blood agar)	BHI 18.5 g Agar 5 g 35ml of defibrinized horse blood was added after the media is cooled.	Oxoid, UK Oxoid, UK Sigma-Aldrich, UK	Culturing *C. difficile.*
Fastidious Anaerobic broth (FA), 2.97%	14.85 g FA	BioConnections, UK	Culturing *C. difficile.*
Brain heart infusion agar, BHI 3.7% and agar 1%	18.5 g BHI broth 5 g Bacteriological agar	Oxoid, UK Oxoid, UK	For plaque assay and spot test of *C. difficile* phages.
BHI soft agar (BHI 3.7% and agar 0.4 %)	18.5 g BHI broth 2 g Bacteriological agar 250 ml Distilled water	Oxoid, UK Oxoid, UK	For plaque assay and spot test of *C. difficile* phages.
Salt (0.4 M MgCl2, 0.1 M CaCl2)	40.6g $MgCl_2$ 0.75g $CaCl_2$ 250 ml Distilled water	Argos Organics, UK Fisher Scientific, UK	For plaque assay and spot test of *C. difficile* and propagation of *C. perfringens* phages.
Brain heart infusion broth 3.7%	18.5 g BHI	Oxoid, UK	Culturing *C. difficile* and propagation of *C. difficile* phages.
BHI - Sodium thio-glycolate broth 3.7% BHI and 0.1% Na-thiglycolate	18.5 g BHI broth 0.5 g Na-thioglycolate	Oxoid, UK Sigma- Aldrich, UK	Culturing *C. difficile* outside the anaerobic chamber and recover electroporated cells.
Media	Composition in 500 ml	Manufacturer	Purpose
Brucella agar (BA) Supplemented with Hemin 0.0005%, Vitamin K1, DHB 5% and thiamphenicol 15µg/ml	BA 22 g Hemin 2.5mg Vitamin K1 5µl 25ml of DHB 221µl of 34mg/ml thiamphenicol was added after the media has cooled.	Oxoid, UK Sigma-Aldrich, USA Santa Cruz Biotechnology Sigma-Aldrich,UK Sigma-Aldrich, UK	Selection of transformed *C. difficile.*
Trypticase-pepton glucose-yeast extract agar (Ackermann *et al.*, 2001)	Trypticase 25g Peptone 2.5g Yeast extract 10g Dextrose 2g	BBL, France Oxoid, UK Oxoid, UK Fisher Scientific, UK Fisher Scientific, UK	Selection of transformed *C. difficile.*

	Sodium Thioglycolate 0.5g Agar 5g	Oxoid, UK	
Trypticase-pepton glucose-yeast extract broth	Trypticase 25g Peptone 2.5g Yeast extract 10g Dextrose 2g Sodium Thioglycollate 0.5g 33.3ml of 1.5g/100ml Trypsin was added into the broth just before using it.	BBL, France Oxoid, UK Oxoid, UK Fisher Scientific, UK Fisher Scientific, UK Fisher Scientific, USA	Recover Electroporated cells.
Fluid Thio-glycolate broth	Yeast extract 2.5g Tryptone 7.5g Glucose 2.75g Sodium thioglycollate 0.25g Sodium chloride 1.25g L-cystine 0.25g Resazurin 0.5mg	Oxoid, UK Oxoid, UK Fisher Scientific, UK Fisher Scientific, UK Sigma-Aldrich, USA Fisher Scientific, UK Sigma-Aldrich, USA	Culturing *C. difficile* outside the anaerobic chamber.
Lauria Bertani agar (LB) agar	12.5 g LB 5 g Bacteriological agar chloramphenicol	Fisher Scientific, UK Oxoid, UK Sigma-Aldrich, USA	Culturing *E. coli.*
Lysogeny Broth (LB) broth	12.5 g LB	Fisher Scientific, UK	Culturing *E. coli.*
Media	Composition in 500 ml	Manufacturer	Purpose
Tryptose-sulfate cycloserine agar (TSC), 4.6% TSA, 400 µg/ml of cycloserine.	23g of TSA One vial of cycloserine (200mg) was added after the media has cooled for the isolation of *C. perfringens.*	Oxoid, UK Bioconnections, UK	Isolation and culturing *C. perfringens.*
Tryptose-sulfate agar (TS), 4.6% TSA.	23g of TSA	Oxoid, UK	Culturing *C. perfringens.*
Tryptose-sulfate broth, 1.5% Tryptose, 0.5% yeast extract, 0.5% pepton, 0.1% Sodium metabisulphate, 0.1% Ammonium ferric citrate, and 400 µg/ml of cycloserine.	Tryptose 7.5g Yeast Extract 2.5g Neutralized soy pepton 2.5g Sodium metabisulphate 0.5g Ammonium ferric citrate 0.5g Cycloserine was added only for the isolation of *C. perfringens*	Fluka, USA Oxoid, UK Oxoid, UK Fluka, USA Sigma-Aldrich, Germany Bioconnections, UK	Isolation, Culturing *C. perfringens* and preparing cryo-stock.
Tryptose-sulfate soft agar, 1.5% Tryptose, 0.5% yeast extract, 0.5% pepton, 0.1% Sodium metabisulphate, 0.1% Ammonium ferric citrate, and 0.4% agar.	Tryptose 7.5g Yeast Extract 2.5g Neutralized soy pepton 2.5g Sodium metabisulphate 0.5g Ammonium ferric citrate 0.5g Agar 2g	Fluka, USA Oxoid, UK Oxoid, UK Sigma-Aldrich, Germany Fluka, Germany Oxoid, UK	Plaque assay and spot test.

Tryptose-yeast extract agar, 1.5% Tryptose, 0.5% yeast extract, 0.5% pepton, 0.5 sodium chloride, and 2% agar.	Tryptose 7.5g Yeast Extract 2.5g Neutralized soy pepton 2.5g Sodium chloride 2.5g Agar 10g	Fluka, USA Oxoid, UK Oxoid, UK Fischer Scientific, UK Oxoid, UK	Culturing *C. Perfringens.*
Tryptose-yeast extract broth, 1.5% Tryptose, 0.5% yeast extract, and 0.5% pepton, 0.5 sodium chloride.	Tryptose 7.5g Yeast Extract 2.5g Neutralized soy pepton 2.5g Sodium chloride 2.5g	Fluka, USA Oxoid, UK Oxoid, UK Fischer Scientific, UK	Culturing *C. Perfringens.*
Tryptose-yeast extract soft agar, 1.5% Tryptose, 0.5% yeast extract, 0.5% pepton, 0.5 sodium chloride, and 0.4% agar.	Tryptose 7.5g Yeast Extract 2.5g Neutralized soy pepton 2.5g Sodium chloride 2.5g Agar 2g	Fluka, USA Oxoid, UK Oxoid, UK Fischer Scientific, UK Oxoid, UK	Plaque assay and spot test of *C. Perfringens* phages.
Antibiotic	Composition in 500 ml	Manufacturer	Purpose
Chloramphenicol	Sigma-Aldrich, USA	30 µg/ml	Amplify pMTL82151, pMTL84151 plasmids.
Thiamphenicol	Sigma-Aldrich, UK	15µg/ml	Selection of *C. difficile* mutants.
Cycloserine	Bioconnections, UK	400µg/ml	Isolation of *C. perfringens.*
Carbenicillin	Sigma-Aldrich, UK	150µg/ml	Amplify pLEICS-02 plasmid.

9.2 Appendix 2 - Buffers, solutions, and enzymes

Table 14. All buffers, solutions and enzymes used during this study are listed in this table.

Buffer	Composition	Manufacturer	Purpose
SMP buffer **Sucrose (0.268M),** **MgCl2.6H2O (7mM),** **Sodium phosphate (0.1M),** **pH 7.4.**	92.4 g Sucrose 0.2 g MgCl2.6H2O 70 ml of 0.1 M (16.4g of Sodium phosphate in 1 litre) The sucrose was dissolved in 200 ml DW and filtered into the above autoclaved 800ml.	Fisher Scientific, UK Sigma-Aldrich, USA Sigma-Aldrich, USA Sigma-Aldrich, UK	Electroporation of *C. difficile.*
Sodium-Magnesium buffer (SM), pH 8.	Sodium chloride 5.8g (0.1M) MgSO4.7H2O 2g (8 mM) 50 ml of 1 M Tris–HCl Made up to 1 L with distilled H2O and Autoclaved.	Fisher Scientific, UK Fisher Scientific, UK Santa Cruz, USA	General phage suspension buffer and tub dialysis.
2M $NaCl_2$ SM buffer. pH 7.5	$NaCl_2$ 116.88g (2M) MgSO4.7H2O 2g (8 mM) 50 ml of 1 M Tris–HCl Made up to 1 L with distilled H2O and Autoclaved.	Fisher Scientific, UK Fisher Scientific, UK Santa Cruz, USA	Loading buffer for protein purification and phage cleaning by Anion-exchange chromatography.
SM buffer without $NaCl_2$, pH 7.5.	MgSO4.7H2O 2g (8 mM) 50 ml of 1 M Tris–HCl Made up to 1 L with distilled H2O and Autoclaved.	Fisher Scientific, UK Santa Cruz, USA	Elution buffer for protein purification and phage cleaning by Anion-exchange chromatography.
0.5 M Ethylene-diamine-tetra acetic acid (EDTA)	EDTA 93.5g Dissolved in 450ml distilled H_2O and autoclaved.	Sigma-Aldrich, USA	Phage DNA extraction and making up other solutions.

1 X TAE buffer	Tris-HCl (40mM) Acetic acid (20 mM) EDTA (2M) pH 8	Santa Cruz, USA Fisher Scientific, UK Sigma-Aldrich, USA	Used for agarose gel electrophoresis.
5 x TBE buffer	54g Tris-Base 27.5g boric acid 20ml 0.5M EDTA pH8 Up to 1L with H2O.	Fisher Scientific, UK Fisher Scientific, UK Sigma-Aldrich, USA	For pulsed-field gel-electrophoresis.
1 x TE buffer	100 mM EDTA 100 mM Tris-HCl (pH9) 1% SDS	Sigma-Aldrich, USA Santa Cruz, USA Fisher Scientific, UK	For pulsed-field gel-electrophoresis.
Lysis Buffer	20 ml 1 M Tris-HCl 20 ml 10% SDS 50 ml 500 mM EDTA 120ml distilled H2O	Santa Cruz, USA Fisher Scientific, UK Sigma-Aldrich, USA	For pulsed-field gel-electrophoresis.
Solutions	Composition	Manufacturer	Purpose
Ammonium Acetate	0.1 M, pH 7.5	Fisher Scientific, UK	Washing phage lysate for TEM.
Sodium dodecyl sulphate	Vary	Fisher Scientific, Belgium	Making up solutions.
Enzyme	Concentration	Manufacturer	Purpose
Cysteine-protease inhibitor	Final concentration of		Diminish the surface protein layer of *C. difficile.*
Lysostaphin	Final concentration of		Increasing the area of osmosis pores in the peptide-glycan layers of *C. difficile*.

9.3 Appendix 3 - Oligonucleotides

Table 15. Oligonucleotides used in this study.

Name	Forward and reverse primers	Target	Expected product size (bp)	Source
CP 16s F	**5'- TGA AAG ATG GCA TCA TCA TTC AAC-3'**	**CP specific 16S rDNA**	**260 bp**	**(Skånseng *et al.*, 2006)**
CP 16s R	**5'- GGT ACC GTC ATT ATC TTC CCC AAA-3'**			
8F	5'- AGAGTTTGATCCTGGCTCAG -3'	Universal bacterial 16s rDNA	1-1.8 kb	(Turner *et al.*, 1999)
1391R	5'- GACGGGCGGTGTGTRCA -3'			
CPAlpha F	**5'- GCTAATGTTACTGCCGTTGA -3'**	***cpa* (αtoxin)**	**324 bp**	**(van Asten *et al.*, 2009)**
CPAlpha R	**5'- CCTCTGATACATCGTGTAAG -3'**			
CPBetaF3	5'- GCGAATATGCTGAATCATCTA -3'	*cpb* (β-toxin)	195 bp	(van Asten *et al.*, 2009)
CPBetaR3	5'- GCAGGAACATTAGTATATCTTC -3'			
CPBeta2 F	**5'- AAA TAT GAT CCT AAC CAA Ma AA -3'**	***cpb2* (β2-oxin)**	**548 bp**	**(van Asten *et al.*, 2008)**
CPBeta2 R	**5'- CCA AAT ACT Yb TAATYGATGC -3'**			
CPEpsilonF	5'- TGGGAACTTCGATACAAGCA -3'	*etx* (ε-toxin)	376 bp	(Baums *et al.*, 2004a)
CPEpsilonR	5'- AACTGCACTATAATTTCCTTTTCC-3'			
CPIotaF	**5'- AATGGTCCTTTAAATAATCC-3'**	***Iap* (ι-toxin)**	**272 bp**	**(Meer *et al.*, 1997)**
CPIotaR	**5'- TTAGCAAATGCACTCATATT-3'**			
CPEnteroF	5'- TTCAGTTGGATTTACTTCTG-3'	*cpe* (enterotoxin)	485 bp	(van Asten *et al.*, 2009)
CPEnteroR	5'- TGTCCAGTAGCTGTAATTGT-3'			
One	**5' GGTAATAACTGGGGAATATGTCGA 3'**	**Close CPAP1 contig**	**?**	**This study**
Two	**5' CGACCATCACGGGTAATCATC 3'**			
Three	5' ATGAATCCACAATTGTTACGCG 3'	Close CPAP1 contig	?	This study
Four	5' GTCTCTTAACACATCTCCGAGC 3'			
ECCF	**5' CTCCATTACTGAGGCGTGGG 3'**	***E. coli* contig**	**418 bp**	**This study**
ECCR	**5' AGTCTCGCGCGAACGTTC 3'**			
β toxin F	5' AACTTAACTGGATTTATGTCTTCA 3'	CP β toxin	317-bp	(Kadra *et al.*, 1999)
β toxin R	5' ATAGTAGAAAAATCAGGTTGGACA 3'			
***etx* F**	**5' TGGGAACTTCGATACAAGCA 3'**	**CP Epsilon toxin**	**396 bp**	**(Gharaibeh *et al.*, 2010)**
***etx* R**	**5' TTAACTCATCTCCCATAACTGCAC 3'**			
iA F	**5' ACTACTCTCAGACAAGACAG 3'**	**CP Iota toxin**	**446 bp**	**(Perelle *et al.*, 1993)**
iA R	**5' CTTTCCTTCTATTACTATACG 3'**			

E. coli contig F	5’ CTCCATTACTGAGGCGTGGG 3’	CPAP1 short contig	418 bp	This study
E. coli contig R	5’ TTGAACGTTCGCGCGAGACT 3’			
CPAP1F	5’ CACGATTCGACATATTCCCC 3’	CPAP1 long contig	3730bp	This study
CPAP1R	5’ GGCAAACAAGCGCTGATATT 3’			
F42End F	5’-TACTTCCAATCCATGAAAATAGGTATTAGAGATGGACA-3’	CPAP1 Endolysin	666 bp	This study
F42Endr	5’-TATCCACCTTTACTGTCAATTACACTCCTCCACAAAACA-3’			
pGEX5‘	5’ CCG GGA GCT GCA TGT GTC AGA GG 3’	pLEICS-02 plasmid	687 bp	PROTEX service
pGEX3’	5’ GGG CTG GCA AGC CAC GTT TGG TG 3’			

9.4 Appendix 4 - Lysis caused by induction lysates.

Table 16. Lysis caused by the induction lysate of 17 *C. perfringens* strains on 27 indicator *C. perfringens* strains. Each induction lysate was able to produce lysis on at least one of the indicator strain.

Indicator strain																		
Phage induced from	CP1		CP2		CP3		CP4		CP5		CP6		CP7		CP8		CP9	
	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N
CP1	x	x	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	Y	Y
CP2	-ve	-ve	x	x	lysis	lysis	-ve	-ve	-ve	-ve	lysis	lysis	lysis	lysis	lysis	lysis	-ve	-ve
CP 3	-ve	-ve	-ve	-ve	x	x	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP4	-ve	-ve	-ve	-ve	-ve	-ve	x	x	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP5	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	x	x	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP6	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	x	x	-ve	-ve	-ve	-ve	-ve	-ve
CP7	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	x	x	-ve	-ve	-ve	-ve
CP8	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	x	x	-ve	-ve
CP9	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	x	x
CP10	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP11	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis
CP12	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP13	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP14	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP15	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis
CP16	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve
CP17	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve

Indicator strain																		
Phage induced from	CP10		CP11		CP12		CP13		CP14		CP15		CP16		CP17		CP18	
	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N
CP1	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis
CP 3	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis
CP4	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP5	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis
CP6	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP7	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP8	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP9	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP10	x	x	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP11	lysis	lysis	x	x	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP12	-ve	-ve	-ve	-ve	x	x	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis
CP13	-ve	-ve	-ve	-ve	-ve	-ve	x	x	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP14	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	x	x	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP15	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	x	x	-ve	-ve	-ve	-ve	-ve	-ve
CP16	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	x	x	-ve	-ve	-ve	-ve
CP17	-ve	-ve	-ve	-ve	lysis	lysis	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	x	x	-ve	-ve

Indicator strain																		
Phage induced from	CP19		CP20		CP21		CP22		CP23		CP24		CP25		CP26		CP27	
	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N	M	N
CP1	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP2	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP 3	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP4	lysis	lysis	lysis	lysis	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP5	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis
CP6	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve
CP7	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve
CP8	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	lysis	lysis	lysis	lysis	-ve	-ve	-ve	-ve
CP9	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP10	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve
CP11	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis
CP12	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve
CP13	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP14	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP15	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP16	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
CP17	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	lysis	lysis	-ve	-ve

9.5 Appendix 5 - Environmental samples for the isolation of *C. perfringens* strains

Table 17. Environmental samples and *C. perfringens* strains. Source, location of collection, and toxin types.

N0	Strain number	Toxin type & toxin produced	Source	Place
1	CP 1	A, α	Chicken faeces	Melton Mowbray farms
2	CP 2	A, α	Chicken faeces	
3	CP 3	A, α	Soil	
4	CP 4	A, α + cpe	Soil	
5	CP 5	A, α	Sheep faeces	
6	CP 6	A, α	Sheep faeces	
7	CP 7	A, α	Sheep faeces	
8	CP 8	A, α	Cow faeces	
9	CP 9	A, α	Horse faeces	
10	CP 10	A, α	Chicken faeces	Iraq-Karbala province
11	CP 11	A, α	Chicken faeces	
12	CP 12	A, α	Soil	
13	CP 13	A, α + cpe	Dog faeces	Victoria park
14	CP 14	A, α	Dog faeces	
15	CP 15	A, α + β2	Dog pound	Samples in the fridge, Kindly Provided By Julian Clokie
16	CP 16	A, α + β2	Duck dropping	
17	CP 17	A, α	Sediments	
18	CP 18	A, α	Sediment	
19	CP 19	A, α	Duck dropping	
20	CP 20	A, α	Soil	
21	CP 21	A, α	Strains existed in the lab.	
22	CP 22	A, α		
23	CP 23	A, α		
24	CP 24	A, α		
25	CP 25	A, α + β2		
26	CP 26	A, α		
27	CP 27	A, α		
28	CP 28	A, α		
29	CP 29	A, α		
30	CP 30	A, α	Lamb faeces	Melton Mowbray farms Collection was conducted in different farms at different time.
31	CP 31	A, α	Ostrich faeces	
32	CP 32	A, α	Horse faeces	
33	CP 33	A, α	Sheep faeces	
34	CP 34	A, α	Horse faeces	
35	CP 35	A, α + β2+ cpe	Sheep faeces	
36	CP 36	A, α	Horse faeces	
37	CP 37	A, α	Unknown faeces from the road side	
38	CP 38	A, α	Chicken faeces	
39	CP 39	A, α	Ostrich faeces	

No	Strain number		Source	Place
40	CP 40	A, α	Cow Faeces (farm)	Walton lodge farm, Sutton Lane, Sutton Elms, Leicester LE9 6QF
41	CP 41	E, α + ι	Cow Faeces (farm)	
42	CP 42	A, α	Cow Faeces (farm)	
43	CP 43	A, α	Cow Faeces (stable cow with number on it's ear)	
44	CP 44	A, α	Duck Faeces	
45	CP 45	A, α	Duck faeces	
46	CP 46	A, α	Horse faeces	
47	CP 47	A, α	Deer faeces	Bradgate park
48	CP 48	A, α	Deer faeces	
49	CP 49	A, α	Duck faeces	
50	CP 50	A, α	Duck faeces	
51	CP 51	A, α	Duck faeces	Watermead park
52	CP 52	A, α	Swan faeces	
53	CP 53	A, α	Duck faeces	
54	CP 54	A, α	Chicken	Ansty (Gorse Hill City Farm)
55	CP 55	A, α	Chicken	
56	CP 56	A, α	Turkey	
57	CP 57	A, α	Parrot	Desford (Tropical Birdland)
58	CP 58	A, α	Soil Mix	
59	CP 59	A, α	Parrot mix	
60	CP 60	A, α	Blue and red Parrot	
61	CP 61	A, α	Duck	Water mead
62	CP 62	A, α + cpe	Soil	Kindly Provided By Julian Clokie
63	CP 63	A, α	Soil	
64	CP 64	A, α	Soil	
65	CP 65	A, α + cpe	Soil	
66	CP 66	A, α + cpe	Soil	
67	CP 67	A, α	Duck	
68	CP 68	A, α	Duck	
69	CP 69	A, α + cpe	Duck	
70	CP 70	A, α	Soil and sediment	Mahananda and Anisha's samples.
71	CP 71	D, α + ε + β2	Soil and sediment	
72	CP 72	C, α + β	Soil and sediment	
73	CP 73	A, α	Soil and sediment	
74	CP 74	A, α	Soil and sediment	
75	CP 75	A, α	Soil and sediment	
76	CP 76	A, α	Soil and sediment	
77	CP 77	A, α	Soil and sediment	
78	CP 78	A, α + β2	Soil and sediment	
79	CP -ve		Pigs	Kindly Provided By Julian Clokie
80	CP -ve		Pigs	
81	CP -ve		Pigs	
82	CP -ve		Pigs	
83	CP -ve		Pigs	
84	CP -ve		Pigs	
85	CP -ve		Pigs	

No	Strain number		Source	Place
86	CP -ve		Fish	Iraq
87	CP -ve		Rabbit	Ansty (Gorse Hill City Farm)
88	CP -ve		Rabbit	
89	CP -ve		Rabbit	
90	CP -ve		Rabbit	

9.6 Appendix 6 – Electroporation settings

Table 18. Shows electroporation of *C. difficile* ribotype 076 with phage phiCDHM1 genome using different DNA concentrations and pre-pulse incubation. (Exponential decay pulse).

C. Difficile **ribotype**	**Pre-pulse incubation**	**voltage**	**Field intensity kV/cm**	**Time millisecond**	**DNA type**	**DNA concentration**	**Transformation Efficiency**	**CFU before pulse**	**CFU after pulse**	**Cuvette gap mm.**
R 076	Room temp.	2.5 kV	1.25	5.2	phiCDHM1 genome	1 µg	Zero	10^{10}	10^{9}	2
R 076	=	2.5 kV	1.25	5.2	=	2 µg	Zero	=	=	2
R 076	=	2.5 kV	1.25	5.2	=	3 µg	Zero	=	=	2
R 076	=	2.5 kV	1.25	5.2	=	4 µg	Zero	10^{10}	10^{9}	2
R 076	=	2.5 kV	1.25	5.2	=	5 µg	Zero	=	=	2
R 076	=	2.5 kV	1.25	5.2	=	6 µg	Zero	=	=	2
R 076	=	2.5 kV	1.25	5.2	=	7 µg	Zero	=	=	2
R 076	=	2.5 kV	1.25	5.2	=	8 µg	Zero	=	=	2
R 076	=	2.5 kV	1.25	5.2	=	9 µg	Zero	=	=	2
R 076	=	2.5 kV	1.25	5.2	=	10 µg	Zero	=	=	2
R 076	Ice	2.5 kV	1.25	5.2	=	1 µg	Zero	10^{10}	10^{10}	2
R 076	=	2.5 kV	1.25	5.2	=	2 µg	Zero	=	=	2
R 076	=	2.5 kV	1.25	5.2	=	3 µg	Zero	=	=	2
R 076	=	2.5 kV	1.25	5.2	=	4 µg	Zero	=	=	2
R 076	=	2.5 kV	1.25	5.2	=	5 µg	Zero	=	=	2
R 076	=	2.5 kV	1.25	5.2	=	6 µg	Zero	=	=	2
R 076	=	2.5 kV	1.25	5.2	=	7 µg	Zero	=	=	2
R 076	=	2.5 kV	1.25	5.2	=	8 µg	Zero	=	=	2
R 076	=	2.5 kV	1.25	5.2	=	9 µg	Zero	=	=	2

Table 19. Shows electroporation of *C. difficile* ribotype 076 with phage phiCDHM1 genome using different pulse duration at ice pre-pulse incubation. (Exponential decay pulse)

C. Difficile **ribotype**	**Pre-pulse incubation**	**voltage**	**Field intensity kV/cm**	**Time millisecond**	**DNA type**	**DNA concentration**	**Transformation Efficiency**	**CFU before pulse**	**CFU after pulse**	**Cuvette gap mm.**
R 076	Ice	2.5 kV	1.25	7.8	phiCDHM1 genome	5 µg	Zero	10^9	10^9	2, 4
R 076	=	2.5 kV	1.25	10.4	=	5 µg	Zero	10^9	10^9	2, 4
R 076	=	2.5 kV	1.25	15.3	=	5 µg	Zero	10^9	10^9	2, 4
R 076	=	2.5 kV	1.25	20.2	=	5 µg	Zero	10^9	10^9	2, 4
R 076	=	2.5 kV	1.25	26.2	=	5 µg	Zero	10^9	10^9	2, 4

Table 20. Shows electroporation of *C. difficile* ribotype 076 with two plasmids using different field strength, pulse duration and DNA concentrations. (Exponential decay pulse)

C. Difficile **ribotype**	**Pre-pulse incubation**	**voltage**	**Field intensity kV/cm**	**Time millisecond**	**DNA type**	**DNA concentration**	**Transformation Efficiency**	**CFU before pulse**	**CFU after pulse**	**Cuvette gap mm.**
R 076	Ice	1.3	0.65	5.2	pMTL82151	5 µg	Zero	10^8	10^8	2, 4
R 076	=	1.35	0.675	5.1	=	5 µg	Zero	10^8	10^8	2, 4
R 076	=	1.4	0.7	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 076	=	1.45	0.725	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 076	=	1.5	0.75	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 076	=	1.55	0.775	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 076	=	1.6	0.8	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 076	=	1.65	0.825	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 076	=	1.7	0.85	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 076	=	1.75	0.875	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 076	=	1.8	0.9	5.3	=	5 µg	Zero	10^8	10^8	2, 4
R 076	=	1.85	0.925	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 076	=	1.9	0.95	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 076	=	1.95	0.975	5.3	=	5 µg	Zero	10^8	10^8	2, 4
R 076	=	2	1	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 076	=	2.05	0.1025	5.2	=	5 µg	Zero	10^8	10^8	2, 4

R 076	=	2.1	105	5.3	=	5 µg	Zero	10^8	10^8	2, 4
R 076	=	2.15	10.75	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 076	=	2.2	1.1	5.1	=	5 µg	Zero	10^8	10^8	2, 4
R 076	=	2.25	1.125	5.2	=	5 µg	Zero	X,10^8	X,10^8	2, 4
R 076	=	2.25	1.125	25.7	=	5 µg	Zero	X,10^8	X,10^8	2, 4
R 076	=	2.35	1.175	5.1	pMTL84151	5 µg	Zero	10^9	10^9	4
R 076	=	2.45	1.225	5.1	pMTL84151	5 µg	Zero	10^9	10^9	4
R 076	=	2.5	1.25	5.2	pMTL82151	1 µg	Zero	10^9,10^{10}	10^9,10^{10}	2,4
R 076	=	2.5	1.25	5.3	pMTL82151	2 µg	Zero	10^9,10^{10}	10^9,10^{10}	2,4
R 076	=	2.5	1.25	5.3	=	2.2 µg	Zero	10^{10}	10^{10}	2
R 076	=	2.5	1.25	5.3	=	3 µg	Zero	10^9,10^{10}	10^9,10^{10}	2,4
R 076	=	2.5	1.25	5.2	=	3.3 µg	Zero	10^{10}	10^{10}	2
R 076	=	2.5	1.25	5.3	=	4 µg	Zero	10^9,10^{10}	10^9,10^{10}	2,4
R 076	=	2.5	1.25	5.3	=	4.4 µg	Zero	10^{10}	10^{10}	2
R 076	=	2.5	1.25	5.3	=	5 µg	Zero	10^9,10^{10}	10^9,10^{10}	2,4
R 076	=	2.5	1.25	5.3	=	5.5 µg	Zero	10^{10}	10^{10}	2
R 076	=	2.5	1.25	5.3	=	6 µg	Zero	10^9,10^{10}	10^9,10^{10}	2,4
R 076	=	2.5	1.25	5.3	=	6.6 µg	Zero	10^{10}	10^{10}	2
R 076	=	2.5	1.25	5.3	=	7 µg	Zero	10^9,10^{10}	10^9,10^{10}	2,4
R 076	=	2.5	1.25	5.3	=	8 µg	Zero	X	X	2

R 076	=	2.5	1.25	5.3	=	9 µg	Zero	X	X	2
R 076	=	2.5	1.25	10.3	=	3 µg	Zero	10^{10}	10^{10}	4
R 076	=	2.5	1.25	10.3	=	4 µg	Zero	10^{10}	10^{10}	4
R 076	=	2.5	1.25	10.3	=	5 µg	Zero	10^{10}	10^{10}	4
R 076	=	2.5	1.25	10.3	=	6 µg	Zero	10^{10}	10^{10}	4
R 076	=	2.5	1.25	10.3	=	7 µg	Zero	10^{10}	10^{10}	4
R 076	=	3	1.5	5.3	=	2 µg	Zero	10^{10}	10^{9}	2
R 076	=	3	1.5	5.3	=	3 µg	Zero	10^{10}	10^{9}	2
R 076	=	3	1.5	5.3	=	4 µg	Zero	10^{10}	10^{9}	2
R 076	=	3	1.5	5.3	=	5 µg	Zero	10^{10}	10^{9}	2
R 076	=	3	1.5	5.3	=	6 µg	Zero	10^{10}	10^{9}	2
R 076	=	3	1.5	5.3	=	7 µg	Zero	10^{10}	10^{9}	2
R 076	=	3	1.5	5.3	=	8 µg	Zero	10^{10}	10^{9}	2

Table 21. Shows electroporation of *C. difficile* ribotype 220 with pMTL82151 using different field strength and pulse duration at Ice pre-pulse incubation. (Exponential decay pulse)

C. Difficile **ribotype**	**Pre-pulse incubation**	**voltage**	**Field intensity kV/cm**	**Time millisecond**	**DNA type**	**DNA concentration**	**Transformation Efficiency**	**CFU before pulse**	**CFU after pulse**	**Cuvette gap mm.**
R 220	Ice	1.3	0.65	5.2	pMTL82151	5 µg	Zero	10^8	10^8	2
R 220	=	1.35	0.675	5.2	=	5 µg	Zero	10^8	10^8	2
R 220	=	1.4	0.7	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 220	=	1.45	0.725	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 220	=	1.5	0.75	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 220	=	1.55	0.775	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 220	=	1.6	0.8	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 220	=	1.65	0.825	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 220	=	1.7	0.85	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 220	=	1.75	0.875	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 220	=	1.8	0.9	5.3	=	5 µg	Zero	10^8	10^8	2, 4
R 220	=	1.85	0.925	5.3	=	5 µg	Zero	10^8	10^8	2, 4
R 220	=	1.9	0.95	5.4	=	5 µg	Zero	10^8	10^8	2, 4
R 220	=	1.95	0.975	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 220	=	2	1	5.3	=	5 µg	Zero	10^8	10^8	2, 4
R 220	=	2.05	1.025	5.4	=	5 µg	Zero	10^8	10^8	2, 4

R 220	=	2.1	1.05	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 220	=	2.15	1.075	5.3	=	5 µg	Zero	10^8	10^8	4
R 220	=	2.2	1.1	5.2	=	5 µg	Zero	10^8	10^8	2, 4
R 220	=	2.25	1.125	25.8	=	5 µg	Zero	10^8	10^8	4

Table 22. Electroporation of *C. difficile* ribotype 220 with pMTL82151 using different DNA concentration at room temperature pre-pulse incubation (exponential decay pulse).

C. Difficile **ribotype**	**Pre-pulse incubation**	**voltage**	**Field intensity kV/cm**	**Time millisecond**	**DNA type**	**DNA concentration**	**Transformation Efficiency**	**CFU before pulse**	**CFU after pulse**	**Cuvette gap mm.**
R 220	Room Temp.	2.5	1.25	5.2	pMTL82151	1 µg	Zero	10^9	10^9	2
R 220	=	2.5	1.25	5.3	=	2 µg	Zero	10^9	10^9	2
R 220	=	2.5	1.25	5.3	=	3 µg	Zero	10^9,10^{10}	10^9,10^9	2,4
R 220	=	2.5	1.25	5.3	=	4 µg	Zero	10^9,10^{10}	10^9,10^9	2,4
R 220	=	2.5	1.25	5.3	=	5 µg	Zero	10^9,10^{10}	10^9,10^9	2,4
R 220	=	2.5	1.25	5.3	=	6 µg	Zero	10^9,10^{10}	10^9,10^9	2,4
R 220	=	2.5	1,25	5.3	=	7 µg	Zero	10^9,10^{10}	10^9,10^9	2,4

Table 23. Shows electroporation of *C. difficile* ribotype 220 with pMTL84151 using different field strength and pulse duration at ice pre-pulse incubation. (Square wave pulse)

C. difficile ribotype	Pre-pulse incubation	Voltage kV	Field strength kV/cm	No. of pulses	Interval between pulses	Time millisecond	DNA type	DNA concentration	Transfor-mation Efficiency	CFU before pulse	CFU after pulse	Cuvette gap mm.
R 076	Ice	2.5	12.5	2	5 Sec	5	phiCDHM1	5 µg	Zero	10^9	10^9	2 and 4
R 220	Ice	2	10	2	5 Sec.	4.1	pMTL84151	5 µg	Zero	10^7	10^7	4
R 220	=	2.1	10.5	2	5 Sec.	4.1	=	5 µg	Zero	10^7	10^7	4
R 220	=	2.2	11	2	5 Sec.	4.1	=	5 µg	Zero	10^7	10^7	4
R 220	=	2.3	11.5	2	5 Sec.	4.1	=	5 µg	Zero	10^7	10^7	4
R 220	=	2.4	12	2	5 Sec.	4.1	=	5 µg	Zero	10^7	10^7	4
R 220	=	2.5	12.5	2	5 Sec.	4.1	=	5 µg	Zero	10^7	10^7	4
R 220	=	1.7	8.5	2	5 Sec.	5	=	5 µg	Zero	10^9	10^9	4
R 220	=	1.8	9	2	5 Sec.	5	=	5 µg	Zero	10^9	10^9	4
R 220	=	1.9	9.5	2	5 Sec.	5	=	5 µg	Zero	10^9	10^9	4
R 220	=	2	10	2	5 Sec.	5	=	5 µg	Zero	10^9	10^9	4
R 220	=	2.1	10.5	2	5 Sec.	5	=	5 µg	Zero	10^9	10^9	4
R 220	=	2.2	11	2	5 Sec.	5	=	5 µg	Zero	10^9	10^7	4
R 220	=	2.3	11.5	2	5 Sec.	5	=	5 µg	Zero	10^9	10^9	4
R 220	=	2.4	12	2	5 Sec.	5	=	5 µg	Zero	10^9	10^9	4
R 220	=	2.5	12.5	2	5 Sec.	5	=	5 µg	Zero	10^9	10^8	4

Table 24. Shows electroporation of *C. difficile* ribotype 220 with pMTL82151 and pMTL84151 using different field strength, DNA concentration and pulse duration at ice pre-pulse incubation. (Time constant pulse)

***C. difficile* ribotype**	**Pre-pulse incubation**	**Voltage (kV)**	**Field strength kV/cm**	**Time millisecond**	**DNA type**	**DNA concentration**	**Transforma-tion efficiency**	**CFU before pulse**	**CFU after pulse**	**Cuvette Gap**
220	Ice	1.7	8.5	6.7	pMTL82151	5 µg	Zero	10^9	10^8	4
220	=	1.8	9	5.8	=	5 µg	Zero	10^9	10^9	4
220	=	1.9	9.5	5.9	=	5 µg	Zero	10^9	10^9	4
220	=	2	10	5.3	=	5 µg	Zero	10^9	10^9	4
220	=	2.1	10.5	6.2	=	5 µg	Zero	10^9	10^9	4
220	=	2.2	11	6	=	5 µg	Zero	10^9	10^9	4
220	=	2.3	11.5	7.3	=	5 µg	Zero	10^9	10^9	4
220	=	2.4	12	6.8	=	5 µg	Zero	10^9	10^9	4
220	=	2.5	12.5	6.5	=	5 µg	Zero	10^9	10^9	4
220	=	1.6	8	5.2	=	5 µg	Zero	10^8	10^8	4
220	=	1.7	8.5	5.1	=	5 µg	Zero	10^8	10^8	4
220	=	1.8	9	5.1	=	5 µg	Zero	10^8	10^8	4
220	=	1.9	9.5	5.1	=	5 µg	Zero	10^8	10^8	4
220	=	2	10	5.1	=	5 µg	Zero	10^8	10^8	4
220	=	2.1	10.5	5.1	=	5 µg	Zero	10^8	10^8	4
220	=	2.2	11	5.2	=	5 µg	Zero	10^8	10^8	4

220	=	2.3	11.5	5.2	=	5 µg	Zero	10^8	10^8	4
220	=	2.4	12	5.2	=	5 µg	Zero	10^8	10^8	4
220	=	2.5	12.5	5.1	=	5 µg	Zero	10^8	10^8	4
220	=	3	15	5.2	=	5 µg	Zero	10^8	10^8	4
220	=	1.6	8	6	pMTL84151	5 µg	Zero	10^8	10^8	4
220	=	1.7	8.5	6.5	=	5 µg	Zero	10^8	10^8	4
220	=	1.8	9	6.8	=	5 µg	Zero	10^8	10^8	4
220	=	1.9	9.5	6.4	=	5 µg	Zero	10^8	10^8	4
220	=	2.1	10.5	6.3	=	5 µg	Zero	10^8	10^8	4
220	=	2.2	11	5.5	=	5 µg	Zero	10^8	10^8	4
220	=	2.3	11.5	6.7	=	5 µg	Zero	10^8	10^8	4
220	=	2.4	12	6.2	=	5 µg	Zero	10^8	10^8	4
220	=	2.5	12.5	6.2	=	5 µg	Zero	10^8	10^8	4
220	=	2.6	13	6.4	=	5 µg	Zero	10^8	10^8	4
220	=	2.7	13.5	6.6	=	5 µg	Zero	10^8	10^8	4
220	=	2.8	14	6.2	=	5 µg	Zero	10^8	10^8	4
220	=	2.9	14.5	6.6	=	5 µg	Zero	10^8	10^8	4
220	=	3	15	5.9	=	5 µg	Zero	10^8	10^8	4
220	=	2.2	11	2.5	=	4 µg	Zero	10^7	10^7	4
220	=	2.3	11.5	2.4	=	4 µg	Zero	10^7	10^7	4

220	=	2.4	12	2.2	=	4 µg	Zero	10^7	10^7	4
220	=	2.5	12.5	2.2	=	4 µg	Zero	10^7	10^7	4

9.7 Appendix 7- Sequences of CPAP1 and CPAS1 phages

9.7.1 CPAP1 (F42) phages' sequence

>NODE_1_length_18390_cov_1218.51_ID_1
TACGAGATAGGAGTCCGTCTCGTGGGCTCGGAGATGTGTTAAGAGACAGCTACACGATTC
GACATATTCCCCAATTATTACCAGTAGGTAAAGCAGCACTCTATTTTAACAAATTGTTTA
CAACTAAATCAATAAAAACCTTGAAATCATAGGTATAAAATGCTAGAATTATGTTAAGGG
GAAATTATACCAAAATAAAAGAGAGGTGTCAAATTTGAAAGAATATATTGTAAAGCTTTC
ACTAGATGAATTATGTACTATTGATTTATGTTTAGATACTAGAATGGTACAAGCTAAGAG
CATTGATGAACTTGAGAAACTTTATGACCTAGCTAACAAGATAAGGGGAGTGGTGGATAG
TGAGGAATAAACATAATATGGGATTAGAAACTTTTCACGCTGTAGTAGTTGAACCTTTTT
ATTATGAATTAAATTCTAAAAATTATAATATTGGCGATGATATATTTGTAATTGATATCC
TAGGTGATAAGTATTTTTTAGAGAGTGATGTTATAGACATCATACCACGTAAAAACGTTA
GGAAGGTGAGAAAACATGGCAAGAAAAAGATTAACATTAAAAAGTAAGAAAAAGCAAATT
GAGAAAAAGAACTTTGAAAAGCAATTTAACAAAGCTGTAAAAAATGGAGCTTATATCTAT
TTAATGGACAAGGATATTCAAAAGAAAGCTATTAAATTATTAGGATATGATAAGTCTACA
CTTAACATAATAGTTGATATGGCAAGAGATACAGACTATAAAAGAAATGCTGACAGGTTT
GTTAAGAATGAACAGAAAAGAAAAGAAAAAGAAGAAAAAAGAAAAGAGAGAGAGAAAACA
AAAGAACGCAAAAAGATAGTCGAAAATTTAAACAAATTAGATTTAACACATGAGAGTAAA
CAAAAGATTATAAGTGACACACATTTAGGGTTATTAAAGTTTGTTGGGAAAGAAGATGCA
GAGGAAAACATCAGAAAGGAATTAGTTAATAAAGTTACTGATTTATTTTTTGGTGAAGGT
AGTGGTAAGGGAATATTAAAAGGAGCATTTCAAAGGAATTTACCAAAGGACAAAATAGAC
AACTTTGTAAATGAAGTTAATAAATTTATTACGTCATCAAATGATGCTGTATCAAAAGTA
TCTGATTTTTACGAAAATATAGATAAATACTTCGAGTTTATCTACGAAGAAAATATGGTT
GAAAAAGGTTATTACACTCACGGGGTGCATGATGTTGAAATGGGTTATATATTAGAGGAC
TTACTAGCGACATTTAAAACGGGATTGAAACAATAAAATATAAGGAAGTGGAATTAATGA
ATAAACATTTAACATATGTCAACGAACATAGTTTAGAAGTAACTGAATATAGTAAAATAG
ATAGTAGAGATTTAATATTTGACATTGAAACTTGTTACGTTGATGAAAAAATGTTAAATA
AAGATTATCCACAAAAAGACAAGAAAAGAAAAGTTTGGGCGTTTGGATTAGGTGCTACAA
ATACGAATAACGTAATATTCGGTTGTGATTTAGACGAGGCTTTTTATACCTTTCAAATGA
TAGGATTTGAACAAGCTAAAAAAGAAAATATAAAGAAGAATAAAAATAAGAGAGTAATAA
CTTTAAGTGTTGCTGTTCACAACGTTGCTTATGAAATTAGCTATATGCAATACTGGTTAA
CTGATAATGGATTCACTTATGTTAATCCCAGCTTACAGACTAACGAATTAGGAATTAGTA
GAACTATTGAGAACTTTAAACCAATGACTTTTTCTATAGTACAGACTGATAATGTTTATT
ATGGGTTAGAAGTAAATTTACCTAACACTTTAGAACTTACTGACAAAGACGGTCATGTTA
ATACTTTTAGCGTAAAGGTGAAGTTTTGGGATACTTTAAAAATAGTTCCATGTTCTTTAG
ATAAGGCTTATAAATTTTGCAATAACTTAGATGAAATGTTTTATAAATTGAAAGAAGAGT
TTAATTATGATAAGATAAGAGAAGATAATCATGTTTTAACAGATATTGAAAGAAGGTATC
TTTATAATGATATCAGAATACTAAAAGAAGTTATAAATGATTATTTTTACAACTCACTTT
TAAAGTTTGAATATAATGATGAAGAGATTTACATAAGTTCAAATTGTAAAACAAGTTCTT
CAATATCATTTAAATCAGCTTTAGAGATAACATATTTTGAAGATAGAAACAAGAAAAAAG
CTTTTATAAATCAATTTGAGATTGAGGAAAGACAGATGCTTTTAAGCAAAATGGCTAGGG
AAATGGAGAATAAAAGTTATGAAGGTGGGTGGACATGGTACAACCCTAAAATAGTTAATA
AAGTTGTAAAATGTAATGGGTCAAGCTTTGATATAAATTCAAGCTATCCATTTCAAGTTG
AAAGTGCTTTATTACCTTATGGTATGCCAACTGAATACAAAGGATATGTTAAACCAGTTG
ATGGTGAAGAAGTAGCAATTTATAATATAGGCTTTGATTATTTTAAACCTAAGAGTAAAG
AGTATGAATTACAAATGATTAGATTAGGAGCAAAAAACTTTTCTGATACTGATTTACCTT
TACTTTATAGAGATAAATTGAATATGGTTCTTATGAATGGTAATTCTTTTATTCATACTA
ATATAATAGATGGCAGAGTTATTGAAATTTATCGCAACGGGTGTGAAAATTTATGCAATT
ATAATATGAGTATTACAAGTGTAGAACTTGAACATTTAACAAAATATTATGATTTTGGTT
ATTATGAAAATGTTGAGGTTGATGGTTTTTTATTTAAAGAAAAAGATAAGATTAAATTTA
ATGGTTTAAAGTTTGGTACTAGCTTAGTATATAAAGCTGAAAAAGGTATTTTCTCAAACT
TTGTAAATCATTTTTATAATTTAAAATCAGAAATAAAGCATAGAATAGATAAGGGTGAAA
AGTTAGACGCTCAATATTCAGCAATAAAAACTATACTCAATTCTTTTTATGGTAAGTTTG
GGACAAGAACTACACGTACAATAGACTATTTAGTAAAAAAAGATAAAATATTTACATTCG

ATAGAGGGGGTAAAGAGAAAAATTTAGAAAGGGTTAAAAAGTTAAAAAATGAAAAAAGAA
GAAACCAAATTTTAGCAAGTGGACAATATATTGAAACTTATGAAAGTGAACCCTTTTATA
AGCCCTTTGCTAGTTTTGTTACAGCATATGCTAGAGTATACCTTCAATCAATGATAATAG
AAGGTTGTGGAGTTGAAAACTTTTTATACAGCGATACTGACAGCTTTTACTGTTCTATAC
CTAAAGAAAAAGTAATTGAAGGTTTAAAGAAAATGGGTGTAGAAGTTCACCCTAACAATT
TAGGAGCTATGGATAATGAGAAAAACTTTAAAGAGTTTAAAACCATAGGGGCTAAAAAGT
ATATGCTTAGAACTAATGACGGTAAAATATCTTGTAAATGTGCTGGTGTACCAAAAGATG
CACAAAAGATACTTTGTGATGAAGGCTTTGAAGAGTTTAAACTAGGTAAAGAAGTCAAGG
GAAAACTAGCACAAATAAAATGTGATGGTGGTCTTGATTTAGTAGAAGTTCAATATATAA
TAAAAGACTTAAGTTTAAGGAGGTAACATGGGTAAGTTTTTTGAAATAAAAGAGTTTATA
GAAGAATATCCGAACCATTTAATACATTGTGTTATAGGGGCAAGGGGTGTCGGAAAATCA
TACTCAACAAAAAGATTAATAATTGAAGAGTTTTTAGAATATGGCAAACAAGCGCTGATA
TTAAGAAGATACAAAAATCAAGCTGAAAGTATGGCAACAACATACTTTACAGATGTATTA
CAAAAGGAATTTCCAACTGTTGAATATGAGTTAAAGGGTGATATGGGATTTATAAACGGT
GAACCATTTTGTTTATTTACAGGACTAAACGGAAATAGTTTAGCTAAAGGTTCATCATTT
CCTAATGTTTATTATATTATTTATGAGGAAGTTATGCCGGAGCCTGGTGAGAAAACTATA
AAAGATGAATATAAGAAATTAGAATCTATAATTGTAACAGTTGATAGATTTGAGGATAGA
ATAGAAGTAATATGTGTTGGGAATAATACAAGTTATTACAATCCCATTTTTGATACTTTA
AAGTTATATCCCTCAACTAAACCTAATAGAGTTGTCAAAAATGAACTTATGGCAATTAAA
ACACTTGAAACACCCAAAGAGTTTAAAGAGTACGCCTTAAATTCTAAGGTTGGTAAGTTA
ACTGTAATGAGTGGTTCATCACGTTATAATATAGATAATGAAAATATTAGTAATGATAGA
TTTAACGTTTGTAGTAAAAAAGAATTATGGGACACCGATAAGTTAGAACCAGTTTTTAAG
GTTCGTGTTGATGTAGACAAGGTAATTAAAATATGGAAATGTCAAAGTGATACAATGTTT
TATTACTGGGTTGATAATCATAATAAAGGTAAATGTGACGAATACTTTTTAGATTTAGAT
TACCAAACAGACGAAAATAAGCATATATCTTTATGTAATAAAGGGCTTATAAATAGAATC
AATTTTAATAATAAGGTTGGTGCTGTTTTCTTTAATAATGCTGAAACAAAATTTTACTTT
AATCAAATTGGATTATTTTTTAAAAAATAATGCAAAAAAGTGTTGACATAATATATAAAT
GTAATGTATAATTAACTCATAAGGAAAAACAAACCAAAACAACAAAAATATTTAAAATTA
AAGGAGAGATTTTAACATGAAAAAATTATCAATTTTCAATTTAGGGAACAACGTTTCAAG
TGGGGTATATTTTAGATTAAAGGATAACAGAGAGAAAGCTGTTATAACTTTCTTAATCAA
TAACATTGATGAGGTTGAACTAGATTTAGTTCACCCAGTAAAGTTAGCAGATGGTAGAGA
AGTAAATGTTAAGTGTGTGGCTGAATTAGACAATGACTTGAAAGTAATTAAAGATACTTG
CCCTTTATGTGGTGAAGGTTTCAAGATGTCAGAAAAGGTAGCTGTTAATGTTTATAACCA
TACTACTAAGAGTGTACAAATTTGGCAACTAGGTAAGACTTTAATAGAAAAATTAATTTT
CTTAGCTGAAAAAAGACCTGACCTTATTAATCACACTTATGAGGTAACAAGATTAGGGGC
TAAGGGTGACACTAATACATCATATGAATTTGAGTATTTAGGTGCATCAGATAAGAAAGT
ATTTGAAGGATTAGAGTTAATCAAAAAGAACGAAACAGTATATTATGAGGTTGGTGTTAC
AGAGTTAGAAAACTTCTTAATTACTGGAGATGAAAAAATATTTGAAAGAAATAAATAATT
TAAAGGTGGGTTAATTCCCACCTACTAAAATTAATATAGAGGTGAATAAAAATGGAAGGA
AATAGAGAGTTAACAGAATTAGAAATCAGAAAGGAATTAGCTTTAGAAAAAGTTAATGAA
AAGAGATTACAACTTAATAGAGTAGTTTCTTTTATAAATCAAGAAGAAAAAGAGTTTCCA
AAATCTAAAGAACATGAGGCTGTAATGTTTGATACGCTTTTAACTTCTTTAGGAATAGAG
TTTAAAGTAACTGAAACTAAAAAGAGAATAATATTTCAATTAGCATAATGTAAAGTATAC
CCCCTTACTTAGCTATATAACGTAAATGATTAGGAAGTCACACGGTAGAACGTGCCTAGC
ATTTTTCCACTTATGACCGTGGGCGTTGTATAGTGAAGTAAGGGGGTATAATATTTTGTA
AGGAGGTTTTAAAATGAGTAAAGAAATGACTCTTGATGATGCCCTTTTAAGAATTGTTGA
ATTAGAAGAAACTGTAGAAAATCAGAACAATGTAATCAGCACATTAAATTCTGAAAAGGA
AACTAATTCAAAAGAAATTGCGAAACTTCAAAAAAGTAACATGGAATTATTCCTTAAAGT
TACTAGCAAACCAAAGGAAGAAAATAATACTGAAATTAAAGAAAATAATGAGTCAAAAAT
GGACAAGTTATTAAGAGAATGGGAGTGATAAAATGGCTGTAAAGGCAAACAAAGAAATCA
TGGAGGCTTTTTTAGAAGAGGCAACCAAAGACACTAAAAAAGTTTTAAGTCAGGTAGATT
TATCTGACAGTAGAGCTGTTTCAGATGCTTTATTCAATTATCAAGTTGTGAAAAATGAGT
TTATAAGTTCCCTTATAAATAAAATATTTATGACTCAAATACATTCAAAAGTTTGGAAAA
ATCCTTTAAAAATATTTCATGGTGGAATGGAAGAATACGGTTATACTATAGAAGACATGT
ATGTTGAGGCATCACAAAAAGTTGGGTTTAAAGAACATTTTAATGGTGGTGATGATGTAA
AGGATATAGTTGGTTCTTTAGTTCCTAAAGTTTACACTAATTACCTTTCTAGAAATTTTG

CCGATAAATACAAAATAACTATTTCAGATGTTCAATTAAAAACAGCTTTCAGAAATGAAT
ATGGTGTAGCAAACTTAGTTAATAAATTATTAGAGGCTAATTTAAGGGGTGCTTATCGTG
ATGAATATAAATATATGAAAAATATGTTTTTCAAATATTGTAGTGGACTAAGCAATCAAG
ATGTTGGGTTAAGTGGTTCACCTACACAAACACAATATATTCAAGACTCTCAAGTTATTA
AATTAAATGATGATGCAAATCTTGTAAAAAATCTTTGTAAATCATTAAAAGCTATGAATG
ACAGGCTACAATTTGAAAGTGATAAATACAATAGCGCTAAAGTAATGCAATTTTCTTCAT
TAAATGATTGTGTATTTATAACAACACCTGAAATGAAGGCTGAAATAGACGTTGATTATT
TAGCAGATGTTTTCAACCTTTCAAAATCAGAGGTACAGCAAAGAATTATACTAATGGACG
AACTACCAACAACTATTAAGAGTGGTGATACTGGGACTAATGCTCATAGCGGTGAAACTT
GTATTGGAATGTTAGTTGATAAAGACCTTCTAAGATTTAAAGATTTTGTATTTGAAACAA
GATATTTTAATAATCCAAACAACTTATCTACTAACTACTTTTTACACAAACAAGGTTTAT
GTGGTATAGTTCCATTTTTAAATGCCATAATATACACTAAATCAACAGAATAGGAAGTGT
GATAAATGGAAAAGTTAACTAATAAGGAAATTTACGATAAATTAATTTCAACCATGCCCA
AGGATTTGGGCAACTCTTTTCCTTCTTATGAAGAGGCACTAAGGTTGAATTTAATAAATA
TTGATATGAACAGTTTTATTGAAACTATATGCAATAAAATAGGAAAAATGTTATATATAG
AACAACATTTTTCAAACCCTTTCAATGAGCTTTTTTATGGCGAAATGCCATTTGGTTATT
CAATCGAGGAGTTGTTCTTAGAGGACTCAACTGTTCAATCATTACTAGAAGGTGATGAAA
AAGAAGTTTTCAACTCTTCTTTACCAAGATTAAACGGGTTAGTTGCAAATAGTAGATTTG
ACAAAAAAGTTAAAATGAGTCTTAGTGTTGAAAGATTAAAGTCAGCATTTTTAAAAGAGT
TTGGACTATATGATTTAATATCTAAAATGTTATCTAATTTAAAAACTTCTATGAGTAAAG
ATAAATACAAAGAAATAGAAAAAGCTTTAAAGGAATTTGCTAAGGGATTAGATTCTAACG
GAGAAAACCTTAATTTTAAACAAGCTCCACATAAAGAAGTTGCAAGTTCAAACTTAATTG
AGAGTATTGTAAAAATTGTAGAAGAATTCACTTTGCCAAGTGATAAATATAATCCTAGCA
AATTTAATACACAATGCTTAAAAAGTGACATAATAATATTTGTGAAACCTGAAATTTACG
CAAAATTAGTAAGTACAGATAGCATACTTTTAAGAGGTGAATATAATATTAAGTCAATTA
GTGATTTACCAAATAACATTACTAATTTGGTATCAAATGAAACGATCAAGCCTATAGCTT
TAATTATGGATAAAAGATTAATGTCTATAATGTTGAATTACAATGGATTAGATAGAATTG
ACGACCCTAATAGTTTAACAACAAATTATTATTACTATGAAAATTATAGTCTACCAGTAA
ATACTTTTTCAAATTTTGCAATTCTAACAAGTGAATAAAAACGTATATTTTTATTCTTTA
CCTTATATTGACCCCTCATATCAGAACCTTTTTAACTTTAAAAGTATAAGTAATCAAAAT
AATTATTTTTCTTCTAAAGTAGTGTGGGTGGGTCAATATAACATAAAGGTAGAGCCTTCA
CGTGTAAGCTTAGTGGTAGACAAGCCTTACGGTTGGTTTATGGAGAATAATGTTAATTAT
ATTTCAGCTTATGATACTAACATGAAAAATATTTATTATTTCATAGACGACTTTGTTTAT
AAAACAGAAAACTCAACACTTTTAGTAATATCAGTTGATGTATTCCAAACCTATCAATTT
GACTTTGAAATTTTAGAAAGTTTTGTTGATAGATGTCACGTTAATAGATGGGACGGTGAT
AACCCAACGAATGAATACGAAACAGAAGATATTTCTTATGGTGAAAATATCATGCTAGAA
TATGAAAAAATAGCTGATATGGGGCGTGGTGTTGTTGTCACTTCTACAGTACCTCTCGGA
AAAGTTGAAACAAACGTTGGTGGTGGTTCTGACGGGACTGGTTCAGCAAGTGGTGATATA
GCAAATGGTATTATATCAGCAAATGGACTTTTATTTGTAAAACAAGAAGAGGGTTTTGCA
GAATATGGGGCATATTTTAATGGTGAATCTTTCAAAACTGGAGGTTATGGTGTAACAGAA
AATTTCCAAACTAAATATTATAGACAACTTGAGCCTTTTCCAGTATCAGAAGAAAAAGCT
AGTCAAGTCACCTATGATTTATTAAATAATGAGTTTGGAATACCAGTTAAAAACGCAATG
TTAAAAGCTAATATAAATTTAAGTGATATTCCTATATATCAATTTGATGTTTGGGTATCA
ATCGCATTTAACTATGGAATGGGTGGTTTAAGTGGATTAAATGCGTGGCAAATGTTTTTA
GCTAATCCAAAGGACACTAAAAATATAGCGGCGGCAATCAAAAGCTTATCCGCCAACCCA
AATAGAAGACAAAGAGAGGGAGCATTATTTGAAAGTGGTGTTTATCCCCAAAGACAAATT
TTAAAGTATGGCCAAAATGGTCAAATAGTTGGATATACAGATGGTAAAGGTTGGTTGCCT
AGTGGTAAAAAAGACGGTAAATATGTAGATAATGATGCTGGGACAAACTGGTTAATACCA
ACAACTGGACAAATAAGTGCATATTACCCAACTTATCCAAGTGGCAACCCTCATAATGGT
GTTGATATTGCAACCCCAACTGGTACACCAGTATATGCAAGTAAAGACGGAACTGTCATT
AAAAGAAGAGAATTAACTACTAGCTATGGGAAATTTTTAATTATACAACATGGTGACTCC
CAAGTTGTTTACGCTCATAATTCAGAGTTAAAAGTGAATGAAGGTGACACAGTAAAACAA
GGACAATTAATTGCATTAAGTGGGAATACTGGAAACTCAAGTGGTGACCATTTACATTTT
GAGATTAGAAACGAAAAGGGAACAGTTGTTGCAAATGGTGTTAAAACTGTTAACCCTATG
CCAAATTATAAGGTGGGTGACAAAGTATGACGATAACTAATAAAGATTATGATACGTGGG
AATCAAAAATGTATGGGTTACCATATGCTGTATATAGTTATTATATTGACTCTACCGTGG

CAGGTGAAAATACAGCTGTCATAGGTGCAAGTGGTGACAATGTTTTTAGCGTTGTTTACA
CACCATTCCTTGATATTGCAGATTTAGAATTAGAAGAAATTCCATATGATAATAAAAGAT
TTGGTAATATTTCTGAAATTAGACCCGAGATTAAAGCAAACCCCCACGTTTTCAGAATCA
AAAAATTATTAAAAGGTTCAAAATTTGTCGGTGAGTTTGAAACTTACAAAGTAAAAAAGA
GTGTTGGTGGTAAAAGAAATTGGAGAAATGAAAGTAAATTATATAATTATCCGTACACTT
ATTTAATGCTAAGTGATGGTATAAATGACCCTATGATTTTAAAGCCTCAATTTTGTTCAA
TTCCTACCTGTTCTGTTGGTATAAAATTATCTGTTTCAGACCGTTGTTCTTATGGTATAT
TCGTACAAACTTATAAGGGTGATACAGACGGAATGACGGAAGCTTTGGTATCAAATGATG
CACTTGAATTGCCTTGCACAAGTTCAGCATATGCAAATTGGGTTGCAACTTCAAAAAATC
AGACAGCACAATCAATACAAAATACCGTAAACCAAACGATTTTAAATGATAAAATAGCGA
AAAATAACATGAATTTAGGTATCGCAAATAGTGTTGTTGGGGGTGTTGCTAGTGCGTTTA
CTGGTAATGTTGGGGGTGTAATAGATAGTGTCTTTGGTGGTGTTGGCTCATATATGGATA
AAAAACATACAAATATGCAGAGTCAATTAACTAGGGAATCGGCTATCAGTTCAGCTTTAG
CTACAGCAAACGACATGAGGTCAACACCTAATACTTTACTTAGTCAAGGGTCGAATATAA
TTTATGGTTTGCGTAATGGTGGGCAAGAGTTAAGACTTTACCGTTATGGATTAACAGAAA
GATACTATGAAAAGATTGGTGATTATTTTGCACAGTTTGGTTACAAGCAAAATAAAATGA
TGGAAATTAATATTAATTCAAGATATTATTATAACTATATAAAAACAATAGGAATTAATA
TAAAAACTAATAAAATTCCTAATAATTACTTAAATATCCTCAAAGGTATTTTTGACAATG
GCACTACAATATGGCATATTGATAATGACGGTGTAGAGATTGGTAATTATTCAATGGATA
ACAGGGAGGTATAAAATGAAAATAGGTATTAGAGATGGACATAGTCCAAATTGTAAGGGG
GCTATTGGTTTACGTGATGAGCAATCATGTATGAGAGTTTTATGTAAAGAAGTCATAGAA
ATATTAGAAAAACATGGTCATGAAGTGGTTTATTGTGGTAGTAATGCAAGTACACAAAAT
GGTGAACTTTCAGAAGGTGTGAGAAAAGCTAATAATTCAAATGTTGATATATTTATTTCA
TTACACATGAATAGCTTTAACGGACAAGCTCAAGGAACAGAGGCACTTGTTACAGTTGGA
GCAAGAAATTCTATAAAAGAAATTGCATCAAGGTTATGCAAAAACTTTGCTAGTTTAGGT
TTAGTAAATAGAGGTGTAAAAGAAGTTAATTTATATGAAATGAAGAACGTAAAAGCTCCT
AATATAATATTTGAAACTATGTTTTGTGATAACCCTCATGACATAAATGAAGTGTGGTCA
CCTACACCATATGAAAAAATGGCTTTACTAATTGCAAATGCTATTGACCCAACTATCAAA
GAAAATGAACTTTATAGAGTTGTTGTTCAATATTTTAACAGCAAAGAAGATGCTGAAAAC
TGTCAACAAGAAATTGCTAAAAGATGGTATTGTTTTGTGGAGGAGTGTAATTAATGCAAG
AAATAATAAATATAATAAATAGTGTTGGTTTTCCAATTGTAGCATGTATGATAATGTTTA
ATCAAAATAGCAAACTATCAAATGCAATTTCAGAACTTAACATAACATTAACAAAGATAC
AATCAGATATTGACAGCTTAAAAAATAATCAAAAACAAGGGGAATAAATTTCCCCTTTTA
AAAAAGGTGGTGATTATATGGGAAAAAGAAAATCTATAATATCTCAAAACCCTAACATTT
TACTAAATGAAAATACAATATGTGATAACCAAACAAGGTTATTTTTACATTATAAACTTA
TGGCTTGTAATAGGTTTAAGTGGGAGAACTTACCGACTGGATTAGAAAGTAGACATATTG
AGGACTTTTTATTTGAAAATGGGCAATGTTTTTTCTTTAAAGATGATAAGTTGGGTTTAA
TGTGTTTACCATGTCATGGTTTGGGTGATTTAAATATCTATGGTGATAATATCAAATTAA
GCATAGTTTCAAGAAATGGCAAATATCAGAACATCTTAGAAGATGGGAAAGATGGTATAA
AAATAAGAGCGAATGACTTATGTTTACCAACTTCTCATTTTATTTCCCATTATGCTCAAA
AAATGGATGATATAGAAACAGTAATAAAAAGAAATTTAAAGCAACAAATGAAACCTTTCT
TTGTTACAGCTACAAATAATAATTTATTGTCAGTAAAAAATATTGTGAATGATGTTGACA
ATGGGAAAGAGGTTGTTATATTAGATAAGGATTTAGGAGAACAGGGGTTTGACGGTTTCA
AGATGCTACAGACTGGTGTTATTTATTTAGTTGATAAATTAGAAGATGAAAGAAAAGCAG
TCGAGAGTGAACTACTTTCATTTTTGGGTTTAGATAATGCTAATACAGAAAAAAGAGAGC
GACTTCTAGTCGATGAAGTTAACGCTAATAATGAATACATTGAAACTAACTTAGATATGG
AATATAAAACTAGGTTATTAGCTTGTAAAATGATAAATGAAAAATTCGGAACTAACATAA
AAGTAACTAAGGTTGTTGATAGCTTTGGAGGTGATGAAGATGGCGAAGTACACACTAGAA
TTAAGGACATTAGAGAATAATTTATTTGACTTTGATTATCCTTATTATGAAGAAGAGGCA
AAATGTTACTTTGAGGAAAAATTTTTAAATCATTATTACTTTCATGAAATAGGATTTGAA
ACTATAGCAAGGTTTAAACAACAATTAAGAGCATACTTTTTGAGAGTTATGCCATATTAT
CAACAACTTTATGAAATAGAATTAAGATGTAAAGATATTGACTTTATGTTAAATAAAGAT
TTGAAAGAAACTTTTATTCGAGAACTAGAAGAGAATGAAATAAATAATTTATTAACTTCA
CAAAATGAAACTGGTAACACTTCTACAAATATAAATAATAATGAAAGCAATAACTCAAAT
AATAATACAAAAGAAAGTTCGCTTGCCGATGGTGTATCGAGTTCAAAAATTGCTGACGGA
TATTTAACAAATTCAAGTCAATCTACAGATACTTATGATGGTAATAGTAGTACAATAAGT

AAAACAGATAATAAGAGTAATTTAAATATAAATAATACTGGGGATAAAAACAACAAACAA
AAAGAAAAAACAGAATTAATTTCCCAGGGTAATATTGGGGTAACTTCATCAGCTGAACTA
AAAGAAAAATGGCAAAGTTGCTTAAATAACATAGATGAACAAATTATACTAGGCGCTAGA
AGTTTATTTATGTATGTTTATTAGGGGTGAAATAATGATAATAATTGATAGAAATAAAAT
AAAAGTTAATCAATTTGATACTTGTATTTTAGATTTAAATGTGTGTGGGTATAAATTACA
AGAAGGTGATAAAATAGAATTTGTATTTAATTCTATAAAAGAAGAAAAAGAATTTAATGA
TACTGATTTAATATTCCCAACTGATGAAAAAGGTTCATTTAATTATTCTGTAACAATATT
ACAAAAAGGAATTATTAGAACTGAAATTATTAACAGTATATGTGAGGTGATATAGTTGAG
TAAAATAACTGGTAAAGTTAATTATAAAGAAGTGCTAAGGGGTGATACTGGGTTTACATT
TAAACCCCATGTTACGACAGACGGTATTTTATATTGGAGTAATAATGGGGATTTACCTAA
CCCATTACCAGTTAATATAAAAGGAAAAGACGGTGACATTATTCAAAATGAATTAATTGA
AAGTATAACAGATATAAATTTACATGAAATAGTTGATAATAAAATAATAAAATTATCAAA
TATTAACTATTATGTTAACGGTAATGTTGAATTAAATAATTGTGAGAAGTTAGTTGGTAT
TTCTCTAAGCACGATAAAAGGTAAAGCAGTCATAACATTCAATAAAGAAAATGTTATAAT
AAAAAACATAAAATTTAAAAATACTACAATGTTATATAAATATGGTGGTAATATTATATT
TGATAATTGTGAATTTGAAGATATTGAAAAAAGTTGTTTATTCATAAGGGGTGGAAACGC
TGAATTTATAAATTGTAAATGTAAGAACATTGGAACAAATTTAAATGATGATTACACATA
TTTAGGTGGTTTAGTTTATGCTGAAAATTGTGAGAGTTTAAATATATTTAATTGCATATG
TAAAAATATACATGGTCAAGCCTGTGTAAGATTTACAAATCTTAATAAAATAAATGTTGA
AAACTCTATATTTAATGAAACTAAATATAGAGCAATAGGCGGTTCACTTGAATATAATGA
AACAATTGGTTTAATAAAAAATAATTCTTTTTTAAATATAGGTAAATTAAACGACCTTGA
TGGTGTTGGCTGTAATGCAATTTATACTTATAGGGGTTCAAGTAAAATAGATATACTAAA
TAATGTGTTTGTAAATGTTATTGAAAATGCAATAGAAGGTAATTATTTAAACATAAAAAA
TAATTTATTTGATACTATAGGTTCAGAAAATTACACAACACCGTCAAAGGAGTGCATATG
GGGTGATATAGAACACATTGAAATGAATCACTTTAAAAATATTAATTTATATCCTATAAA
TAAAAGCTTAGGGGGTTTTACAATTAAAACCATAAACAATAATATATTTGAAAAAGGAGA
TATATCAAAAACAAATGAAGCAATTAGATGCCTAAAGTTTAATGCAAACACAGTAATTTC
AAACAATATATTAATAGGTTATGATTACTTATTAAAAATTGAAAATAATGTTTTGAATAA
TAGTAAAGTTTTTATAAAAAATGATTATAATAAGACATTAAACCCTAAAGGTCATTATAA
TAATGTTTATATACTTAAAAACAACTTAATAGAGTATAAAAATGACTATGAAAGTTTTAC
TAAAATAAACGATATAAAGATAAATAATGCTAATTATGTCAGTTTCAATGATGGCATATT
AAAACTAAGTCACAATAACACATATGACAGTTTTATATATAAAGAATTTAATGTAAAAAA
TGATAATATAATAATGGTTAATTGTAACTTTATAGGTGAAAACAATGGTGGAATAAGAAT
TGTTCCATACAATAATGATAAATGGGATTATGTAAATTGTAGAGAATACTATAAAGAATC
TAATAATAATAAAGTTGATTTACATTTTACAACAGTAATGAATGGTAAATTTAGAATTGA
AATAATAGACCAAAATTATGAAAAAGGTAAAGAGTATGAAATATCAAATATTGAAATAAC
TTTAAAAGAAAATGGAGGTTTTATAAATGAATAATATCAATAAATTAAATCCTATCGACC
TTAATTGTAGCATCTTTACAGTTTATGATTATACAGGGTTATCTATGCAAGAAATTTTAT
GTCAATTCTTTAGTAAAATAAATGAATTGATTGACTCACAAAATCAAGTTATAGATTTAA
CAAAATGGTTAGTTGGTCAAGGATTAAAAGAAGAAGTAGCCAAACAACTTAATCAATGGT
TAATTGATGGTACTTTAGCTAACATAATAAATGAAACAGTATTCAGAGATTTAAATAATA
AAATTAACTCTAATATAGAAAATATTAAAAACAATAAAAAAGAAATAGATAAACTAGTAA
CAGTTTGGAATAACATTAAATGTTTTAATGTTGTTGATTTTGGAGCAATAGGTGATGGTG
AAACCAACAACAATAAAGCCTTTGAAAATGCTATAAATTCATGTGTTGAAAATAGTGTAC
GTGGTTTATTAATTCCAAGTGGAAAATTTTATATTACAGAGGGTATTAACACAAAGGGTG
TAAAAATCATAGGAATGGGTGACCCCTATATTCCATTTTTAGAATGGGAATATACAAGAC
CAAACTCAAAATTAGATGAATATAAAAAATATTTAAATAAATGTCGTGGTAGTATAATAA
CATCAGATAAAAATATTAATATATTTACTAATGGTTTATATGCTGAAAATATCGGTATTT
TTGGTAATAGAAGAGCATTATCTCAAAATGGTATAGGTCAAATGGACGGTGGGTTTGGTA
ACTGGATTGAAATAGAAAAAGTTAAAATCCATGGATGTGGTAATAATGGTATTAACGCTG
AATATGGTTTAATAACACCTATCATAAACCAATGTTGGATGTATCAAAATGGTAATAATG
GAATAAGAATTGGTAAAAATAAAGGAACTTACACAGGAGAAACAAACGCCTTAATAATAG
AAAAATGCTTTATTAATAGAAATGAAAGCCACGGCATTTACTTAGATGTTAAAGGTAGAG
CATATTCTATTAAAAATAATGACTTAGAGCAAAATGGTGAAATGAGTGACCCCTTGAGAA
ATAATAAAGGTACTGATTATAATGATATAGTTTATGGTTGTTATATGAAGGTTGAAGGTG
AGGGTGGATTTACTAGTGGTTCAATAGATTTTAGTAATAACTATTCAGAGGAAACCTTAG

GTTTATTATACTTAGAAAGTCCTGATAATAAAATATGTCAAGGTGTAAAATTTGAATCTA
ATATGTGGCGCCCTTATAATCAAGAATTATATTCAAATGGTTTACTACTAAAAGGTTGGA
TTGAAGGCGTTAAAATTGGTAATAATAATATGTACGGTAGACATAAAGTAAGAGTTATCA
ATGCAAATACTTATGGTATAGAAACTGACACAGATATAACAAACCCAGTATATAAAAATA
AAAATATATCTATCGAAAAACATTATGACTATAATGGTACAATGGTTGACTTTAGAAGTA
CTGGGAGAGTTGAAAAATATTCTATTGAAAATATGATACAATCAGCATCTTATGATGGTA
GCACATATACCAACGTATATTTAAATAAACCTAGCGTTCCGTATGAATTACAAGAGGACG
GACAAGGTAATAAATTAATTGGCACAACATTTTTAACATCTGACGGTACAAGTATCGGTT
TAGTTGTCGACTTCTCATGGACTCATGGATTATTTAAAATAAGAAAAGACAGAACATCAT
TAATCAAAACTGGTAACGCTAAGTTTATGGAGAATGGTGGATTTACAACAATAAATGGTG
GTAATACACCAGTAAAAATCTATATAGACCATGACAACGAAATAGTAATTTTATAAAAGA
AAAAGAGGGTTTATTTACCCTCTTTTATTCTTTCTTTTATATCCTCATAAAAGTCGATTT
GTTTTTCTAGTTGATTTATGTTGTCGTAAGTGTCGCTATAGTTTTCAAAAAACATTTTCT
CGTATGCTATTCTTGTATTAAGTTCTTGTATTCTTGTATTTAATAAATCATTTAATACAG
TTTCTAAACCTTCCTTATCTTTACACTCATAATTTATGAAGTTATCAATAAATTTTTGTG
TGTAGCGTGACCTTCTTAGTAATGTATAAATCATATCTAATTTGATTTTTCTGTCATAGT
CTTCTAATAAAACACATCTTTTAGAATCTATTTTTATTAAATTTAATAATGTTTTGAATT
TCATTTTTTAATCACCTCTTAATATAATATACAGCTTAAAAACATTACAAATAATAAGCT
TGATATAATGAAAATTGAACTTGTTAAAAATGTATTGTTATATAAAAACATAAATGATAT
ACCAACTAATATTCCCATAATAATGACTAATATTAATTGAATTAATTTTTTAATCAATGT
TATCACCTCTTTACATAATATAAATAACCTAGAACGAATAAATTAAATAGTAACCAGTAA
CATATTATTTTTAACACCCTAAAACCTCAATAATAAAATATAAATTATCTAAAACAATAA
ATAATAAAATAAGAATTAATAAATTAATAAATCCATTTACATAATCCATAATATCACCTC
TCTTAATTGATACTTTAATTATAACAAATTATGTTAAGCTAATCAATGCTTTTTATTGAT
TTAGTTGTAAACAATTTGTTAACTAGAATGACAATAAAAAAGAGGCATAAAGCCTCTTGA
ATTTAGTAAATGTATAATAAAGTTTCTATTTGTTCTTTTCTTGAATGTGCTATTTTGGAA
TGTTTAAGTTCATCAGTTAAATATTTTAACATTCTTTCAATTCTTTGTGATAAATTATAA
AACTCTTGATTAGATAAATTAGACCCTGTATAATAAAGTTCCTCAATTAATAAATTACAT
CTCTTAATAACTCTGTCTATTTTACACATTTTAACCACTCCTTGTTTTGATTTGTTTTGT
TTGTTCCTTATGTCTATATTATACCAAATTATGTTAAGCTATTCAATGCTTTTTATTGAT
TTAGTTGTAAACAATTTGTTAACAGATAGTGCTGTTTTATCTACTGGTAATAACTGGGGA
ATATGTCGAATCGTGTAGAGGGTCGAACAG

9.7.2 CPAS1 (F43) phages' sequence

>NODE_1_length_152340_cov_119.513_ID_1
TCTTAACATCAGTTTTGTATGCTCCAGCCATATTACCTACCATTTGACTATTAGCCCCTT
GTATAGCGTTAGCACCATCTACTGCTGCTTTACTTTTGGTTGTAACTAAATTGGTGTGGT
TAGTATTATCAGTTTTATAGTGTTCGGCTTTATTATTGGTTTCTTGTGAAGAAGCCTCAG
TTCTAGCCTCTGCTCCATTTTGACCATTAGTCACCTTTGTACTTTCGCTATTTGCATGAG
CGTTAGTATCATTGGCATAGTTATTCATTTTAGCTTGAGTTTCTTGATTAGATAGACCAA
CACTATGATTCATAGCTTGTGTTTCAATAGCTTGTTTACTTGATAAATCATTTATCCCTT
GTTGCAATAAATTTTGCGAATTTTGTATTGCTTGTCTATATGTAGCATCACTTTCAGTTC
TTAATGTTTTTTCTAAAGCAATACTAACCACACCAATTTAGTTACAACCAAAAGTAAAGC
AGCAGTAGATGGTGCTAACGCTATACAAGGGGCTAATAGTCAAATGGTAGGTAATATGGC
TGGAGCATACAAAACTGATGTTAAGAATTTTGATGATGCTTGTAAAACTAAGGCTTTATC
AGCTTTTGAACTTATGCAAAAACTTAATGATATGATGGCTCAAGTAGGGTTAGGATTAAA
TTCAGCAGGATTGCCCAATCAATTCGACCCTAAACAACCTAGCGTACCTTTAAAACCAGG
ATACCAATGGAAGAAAGATGGTGTCTTCTGGTCTGCTTGGAGTGAACATAGTATTGATCC
GATATTACCTTCAAACAATCCAGTACAAGTTGACTATTCACCAGAAGCTAATGGACATGG
TGGCAAAAAAGGAAATAAGGGTAGTAAAGGTAAAGGTTCTAAAAATGGTGAACATGTAGA
TATTAAAGATATCGAAGACAAAATCGATGCTTACAAATCTTTACAGGATGCTATAGATGA
TGTTAACAATGAATTAGAAATAACAAAAACTCTTGAAGAAAATGCTCAAGGTGTTAATAA
GCTTCAATACATGAATAAAGAAATAAATCTTTATAAACAAAAGAAAGACGCTATAAATAA
TTTAATAGCTGCCAAACAAAACGAAGGTAGATATTTAGAAGGTGTCTTAAAAAGCAATGG

CTTTAACGCTTCTAATGGTAATATATCTAACTACTATGATAGATTAAAACAAATAGAAGA
TGAAGTTAATGCTATGGATAATTCCAATAAAGCTAAAGAACAAGCAATTAAGAATTTCAA
AGATTTAAAAGAAAAAGCTGATAAATATTTTGAAATAACTTCTAAGGAATTACCTAAACT
TAATAATGAATGGTATGCACTAGCTAAAACCATTAAAGAAGTTTCTGAAAAACAAGTTAA
GTTAATGGGTGATACTGAAAGAGAAATGACTAACGTTGTTAAAAATCAAGTAGAAGAACG
TAAAAAAGCTTTAGAGGATGAAACTTCTAAAATAAAAGAAGAATTAAAGAAACAAAGAGA
TGAGTATAACAAGAAATATGACGATGAAAACTTTGAGAAAAACCTTAAAGAAAAGCAAGA
TAAGTTAAATAAACTTAATGCTCAAATAGATTCTGTTAGAAGAGATATTTCATCTGAAGG
ACAATCTAAACTTAAAGATTTGTTAAAACAAAAAGATGACCTTGAAAAAGAATTAAATAA
CTTTATTAGAGATAGACAAAAAGATCAAGGCAATAAAGCTTTTGATGAACAAATGGATAA
GTTAGATAAACATAAAGATGACAAAATAAAAGAAATGGAAAAGACTTATACTGATGAAAA
AATAGCAGAATTAGCAAAAGGTATGATTCAAAAAGGATTTGTTGAAATAGAAGGTCATGT
TATAAAACTTAGAGATGCTTTAAATGATTATTATAAAAAGAATGGCGAAGTATTTGCAGA
TAGTAGTCTTAAAATGCAAGAATACATAGATAATCTTGAATTAACTAAGAAACTATATGG
TGAATTAACTTCTATTAATAACAATCTAGGAGTATCAAGTTCTAATATTAGATATAACAG
TGGTAACAATGTTGTACAAGCTATTCCAAAGGTTGCTTCATTTATGGCAATACCTCAAGG
TAGCAAAAAGGCTAATATCAACATCAACACACATTTAAATGTTGGTTCAGTAAATAACGG
AACAACAACTGATGATATCAAATCTATGTTAGATGAAAGAGATAGAAAAATTATTAATGA
GATTAATGAACAACTTAATAGTTATTAATACGTTTTAGTGGTGTACGATAACACCACTTT
AATATAAATAATATTGAATTAGTATCTGTATTAGTTGAATAATGTTTATATTAAAGTTTA
GTAATGTTATAATTAGAGTATAACTATTGAAAGGAGTAATTTTAAATGGTGTATAGAACA
TTACTAAATCCACAAATAGATTTTGTGTTTAAAAAGATATTTGGTACTGAAAAGAATAAA
CCTATATTAATTAACTTTTTAAACGCAGTAATAAAACCAACAACTCCAATAAAAGATGTT
GAAATTAAAAATAACGATATAGATAAAGACTTTGTAGAAGATAAATTTAGTAGATTAGAT
GTAAAGGCTACAACTAGCAATAAGGAACATATAAATGTTGAAATCCAAGTAAAGAATGAA
TACAACATGATACAAAGGACATTGTATTATTGGAGTAAAATGTATTCAGAACAAATACAA
AATAGAGATAACTATAGTAAGTTAGAAAGAACTGTTTGTATAAACATATTGAATTTTAAA
TACTTAAAAAATGATAAATATCATAATGCATATAGACTAAAAGAAATAACTTCAAATGAA
GAGCTAACAGATCTTCAAGAAATACATTTCATTGAGTTACCTAAATTTAATGAAATAGGT
AATAAAGAATATGTTGAAAATGTTGAAAAAATGGATGCTCTAGAAAAATGGTTAGAGTTC
TTAGTTGAACCTGAAAGTAATACTGTAAGACAACTAGAATTAAGCAATGAAGAAATTAGA
TTAGCAAAATCTGAATTATATAAGCTTAGTATGAATAGTGAAGAGAGAGAAAAATATAAT
ATGAGAGAAAAAGCTATATATGATAGAATATCAGCTTTAGAAGGTGCAAGAGAAGAAGGT
AAATTAGAAGTAGCTAAAAAACTATTAATAGCTAATGTAGATATGGATATCATAGTTTCT
TCAACTGGATTAAGTGAAGATGAATTAATTAAAATAAAAAATGGATTATAGAATATGTTA
AAGAAATATTTATAGAACTTAGATTAAATCTAGGTTCTTTTTTTATTGTAAAAAATTGAT
TAAAATTGTGTTTTTAACTAAAAATTGATGTTATAAAATGCCGAAAAACCTAGTGTTTTA
CAACATGAGTTTTTATAAAATTACTCAAAAATATATGAAAGGTGGTGGAATAATGCAATT
TAGAAAATTCTATTTTGAATTCGATGGAAAGAAAAGTAAAGATAGAAATTTAAAAATGGT
TGTAATTGATAATAAGGGTGAAGAGGATAAGTTTGGTGTGGAACAAGAGATAATTGAAGA
AGATAATGGCACTGATACACCACTTTTTCTAGGTATTAAAAGAAAACCTCAATCTCTTAA
AATATCTATTATGAAGATGAATAAATATAGTAGACCTTTACCATATACAGATAAGGAATT
AGAAGAAATATGTAGGTGGTTATTTAAAAAAGAATATAAACCCCTAAAAGTTTATGACCA
AACAGATTTAACTTATTATGTAATATTTACTAAGGGAACAGATTTCTTTAATTGTGCAAA
AGAAGGTTATATCAATCTTGAAATGAGATTAAACGCACCTTACGGTTACAGTAATTTATT
TAATAATGATTATTGGATTAAAGGCGAAAAAATAATAGATATTTATAATGGAAGTGATAT
AGATAATTTTATATATCCTGACATTGAGTTTGAATTAAGAGATGATAGTACAGATTTAAC
TATAAAAAACCTTTCATTGGGTGAAACTATGGAATTTAACAATCTTGTTAGCAATGACCA
TATAATGATATACAATGAAGGATTAAAAGATATGGTTAGCTTAAAAGATAAAAGTAGAAA
TATATTTCAACATAGTAATAAGAAGTTTATTAAATTGCAATATGGATTGAACAGAATACA
GGTCAAAGGAAATTGTAGGATAAGATTTTTATATCAATATCCAATAGGATTTAAATAAGG
AGGTGATATTTAATGTTTAGAGGTGTAAGCATAAATCCAAAAGATGATGAGCCTAAAGCT
GTACTATATAAGAATAGAAGAGAAGAATTATGTGAGATTCCAAATGAATATATAGAATCT
ATAGAATATAAACTTAGAGATTGTTTTACTATGACTTTAAGTGTTCCTTCTAAAGTTCAA
AGAAGAGGAGAGACAATTGATAATCCTTTATTTGATAAGTTTAAACCTAAAAGACAAATT
GTATATAATGGCGATAGATACGAGATATGTGGAGACTTCAAAGTGGAATCCAATAAGCAT

ATTAAGAAAAAGAAGTTTACAGCTAAATCTTTTGAAATAAATCTAAACAAGAAAGATGTT
GCCGTACAAGAAGGAACTTTTCAATTATATAAGTCTAAAGATGATAAAATTGATGTTGAA
GAGGGTGTTTTAAATTGGCTTGAAAATGAAACAAGTTGGAAAGTTGGCTATATTGATCCT
AACGCAAAATCAACTATTGGTTTATTTAATGAAACTGTAGATATAGATTTATATAATAAT
TTAAATGTTAAAGATGTTCAAGTAGATAAAGTTTTATTTGATAAAGATATTTATATTAAT
ATCCCTAATCAAGCTCTTAATTTTAGTATTAAATATAATAATATTGTTAGTATGGATTCT
AAAAGTAATGTAACTAAAACAGAAAACTATGAGCATAAGTTTGAAAATTTTGCAGAAGGA
ATTAGACATATTAAAGCTTCATATAGCATTGATAATAGTTATAATACTGTAATTAGATAT
GAATTCACATTAATTAATGGATTCGTAAAAAAAGAAGTTGAGAAATTTACATATTTACAA
GGATTAGATGTCAATTTCAAAGATATTGCTTTAACGTATGAGACAGGGAATAAAGTAGAA
AAAACTAAAACTAAATATAGGAGTTTTGAAAAAGGAATTCATCAATGGCTACCTTTTTTA
AGAGACATGGTAGAGAAAGCATATGATTGCATATTCCAATTCGACACTATTAATAAGCTT
GTGAATGTATATGATAGGCAAACTATGGGAAGAGATAATGGATTTTATCTATATTATGAC
CAATATCTAATGAAAATTGATAAAGATTTAAAATCTGATGATATAGTAACTAGATTAGTT
ATTGAAGGTAAAGATGGATTAAGTATAAATGGTGTAAATCCTTTGGGGACTAATTATATA
GAAGATTTTACATATCTTTTAAAACAAGGAAACATAAGCGATGAATTACAAATATCCCTA
CAAAGATATAATAATTATATAAATAAAGTTTTTGATGAGTGGGATTCATATAAAAAGAAA
AAAGACGAAAAAAACAAACAAAGTATTTATATAGAATCAAAACTACAATTAGTTAGGGAT
CAATTAGAAGTCAAAAAATCTATAAAAATAGCTTATATAAAAGCAGGAGAAGATAGAAGT
TTTGAACAACAAATGGAATTCAAACAACTTGAAGCAGAAATAGAAGTATTAAATAATGAC
TTAAATAGTCTTATAAAAACTTTAAATATATTAAAAGAGGATATTAAGAAATTAGATACT
AATATGGGAAATTGCAATAGTTCATTAGATAAAAAAACAGCTAAAGATGAACAAGGGGTA
ATTTTTAGCGAAGAAGATTTAAATGAATTGGATGAATGTATTTATTCACTTAGATTATCA
GATGATTATTATACTGATGATAAAGAATTGTTCGATAATGCAAAAAGAGTATTGAATGAA
AGAAATATGTTACCTATTGATTTTACAACAGATGTGGTTGGATTAACAAGACATCCTAGA
GGTTGGAAAAACATCGTTAAATTAGGTGATGTAGCACATATTATAGATGGAGAAGAGGAA
ATAGAAGGCGGAGAGGTCAGAATAACAGGATTTAAATATATTCCTTCAAGAGAAAATTCT
CAAGCTAAAATATCGAATGTTGAATTTAATAATTCTAAGTTTGTATTACATGATTTAAAA
ACAATAAGAAATATAAGTTTAAACAAAATAAATAGAAGTGCTAATGCTATAAATTTATAT
AGAAATACATGGATAGATAGTTCTGTAGCAACTAATAACTTTAAAAATATTCAAAAGTAT
GGGATTCAAGCTAATTCTATTCCAATAAAATGTAATGAAAATATAAATGAGCTAGATATT
ACTGGCACTGGTGTTTGGTGTACAGATAAATCAGATAAAACAAATAAAAAACAATTTTAT
ATGGGTGCTGGCTTTTTTGCAGTAACCAATGATAATTGGAAAACTTGTAAAACGGTTGCT
GATGAAAAAGGATTAGTTGCAAAAAGTATTATAGGAACAGCGATATTAGGAGAAAGAATG
AAATTAGTAAATCCTAATAAATCTATAAAGATAGATGATTATGGTATTAGTGTATATGAT
TCTAGTAAAACACTTAGAGCAAGAGTAGGTATATATAGCATCAGAGGAGAAAATAAAAGT
AGCTTAATTCTATATGATAAGAATGGGAAAGTTGTATTATCAGGAGATGGTATGTTACAA
AATGATAGTTTAAATTTTTGTGATAATATAGATCAATCTCATCCTATGGAGTTTCCTATA
TATTTATATAAAAACATAGAATTAAGAGAAGCAAAATTATTTTTACATTTATCTAAATAT
AGAGTTGGATTTGAAGGTGTTGAAGCTGGTGGGTCTATAATTAAAACTACAAATATGAAT
AGCGGAGTTTTTACTTACTTATCTAGTGCCTCCAAAACACAAATAGGTGGAGAAAATATA
ACGATAAATAAAAGTGATATAGAGGATTTGTTAAATGATAAAAAACCTTCTATACAAATA
GACAATAAACATTATCATTTAGAAGATAATCACAAACATGAATTACCTAATCATAATCAC
GTAATTCAACTAGACAATCATAAACATGAATCAAAGTATAAAATTATTGAAACTACAATG
CCAACTTCTATAGGCGTTTATGTTAATGATAAATTAGTTGCTTCAAATATAAATGAAGAT
TGTAATATAGAAATATCTAAATTTTTAGAAACAAATAAATTAAATATTATAAAGATAACA
ACTCAGACAAATGGTAGAGTAAATTGTTTGCTGTCATTGTCTGAGTTTATTAATTTTTAA
GGAGGAATTTATTAATGGCAGAATTAAAAGTTATAACTCAAAAGTTAGAAAACAAAGATC
ATGACCTTATCGGAGAAGTTAGAGGTGATGTAGATGTTAATATGGAAGCTTTAAACTTTC
AAATAAATATTTTTGATAAAAATAAAGCGTTATCAGATAAAAATAAATTAAGAAATACAA
TAAATGATTTTTATTACAACTATTTTAAACCAGCTTTAAAAGGAACTGAATGGGGATTCT
TAGTTTTAGAACCTATATCAATAACTGGTGTTAGTAATACGGTAATTAAAATTGGAGATA
AATTTGATAAAAAAGAAGGCGTACTAGCTATTAGTGCTATAGACGGAGACATTACCAAAG
ATATTAAAATAACAGGTGAAGTAGATAGTAATAGAGTTGATAATTACAACCTTACTTATG
AAATAAAAGATAGTGTTGGTAATAGTATGACTGTAATTAGAACCGTCACTGTAAGAACTA
ATAATCCTCCAGTAATTAAAGGTATAGAACCAATGACAATTCAAGTTGGAGAAAACTTTA

ATCCAAGATTAGGCGTAACAGCAGAAGATACTGAAGATGGAAATTTAACAGATGAAATAA
AAATAACAGGCATAGTAAATAATCAAATACCTAATGTTTACAATGTTACATATGAAGTTA
CAGATAAAGATGGTAATTTAGTAAGCAATATAAGAGCTATTACAGTAGAAGAAAAGAAAA
AAGAAACGGAAGAACATAAAGAGGTACAAGTTCTTCCTGAAAATCATGAGATAGCATAGT
GTTATAAATAATATGCTAGGGGAAAGGAGGAAAGAATTTGAATAATTTAGAACAAATAGT
TTGTCAGTATTACAAAGATAAAAATGGTAATCCAATGTCTTATCATATACGTAGAAAACA
TCAGATTTCACCTAAAAATTATCAAATACAGCTCGATGGTATTCCAGATGAATATCGAGG
AGTTGAAGTAATTGAACCAATAGGATTATATAGGGTTTATAATGCAGATGAAATAACAGA
AAATAGTTATTGGGTTAGAGATGATGGAAATGTTTTCTTCCATGAATCAAGAGCATGTCA
AAATGTTATGCTAGATTATTATAGTATAGGACTTCCTGTCGTTGGAGCTGGTAGAATATA
TACTCTATTAGATGAAGAGGGGAATGTAATTGAAACTCTTGAGGATATATTAGAAAAAGG
TAAAACCGTTATTGATGCCTTAAAAACAATGAGTGATGTTATAGTAGCAATAAATGATTT
AAAAACATCAACTCATGAGGCTATAAAAGTTATTAGCACATTGGATGATACAATAGATAG
AGGATATGAATTGTTGGCTAAGTTAAATGCTGTAGAATATATTCAAAGACCAGAGTTTAA
TAGAACGGTAACGCAAATTAATGGAGATATTAAAAAAAATAAAGAAGATGCAGATATCAA
TATAAAAAAGATAAATGACAATATTTCAAATATCAAAAAAAATATGAATAAACAATCATA
TATATGTGACAATGAAGAGGATATTGGTGCATGTGTTAATAATGCTATTGCAGAGGGATA
TAAAACAATTAAAATTCCAAGTGGACAATATAATTTAAATACTTCAATAATTTTAAAAAG
TAATGTTGAGCTGTTTGGAGACAAAGATACAATAATCACAACAGACAAAAATATTCCGAT
AATTACAACTTCAAAAATAAAAAATGATTGGATTAACACTTGTTATATTCACGATTTATG
GTTAACCAACCACAATAAAGATTTACAATTTTATCATATGGATTTATGTAATGTTAATAT
GACAAAAGTTGAAAGAGTTAGGATAGAAACTGATACTACCAACTCAACACATAATGTAGG
AGGTATTACTGTATATTACAATGGAGATTATATAGGAGAAGGAGGAGCATATAGTCTTTG
TATAGATAAGTGTGATTTAAGAAGTTCTTCTATATATCTAGGGATCACTGATTGTTACAT
AAGTAAAACTAATATATGGGGAAAAAATAGAGATTTTGCACTATGGATAAATTCATCCTC
ACAACAAATATCAGATTGTCAATTTGTTGGTGGACAAACATATGGTGCTATATATATAAA
GAATAAAAATGATTATGATGTAGAAATATTAAAAATAAATAACTGTTATTTTGATGGAAG
TTATGAGGGAATTCAAAGTGGAATAGGATTAAATGCACATAAGATGAGAAACTCTAATAT
TTCAAATTGTAGTTTTTGGCATCAAAAAGATAGTGCCATGTATTTAAAAGATTGTTTTGG
AGATACAATAACTAATTGTAATTTTAATCAAAATGGTAGCAATAATAAAACTCAAAATTC
TAATAATGTACAAGATGGTATATACGATATTGTAGCCGAAGGTTTATTTCAAGCTAATAT
TATATCTAATAACACACATAGTTGTAACGAAAGATTTTACATAAAACCTAAAATGTACGA
CTTCAGTAGAGTAACAAGGTCTAGTGAATGTGTGTTTAGCAATAACATTATATTCAACAA
TGGGCTTTATGATTCTGACAACCCAATTAAAGATGTTGATAATGTTAGAGGTAACAATAC
TGTATTCTTTAATATAGGAACTTATAATAGTTGGTATGACATATCTTTACCTACCAATTT
AAGTATTAATGGAGAATTTAAAGGTTTAGGAACATTGAACAATCTTAATTTTGGTGTAAA
TATAGAACCTTCAGCAACATTTTTTGAAGGATATACTGGATTACCCGTAGATAAACATAT
TTTAACTAGCCAAGGGTATATAGAAAGGTTTGCCGTATTGCTGGATAAAAGATTAGAAGT
TGGAGATGCTGTGTTTACATTAAAAGTAAATGGACAAGATAAAGCAAGTATGAGATTGGG
AAATTATCAATGGAACATATCCAATTTTACAAGATTCCATGTTAATGCTGGAGATGTGTT
ATCTTTAGTTTGTAATACAAGTGGAGTAACTCAATCTTATAAGTGTAATGTCGTAGTAAC
TATAAAACAATAGTTTAGTTAAAACATGTCTTTTAACTAAAATAAAATAAAATATAAAAA
ATGTAAGGCTAAGGATTAATTTCTTTAGTCTTTTTTATATGTAATAAAAAATAAAAAGGA
GATGTTTATATGTCAATAGTAATGAAATACAGATTTGATAATGATGACCATTGGGTGGGT
ACAAATAATCCTGAATATATTGTAATTCATGATACAGGAAACTATGATGATACAGATGAA
GGAAACGCAAATTATTTTTGTACAGGACATAGAAGAGCTTCAGCACATTATTTTGTTGAT
GAAGATTCTATAACTCAAGTTGTTAGAGAACATGATAGTGCTTTTCATTGCGGTGATGGT
TACGATAGATATGGTATAGGCAATAGAAATAGTATAGGAATAGAAATGTGCAAAACTAAA
GGGGATATATCAGAACAAACTATTGAAAATACTTTATGGTTAGTTAAAGATATTCAAAAG
AAATATGGAATACCAAATGAAAAGGTGTGCCGCCATTACGATGCTTCTCGTAAGATCTGT
CCTCAAACTTTTTCACCTAATAATTGGGCTAGATGGTGGGATTTTAAAGCTAGATTAGAA
GGGAATGAAATTCCACATGTTGAGACTATAAAAAAAGACAATCCTTCTTGCTTTTATGAA
AGTGATATAACTAAGACTAATGCAACTATAGTTGGTGAAGGAAATATTCAAGTATTAGAT
GATAAATGTAATCCAATAAAAGGTAGATATATTTCCAGTTTAGATAAAATATTTGTATTA
GGAATTTATCCTTCATCTAAATTTATTGAAGTAATTTATCCAGGAGGAGATAAAAAATAT
CATGCTTATATCTCTATAGATAATTATAGTAGAATAAGTTTTGATTATCATATGCAATAT

CAAAATGATGGTGGTGACACTTATGTTTGGTGGAGTTCTAAAGATGTAAATAAAACAAAT
CATAATGAAATATTATCACCTAATAAAAAAGCCTCTCCAATGTATAGAGAAGGTGGATGG
TTACGTATAACTTTTTATAGAGAAGATGGTACTCCAAGTGATGGGTATGTTAGATATGAA
GGGCAACAATCAGAAAAATTCTATGAAAAAGCTAAAATAAAACAAGGTATAGTTAAAGTT
GATAGTAATCTAAATGTTAGAGATGATGTAAATGGAAATATTATAGGTAAAGTATTTAAT
AATGAAAGAGTCGCTATAGTATGGACTGAAACAGGTTGGTATTATATAGAATATGATACT
TCACATGGTAAAAAAAGAGGCTATGTAGATGCTAATTATGTTGAAATAGAATAATATTGG
AGGATTTAATATCCTCCTTTTAGATAATAATGGAGGTATTAAAGGTATTATGAACTTAGA
AGCAGTCAAAAATGAATTAGACAATGGGGGAAGGTTTGTAGGGGAATATAAAGATATAAG
TGAGTTAGTAAAATATCTAGAAAATATTAATTTGTCCTATAATTTCACACATAAAAAAAT
AATTGAAATTTTTAAAATAAATAATGATTATATATGTGTTAAAGAAGGAATAAAAAGAAA
GGATGTGGAATAAGGAATGGCAATGTATTTTATAAAAGATAGTTTGGTTGAAGACGATAT
TAAATTACAAAATTCTTTGAAAGCTACCAAAGAATGTATAAAAGAAAATAATAAAAGTAT
TAATTCAGCTTTAGATATAACAAATAAATATGCTGATAAACTTAAACAAAGTACAGAAAA
AATTGAACTGCAATATGCTAATACATTAAATGACATCAATTTAAAAATATCGGACATAAC
AATAAACAAAAAGAAAAAAATAATTTGTTTTTATACGGATGTAAATATTAATATAGAACA
AGCTAAAAAAACAATTGGTAAAATTAAAAAGTTGGGAGCAGAGTATTTGTTGTTATCTAT
ATCTATAAACAATAATTTATCATCAAATTCTCCTTCAAGATGGGTAGAAGATGAATATTC
AATACAATTAATAAAATATGCTAAAGATATAGGATTAAAAGTGGCTTTAAAATGTCATAA
TGTACCAACAGACGGTGATGGTACTAATAATTATAAACCTAACAATGCAGAAAAATGGTT
TAAAAATTATAAAAAAATAGTTTTAGCACAAGCTGAAATTTCAAATAATGAAAATTTAGA
ATATTTTCTTATAAGTAACGAGATGAAATCTGTTACAACGTTTGAATTTAAAGAATATTG
GGTAGATATAATAAACACTATTAAGAATGAATATCCTAATTTAAAAATAGGAAGTTCTGT
GAATTTACCGGAGGCAATGACATATTCTCTATTTAATAATTTAGATTTTTATGGATTTAA
TTTATATCCTTGTTTAACAAAAAAAGGAATGAACTCTACAGATAAAGAAATAAGAAGTGC
TTTTTACTATGATTTAGAGGGATATGAAAATATTCCATTTATTAGCAATATGAAGAAAAA
TAATAAAGAAATTTGGATAACGGAAACTGGTTGTCAACCTAGAGAAAACGCATTAGAACA
TCCAGCTAGTTGGAATGATAAAGCTAAGTATGATGAAAATGTTCAAGAGTTATACTATAA
ATATATTTTTGAGATGTTTGGAAATATGCAATCTATTGAGGGGTTAGCGTTGTGGGTGTC
AAACGAAATTGATCCTTTTACATTTATAGGAAAAAAAGCAGAAAAAATAGTTGAAAAATA
TTTTGGAGGTGATCTATAATGTCGCTACCTAGTGATATTAAAATAGGGGGGTTACCAGCA
TTGCATGAAGGAAATCATAATAGTAATGGTAATCCTCACAATCAATATGTTATTAAAGAT
ATAGAATATTCGATTTCTCCAACTAATGAAATAAGTAATGCTTTTATATTGTACGAAGTT
AATCTAAAAGAAGATTATCAAAAAGATAATATAATATTTACTATATCATCTTTAAATAAT
GATAGTATTTCTATATTTAATTATACATCAGTTAATATTCCTAAATCTGATCCCCATATT
GGTGTTAATGAAATTGTCCATTATGGGAATAGCCTATCAAAAAATGTCGGATATAAAGTT
AATGGTAATAAAATATCAGTTTTTTTTATTAAAAATGATTTTAACTATTTAATACAAACT
AATTTAATCAAGAGACAAAATACTTTAAATAAAGAAGAGATAGATATAGTATTTAAAGAT
ACACAATTAAATGAAAAAATAAAGTTAAGCTATATTAACGAAAACAATTTTAATATTTTA
ACTTTTCCTAAAGCAGAGAATAAGTACATGAAATTATTTGATATTGAAATCAAAAAAGAA
TGGTCTGAGAAAACTATAAAATTTGATATATTAACAGATGAAGTGGATATTAACAATATA
ACATATGGTACATATATTATAACCATAAAGAAAATATCTGAAAAATATATAATAGATTGT
TTACTTATAAGCGGTGAACAATTAATTCCTCAAGATATCTGTTTTGCTGAAAATGGTGAA
TATATAAGTTGTTATTTTTTAAATAGATTAAACAAAGATATATCATTTAATATTTTATCT
CTATCAGGTGTATCTTTTAAAGAGTCTTATATATACTTAAACAATAATTTGTGTTATAAT
GATAATATAAATAAGAAAGAATCTGTAAATTATAGATATGGTTACAATGTAATGCAACAT
ATTTATACATTTGATGATTTAGGTAACAAAGTTGCAATAGGTGTACATTCGGATGGAAGT
GTATATACTCAAAAAATAAAATAAAAAATATATTTAGAGGGGATATGATGTTTTTCATGA
GAAAATTTTGTTTATTTTTAAAAGCTATACCAAAAACCATATATTTTAATTTTAAATATC
TACCTATTAAACAAGCTATAAAATTACCAATATTAGTGTCTAATAGAGTATGGTTAATGA
CTTCTAAAGGAAGAATAAATATATTAACAAAAGATATTAAATTTGGAATGATTAAAATTG
GTTTTGGGGAAGTTGGATTGTTTGATCAAGTAAAATCGAGAAGTGTGTGGCATGTTGTAG
GAGAAGTTATTTTTAAAGGTAAATGCAATATTGGACACGGTTCTAAAATATTAGTTGGGA
AAGATGCTATTCTTGAATTTGGCAAAGATTTCGATATAAGCGCTCAAAGTCAAATAGCTT
GTGAAAAACACATATCTTTTGGAGACAATTGCTTACTTTCATGGGATATATTAATTATGG
ATACAGATTTTCATAAAATAAAAGATATTAATGGTAATATTATTAATCCTAAAAAAGATG

TTGTTATTGGTAATAATGTATGGATTGGTTGCAGAACTACTGTTTTAAAGGGTGTAAAAA
TTAAAAACAATACTGTAATTGCAAGTGGGACAATGCTCACTAATTCATTTAACGATGAAA
ATGTAATTATAGGAGGAAGCCCCAATAGGATTGTAAATAACAATGTTACATGGGAGTTTT
AAAAAATAAAAAAGTATTAAAAGACTAAAGAATTAATCTTTAGTCTTTTTTATATGTAAT
AAAAAAATAAAAGGAGATGTTTATATGGAAAATAAAACAGTATTAGTAAAAGAAAGATTA
AAAAATCCCGCATTTTGGTTTGGTATGCTTGGTGTAATATTTAGTGCATCAGGAGTAGAT
TTTAATACTTTAACTTCATGGGGATTATTAGGAAAAGCGCTATTGGATATATTAGAAAAT
CCAGTTGCTATAGTTGCTATACTTATGGCGATGTATGGTATATGGAATAATCCAACGACT
AAAGGATTTAAAGATATAAAATAAATTTACAATATAGAAAATATAGTAACAAACTATGTT
CCTATATTTTAAAGATAATTAATAAATATCAATTCTGTTGAAATTTTGAATTTTAAACCA
CCATCATATTATAATTTACTAGATTATAATAATGTTTGAATTTGATTTTTCAACAGAATT
CATTTACATCTCATTAGGTAAATATTTTACAATTTAACTTCGTAAAATATAGATTTTGCG
AAGAAAATATGTTATAATGTAGTAAAATAAAGATTAAAAATGAGGTGTTTAAGATGAAAT
ATGAACAAAATATTAATGAAATAATATCAAACAATATATTCAATAGTAAAGATATGGATA
ATTTGAATGATATATATAATAATTACGACAACTTGGGAGTTACTAGAGATTATGAAAATT
ACATAGAACATATTTATATTTTTTATAATAAAATGAATATGTCTATAGATGAAATAGCGA
ACATATACGAAAAAAATAAAAGAATTATCCAAATGTGGATTGAAGAATTAAAATTAGATA
GCTTCAATTCTGATGAAAAAATGAATTTGAACGATAATTCAAACATACAATTAGAGTTGG
ATAAAAAAGTTGGAATCAAAAAAATAGAGGAAAACAAATTGCCTAAAAGAGTTTATGATT
TTATATCCTACTTAAAAAATATAAAAGAACAAAGTCCTAATACTATAAAAAATTATACAT
ATGACTTAACATTATTATTTAAGTATCTAGTTGGAAGAAATCAAGATGTTGAAAATTATG
ATAATGTTGGCATAGGATATGTTGATGATAACTTTATAAGAAGTATAACATTATCTGATT
TATATGATTTTTTAAACTATGTAGAAGTAGATAGAAACAATAGTGCTTATGCAAAAGCTA
GAAAAGTTGCTACTTTAAAATCTTTCTTTAAGTTTTTGAATGTAAAAATAAAAATAATAA
ATGAAAATCCTACAATAGAATTGGAAACTCCAAAAATAAAGAAGAGACTTCCAGTTTATT
TAACTTTAGATCAAAGTAAAAAAGTTTTAGAATCTATGAATAAAGGGAAGAAATACTATA
GTAGAGATTATTGTATATTTGTACTATTTTTAAATTGTGGAATGAGATTATCTGAACTTT
GTAATATAAAATTAAAGGATATAAAAGAAGATACTATAACAATTATAGGTAAGGGAGATA
AAGAAAGAACAGTTTATTTAAACGAAGAATGTATAAAAGCAATTAATAATTATTTAAAAG
ATAGAAAAGAATTAAATAATTACAATGAATATTTATTCTTATCAAAAAGAAAAACAAAGA
TAACTGCAAGAGCTGTTGAAGATTTGGTGAAAAAGCATATAGAAAATGCAGGATTTAAGG
ATAAGAAATATACTCCTCACAAACTTAGACATAGTGCAGCTACAATGTATTTGAAAGAAG
GGGTGGATATAAGATTTATTCAAGAGATATTAGGACATGAAAATATATCTACAACTCAAA
TATATACACATGTAGATGATGTAGAATTAAGAAAGATAGTGAATGATAGTCCATTATCAA
AATAAAATAAATTTGAAAATTATTGAAGAATCAAGATTAATTTCTTGGTTCTTTTTTATT
TATATAAAAAATATTAGAAAGGAAGCGTTTATGAGATATGGCTTTAAATATGGATAGGTT
AAATAAGTTTTTAGATTATTTAGAATTAAAATATATAGATAATATTTCAAAGATTAAAAC
ATATAGTACATCTGACAAGATTTCATTTCAAGATAAAGATGGATATTTGTATTTTCTTAA
TATTCAAAATTTATCAACTTTATATAGAAGAAAAACTAAACCTGCAATATTTTTTCAACA
TAATATTTATACTTATGACAATATTAATAATTATTTAAAATTAAATAATATTCCTTTGAA
ATTGATGACTATAACTCCTAAGAATGCCATTTGTAAACTGAAATGGAAATGTTTAATTCA
CGACGTAATATTCGATAGAAGTTGGAACGTTATAAAAAATGGTTCAATTCTTTGCCCAGT
GTGTGAAAGAAATTCATTTAGAAAAAATAGGTGTCATAAAATTGAAGATATAGTTATAAG
AGCCTTAAAGGATTATAATATTCAAATTTTAGATGATAGGTATATTAATAATGAAGAAAA
ATTATCTTTTATATGTAACAAACACAAGGATAAAGGGATTCAACATAAAAGTTGGGGTGG
AATGATATCAAAATCACATCCTTGCATTTATTGTTCTAAAGAAAAACAATTATCTAAAAT
TAGATATTCACATGATGAATTTATAGAGAAAGTAAATAAAATACATGGTGATAAATATAA
AGTTATATCTAATTATATTAAAGGTAGGGATCATATCAAAGTATATTGTAACAAATGTAA
TAGTATTTTTTCTATTCGTGCAAGTCACTTATTAGAAGGACACGGTTGTGGATTGTGTAC
AAAATCTATTGGTGAAGAAACTATAAAAAATATTCTAGATAGACATAAAATAAAATATAA
AAGAGAATTTAGATTTAATGATTGTAGAAAAAGTAAACCTTTACCATTTGATTTTTATTT
AGAAGATTATAATTTATGCATTGAGTATCAAGGAGTTCAACATTATAAGCCAGTTGAAAT
TTTTGGTGGAATTGAGCAATTTAATAAACAAAAAGAAAACGATAGCTTTAAAAGAACTTA
TTGTAAAACTCATAATATAAATTTGTTGGAAATACCTTATTTTGAAAGCAATATAGAAGA
TATGCTATTAAATAAAATAAATACAAAGGAGGAATTCTAAATGACAAAAACTAATATAGA
AAAAAATATGGTACGTGAAAGAGCTTTAAAGTTACCTGTGGTCACTGATGAAATGTACAA

TGAGTGTAACTATGAAAACAGAGAAATGGTGCAAGAATTTTTTGAAGTTAAGTCTCAATT
AAGCAAAGATACTAGAACTCAATATAAATCTGGATTAAGACAATTTGTATATTGGTTACA
TACTAGCTGTAATGATAAGCCTTTTCATAAAGTAAAGAAAAGAGATTTTGTAAGATATAT
GAGCTATTTGGTTAATAGAGGGATGTCAAGTAGTGCTTTAAAATTTAAAAAATCATCTGT
ATCATCTTTATGTGGATATATAGAAGATGTAGTATCAGAAGATGATGAAATGAAAGAATA
TAAATCTTTTAGAAATTTTACAAAAGCATACAAAGATATACCTAGAAACTATGTTTATGA
AAAAATTCCTATATCAGAAGAGGAATATAAAATATTGATAGATGCATTAATTGATGATGA
AAATTATATGGGTGCTGCATGGGTTGCTTGTGCATTTAATTGTGGTGCAAGAAGAGGAGG
TATTAGACAATTTGAATCATCAATAGCCGAACAAGAAATACCAGAGGGGAAAACTTTTGT
TTATTCTAATTATGTAAGAGAAAAAGGTCGTGGTTCTGATGGTAAGAGAGTTCATTATAT
GGTTAATGAAGAAGCTTTAAGATATATTAAGTTATGGCTAGAAAAAAGAGGATATAATCA
TAAGTACATATTTACATGCAAATATAATGGAGAAATTCATATGATTAGCAGAGAATGGGC
AAATGAATTTTGTGCCAATACTTTATCTGATATATTAGGTAGAAGGATAAATGCACATTT
ATTTAAAGGTTCATGTATAACAAATCTTTTATCAAAGGGTAAAGATATAAAGGTTGTATC
AAAATTTGTAGCGCAGCACAATAACATCTCGACTACCTCATCATTCTACGATCTTAGAAA
TAATGATGAGGAAGCGAATCAATTATTTAGTTAAAACCTTCTAATCAAGTAAAATATTTG
ACTTGAGAGGAGGTGACAACAATGGAAAAATTTATAATGGAGGCAATAGATAAACATGGT
TTTCCGATTGTAATGTGCCTCTTAATAGGATATATAGCTTATAAAATAGTAACAGATAGA
ATTAAAAAACAAGATGAAAGAATAGATAAAATGGAAAATCGTAATCAAGAAGATAGACAA
TGTTTTTTAGATGAGATTACAGCTTTAAAATTAGAAAACAAAGAAGATAAACATATGTTT
AAAGATGCTATGAATCTATTTAGAGAATCAGTTAATGAATTTAAAAGTTTTAATAAAGAG
ATAAATTCAAAAGTTGATTCCATCCAAGATGATGTTAAGATCACTAAAGATGATATCACA
GAAATAAAACAAATTATAGAATATAAAGCTAAAGAAAAATAAATTAAAAGAGTAGAAGAA
AACTCTCCTACTCCCAATACAACAAAAGACAATATAAAAATTGTCATACTTATTATAATA
TTTTTTATTGTTAAAGTCAAGGGTAGAATTATTAATTTAGTTCTACCCTATTTTTTTGCT
TTTTTTATTTAGTCTTTCCTGTAGTACCAAATCCTCCCCTGTCTTCATTATTAAGTCTTT
CAACTATATTAAATTCTATTTCAGGCATTGATTTTTGAATTCTAAATTGTCCTATTTTAT
CACCTTTTCTTATCCAAGTACCCATGACAGTAACTTTATTACCTTCAATATTAACTTTTT
CAGTTGTTTTTGCCATTGTACATTGTACTGGAAAATGCCACTGATCATTATCTCCTATAT
ATGTATCATCCACAATACCAACGCTATTAGTCTGTATAACTCCCCAAGTTTTAAAAGTTG
AGCTTCTAGGTGCTAAACACCCTTCCCATCCTTTAGGTAATTCTAAAGCAAATCCTAGAG
GTATCATAGTATAACCCATATATGGTACAAACACATCTTTATTAGCATACACATCAATCC
AATTTCCTTTAGTTATTTTCTCCATTTTAGTTGCTCCATCAAAATATTTAATTCTTAATT
CCATAATTTATTATTCTCCTTTTCTATCTTGTAATATTTTTTTATTAACAGGGAGAATTA
ATATCTCCCTTAAATATATTTATTATTTTAAATCTACTATATATTGATTCTCAGAACCAT
ACATTGGATTTTCACATTTTTTATCTTTAATAAATTTACCACATATGATTTTGTCTAGGT
ATGTAATTATTTCTTTGTTTTTAACTTCATCCCATTCATAACCTGTCCATAGATAAATGC
TACAATCTGGAATTTCTTCTTTTACTTTTTTAACAATTCTAGTAGCTTCTTTAATATTAA
ATGGTGCTAACGGTTCACCACCTAATACAGATAGTTTCTTGCTTTTCTTACATGTTTCAA
CTACTAAATTGCAATCTAAATGATAGTTTTTGTCATATTCCCATGAACCTTTATTATGAC
ATCCTTCACATCTATGTGGACAACCTTTAAACCAAATAGTAGTTCTAAGTCCATCTCCAT
TTAGAATATCAAAATCTTGTATTCCTTCTCCTATATATTGTCCATAATGCACTACTCTAT
TTTGGTTTTCTTGATATTTACCATCATTAACTCTTGTACTACCGTCAATTTCATAATAAC
TTAAATATCCACACACTCTATCAACAACTATTAATTTTGTACTACCACAATGATTGCATT
TAGGATTTTTAGGATCATATTCTGTTACTGTTTTACCACAATTTTTGCAAGTAATAGAAT
CAAAGTTAACACCTTCATATAATCCTAATTTCATAGCTTTTCTAGTTGACTGACTAATTG
CTTCAAGATTATGATTAATAGGATATTCAGTATAAACTATATGTCCTCCATTTGCTAAGT
CAAACATAGGTTTTTCTATTTCCATCTTTTTGAATGGATTTATTTTAGCTGCTACATTAA
CATGGAATGAATTCATATAATACTTTTTATCTGTAACTCCTGGTATTAAACCAAATTCTT
CATAATCCTTATTTCTACAAGTTTCCGCATAACCTTCTGCTGGTGTAGAATATATAGCAA
ATAATAATCCATATTTTTTTATAGCTTCATTTTTCCATTTATTAAAATGTTCTAATACTT
CAATAGCAAAAGAATTATCCTCATGAATTTCTTTACCTGTCATTAATAAACTAGCTTCAT
TTAAACCAATATATCCGTATGACCACGTAAAAGTTTTTATTGCTTTTTCAATAGTTTCTT
CTGGTTTTAATTTAATGTGACAACCACCTTCACAGAAAAATAAAGGATTTGTAGATGCTT
TAACATCTTTCATTCTATTATATGTCCATAAATGACCTTCTGTAGCTAGATCAAAACATC
TTTTAACTTCTTTAAAATAAGCTTCTTTATTTCCTTTATATTTAATAGCTGGTTTAACTG

TATTAATAGTTATAGCTCCACAATTAGCTCTACCATTAAATATAGCTTTATCATTTTTAT
CTAAAGGTTTTATTCCACCTTTTTCAAACCAAGGTGAAAGATACGCTCTACACATGTTTA
CCCAATATCACTATTGGCACTGACTATATCTTCTCTCCGATTCACCACATCATCAACAAG
CCCCTTCCTTCGAATTGGTGCTTATCTCCAACCCTACGCAATTACACTCATCATTGCTAG
TCGATACACATTTATCAATTTATATAATAAATTGAATTTAGCACGGTCTCAACTTAATTA
TATTTTATATTTATAAATAAGTCCTAACCGTTAGCATGATAAACAAATATCACACACCCT
ATAAGCTAGGTTCAAGGGGTTTTACATGGGCTAGTAGATTACAAACCCATAGGTGCTAAC
GCATATCCATACTTTTTCCACACTTCACCACACCAACCATTATCAAGTGATAAGAAATCA
GGATATTCACGCTTACTTCTACATTCTATTGCTAATTTATATAAATCTTCATTTATTCCA
CCTTTACCATGTATCTCTTCTCTATAAAAGAATACTAATTTAGGGAAAATAGCTGTTTGT
CTATATTTACCACAACCTTTTAATCTAGTTTCTAATATAATCCTTGAAACCATTCTTGCC
CAAAAACTTGTTCTATTACCGAAGGCAATAGTTTCAAAGGGTACTTGCATATTTGAATTA
GATATAGAATTAAGTCTTGTTTCGATACCATTCCATCTTTTTCTAAAAGCTCTCTCAACA
AAATTCATAGCTAACTTTTCTATTCTATCTTTAGGAACTAAATCTCCAACTTGCTCCGTA
TAATATTCTATGGATTTATTATAAGCATTTTCTAAAGCATCCTCTAATACATGATCTATT
TCATTTATTGTGAAACCACCATATTGATTTGCACTAGCTTCTAGTAGAACATCTGATAAA
ACACCTAAATAAGCCTCTATGCAGTTTGCATCGTCATATTTTAAACCATTAATGATAGGA
TTATTGTCAATAACATTCTTCATATCATATAGACAACAATTTATACCTTTAAAGAATCTA
TCGGAAACATCATGGAATTTTATATCATTCTTTTTATGTGCTTCTGCTAAATGTTTGGGT
AGTTCATGATTTAACATTTCATCTGTTATGTACATATCTAAAATTAAACCTTTTTTAGTG
GCTATTAAGTCAGAATTTTTATTAGCGTTTTCTCTAGTTTGTTGATTTAAAATTCTTGTT
GTTTCACTTCTCTTTGCTAAAGTTCTCTTATATTCTTGAACAGCTCTATAACCTTCATAT
TTAATAGCTGTAGTTTTATCACCATTTTCCATTAATAAATTAACAACCTTACGTTCTATG
TCTCTAACCGTTACCTTATCATTATCAAAATTAGTGTTCTCAATTTCTTTGGCTATCTTT
TTAGCTATATGTTCTTTACCTTCAGCTTTCATAATAGCATTAACTATTTTATCCCTATTA
AAGTTTACTTCTCTACCGTCTCTTTTAATTACTTTTAAATCTTTTATCATATATGTATAT
TCCTCCAATATTTTATTTTATATGTTTCTTAGTTGATATTTAACGAATTGTCTTAATACT
TCTATTTCTTCATAAGTTAGTGTTGGATTTTGTTCTAAATAATTTTTAAATTCATCTTCT
ATACCCTTCTTTTCGGTTTGTCTAATTTCTTTTTGAAAGTTTCTTATATTTTCTTGATGA
GAGTATAGTTTTTCTTTATCATAATCCTCTTTTAATATGTATTTATCTATCATAAAATCA
ACCCCTTTTATTTTATTTGGACATACCCTTTAACATGAGGTTTACCCATGTTATTAACCC
TTCTTCTATATTGATTTACACTTTCATCACCTTCTTTCGGTATTAGAACATCCAATCTAT
TATAATTGTCAAAATGACTTCCTCCTCTATCTGCTACAGTAAATAAACCATAACCTTCGA
GATAGATTTTAGTTCCTAGAGGATATACATTACTAGCAACTATTCCATACTGAAGAGGTT
GTCCAGTACAAGTTACTGTATATCCTCCATTTTCATCTTTTGTATTCGTGTAAAAACTGA
TTATAAAATCAATTTCTTCAACTTCTCCAGTTAGCCCTCTACCTGATATTTCTTCTTGTT
TCTTTTGTTCCCTTAATTTCTCTTCATTTTGTTGTTTTAAACCTTCTCCAATCTCTTTTA
ACATTTGTTGTTTCTTTAATTGTTCTGATTCTTCTTTAATTTTTTCAAACTTATTATCCT
CTAAAGGATATTGGTTGTCTTGTATGTTCAACTTAAATTTATTATGTAAATTCGATTTAT
CTAATACTTTATCTGTTATTCCTAGTAGAAGAGGTAAACAACACAAGACACTTACTATCC
TTCTTTTCATTCAATCACCTCTTATATATCTATTCTTAATATTTCTTTAGACTCAATTTG
ATGCCTTTTATCAAAAAACGCTGTAAAGTTTTCTCTATTATTAACATATACATTTGTGAA
CATAATAATATCTAATTCCTCATTATAAAATAATATAGTATTCTTGGTAGGTTTTAACCA
ATAACCATTTGATAACCATATTTCTACACTATCAAGTAACCCATCTTTCTTATATTGTTT
TAATAATTCATATTTCTCCATTTATATCACCTCATAATAAAAAATATAACATTCTACTTC
TTTGTTGTTATTATTAAAGATAACGTTGGTTGAGATAATTTTGTTAAATTTATTACTATT
CAACCATTTTTGAATTTTCATTGTTACAAAAGTCTCATCTATTCCAGAAAAACATATCAC
TTTCATTTTTAACTCCTATTCTTTTAATCAATTCTTTAATTTATTTTAATATGTCATACA
ACCATAAGGGCAATATCCTTCATCATCAACAAATTCTTGATAAGCTGGTGAACCTTGAAA
CTCTCCAACACATTCTTCATGATAATTATATATTAATTCAGTTCCACAACAATTGCAGAT
ACTTTCTTTATTACAAAACGTATTTAATTTATCTTCTAAAAATTCTATGTATTGATCTTT
ATTATTTGCTAATTCTTTTATTAATATATGTAATAAATATTCTCCATCTTCTTGATTTTC
TTTTATTTCTAATATAGATTTAAATAATATATCTGGAAGCGTTGTTATGATTGGTGGATT
CTTTAATTCTTTAAGCTCTAATATTAATTGATTTTGTTCTTCAAATTTTTCTTTAATTGT
TATATCCTTTTCAAGTATTATATCTAAAGCTCCTTCCATAATTTGTTCCATATCTTTATT
CTTTTCTTCTAATTCAAACATTTTTAAATCCCCTTTTGTTTATATATTTGTCTTTCGACA

TTTTTAATTATATACCTCTTTTTAAAATAAATCAATCATTTATTTAATTTATTTCAACAT
TTAATGATTTTATATCCAACTTCATCAAATATTTCAGCTACTATGTGTCTATGGCAGAAA
TCGTTGTTGGACTCATAACACAGTAAACATATGTTTACACCTTTATCTAATAAGCTTTGT
ATTAAGAGTAGTGTATTCGCAGATTTGATATTATCTAATTCATCTAAAAACTTATTTCTA
AAATCACTCTGAGATACTAAACCGTTTTTATATGCTTTTAATAAACCTTCTGAAGGTGCT
AATTCTAATAATTCTTGAATCCCTTCTTTATAATAATTCATATGTGGCGGTCTTTTTCTA
CATATCGCTACAGTAAAAGCATTTAACTGCTTCCATTTTCTCCAATTACCAAAATAACTT
GTATATAATATTCCTTTACTCATGTTTAATCACCTCGCTTAAATCATTCATAAATAAGCA
TATTATCCAACAGAAACAAGTTATTTTTAACGTACTAATATTTGATTGAAACCATACCAA
TATCATACATAATACTTCTATGAATAAGAACAAGGTATTTAATCTCTTTATTATTTTATT
CATTAATTTTACACCTCCCTTGGATCTATTCTTATAATAGTTTTATTTATCTTTTTAGCA
TAATTAACACAATTACCAGTTCCACCTTTACTTCCATCCCATACTGCTATAACTATATCG
GCTTTATCTACCATATACATATTCCTCTTCTGCATTTTAGCTGGATGGTATACTTCATTT
TCTATTCCTTTTAAATGATACTTTTCCAATTCATCTACAAATGTAACATCATCAGCAATT
AAAACTTGTTTATAATATCTATCTACATCTGTTTTATTAAACCATTTATTAGCTTGATTT
TTAAATGGTATTGCTACCTCAAGAGTTATTCTTTTAGGATATTTAGCTCTTAATACTTCA
CAACTTCTGAAGAACATTTGGTCTATACCTAATGCACCACCCTCTATGCAATTAATAGTT
TCATTAGGATTTTCTTTTAATATATCTTCTACAGTTTGATAAAGCTTTAACATGATACGT
TGATTCTTATCAGAATTCCAATTATATCCTCCTAAATTAGGATTTGAAGGTCTATGTCCT
GTTACACAAACATTAATCATAATATGTATTCTCCTTTTATTTTATAAAATTATTTTCATT
TACGAGTAAATTATTTTAGATATAACTTTAAACTATACTTAATTCCAATTCTTCAGCTTG
TTGTAATAATTGAGCTGCTTGATCTTTTATAGACTGTATTTCTCTTCTTAATACATCTTT
AGATATTTCTAAATGTAATTTATTATATGATATTAATTTACTTGTTTGGTATCTATTATT
ATCAGTCATTATATAAGGATTAGGATCTTCCCACCATAATAATTTATATTCTATGTCTTC
ATCTTTATAAATTAGATTTAATGAGACTATATCATTATATTTATTAATTCTTTGTAATGG
CGTTGAATTATCAAATCCTTTATATTCTATCTTATCACTTAAATTTATATATGTATTAAT
TTCCCCATTTTGTATGTATAAATCAGTAAATGATTCTACTGGAATGAACATTCCCTCACA
ATTCTCAAAAATTAATTCAATCCCTAATAATTCTTTTGTATTTATTTGTTTGTTATCCAT
ATTTGTATTACCCCACTTTGATTATTTTTATCTTTAAATTGTAGATTTTAAATAGATTAA
TTATTTAATTTATTTTGAAATATAATGTCTACAAATAGCTTGTTTTGGTTAAAATATTGA
CTATTTAGCCTTAATTTTACTCTTATTGTATCAATCTACGTTTAATTAATCATTTATATA
CCATTTATTTCTTATTTCATTATAAGAGAATAATCTAAATTCATCATTTACCTTATCTAC
AATTTCACGATTATTTAAAACATACTTACTAACACTATTTCTAGTTTTAAAACCTCTTCC
ACTAACCACACTTTCTATTTCTTTGCCATCTTTAAGAGATTTAAAAGCTTCTTCAAAGCT
ATATTGTTTTCTTTGAAGTGTAAATAATATAGCATCGCAAAAAGCTATACCGTTAAATAT
TTCACCATTTTTAGTTTCTATAGATATAACTTTATGTGAACATTTTATAATATAGTTTTC
ACTTTCCCAAGTTTCACCTTCTTTAATGTTTGTTATTACTTTTCTAAAAGTTAGTTCCTT
TATATTCTTGGCTTCTATTTTTTCTAACATTTCCTCTGTCCAACTCCAATCTTCTATATC
TATATCAATATTATAACCACCTGTAAAAATATCGGTTATTATAGCTTCTTTACCTTTTAG
CGATTCCATATAACCACTGGCAAAAAATGCTCCTCCATATCTTTGCCCAACTTCTAAATC
TTCTCTTATTTTAACTTTGTCTCCTACTTTTAACACTATCTATCAACTCCCATTATTTTA
TTATTTAACTTTTGATTTTTGCTAATTCTAAAGTCTGAAGGTTTATAAAAATGTATTCCC
ATAGTTGTATAAACGGTTAAATGCTCTTTTAAGTTTAATTGTGGCATACCTTCACAAAAT
ATAGCAAATATTTTATTATCCTTTCTCAACGCTTTATTACTATAAGCTCCCTTAATATTC
TTATTGGCTTGATAAACTTTAAATTTACACATAAATTCCTCCTAATACATCTCTCAATAA
TAATACTAATTCTTTATCATTAAAAGATTTTATATTTGAATATTTGTTTTTAGAAACCCT
TTTACTTATATTTTCTCGCCATTCTTGATATGTAATATTATTAGAATCTCTCGTAGATAA
CCACATTCTTCTAAGTATGTCTTCATAATAATTAATCATTATTTACTCTCCTTATTTTAA
TAATATCCATTTTCTCTTTCTTCTTCATTTTTAGGCTCTCTAAAACAAGTATTGAAATTA
TCTGGTATTATATCATTATCCTTGAAAGCTTGGCTCATGTCTTTCCACTCTTGTTTAGAA
TCTAAGAATACCTCCAATTCATTAATAAACATTGAATCCATTCCTTCATCATCTAAAAAT
CCAACTATTACTTCTCCTAAACCACAAGTATTAAATTCATTTGATGTACCTTCCCATGTT
TGCTTTTTATTTCTAATTTTCATTAATTATTCACTCCTTAATATAAATCTGATTCTTCAT
CAATTTCATATGTAAATCTACCATCTATATTATTTAATATTCTTTCTTCTATGGATTCCA
TATTCTTAACTACATCATATATAGTTTTATATTCTTCTTTTTTAAGTTGTTGCTTTAATA
TGTATTTAAAAGCTTGTCTTAAAGTTGGATGATAAGTTATTTTCTTAATACCATATCTTT

CATTCTCTTTATGTTTAATTAACCATGTTCCTCTATTATATAATTGTTGTCATCTGATTT
AATTATAAAATTATCTAATACTTTTAATTCCATTGTTATTTCTCCTTGCTACTAATTATT
TTAAAAATATATTTATTAATAATATTGCTACTCCAACTAAAATCGGTAATATTATAGCTG
AGTTTTTGATAGTAAAATCAATATCTTTCTTATCATTCTTATATTGAAATGCTACCCCAT
CTAAATTTCCGAATAATTCTATTAAACAGTAAGTTCCTAATATTAATATTAAACCTTGTG
TCATCATTTTAAATTCCTCCTTAATTATCTTTAATTATGTCAAATGGTTTGTCTGATTTT
CCTTTAAAATACTTTTCTATCCATCTATAACCCTCCCCTTCATAGCTATATTCAAGTTCG
CAATAATATTCCTCTTCATAACATTCATAGTAATTATCATATAACACATACGTTTTGAAA
TGTTCACAATCGCGACACTTTTTATTTATACAATGTCCAATGAGTTTTTTAGCTTTTCTT
TTATATCTTTTATTTTTCATTGTCTATATCTCCTATTACTTTTTCAAATTCTATAACCCA
TACCCACGGATTGGCTTCCCAAGAATATTTATCTTCTTTAATTGTCATGTCCCATATATC
AGCAAATCCAGTTTTATAGTTTATTTTATGATTAACTAAACTTCCCACATATTTTTGTTC
ATCCTCAATCTTAGTACCAAATGGTTGACACCACGATTGTTTTTTAGCACCTTCTTTTTT
AGCTTGTCCTTCATCTATATCTTGTAATTTTTCAACCTTAATACTTTTTACACTTAAAAA
TATTCTCGCCAATTCTTTAGGCATGTGAATTGAAGGTCTCCATTTTATAGCACTATAATC
CCAATTATCATTTTTAATTCCATCTGCTCGATAAATAATTATGCCTTGTTCTTCAATTTC
CTCTGCATCATTCAACAAATCACCTTTAAACCACGTTTCTTTTACATAAAGAATATCACC
TATTTTATAAGGTTGTTTTATATATTCAACAACATCTTTAAATTCTTTATCTACATATAC
ACTACCTTCAGCATTAAGCGTATATAATCCATTCTTTTGTTTTTCTATGTATTTAGGTAA
TTTAATAATTCTTCTAGTATAGATCTTTTTATTATCTAATATAGCTTTAACCATTTGTGT
ATTAAATAAAATAGGTTTCATTTAATTATCACTCCTTAATTTAATATGTATTTGTTTTAT
TAATTTTATAATGCTATCGTCGTACCTATTATAATTCTGTTAATATATATCATTATTTAT
TGATAATTTGTAATCTTAATTCTAATTAAAACAGTTATTTTATAACCTTTTAATTCTTTA
ATTTATTTTAAGAATTACTTCATTTCTTTAATTTGAATTGAAGCTTGATTGTACTTTTTC
TTTCCCTTTCCTACATGCTTAACAAAAACTAGCTTCTTTTTCTTTTTATACCAGAATACT
GTGTCATATTTCATTTTCTCATATTTGCTTATGTAATTTAATCTTTCCATGTCATTCTTA
TAAAATTCTGTCTTTTTATTATCTTCATGAAATTCCTCTGGAGATTTATCAAATGTTGCT
CTATTATCAACCCACATATTAATACTATTTATAATATCTTCTATTTTTAGTTTACCAAAC
ATGGATTGTAAATCAGATGTTGTTTGTTTGTAAAATCTTCTGTCAATTTCATTTTGTTTT
AACTTATTAAACAACTCTTTTTCATACTCATCTTGTTCTTGTTGTGAATCAAATACCTTG
TTATTAGATAATTCGGTTTCATGTAACAAGATTGATCTTTCCAAGTCACACTCGCTAAGT
TTATTGTTGTGATATACTTTCATTTCTTTTAAACAATTTAATGCAAATGAAATTTCTTTG
GTTATTTCTCTTACGTCTCTATCTTTGTTCTTTTCTATGTATTCTTCTTGCTCTTTTTCT
ATGTTAGATGCTACGTTTATTTCTTTCTCTCTCCATATATCAACTATATCGCCATTTCTT
GTTATTCCTTTAAATCCTATTGTTGCTATTGTTTTATTGTTTTTATATTTTTCTTTTACT
GTATCATAAAATTCTTTCATTGCTTTAAAATTACTAACATCTATATTGCTGCAATTAACA
TTCTCCTTATCTCCATTTATATATGTAATAATAATTTGATATTTAATGCATTTTGCTTTA
CTCATTTTCCTCTCTCCTTTTATATTGCTTTTGTTGTATTTTTGTGTTTCTCAACCTTAT
GTCTTTATTATAATACCGTATTCATATAATGTCAATCATTTATTTAATTTATTTCAACAT
TTTTCACAAAATAAAAACAGCCTAGATTTTACTCTAAGCTGCTCTCTTCTTGTAATAATT
TCTTTAGAAATGTTTGCTTGCTTCTTACGTTACTTCTTGATATTGCATAATGTAAAGCTT
TATTTTGACTACATAATATACATTTTTGTTTGGCTCTAGTTAACATAGTATATATCATCT
CTTTATTTAATAAACTATAATGTGAATAATCTACTCCGCATATAACATATTTGAAACCAC
TTCCTTGAGACTTATGTACAGTGATTGCATAACCAAGTTCTATTGAATTTAAATGTGATC
CTTTTACAACCACTTCTCCTATATTTTCAAAATCTATGATTAATGTTCTATTGTCTGCAT
CTACTTCTTTTATGATTCCTAAATTACCATTAAAAATAGGTGTATCTACTCCTTTAGTAT
TAACTGTCTTGTAATTATTTTTTATATTTATAACTTTATCTCCCTCATATAATGAATATG
GAGTTTTTGAACCTTCTAGTAAATTAATACATTTTGGCTTTTTGATTAATCCTTTCTTTC
TTTTATTTATTATTTTTTGAACTTCTAAATTTATTTTATATGTACATGATTCCCCTCTAC
TTTTCATAGGTACTAAAATTTGAAAATCCATAATATTATCTATATTACTCAATACTTCTT
TGGTATAATCTATAATTTTATCAAAAGTTTCATTTTTATTGTTATATATATCTAAAATTA
AATCCTTAATCTCCCCTCTTGTTTCAACTCCTGTAAATTTAGAATCAGTTATTTGTTCAT
GATTACGAATCTTCATACTTTCTGTTATTATAGCACTTTTAGCAGCTTGTCTATGTATTT
TTGTTAATGTAGCATGGGCAATTATATTACTATCTATTATATCTTGCATAACATTAGCTA
CACCTATAGATTCTAATTGTCCAGTATCTCCTAACATTATCAGTTTTGAACCACTTTTAA
TAGCTTGGATAAGTTTATAGAATAAATTACCGTCTACCATAGATAATTCATCTAATATAA

CTATATCTGTATCTAATTGATGTTTATAGTCATATGTAAAACCTTTTGATGGATTGTAAC
CTAATAATCTATGTATCGTAAAACCTTCTTCTCCAGTAACATCTGTTAAATTAACACTAG
CTTTACCACTTAAAGCAGTTTGGGAAAATTTATAATCTTTTAATGCAGCTAATACTCCAG
CTACGGTAGATGTTTTACCAGTACCAGCAGAACCACTTAAAAGAACTACATTGTTTTCTA
ATATTGTTTTTATAGCTTCTTTTTGTTCATCTGTATAATTCCACCCTTGTTCCTCTTCTT
GATCTTTTATATCATTTAACCAATTATTATATTTAAAATTATTTGTTGCATTTTTTAATC
TTAATAATTCTGTAGCTATGTTCTTTTCAAGATAATAATATTTTTTTAATCCTAATTTTG
TTTTATCCTTATTACACCACATTTTGGTTTCTGACATTTCCTTAAAAGCCATATTGAGAT
TATCTTCTGGTATTTCATAGCCTATATTATCCTCTATACATTCTAATAAATCATCTATAT
CGACATAAGAATTCCCTTCCATAGCTTGAGATTTAAGATAGTATTCCATATATGCCTTTA
TTCTATTAATTGAATACTCTTCACAACCTCCATTTAATGCTATTTCATCTGCTTTAGCCC
AACCAATTCCATCAACATCATCTGCAATTAAATAAGGATTTTCTTTTATTTTTGCAATTA
CAACATCTGGACTACCATAAGATTCAATAAGTTTATTAATCATATTAATAGTCAAACCAT
AACTATCTAAATATACATAAGCAGAACTATAATCTTTTGTATTATTATATTTTTCTATTA
TATTTAAAGCTGTTTTTGCACCTATTCCATTAACAGTACATAGTTTTTCAACATCCTCAT
TTTCTATAATTTTTAAAGGATCTTCAAAACTATTGAATAAATTTTCTACTTGGTTTTTAG
TTAAAATCTTATTGAGAAAAATTTTTTGGTTTTCAACCGAATCTAAATTTATATTTTTAC
CTATATATATTAATTTATATTGCTTACCATATTTATCATCAATAATTTCATCAGCTATAA
TGGTATATATTTCATTATGTTCTATTTCACACATATTCCCTGTTATTGTTATAGTTCCCC
ATTTACTTACGTCTGGTTCACCTTGTAACACTTCTTCAACAAAAACAGAGAGTATTCCGT
ACTCTCCATTATTGATTTTACTCTTTGGGAAAAGTTGTTTACTTAATTTTACTTTACATT
TAATTCTATTTATATTATTATCCATATTAATCACCTTGTACCCTTTCACTTTGTAATGTT
AAAATTCCATTTTCATCTATATCAAGTATTTTTTGAACTGTGTGTTGATAGATACTATTC
TTATATTTTTTAGGTGCAAATTGATCCCCTCTTCTATAACCAGTAATCATTAATTTAGTA
CCTCTTTTAAACCATGATTCTTCTAAAGTTGTTTTCTTATCTTCTTCTTCATCATATATA
GAAATTTGTTTATCATAAAAAGTAAATTGACCACTATAAAATTTAACTGTTACTACACCC
GTTGGTGTTAATAAAGCAACACTATGTTTATTTTTGTCTCTATCTAAAACAGTACCAACA
ATCCTAGTTAGTTCAAACTTAGGATATTTCATTCCATTATGATAAGTGAAACCAACAATT
CTGGCTTCTTCAGGTAACTCTGTAAAATTGCTAATTCCATATGTTTCTGTATCAACATTT
ACTAATTCATGTTCACTGTAATAAAAATTCATTGAATCCATTTCCCATTTACTAATACCA
CCTTGCATAAATTTATTTTTTAGTTGATTAAAACTATCATTGTTTAATAGATTTAAACAT
TCTTTTGATTTATACCATTTAAAGAAAGGCTTCATTAACTCATCATAAGCTTTTTTAAAG
CTGTATTTACGACTTGTTCCTAATGCTATACATACATTACCTTCATCATTATATATATAT
CCTTTGTCCTCTCCCTCTTGCATTAAATATTCAAAATGCTCCATAAAGAAGTTGGTTATA
TATTCAGTTTCTTCTTCATCTTCTCCTTGTAATATATACCATTTAATACTTTGTTTTTCT
TCATCTTTAAAAAACGGTAAAGATTTAATATATTTATTAAAATTATATAATCTTATATAA
TCTTTTAATTCAGTTGGGATAATCCCCATTTCTATAATTTTATCTATTGTACTTTCTCTA
ATATCTAACTTTGCTTTTGGTGGATTTGTCATGGTAATATATTTTTCTAATAAATCTTTT
CTATTTATATTACTAATATTATTGAAAGCTCCAGATTTAATTAGGGATATCATAGCACCA
TTTGATACAATAGATTGTTGCTTTTCCTTACCATTAGAATCTACTACAGTTCTTTTAGTA
AGTATCATTCTTTGATAAAAATCTTCTATAGAAGAATATGGTCTATTGTTTATTATGTCT
GTAGCCATATTGTTATTTACGTTCATAATTCCTTTTAAACCAAACATTATTTCATTTTTA
TTTTCTATAGGAGTAAATCCTTTTTCTGCTTCATTAATGTCTGGTAAAGATATTGTAATT
CCTCTTTGCTGTAATTCTGATATGGCAGAAGCAACTTTACCATATTGAGTAGTTTTTTCT
TTTGCTTTTATATCACTATCTTCTACTTCTTCTAGTTCTAAAGCCCCCGATTCAACAAGT
AGAACAGATGTATTCCAATAAATAGAAGGGAAATATTTTATTAAATTTAATTGTTGTGCT
AAAATCCAAGTATATTCAACTGAGTGAATGACACTGAATCCATAACCTTTTTGCATTGCT
ATTTGCACATCCCATATGTAATCTAATAGTTTTTTAGAAGTTCCACATGCTTGTCCTTTT
TCATAAAATAATTTATGTGCCTCTTCAAACTTATCACCTATTTTTTTAGCTACACCTTTT
CTTAGAATATTAGATTCAACTACATTAAAATTAGATATATTTTTATCCATAGACATTAAC
ATCATTCTTTCTTGAGTTGAACATACCCCATAATCTTGTGCTAGATGCTTTTCTACTATT
GATATATCTTTTTCAGATAAACCAAAATCTTTCATTTCTTTATACCATTCATTTATATTT
ATTTTATTTCTAACATATATATCCATAGGCTGATCTTTACCTTCCTCAGCCATTAATCTC
ATTAAATTATTACCGTTCATAGCCTCTATAAAGTTTTTAGGTTTAATAGATTTTATAGCT
TGTTCTCCTACTGGTGAGTCATATTGAAATGCAGAAATTAGTAATCCATCGCTTAATTGT
GACCATAATTCTTCACTATCTCTATCTATAACGTCTGGATGTATATATTTATCATAGGTA

TTTCTTAAAGAATCTTGCCATTGAATTTTGTTATATTCTACTAACATCTCTAGTGTTTTT
TGTATCATTGAGGCTGTTTTAGTATTAAGAAAGTCATATTTTAAATCTCCAACATATTCA
CTATGATGTAAATCAAATTGTGATACTATTTCACCTGATGGAGTTCTCATTTTAGCATTA
TGTTTAGTAAATTCTGAGTTAACGGGAAGTACTCCACAAGCATGTGATGATCTTTTATTA
ATTAATCCTTCTATTCCTAAAGCTACTTCTAAAAGACTTCTTCCTTTTTTCTCTGACCAT
TCATCTACTATGTTTTTAAATTCAGTTACAGGTTGTCTATTCTTTTTAGGATTGCCATAG
TAACAATCACTAATTCCCCAAACTTTACCTCTTTCAACTGGAATTAATGAACTTAAATAT
AAACCTATATCTCCATTAATTTTTAATCCTCTACAAGCTGTTTGTATAGCACTTTTTGCT
GTTTCTGTACCAAAAGTACATACTCTAACTAAATCTCCACCTATACTTTGATAATATTTT
TTAACGGCATCAAAAACTTTATTTCTTTTGTGTGATGGTATATCAATATCAATATCGGGA
TAATCCGGACGCTCAGCACTAATGAATCTAAAATGAGGCATTTCTACACCTTGTTTTAAT
GGATTTATTTGTGTAATTCCAACTAAATAATCAATAATATATCCACCAGCACTCCCTCTT
CCTGGAGGAACTATTGCTTCAGCTTCATTCCAAATAATATCAATTATTTTTTCCATTGTA
GTGAAATATCCAGATACAGGTTGTTGTTTAGCTTCAGATATACCCAATAATTCTTTACAT
TCAATATTCATTCTTTCAAATACTTGATTATATTCTTGTTTATTTTTAATTCTTTTCTCT
ATTCCATTAAAAGTTAAGTTAATTAAATATTGATTATAAATATTATCCTCACTTTCTATC
ATTGCTTGTACATTATCGTAATCTTTAGCATATTCATATAATTCTTTATGTTTTTTCCAT
GTAGACTTTGGAGGAATTGGAGTTAAAGGAATCTCTTGATCGTTAGCAAGATTATATCCT
ATTACTTTATCTTGTATTTCATATGTATAATTAATAGCTTGTTCAAAATCTTGAACATCT
AAATACCTCATATTTTTCATTAAATCTTCCATAGTAAAGAAATGTGTAGATTGATAAAAT
TCTCCTACTTCCCTATTACTATTACCTTCTTCATTAGAAGTTAAGTACGCTTCATGAATG
CTTTTATTATCCAATGTTAAATAATGTGAGTCAGTTGTAATTATATGTTTTAATCCATAT
GCTTTTGCTATTTGGATTGCTTTATTGTTGAAATCTATTTGCTCTTGATTTAATGAAGGT
TGTAATTCTATGTAAAAATCATCTTGTCCAAAAATTCCTATACACCACATAATAAAGTCA
TCTATTTCATCTTTTATGTTTTCTTGTATTATTTCATCTTCGGTGTTTAATAAAGTGGTA
ACAAGTCTAGGAAAATATCCTCCTAGACAAGCTGTAGAGCCTATTAAATGCCCTATATTA
TCTCCTACTACTTCTTCAAAATCTGAATAATATGTAGGTACTCTATCCATGTTTTTAAAA
TTAAACATTCTTAACCAAGCTCTAGTAGATAACTCTCTTAACATTCTATGTCCTTCATTA
TCCTTCGCTAATAAAAGAAAATGATAAAAAGAAGTAGAATTACTATCATTTATTTCTTCT
TTCATTGTCTCTTCATCTACTAAATAAATTTCATTACCTAAAATAACTTTAAAATCTTGA
GGAATTTTACCTTCTTTTTTTAATTCTTCAACTGTTAATATGAATTCAATATGTCCACTT
AAACTCTCATGATCCGTCAATGCTAATGCTTTTTGTCCTAAGCTACTGACATATATAATC
ATATCTTTTATTTTATTTATTGAGTCTAATAAACGTAAGTTACTATAATCACTATGATTA
TGTAAATGAACAAAATGTTCTTGTAGTTTTTGTATGTACTCTTTTGAAAATGGCATTATG
TCTCACCTCTAAAATTCTATTTTTCTTCCTTCTGACACTTCAAAATCTATAATTTCTATT
TGAGGATATTCTTTACCCTCCCATTCATTAATGTTAAATTTACCTATTATATTAAACTTT
AATTTTTTAGGAGTTTTTTTACTTAAACCTCTAGTTGATTTTAAAATCATCTTGTTATAT
ATATTTTCATTGGCGAAAAATTTAATGAAAGTTATTGTTTTATCTCCAATGGTTTTGTTG
ATTCTAATAATATTCTTTTTAGCACCTAAAAGTTGTACTTCTTCTGGTTTTACATATATA
TCAGTTATTGCAAATTGTGGCTCTTGTAATGTATTCCCCCACACATCTTTCCACTTACCT
ATATCAATAATATTTTTAGGTTTTAATCTACCTACAGGCATTTCATAATCAACCCAATAA
CAATCTTCTATTTTCATATCTTTTAGTAAATTATTGGTTGTTTGTACTAAATTGTTTATA
TTACTTTTATTTATGTTGAAACCAAAAGCGTTATCATGTCCATTAATCCAATTAAATAAT
TTAGTATTATTTAGGAATTCTTGGAGACTTTCAATAGAAGATAATTTATAATTTCTTCCA
CTACCTCCATAAGTATCGCTTTCTTTTTGTCTAAGTAGTATTACAGGTCTTTTATATGTA
CTTGCTAATTTGTTAGCAACCAATCCAGTAAAAGTTTTTTCTAATTCTTGTGTAATGTCA
ACTATTATCATTTTATTGGCATCTAATTTTTCTTCGTTGATTTTATTATCTACTAACTCT
ACACCTTTTTTTACTAATCTATCTTGTCTACCTTTTATATTTTTAATTTCTCTTGCCATA
TATTTTTGTAAGGATTGTAATTCTATAGGAGGCTTTTCTTTTTCGCCTTTCTTCTTTCTA
GGTTGATATTCTCTATCTTCTTTTGTGTTAATAAAAGCCTTAAAAACATCTGTTTTTTCC
TCTAGCGTCCCCATTCTTACAACACCATTAATTAGTGGAGCTATTTTCCAACCAACATCT
AAAATATTAAGCTTGTCTAGGTTATTTTTACTCATTATCTCTTTGATAAATAAATTACAA
TTACCTTTATTTATTTTATTTAATCCATTTAATACTAAATATCTTGTTTCTGGATTTCTT
AAATCCATATCATCTGCTATCATTCCAAGTGCAGCTAAATCTAAATAATTATCAGCTTTA
TTAAATCCATATTTTCTATCAAATTCTTTTAGAAATTTATAAACAACTCCTACACCTGAT
AATGTATTATTAGGATAATTTCCATCTTGACAATTAATAATTATAGTATTATCAATATCT

TTCCAATCTAAAGGATTAAAATTATGGTGGTCTAATATAAGAATATCTTTATCTTTTAAT
TCTTTACATTGTTCCATATCACTACTACCAGCATCAGGTACAATCAATAAATCAAATTCA
TAGTCTTTTAATCTATTAAGTATAATTCCATGCTCTTTATTTTTATGGATTGAAAAAGTT
ATATCGTTATTAAAACCAATATCTCTTATGTAATTTATACATGAAGCTGCTGAAGAATAA
CCATCAAAATCACTATCATCTATTACGTGTATTTTACTTCCATTTGTTAAATGCCAATAT
AACATATTTAGTCCTCTATCCATGTTATTTAATAGCATTCCATCATAAACACAATCACTA
TCTAAATTCATTAATTTATTAGGATCTTCTACACCCCTCTGTTGTAATAATGTATATAAT
AAATTATCATTATTTAAACAATCTTTATTTTTATTTATAACTTTATATTGCATCTAAAAT
CTCCTCTTCTAATTCATCTGTAGATTCTATTAAGAATCTTTCATTTAATAATTCTTCAAA
AATATCTTGTCCTTTATCTATAGGAGCATCTTTGTAATCTATACGGTCATCCCAACAAAA
GATAACTGATACATTACAATAGTTAATTAGCAATGACACTACTTTTTTTATTTTTTTAAT
ATATTGAACAAATTCTTTATATTCTTTGGTATTCTTATATTCTGGTTTCAGATATTCTAG
TCTATATTGTTTATCCCAAGCCAATATAACCTCATCTACACCTAAATAAAGAATTAAATC
TCTTTGCTGCAAAGTAAAATTCATTGACATTGTTGCTAAACTAATATTATTATTTTGACC
ATAAATACTCCCATATTTAAGAACCGATTTTTCACTCTCAAATAAAATAACTTTTCTTGT
TTTTCTAATATTATTTTGGTTTTGATAAATACCATATAAATTCATTGCTGTTGGATAACG
ATAAGTTAATCCTTGTATTGTAACTGGTATATATTTTTTACCTTGTTCAATTTCTCTCTG
AAAAAAATTTCTACCCCTTATCCCTACTAAAGAACCATAAATATCTCTATGGGGAATAAT
ACATTTGAATTGGTTAAAATAAAATTTAATACCAAAATAATCAGCTATATCTTCATGAAT
TCCTTCTTTTTCCCATTCATCAGGATAATAATTATCAAATATACTCAATACATTCTCATT
AAAAGAAGGTAGTTGTTTTATTTCTTTTTTTGTATTATGAAAGGTATGTTTTTTTAATAT
TTCTAAATCTGTGTTATCAGTATGTATCTTTAATCCTATACTTCTTTTTTTATGTAAATC
TATTCCCTTAATATCAGCTACATATTTAAAAGCTTCTGAAAACTCACAACATTTTACTTG
TGATATTAAGTCATATAAGCTCATAGAACCACAGTTTGTATAACACATGAAACTTTTAGT
ATCTGGATAAAACCATAGTTTATGTTTCTCCCCACAATGACATATTGTTTGAAAACGAAG
ATTGCCTTGTTTATCTGGAATAGGTTCATTACTTCCCAGATCTTTCATTATCTTTTTTAC
ATCTTCTGTGGTGATTTCTTTTAGTAATTCATCCCTATCCATCTATTTCATTCTCATCCT
CCCTTTTAATTTCTATATCTATCTATATTAATTTGAGTGTATTTGTGGTCTGTACAAAAC
ATATCTATAAAACGCATATTACCTAAATTCTGATAACCCCAAATCTTAACGTTTTTAATT
CTTCCTCCTCTATTTTTATAGAAAGAATAACATACATTAGGTACTTTCCCCTCAATTAAT
CCATTTCTTCTTTCTAATTGTTCTAATATAGGTTCTATTAAATCTAATTCTTTTTTTGTG
GGTTCAAAAGTAACTAAACCAACATCAGCTTTGTTGGGTAAACTTCTAGCCCCCTTTACC
GCTCTTTGATCTCTTATGTTATCTCTTCTAGCTTCATCTGTAGTTTGAGTAAAAGAAAAG
AATGCGACATTAAATTTTTTAGCTATGTTTTTTATGTTTCTTGATAAGTTTAATAAGACT
TGATCTTCTCTAGCTCCCATACCTCTAGTTTCTTGTATAAACTCAGATACTAAAGCATTA
TTTAATTCTATGTAATCTAATGCAACTGCACAAATATTATATTCAGTAACATACTGTTCG
ACTTTATACCATAAATATGTAATATCATAATCTTCTTCATCTATTAAAAATAACTTAGTT
TTCTTTAGTATTTGAATAGCTCTATCAACTCTTTCTTCTTCTTCCTCTGTTAACTCATTC
ATTCTGATTCTATCTTCATCTACACCTGATACAAAAGCCCACATCATAGGTTCAATTTCT
TCATAAGTATCCATTTCTGTACCAATATACAAAGCACTATTTCCTATTGCTGCTTCATTC
TTTTTAAAATCTTTAATTTCAAAATCCCAAATCTCTTCTGCTGTTATTTGTAATAATCTT
TTAATAGCTACTCTTGTTTTCCCCATTCCACTATCTCTAGTCTCAAGATAAAAACCTTTT
CTTCTCCATCCTCTAGTTATAGTATTAAAATATTGACTCTCTAAAGGAAAACCAAAATTA
GGTTCTTGTTTTAATTGTTCTCTCAATTCCTCTGCATTGTCTCCACATTTTCTATCAGTT
TCATCTTCTTCTACTATAAATCTTTTCTTAGATTCTAAGTTTTTTTTGTCGAAATACTCT
AATAAATCTTTAATAGACATATTATCTAATCTTTCTAATTGTCCACTTGTTATATTTGCA
TCTAGTTCATTCTTATCTAAAATTTCTCCTACATCCGTACCAGATTCAATTTTACTTCTT
AATATAGACATTTTAATTACTGTATTATAATAGTAATCAAAATTAGCTAGATTAGAATCC
TCTTTTAAACCTACAATCCATTCTACTCCATCAAAATCTTTTTCAAATATTTGAGCGTAG
TCTATTGGTCTTGAATTATGTAAATATGTTTCTACATCTGCTAAAGTTATTTCTTCAGCA
CCTTGTATAGCTAAATTATATATAGTTAAAAACAAAGTCTTGTGATATGGAGTAATAAAA
TCTAACTCACTTAATTTATATTTTCTTGATTTTATTAACTGAGGTTGTTGCATTAAACAA
CCTAAAATTTGCATTGAAGCTCTATTATCATAATATTTTTTTATTAATTTACTTTGTCTA
CTACTCTTCATCTATTTCACTCCAATCAATGTCTATATTTAACTCATGTCGTTTAAATTC
TTGCTTGTTATTGTTACTTACCTTAAATGTTTTAATTTTTTCTTGATTAACAAATAGCTC
TGCTTTTTCTTCTAAATCAAATCTATTTTTATAATAATTTTTTGCCTTATCATAAAAATA

AGGAACTATTCCCAAACCTACATTATCCATTACAGAATTCTCTAATATTTCATAATAAAA
TTTAAGAGTATATTTAATACCAGAATTAGTAAAATTATATTTACTTTTATAATCCTTAAT
TTGACTTAACATTTGAATAGTAGGAGTATCAATTCTATAAATTTCACATATTGTTGTTAT
TAGATTTTTATATTCCTCTAAATCTTCAAAACATTCTATGCAATAATAACTTCTACCATG
TTTTATTGCATTTTCTTTTTCGTTTTTAGCACCACACTTTGGACATTTTACTAAACTCAA
TCTTCTCTCCCCTTTTTAATGAAATAGAGGCTAAATAGCCCCTATCACCTATTCTTCTAA
TTCATCTAATAAATCCTCAAGATCATCTAATATACACTTTAATGCTTCTAATTGCTTGTT
TGTAGCTTCACTAACAGTAACATCTCCTAAATGTTCTTCTACAATTTCACCGTATTTATC
TAATTCTTCTTCCCCTATTTCTTCTAAAGCTAAGTATTTTTCTTTTATTGTTTCTTTGAT
TTCTTCTAATGTCATATCTTCTGTTTCATATATTTCTTGTTGTTCATCAAAAGATACACT
TTCTAATCCTTTTTCATCTATTTCTCTTTGAATTCCTTCTACAATTACTTTTTCAAGATT
CTCTGCTGTATATTCTGGAATAACTGTATCCATACATGTAAATCTACTTCTAGCAAAGAA
ATCTTTTGTTTCTACTAAATATCCACTTGAAGGTATTTCATTTCCATTTTCATCTACGCC
ATTTGATTGTAAGTAAATAACTATATCAGCATTATCTTTAATTGGATCAATGTTTCTTTT
ATCTCCCTTTATAGTGTATTTATCTTTATCTTTATCATAAGATTCATGACCTAAGAACAT
AACTGTATATCCAAGAGAAATAATACTATCTACCCAACTGTGCATTTCATCTTCATATGG
TTGCCAAGCACCATATCCACCCTTAGCACTTGCTATGTCTCTAGCATCATATTTACTAGC
CACATATTCTCTACAATATCTTCCGACATTTTCTATACCATCAATAATAAGAGTAATTTG
TTCGCCTTGTTGTAGTAGTTTTACAAAGTTTTTACCCGCTAACTTTTTAGCATGTTTTCT
TGCTTGACTCCAACTTCTAGTAGTTAACACCATAGCACCATGTACACCGTTCATTCCTTT
TTCAAATGGCATAAATACTGGGTTTTTCATTCTTGAAGCTTGTAAAGTTTTTCCTAAGTT
ATTACTTCCATAGACTACTATAACTTTTCCTCTTAAATCTGCTGATAGTTTAGAAACTTT
TGCTTCACCTTTAAATTCTTTATTAAATAATGTTTGTAATTCATTTGATAACATATGTAT
TTCTCTCCTTTAATCTTATATATTTTTAAATTTTGTTTGATTCTTTAATTTATTTTAAGA
TAAGGAAGGAGATATTAAAACCTTCCTTATACCATTTTATGTATTTATTAGAATTTAGGT
CTTCTTCTTTCAGTCTTTTCTGCTTTATCTTTTCCACCTCTTAATCCTGTTCCTTTTGAA
CTTTCTTGTTTAGCCTTGTCCTCTTTTTCTTTTATATCTATAGCTCTTTCTTTTAATGCC
TTTTCTATTGCTTCTTCTTCCCATTCCTTTTCATCATCTAAGACTTCTCCACCTGTAGCA
ACAAGCTCATGTATTGTAGTTCTTTTTTCTTCGACTTTGGCTCTACCTAATGAACCACCC
TTCTTTTTCTTTTCTAATATACTTATGTAATTTATATCTCCCCAGAAATTCATTGAATCT
CCTTCGCTAACATTTTCTCTGATTTGTTCTGCAAAATCAAACTCACCTTCTTCATCTACC
ACTGTACCAGCTATTAAAGTTATTGGGAAAGCTTTACCACCATAGCAAGGTACACATCCT
TTAATCTTAACTCTTCCTGTTTCTTCTTCATCGTCACCATTTTTCTTAACTTCTTCTTCT
ATTTCTTGAACATACATTTCTATATCAAATGTTGCTTTGTAATCTTCTTCTTTTATTTTG
TTGTTTTCGTCAACATAGATATTTCCAAAACCTAAGTCTACTGAAATAACTGATTTACAC
TCTTTTCCGTTCTCTGGAACAAATAACTCCTCTCTAAATTGTGGTGTGAAATCTCCTGAT
CCATATACTCTTATTACTGCTGCTTCATCTTTGTCTTTAGCCATTGTTAAGTATTCTTCT
TTTATAAATTTATTTAATGTTTCATAAGATTTTTTTACTTTTCCTTCTTTTGTTTTTTCA
CCAACATAAACTTTTAATTTTACCTCAGCTTCTCCACAAGCTACTACTAATGATCCATTT
ATGTATTTTCCATTATCACTTTTACCTTCTTTTAATTGATGTTCTTTAACTACTCCTACT
AATTCTACCTTGTTCATTGCTGTTCTGTTTTCCATAATAAAAATCGCTCCTTTTAATCTT
TTAATTTTTTTGTTTAATTCTTTGATTTATTTTAAGATAAGTTTTTGTTTTAAATTATCT
CTCATACAATCAAGATAACCACACCTTTCTTTAAGGTTTTTTATGTCTTATGTCCTTCGA
CAATATTAATTATATTACTTTTAATTTAACTTGTCAATCATTTATTTAATTTATTTCAAC
ATTTTTTTTATAAATGATTGTTTATCTTTGGTATAATATTCCTCAAACTAGCCTATATTC
TCCATTTCAGCTATTCCATCTAATTCTATAGTATATTTACCCTTTGAAGATAATTTAGCT
AATTGTATAGTTGTAGTATTAATTTCTTTAATTTTACCATAATAATTAGCTTCATTGACT
TTAACCCTTACAAAATCACCAATAGTATATTTCTTACCATCAAAAGTAATGCTTATTTCA
ACTTTGTATTTCGATCTATTAATAGTTTTCTTAGATGATATGCTGTTAGCATGTACTTGG
TCTGTACATAGTCTGTCTGATAATGGTACAGTTTTTCCAATGACATTGTTACGTGCTTCT
TCATTAAAAATAGCTCCATAAATTTCTTCACGCCATCCATCATCACATTTTATAAATCCA
ATTCTCACACAACATTCTAATAAAGCTTCTTTAGGTACAACTATAGGTGAATAATAATAT
GGAGTTGAAACAGAATAAGTTTCACCCTCTAAAACCTTCTTAATTTTTGTAACTTCTCCT
TGTCTGAGCATAATCCCGTTTCCTTTATAATTCTTAATAACCTTTAATTTTGTTCCTACT
TTTAAATCTTCTATTTTCATCTTTTATCTCCTCTTATATCTATTAATTATTTAATTTATT
TAAAATCTTAGATTTAAGCAAACTAAACCACATTATATTGTATATATGTATTATTATAAA

CACTATATGTTGTGTTAATTTAACCTTGATATAACTTAAACAACTCACTGATTTTCATTT
TGTTTTGTTTTGCTAAATCTGTAACACATGAACATATATTATTAAACGTGCTACAACCAA
CTTCTATTTCTAAATACTCTAATAAACTTGAATATGTTGGAATATATTTTTCTTCAAATA
ATTCATCCATTTCAAAATCTTCTTTGTTCCAATCGTGTTCTTGCCAACATTTCAATTCTT
TGTTATTAACTATTAATTTAGAATTTACTTTTCCTAAATTACTTCCACCACCTTGTCTCC
ATCCACAATCTACCCAAGTGTTTTCAATATCTGGTTCTTCACATAATATTTTAAACTCTT
CTTTAGTTAATTCGTGAATTGAAAATCTTTTACCTTTGTAAACCTCTTTAATTTGAATAC
CCAATCTATTAAAGTCTTTGGATATATCTCCACTTGTTAATACCTCTACCATTTTTGTTT
CCATAATTAAGTTCTCCTTTATATGTATTTAAAATAATTTTATTAACCAACCTAAAATTG
CTATTGGTATAGTGAATTCAGCAAACACTAATCCAATTAATACATCATAAAGCATGGGTA
TATTCTTACCAAACCATGATGATATGTAATCTACTGATAATCCTCCTACAATTCCGTTAA
ATATTAAAGCCGCTAATATTATTCTTCCACCTAATTTATTAACCATAAATACACTACTCC
TTCATATTGATAGCTTTTATTTGATTTTCATATATCTTATTTGTTTTCTCATTTTTATCT
CTTAAATACTCTTTTAAATAAACTTTCATTTCAATTTCTAATAATTCTTGTTCCTTCTTA
GATAACTCTCTGTCAAAAACCTTAGTTATATACAAATCGGCAAACGATGTATCTACAGTC
ATTTTTGATGAATAGAAATAACCTTTTTCAGAAACCCAAACATCTCTTTTAAAAGTAAAA
TCACCTTCTAATTGATTGAAACTAAATTTAGTATTAAATTTGTTGCTACTATCACACTCT
AAAGCTAAACAACCTTCCCAAATTTTCATTGTTATTCCTCCTCTAATTCGAACATTTTTA
AAAATTCATCTAAAGAAAAGCCTTTTTGATTACAGTCTTTCCATAAAGTATTACTTGTTA
CTTCAATTTTAACTTGATCTTTGTCAAAAGATTTTAAAACCTCATTATACATCTTTATTA
ATATTTGTAATTCTTCTTTGGTTTTTGGATAACAACCCTCCTCTCCTTTACCTGTAAAGT
AATCGAAATAAAAATATACTATTTCATCTATTGGTGCTTCCAATCCTATTCTAAACATAT
TATATATATTTGTTTCTTCATTTAATAAATCATAAACATAATCCTCCATACAGTCATACC
AATCAAAAAGATTATCTATTTCTCTGTCAAAAAACTCAATAGCTAGTGATATATCCTCAA
TTTCTTCATTTATTAATTCCTTACCTTCACATCTAAATATTGCTTTCATATTTATCTCCC
CTCTATCCAATTAAAATATTAATCCCATTCATAATCATCTATTTCAATTTCTTTTTTACA
ACTAGGGCATTTAATCTTTTCGCCAATCCAATCACATGGATAAACACTATGCATTAAATT
TTCAAAATCATTATAAGATATTTCTATTTCATACTCACAATGAGGACAGTCAACCCTTAT
GTCAGTTGGTCTTTGAATTATAGTTATTGTATTCATTATTTTATACCTCTCTTCTTATAT
AATTCTTGAATAGATTTATATATTTAATTCTTTCTTTATGTTGTTTATTTCATTATTATA
ATTTGTTAGCTGTTTATTTATTATCTTATTCCTTGCTTTTATTAATGCAATATAAACTCT
TTCGGCTTCATTCTTTTCATCATTAAATAACAACTTCTCTTTCTTTCTTGAATTATTATC
AGTAGTAAATAATAAATCTACACTTACTTTATATGTATCGTCTCCTTCTCTATACTCTTT
CTTATTATAAAAATCATCAGCTTTTAATCTAACGATTTTCTTTTTTAATTCTTCTATTCT
ATATAATTTCTTTTCTATATCTTCAACACTTTTCATCAGAATATAATCTCCTTTATTCTT
TCAGATAATGGCTCTTCATTCTTCATAAAGCTTTCCATATCTTTTATTATATCTATATAA
TCTTGTCTTATATTCTTCATCTCTTTATACATGGCTCTTGCATATATCTTTCTTGTATCT
TCATCTATTCCTTCAAATATATCAATAAGTGGTTCGTCTCTAGATATATACAATGGTTCA
TCTGTTGTTGTAAATGTAGAACCATATGATAAGTAATCAAAATATTCTACTCTTACTTTT
ATTTCTTCTAATTTACATCCTTTTTTACTTATAGCTATAGCTTGTATCTTTTTATTTATT
TCAGATAATAAGCTTTTATATTCTTTATAACATTCCAAAAACTTTCTTAAATATGTTGGT
GTTTTAGATTCTAGGTCTTTTAGATATATTGTCTCTTCCATAAACTCATTTATATATATC
ACATTTACTTTCCTTTCTTTGTTATATTTTTATAAAAAGTTAATTTTATTTAATTCTCAT
CATATATGTATCCATTTATTCTGTTATAGGCTATGTCAAAATATTCATTATCCAATTCTA
TTCCGATGAACTTCCTATTATTTTTTAAACAAGCTATACATGTAGAACCACTTCCCGCAA
ATGGATCTAAAACTAAATCATTTTCATTAGTTAATCTTTGCACTAGCCATTCCATTACAG
CTATTGGTTTTTGTGTAGGGTGTGAACCTAATTTCTTTTCGGATTTTGGAGTTATACCAC
ATTTTATTTCAGGTCTTTCATAAGATTCATCTATCCTATTAAAAGTCCATTTTGCACCTT
TCTTAACTGCCCAAACAGCAACTTCATAGTCAGTAATAAATCTTCTATCTCTATTCCTTG
GCATAGGATTTGTTTTCTCCCATCTTATTAAATCCTTAATTTCAAATCCATTTTCTTCTA
AAGCTTTAGTAATATTAGTCATATTCTTCCAATCATTAAAAATTACTATATTCCCACCCT
TTTTAAGCAATGGTTGGGCTATTTCTATCCAACTTATTAAATCAAAATCTTTATCCCATT
CACCAAAATCAATTCCAGCTCTCCCCATTGTTTTGAAATTGTTATCTCTTGAAATATTAT
ATGGAGGATCTGTTATTATAGCATCTATACTTTCTTTTTTTATTTTTCTCATGACTTCTA
ATGAATCACCTTTGTATAACTCATATTTCTCTGTTTTTATCATAATTTCAATTCTCCTTC
CAATTTAATTCTTTAATTTATTTTAAAATGCGAATTTTATTTAATGTCAACTATTACTTT

TTCATCTTCTATATAACAAATATTTGTTGATTCATTTCTTGTTAATATCCTATCTATAAT
CTTTTGAAGATCCCTTATATCATCTTCTGTTATATCACTTACTATATTGTCAAACCAACC
TTCATACATATTATTTTCATACTCTCTTTCAAGAGCATCATCTAACATATCTTTAGCATC
TATATTAGCTTTATATTGAGTTGTAGTATACACTCTATCAACTCTACTTTTATAATATTC
AAAATCTCTTATAAGATCCTCTTTTGGTACTACATAACCGTCAACAAGAAGCATATCTTC
ATCTTTTAATTCACTTAACTTTTTACTCATATTATTATCCTCTCCTTATTTTCTATACCA
TTTGGGTAACAAATTATAAACCATTATAGTTAAATCTATTGGAAATAAAATTATATAACC
TACTGACTTAAATAAACTAATAAATGGGTTTATAAGGTCATTTAAATATCCTTTAAAGCT
ATGTTGATTAGGTATTAAGACATAATTTTTAGTTTCAAATATAATATAATTCTTCTTCCT
TGCCCATTCTAAAAATATTTTATTAGCTTGTCTTTTATTCATAAATCAAACCTCTTTTAT
AATCTTCTTTTCTCTATTCTAAACTTACCCTCTTCTCCATCTATGTATATATAATTATTA
TCTACATAATATATAGTTCCTTTTCTACCTTTATGTAATATTGTTGTAACTATAATATCA
TTGCCATAAAATTTGCCTTTAAATACTAGATCACCGACAATACATTCTTGTTTTAGTTCG
AAATAGTCCCCCACTTTAAATCTATCTTCCTCAATTGGTAATGGTCTTAACTCCAGTATT
TCTTTATAAGGTATTATATGTAACCCATCTTCACATTTAATAGCTATATTACTACGAGGA
CTAAAATTTAATATTTGACCTATTATTCTATCCTCGTTTTTGCGAATACACAGATATCTA
TTGTCTACTTTTATTCCAAACTCTTCATGTAACCATTCATCTTTTTTAATATTCTCCATT
ATGTATCTCTCCATTCTTATAATATTAATCCTAAGATTATTAAACTAAATCCTATAATTA
CTGTTTCTATTAATCCATATTTGCTATTACTAAATATCTTCTTTATCATGTTTTTGTCTC
CCTATAACCAAAGCTGAAAAGAATTCGTTGTTATTTATTTGATATTGTATTTCTACTTCT
TGTTTATCTTCTTGTAATATATTTACCTTTTCTATTAAATCTCTATAGAAATCTTCTACA
TCTGAATTTACTATCATATACATTCCTTTTATTTTCATGATTTCACCAACCTTTTAATTA
TTTAATTTATTTAAACTTATAGAATAGAAGAATTTTAATTATTCCTCCAAAGCACATTCT
AATTCGTCCATAGCTTCATCATATTGTAGTTGGTTAAAAGTAATCTTATCTATATTATTA
GCATTTATATATTCTAAACCTTTTTCTAAACAATCTTTGCATATATAATAATATGATGCT
TCCTCATCTTCTAACCAAAACCTTGCTCCATTCTTATATTCATGATCTTTACAAAAAGAA
CTTTCGCATACTGCACAAGTTTCACCATATAATTCATCAACTTTATTGTTGCAAATCTTA
CATTTACACATATTTAAACACCCCCCACTCTATAATTTATTTTATATTAACATAATTTTG
GTTAAATGTTAAGTTTTAATTAAAATCTTCTAGTATTTCTTGTAGGTTTCTAAAACTTCC
ATCTTTCTTCTTTATATGTAATCTTAAATTTTTATATTCTTTAGCATAGTTTTGTTGTAT
TTTTGATAATACCTCTTTCAATGCTAAAGTTGTTTCTTTACTATTAAACATTTCTATCTT
ATCCATTGTTTATTGACCACCCTTTATCTCTTAAATACCCATAAAAATATTAAATACTAC
TATTTTACCTTGTTATGGTTTAATTAAACTATATTCTATTTTCTTCTTTTAAAACTTCCT
TAACTATATTGGGTATATTCTTACCTATAGTTCTTTTAATAATCTTTTCTTCAATATTTC
CTATTAATTCAGGTTCTTCTTTCATAATATCTTGATAAGGTCTATCTTTAAGCTCTTTTA
AGATTGTTCCCATATCTTCTATTGCAAAATCCTCTTTTAATAATCCTTCATCAACTAATT
TATGTATTAATTTACTTACTCTAGCTTTGGTTAAAACTGACTTTATAGTTTCTATCACTT
TTGAATCTACATTTGGATTCTTAGGCTTTTTCTGTTGCTGCACTTCTCTAAATTTATCAC
TAACTAATTTAACGAAAACTTGTCTACCATGATTATCAAAATAGCTAACATTCTTAACTA
CTATACCTTCACCAGTATTAGGTTTTTCTGTTAATTCTGATTTTCCCACAAAACTCATTA
AATGTTCAAATGATATGTATTTTCCATAGTAAAAATATTGAACTGTTAATAATCCTAATC
TCTGTGCTTCTGATTTAACTATTTCATCAGATAAATACATTTGCTTTTCGTCATCCCAAA
TACTAAATAGATAAAATTTATAATAATTTTCTTCCTTATATATAACTTTATGCTTTACAC
ACCATTCGCCAAAATATCTATAGTTTGGATTTAACTTTTCTTTGATTGGAACTATATTGC
TTAAAACCCAATCATAATATCCTCTTAATCTATTTTCTTCAGTTAATAAATTATTTCTTG
AATAACAACTTACTCCTAAAGTATTTTCATTGTCTATACAAAAACTTGCATTAGCACCAT
CTATCTTTTCAGTTATTGAAATTATATCTCCTTCTTGGATTACTCCTTGAGTTGAACTTT
TACCGTATCTCACTACATCAGTATATTTTTTAATTTCTTTCATATCTTACCTCTCCTTAT
TTCAATCAATTATTTGATTTATTTTAAATATTCAATAAATAACAACATATTATTATTGGT
ATTTTGTTAAGTATTTAATTTGTTTTATGAATGTTTTAATCATTATCATAGTTTTTATAT
CCGTATGTTCTAATATATACAATATTATCTCCAAAACCGCCTCTGTTAAAATTATGTAAT
TTATATATTGTCCCATATTATTTTTAACAAATTTTAACATACATTTTAATAATATTACCA
TAAAAGTAATTATATCGTATTTTTGTTGTAAATTATCATATTCCCCCATTTTGACCTACC
CTCTTATTAAATATATAATATTTAATAAGGGTAACGCTTGGTAGGTAGGGGTGGGCTTTT
GTTGTTATCTGTATTCATTTTAAATTCTTTCTAATGTTCTTTACATTCTCTCATCATTCT
TTCAAGTTCTAATTTTTGAATGTCTTTCTTGTAGTTATCAAATGTACTTCCAGTTCTATT

ACTCATTATCATTCCGTTCTTATCAAATTTAACCTTTGTTGGTACTAATAATATAACCTC
ATCGTCTTTTAGATCTTCTCTCATTTCTAACCAGTCTTGCTTATAAGACTTATTCCAAAT
ATCCATGTTAATCATATGTATTCAATTCCTTTCTTTTTATTAATTCTTTAATTTATTTTA
ACTTCTGTCTACATTTATTATTATAGTATCATATTTATGTTATGTCAACAGTTTATTTAA
TTTATTTTAACTTTTTTTATAAGACTTGTTCCCATATCTTATTAGGCTTTCTTCCTCTAC
TCCTAATACACCTAAAATCTATATCTCTATATTTATGTATAAGCATTTTTCTCTTTAATT
TGAAGTCATTTAACAGTTGACCTTTTACATCTATAACCTCTACATGTTTATCTGTATATG
TAACTCTAAAATCAGCTTTATACTTTATAGGTCTAATAGCTTTATCTTTATATCTAAACT
TCTCTTGCAATTCATACTCTGGTTGACATTCTATATTCTCAACAATTCCATCCTCTTTTA
ACTTTAAAAGATATTCATAATACTCCATTTCCATAGTAGAATCCCATTTACGACCATATT
TAACTTGTTTTTTATTATTATATTTGTTTATTGCCATATAATCTTCCTTTCTTAATGGTA
GTCAAATGTTAATTCAACCACCATTTTTAATTAATACTTTAATTTATTTTAACTTTTAAT
TCTCTTTAAAATTGCAGTTTTAATCTAACCACCATTCGTAAAACAACCACTCTAATCCTA
TCACTATTAATAACATCAATATAAACATTCTCATATTTTACTTCTCTAAATTTGATTTAA
CTCTATTTATAAAGTCTTTTGTTTCACTATAGATTATAAATAATAAAGTTGGTATAAATA
GAGTAATTATACATCCAACAATTATTTTAGGTATTAAAAGACAAATAGAAATTATAAATA
AATATGTCAATAGGATTATCGCAATATCTTTTGTTTTCTTCTTAACTTTATCAAAATTAA
TACTCTTTCTTATAGCTCTTAAATATCTCCTCATATTAATCCACCTCTACGCTTGTTATT
AAATCATTCTTAGATCTTGTATTATAGATAAAATTACCCATATTTCCACACTTCCCGTAA
ACATAATCGAACCACATTGTATCGTCTTTAGGACTTATATCATCTAATAACACTCTAATC
TCTTTACCTTTCTCAGTATGTATTGTAAATCTATCATCTTTAGTAACATCAATAATAGCT
GCTACAACTTTAAATCCTTCATTAAGTTTAATTCCTATGTTCCCTTGCGTATTTCTACTG
CTCTTAGAATTAATTCTTTCAGTATTAAATACTAATCCCTTACCTTCAGATGATAATATG
AATATATCTTTATCTTTTTCTATGTAATCAATACTTATTAATTTACTATCTGTATTAAAA
CAATTCATTAGTTTTTTATTATTAGACATAAATGATTTAATATTTACTTTAGCAACTTTT
CCATTTTCAAATACAGAAGCTATATATCCTTTATTAGAATCATCTACAGAAACTGTCTTT
ATAACTTCTTCTCCCTCTTCTAATTGAATTAATGAAGGTATAAACTGACCATATGCAGAA
GGAGTTAAATCTTCTAATTCATATACTGGAATTTTGTATCTATTTGCCTTGTTAGTGAAT
ATTAATAAAGTTGATTTGTTATTTGTTGCAATATCATCTATTATTATTTCTCCTTCTTTA
ACCTTATGGTTATCTGATTGTTTTAAATGTTTTTTAATATAATTCTTAGTATATATAATT
CTACAATTATAATCTTCTATTAAATCTTTGTCATTATAATCAAATAAATCATATGTAATC
CCAGATATTCTTTCTCTTCCATAATTCTTCTTAACTCTCTCTAAATCATTTATTATTATT
TTATTTATTTCATCTTTAGAATTAATTTTATTTATTAAATCATTAATTTCTGTCTTTAAT
GTTTTAATAGCCATTAATTGTTTAGTTATATTTGTAGTATTTATATTTCTTAATTTCATA
TTAGCTATATATTCAGCTTGAACACTATCTATATTAAAATAATCTTGTAAAGAAACAATT
ATATTATTATCATCAGATTTCTTTATTATATCTATGGCTTTATCTACATCTGCTAATATT
TTCTCAAGTCCTAATAACAAATGATACTTTTCTTTTTTTTGTTCGAGATCATAGCTTAAT
ATTCTTTTTACTGTATCTTCTCTAAACTTTATCCATTCTTTTAAAATTTGAGGTATACCT
AATACCATAGGTTTATCATGATTTATAACCGTAAAATTGCATGAAAAACTATCTTCTAAT
GGAGTTAATTTATATAATTTTTTCATTAAAGAATCTATGTTTACATTCTTTTTGACATCT
ATAGTTATGTTAAGTCCTTTTTTTACCTGAATAATCATTAACATCTACAATATCTTTTATT
TTACCGTCTTTAACCAATGTTATTATTGAATTAATAATTGATTCTCTAGTAGTTGTATAA
GGTATTTCTGTGATTACTATTGAATTATTTTCTATATGATATTTGCATCTTATTTTAATT
CCACCTTGCCCAGTATTATGTACCTTTTTTTAGAGATGCTTCATCATATACTATATATCCC
CCTGTAGTAAAATCAGGAATCATAACTTTTAATTCTTCTCCTTTTATTGCATGTATAGTA
TTATCTATAACATCAGTTAAAGGAAATGGACAAATATTAGAAGCTATTCCTACAGCTATC
CCTATGTTAGGTTGACATAAAATAGCAGGAAATGATACTGGTAATACCTCTGGTTCTAAT
CTAGTATTATCATAGTTATCTTGCATATTTACAGCATCTTTGTTTATATTTGTTAAATAT
TCTTGAGATATTGGTGCTAACCTCATTTCTGTATATCTTGCTGCCCCTGGTTGAATTTCT
TTTGAAGTAATACTACCAAAGCTTCCCTTACCATCTATTAAGGGATAATTAACGCTATCA
TTTGTTAATCTAACCATAGCTTGATAAACAGAACTATCACCATGAGGGGAAAACCTTAAA
ACACTTCCTGACGCATTTATTGATTTAGTTCTATTTTTATTATGTTGAAGTCCATCTAAA
TTCATTGACCACAAAATTCTTCTATGTATATTTTTATTTCCATCCCTAACATCTGGTATT
GCTCTCTCTAATATTACATATTCAGCGTAATCCATGCAGTTTTTATTTAATAAATCGTGT
AATTCTATTGCTTTCATTAATTGTTTCTCCCTTCAAAATGACTAAGTATATAATCTTTTC
TTGGATTTACTTTTTCACCCATAAATAAATCTAGTGTCTTTAATGACTTTTCAACATCTT

TTAATGTTATTTGAGTTATGTTTTGATATCCTTCAGATAAACATAAAGCCATAGCTTCTG
ATGATAATTCGGCTAATCCTTTTATATAATGTATTTCATATTTCTTATCTTTTAATTCAT
TAGATAATAATTGTTGTAATTCATCATCATTGATAGCATAATATTCTTTATTGTTGCAAC
TAACCTCATATTTAGGAGTTTCACATACATATATTCTTCCTTCTTTAATTAAAGTTGGAT
TTAATGTTTGGAACATAGATAATAATAATGGTAGTATAGAACCTATTCCATCAAAATCCT
GATCGACTATTATATATATCTTCGAATATCTTAAATTATTTATATCAAAAGTGTTTAAAT
CTTTATTTTCCTTGCTTTTCACTTCAATCCCACAACCTAATGAACGTTGAACTCCTAAAA
CAACATCATTCTTTAGAATCTTATCAAGTTTAGCTTTAAAACAATTTAATATCTTCCCCC
TTAATGGATATAACGCATGTATATCTCCTCTTCCAGACATTAAACTCCCTAATGCAGATT
GTCCTTCACATATACATAGAATATTTTCTTCTTTATTTTTGCTTTTACATTCTATTAAAT
TAGGTATTTTTGAAGTAATAGTTACTTTTTCATTTAGTTTTTTCTTTAAGGCTTTTTTAT
TAGCTTCTGATTTGCTATTAAAATTATGTACATCTATAATATGATTAGCGAACTTCTTGA
ACTCTTGTGAATTTTCTAATTCAAAAAGTTCTAATAACTCTTGAACATATTGAATAGCTA
TAGTCCTATATAATGGTTTTTCGGTAGATAGCTTGGTTTGATTAGCAAACTCTGATTTAA
CACTCATAACATTACATATAAAACTAATACTATCTTCCACATCTACAATGCTTATACTAT
CTGTTTTATTTTTAAATAATTTATTAGTTTTGCAATATTTGTTTATGTAATTTCTAACCC
CTTCAAATATACCTCTATTTATTGTCCCTCCTTTTGGTAAAAAAGTTATGTTTAAAAATG
ATTCTTGAACTGGTTCACTAGAAGTTGACATAACTAATTCATATCTATTTAATTCATCAT
TTAATGTAAATTCTTTTTCTAAACCTATAACTTTAGCTGAAGTATTATTGTTTGTTATTG
TGTCGAAATATTCTTCTATACTATCATAATGATACTCTTCATGTTCTTCGTCTCCAAATT
CAAATATCATTTTAATTTTATTATTTGATCCAGCTAATTGATTCAATATTGTTTTAATTT
GTTCCTTGTCATATTTATACTCTCCAAAAACATCTTCATCTAATTTAAAAGTTACATGAG
TTCCAGTTATTCCAGTATCATTATCTTCTATTTTAAAATCTTCTTTAATTTCTCCTCCTT
CTATAAAAGTCATACTACTTTTTATTTTTTCTCTATAAGATTCTATATGGAAATATTTAG
AACAATAATTACTTACAGTTAATCCCAATCCATGCTCACCCGTTGTTATTTTACCATTTT
CTCCATTTTCAAAGTTAGTACCTCCAAATAATATAAGTAAGAATATTTTATAATATGGAG
TTCCATCATCTGATGTTAATTCTAAAGGAATTCCTCTTCCAGTATCTTTTATTGTTAAAG
TTTGCATATCTTTATCAAGTTTTACTGTAACAATTCCCTTTTCAAAGTTATTATTTATTT
CATCTGTAGCATTCGCCACTAATTCTTTTAATGGATGTATAAAATTGGCTTTACTTTCAA
ACCAAACAGAAACATCTCTTCCTTTTTCTCTTTCACTAAGAACCCTAGCTTTATCTCTAT
TATTCATCTGTGTTCCTCCTAAGCTATCTTTTTGTTGAAATCTATATCTTTTAATTCATT
CTTTAATTTATTTAAACTATCGCTCTGTTTATACTTTAGTGGCTTATATCTATAACCATT
ATTATTAAAAATATTTAAGTATTCTTCAGATAAATTCTGTATTACTATTTTAGATTGTTC
TTCTACAATTTCTTTCATAGTTTTATTGCGAGTTTCAAAACCACAATTGCTAAATTCGTT
ATGTATTTTATTTCTTGTTGTAACATCCCAAGCTATCAACCATTTTATTCCCTCATATGA
TAAAGGTACGCTTCTACTTATTCTTTTATGGTGATTTTGTATTCTACAATTTATATTAAC
CATTGGTCTTTTATGATCTTTTATAGTAAAACCTTTAATTCCAACAGTATTATCTTTTCT
TCTTTTAGCGTTTTGAGTATTTTCAAAATTATCGGCAACTCTTAAATTTATTAATCTATT
GTCTAATGGATTTCCATTAATATGATCCACAATAATATTATTATTTTTTTTGTCATAAAA
CTCTAAACCACATTTAAAAATATTCATCAAAAACATTTGTCTACCATTGGTGTCAGGTTT
ACTAATAAAAGGATACCCTCTACTATCCAACGCTAAAGTTCGATTTATATTCTGTAGGAG
TTTTAAACCTTTTTCATCAACCAATACGTCATATCTTTTATTTTTCTTATATACTTCTAT
ACTATATGTATTATTGTTAATTTGTTTATATTTATTATTAAAACCAGTTTTAGTTTTCTT
GTTAAATTCTATAATTGCCACCTTTTCTTTGCAGCCACATGATTTCGGTGATCTAGTAAG
CTTTTTAGTTAAATAATAATATGATTTATTTACTTCTTTACCACATTTGCATATACATCT
AAAAGTAGGAATTCCTTTATCACTCTTACCTAGATATTTCATTGTATATAAATCTTCATA
TTGTTTATTTTCAAATTGTGTATAATCTATCTTTGTCCCCATTTAATTCTCCCTTCTCCT
ATTTTGACACTATATAATTAAAGTTTTCTTCATTATCTTTGGTAATTCTATTTTCATATT
CACTCTTGGTTAAAAATCCATATTCAAAATATTTTGGATAGAAACCATCTCTTATTATTA
GTTCTTCTAATTGCTTTTTATACACCTCTTCATTAATACTCTTAGGAAATTTTGTACAAT
CTTTTAGATTTATTTCAGTAGAATATATTACTTGTCCATTGAGTTTAGCCCCATAATATA
TTGTCATTTGATATATCACTTATTTCTCCTCCTAAACATAATACTCTTTCATATCATCTA
ATCTTAAACAACCAAGTTGTCCACCTTTAACATCTATTCCACAATCTATATAAATTGTCC
CTTTTCTATGTAATATAGTGTTTCTACCCTCTTCTATAGATTGAACTCTATTGTGTCCAC
ATATTTGGATATAATGTTTATAAGGTTTTTCTTTGCCTATTGTATATCTATGCCATAAAA
AATTATCACCTAACACATCAACTAAAGTTTCTAATTCATAACTATCACCATTAGGAGGAA

TATAAATTCCAGCATGAGATAATATATATTTATCTTCTAATACTACATATTTAGGTCTAC
TTTTTAGATATTGATAAATAAATTGTTGTTGCTCTGGGGTTTTCATATTGAATTGTGTAT
AGGTTTTAAATCCTCCATTACAATACCAACTATACCCATCTTCAAATCCATCCACATAGT
CTATTAAAAATCCTTCATGATTTCCTTGTAATAAATGTATATTCTTATGTTTTAATATAT
AGCCTAATATATTAAATGGTTGTTTCCCTCTATCAAATATATCTCCTAGAATATATAATT
CATCATTATTTTGAAAATCAATAAGTTTTAACATCTTTAAAAACTTAGTATAATTCCCAT
GAATATCACTCATAACATATGTACTCATTTTACTTCTCCTCTTTGTTATTATTTAATTTG
TCTTTAATCTTGTTGATTACACTAGAAATTAAACACCATATTCCAAGTCCTATAAAAACT
ATCAATAACAATATTAAACATATTATTGTTAACCAATCTTTAAGCGTTATAATTGTTACC
ATCACTTTTCCTCCTTAACACATCTATAATATAGTTTTATAGTTCTTCTAATCCCCTTCT
GCATTTGTTTAATAATTTTATTTCTTCTGTTGTATTCGATTATTATATCAGTAGGATCAT
ATAAATTTATAGGTTTCTTCATCATATTTGCTATTTCTTTATATAAATTATCAACTTTAT
TAATTAACTTCTTATCAACTGGAAAGACTTTGTATTTATCATATTTATCTTCTTGAAGAA
AACAAATATCTCCATTATTATAAAAATAGATTTTTCCAAAACAATTCCTAAAAGCCAATA
AACTTTTATTCTTATTCCATTTATCTCCTATTTTAATATTTCTTTGATATTTATTATGTA
TTACAATAACTTCATCTTCATTTGAGACTATAAATTTATGTCTCTCACTTCTAATATTCA
TTTCTATTTATCCTCCTTATTTATATGTATCCATAACGCTACAGCTATTATTATTTTTAA
TATAGAATTACTCTTATAAACTTCGAATTGATTAAGCCATAAAACAAAATCTACTCCTAT
GCTAACAAAGAAAAATAAACCTGCCCATTCACATAAACTTCTCATTGCCTTTTTAACATC
ATTTTTAATTTTTGCTATTCTATCTATAATAATCACCTCTTATTCCAATATTTTACCTAT
TATAAATAATCCTACATTCAATCCGACTATTGTTACACACGCAATAATACCGCTCCAAAT
AGAATTATCATTTATAGTCCAATCTTTAACTAAATACATTAATATGATTGCAAATATACC
CGATAACAACATAATGCTAAATTTACCAGTCGATATTAGGTCTTCTTTTAATTTTCTTTT
CATAAGTAATCACCTCTTACTTACCTAAATTATAAAAATGGTATCTACATACTTGCTGAT
ATTCTTCATCAACATTGCTACCTTCTACATTCACCATATCCCCTCTATGCACTGGTTTTC
CATTTCTTAATAATAAATTATGTGTAGCTTTCTTATTACAATATCTACAAGTAGTTTTAA
TCTCTTGAATATCTTCTGTGATAGATAATAGTTTAGATATCGAAGGGAATAAATCCCCTG
TGTAACTACTTTTTAGACCATAACAAATTATAGGTGCTATTTCAGATAATTTAACTAAAG
ATTCTATTTGTTTTACTGTTAAAAATTGAACTTCATCAATTAAAATTATGTCTAGGTCTA
AAATTGGTTTATCTTTAAATATTAAACTAAGATCATCTATATTCTCATCAACTATGTAAC
ACTTTAATTCCTCATTGGACATTCTCGACTTTATAACTCCACAATCCCTACTATCTTGCT
TTGCTTTTATAATAAAGACACTATTACCATTAAACTTATACGTTTTTGCAGTAGAAATTA
AGTTTAAACTTTTTCCACTGAACATTGTCCCATATTTAAAATAAATTTTATTATACATTC
TATTCTCCTATCTTTGTTTTATTTCTTTTAATTTGACTTTAATTAATTTAGAAACTTCTT
CAAGCTTATTTTGTATTTCACTAAAATCTATTTCAATTCTTTCTTTATTATTTTTTATAT
TGTAATATTCATACATATTTATTCTACTTAAATTATCTTTTGCTTCTGAAATCTCATACT
CTAAACTATCTATTCTATCTAAATACTTGAATTCTTCGGTTGTACTATCACTTAAATTAC
TACTATTCCAATGTTTTTTAATACTCATTATTTTCACCTACTATTTTAAATCTATTTTGT
AAAAATCCTCTTTCAACTCCATCATTACATATTACAAAACACCATTTTAAATCATCGTCT
TGTATTTTGATATCGTAAATTTTATTTTTTGTAAGAAGAATCTTTTCTCTAGTTCCTTTT
ATACATATTGCTTTATATTCCATCATTATCTCCACTTTTTCTTTAATTCTAATTCATTAT
AATATTTAATAATATGATTCTTTATAATTTCTTCTAATTCATCTTTTTTCATGTTTTCAT
ATTTATATAACAAAGCTTCATAAGGTTGATTTCTGTCTATATAGAAGAATTCTCCTATTT
GGAACAACTTATATTTATAATCTATTCTACCTGCACCATATCCCTTACAATTAGCCCATT
CTATAACACTAAAATTATATTTAAATCTTTTATTTTGATATGTAAAATGACCTTTCAAGT
ATAATACCCTCCTTATATGTATTAATTATTTAATTTATTTAAAATTCTTGTTTTATATTT
TATAATTTATTTTATTTTTTAGTTAAACCAATTAATTGTAGTGTGTATATAATTGTAGTT
CCAAGTAAAGCCATCAAAATTATCATTACTAATCCAATAATTATTCTTATTATAAGTTCA
TTACTTATGAATTTTTCTAACGTTTTCTCAAGACAATCTGCAATAACCAAAAATAAGAAT
AAAATCCCTACAATTCCACCTATTGTTGAAAATCCTATAACAAAGACATAAAACACTCTT
TGAAATATTTCCCAAAATGTTTGCATACATTCACCTCTCATTTGTCTTTAAATTTATACT
GCAATTTTAATCCCAAAGATCCATAAAATATTCTGCAAATAAATTCATTCCTTCTTTATA
TTTTTCTATTCTTTCTTTTTCAGTATCATAATCAAAATAGAAATTATCTCTTTCTATAAC
ATATTTAAAAGCATATATCATCTTATCTAGGATATCATTCCATTCTTCCTCTCCACTTAA
TTTAGAAGGATAACTCATTCGCTCCATGTTTCTATATTTCTCTAGTCTAGGTAATATGTA
TTTCGCTAAAGTGAAATCTAAACTCCATAATTCGCTATCTTGTATTTTAGCTAATCTCTT

TTTCTTTGCTATTCTCTTATTCATTAATTTGCACTCTCCTTTATTCTGATAAATTATCTA
CTTCTATATAGTCTATGTTTAAAATATACAAAACTTTCTGTACAATTTCATCTATGGTAT
CACAACAATCTCCGACATATTCTCCGTCTAAATATACTGTTTCTCCATTACTAAATAACT
TATGTTCCATCATATTTTTCCTCCATTTATTTAATTTATTTTTATATTTACTTAAAAGAT
GTGTTTTATTTAGTTTTGTTTTTATGTATATAACAGAGAAATCAATCTCTGTTAGTTCAA
ACATGGTCTTTGTTGATATTTAATAAATATATTTTTGATTGTATTTTTTAAAGCGTTACC
CAAAGCAAAAGGAACTCCATTTGCAACTTGTTGCTGTTTATTATTTAAACTTCCAACAAC
TTTAAAGTTTTTATCAAAGCCAGATAAAGCCAACGCTTCCGCTACACTTAAAATCCTATT
GTATAATGGATGTAATATTTTTGATTTTCTAAAATTAGTTAATGTGCAACAAAGATCATT
TAAATTTAACCTTCTATATCTATTACTGTGACACCCTTTCCCTCTTATTGGTTCTGGTAT
GTCTTTAAAATTTCCTCCTTGTGGTATATATTTCATTTTTTCAATTGAACTTTCTTTACA
TTTAGTTATATCATTAAAGTTATACCAATCAGATGTTACTTTACTTAAAGCTTCTTTTAC
TAAATGTTTTTCGTTTGTTTCTTTTTCAAACTCTACTTCACCAATTATGCTTCCTACGAT
AAATACTCTTTTTCTAATTGTATAGCCACCATAGTCACAATCCTTCATAATTTTATGTTT
TATATTAAACTCATTTAAACATTCTTTTAAAGTATCATAATAAAAACCATTTTTTGCTGT
AATGAAACTAGGTACATTTTCTATACCAAAAACTTTACAGCCACTTTCTGTAACTATTCT
AATGTATTCTATAACTAAATCGCTTTCTTTGTGTCTTTCTATTCTGTCTTTGCTTCTATT
TGCATTTGAGAAAGGTGTACATGGTATTCCACCTATGACTAAATCTATATTGTCACTATT
AGACAATTTAAAATCATATATATCCATTTCTTTAATTTGCTTTCCTATATTCTCTCTATA
ACTCATACATGCATCTTTCATTATATCTGTAGCAATTTTTATTTCAAAAAAATCATCTAA
ATAAAATGGATAATCCAACATTCCACAACCTGAGAATAAAGAAGCAATATTTATTTTTTT
AATTATTGTTTGTGATGTTTTATCTTTATTATTTTTTAATTTAACCTCTACTATTATTGA
TTTGTCTAAAAGTTTTATATTTAAACATTCTCCATCTTTTATAGTGTTTTTTATCTTTTT
ATTTCTTAAATCTATCAAACTTTTATATACTGATTTATTTCCTATTTTCTTTCTACTTAC
TTTTAAACCATTTTTATCTTCTGATTTTATTATAAATATTTTTTTACTTTTATTTGAGAT
CACATACTTATAATATGTTGCTACTTTTATATATTTGTGATTAAAGCTAAAAGTTAAACC
TCTATTTGATGTTTTTATTTTATATTGAAAATTCACATAAATCACCTCACAAATATTATG
AGCGGCACCACCTTAATTTTCAATATAATCTTTAAATATTTTTTTGAAATTTGTCTCTAA
CATTGGAACTACGATTCCTCTTCCAGCTCTTTCATATAATTTAGAATTGGGCAGTCCCGC
TCTTTTTGCCTTTATAAAATCTTCATCTTTAAATCCCTGACATCTCCAACATTCTAAAGG
TGTCATTTTTCTAACTTTATCATTTATTCTTATTTTAGGTTGTCTATTACCCCCATTCAT
AGAATTTAAAGTACAACAGATACCTTTTTGGTCATAAACTCTTCTATTACATTCATTTCC
TTTGGTATTTAATAATCCTAAATATATTAGTTTGTGTTGTTTTTCACCATTATATATATC
TATTGTATTTATGTATTTATCTTCACTTAAAAAATATGTATTATCAACTTCATTTTCTAA
TATATCAGTTATGTATGTTTTTTTATTATTTGTTATTTGCATTTCAAAGGTTTTATCATC
AACATCTTTTCTTATACTTATCATTATTACTCGTTCTCTGTTTTGTGGTACACCATAATC
TTTGCCATTTAAAACCTTCCAATAATTATTGTATCCTATTTGCTCTAACGTTTCGCACCA
CAAGTCAAATAACGGTTTATGTTTTTTACTAATTAGATTTTTAACATTTTCCATCATTAA
ATATTTAGGTTTTTTATGTTTAATAATTCTTCTACACTCCCAAACTAAAGATGATTGAGT
TCCAGATCCCTCTTCAAAACCAGCTTGTTGTCCAGCGTTAGAAATATTTTTACAAGGAAA
CGAATATGTAAAGAAATCAAAATCTGGTAATTTATTTTCGTCTATAAGCCTAATGTCTCC
AAAATTTTTATTTCTTATATGTGCTTCATATAATTTTCTCAAATCATCTTCTTTTCTAGG
GATTTCAGATTTTCCAGTTGAAAAATTATAAGCTATATGCTTACTGTTAATTTCCTCTAA
CATTTCTTTTTTTGTTTTATATTCAACCTTTTCATTATTATTATGTATTGAATCGTAAGC
TATTATAGCATATTTATCCACATCCATTGTTCCAACAACTTCATAATCAACATTTATATT
TCTTAATGCCATTGATTGACTACCTATTCCCGCAAATGCCTCAAAAACTTTTAATTTACC
CAATAAACTTCAATCCTTTCTATGTTATTATTTATATAATTTATTTCAAAATATAATTAT
TTTATTGCTATTATCCCTACAAAATTACACCATCTAAAAAATACTTCTACATCTTTGAAA
CCAGAATTTTTCATTAATTTTATATTTTCATCTAGTGTATTTGGTGACATTACTCCTCTT
ATTGCTTTAGCCTTGCCTATTACTTCAACTTCTGTAAGACCGTTGTCCATTTTAAAATCA
TGATAAAGTTCTGTAAAGATTTCATCAAATTTCGCATTGCTACCTATTATTTTTTCTATA
ATTATAAAAGCCCCTCCTATATTTAAACCTTCATATATTTTTTTTAATACTTTTAACCTT
TTTCTTTTAGGTATAAACTGCATTGTTAATATAGAAGTTATAAAACTAGCATTATGTATT
TTCACATCCTCATTACATATATCTGCATTTAATATTGAAATATTTTTATCTGTTTTATTT
TCAACACTTTTTTCAATCATATCTATTGATTTATCTATTCCAAAATAATGCACTTCTTTT
TCACTATGTCTTGGTATAATATTTTTAAAAACCTCACAGGTAGAAGATCCTATATCATAC

ATATTTGTTCCATTTTCTAAAAACCAATCCGACATTTCTGCTGTAATTTTATGAAATTCA
TCATACATTGGTACAGATTTTCTAACATGCGAATCAAATATATTAGTTACTTGTTTGTCA
AATTTAAAGTTTGCATTCTTGCTATATGTATTTTCATCAATTTTCATAATTGTCTTTATC
CTCCCTTTTACTAAATTCTACTTCAGTATTACATTTTTTATTTGAATATTTTAAATATGT
AACAACCCCTTTTCCTTCACCATAGACTTCATCATAATTGAAATCTGATTGCCAAATTAA
TTTATTTCCACAATACCAACAGTCCATACTATCACCTCTTAATCTCTATTGTATATTTTA
AATGACTTATCTAAATCTGATATTGTTGTAAATTCACTTCTAAATAAGCCTTTTATTATT
ACTCTTCCTTCTTTATCAATATCTGTTATTACATATCTATCTCCAACTTTCATCCATTGG
CAACAATCCACGTAATCTTTATCTATTAATTCTACAATATCATTTAACTTATATTTCATG
GTTTATCCCTTCTTTAATTCTCTTTAAAATTAGATTTTTAATTAATACTTTAATTTATTT
TAACAATATAAATATTTGTTAATCTACATATAATGAAATATAACCATTATTACCACCTCT
TTTTTATTATTTGCTTTCCACAATTATTATATTACACCTTTGATTTCATATTGTCAATCA
TTTATTTAATTTATTTCAACATTTTTTTTAGATTTAAAGACTGAAATATTTACAGCCCTT
AAATAAACTCTTGTTTTATTTAAAACTTCTTTTATCTTCTATAAATATTGAATTGTTTTT
TATAAAAATATTAGCATATTCACTATCAGCAACCATTTCTAATATAGTTTTAAAATCCTC
TAAATGTTTTGTGGTTAAATTTTTATTTTCAACAATTTTATCTAATCCAGCTAATAATAC
GATTTGTTCGCCTAATCCATAAGTATATATATCGCTTGGAATTAATTGAGTTATATTAAA
TAATATTTTTCCATCAAATAATAATGATTTTTGATATAAATCTAAAATTTCTTGATAAAA
AATCCATTGTTTACTTTTTATATTTTCGCCTTGTATGGGATTATACCCTTTAAATACTGT
TGCTATATTATTAACCTTTTGAATATAATTCATGTAAAATACAGCTAATATTAAACATTG
TCTATAAGTTAATTTTTCAAATACTTTTAACAAAAAATTAAACTCATCAGCATCTAAAAA
GTCAACAAATCCAATGTTAGCCAACATATTTCCATAAAAAATTTGTTTCATTTCTTGAGA
TTCTCTCTGACTGGCCACTACTATCGATTCAAAAACTTCTTCAGCATCTGTTTTACCATT
AAGTTTCTCCTCAAAGAAATCATCTTTTCTTAATTCATCTCCATTATTTATACGTTCAGT
TATTCTTTCTATAGAACTAACCATACAATTTTCAACCCTTTTCTTTTCCCATTCAGTTAA
TTGACAAGGATCTATTTTCTCTGCTAATGCTTTTATAGTTTTTCCACAAACCGTTTTAGC
TACTGTTCCAGATATACCAGTTGGATCTGATAATGCCATATTAACAACAAAAGCGATTCC
CTCCTCAGCATATTCTTGAGTATTATCTATTATTTTTTTAGCGCCTTTCTTAATTTTCTT
ACTATTCATATTTTCAATCTCCTATTTTTTATTTATCTAAATTATCCACATGATAATCTA
AAAATATTATCTCGCCATCATTATAAGCTTCAGAACTTATTTCTACTCTTGGATCATAGT
TATTATTCTCTGTTAAAACTTGACATTCAACTTTAGCTGAATTAAAATCTAAGTTATTTT
CTTCCATTCCTTCTTTTATAAGTTTAATCACTTTATTTGAATATTCATCATCAATAATAT
TTTCTAAAAAATTATTTAAAAATTTATCTAATTGCACTTTATATCACCTCTTTTTATATT
ATACCATTATTACCCACTTTAAATAAATCGAATAATTTAATAAGTTTTATAAATATCTAA
TTTCTATTTCTATATCTAATTCTTTGAAATGCTTTATAATTATTTCTCTAACCTTTCCCC
ACTGTAATCTATCCAATCCACAACCAATCTTAGGTATAGCTAAATATTTTATATTATAAT
CCTTACACATATGAGCCATATCTTCAATCGTAGGTTCTATGGTTTTATAAGTTGGCTTAC
TCCAATAATTTTTTTTGGTTACTAAATTAAACACCATTTTATCAGAATTAGAATATGGAA
TTACACATGGAAAGTTTAAATGATTTTCTTCAATCACTCTTTTACAATAGTTTTTCATTC
CTCTAAATTGCTTATCAAATACTACAGCTATTCCTTTACCCATAGCACAATCTTGACTTA
TACAATGTGCATAAGCATATTTTTTAGGGTCTAATTCAAATAAATTACCTTTCTTTTCAT
TTAGTTTCATATTATCTACACTCCTTATATTCATTAAATTTATAACATACTCCATCCAAT
ACAATATGACAATTTTCATTATCCATAATTAATCTTTCAGTTTCTATATCACCATCAAAC
TGTATTAAGTGTTGTATTTCTTTTATGTATTCACTAAGCTTCATTAAATTTCATCTCCTT
CAAAACTTACCTTATTTGAAGAATATCCAATATCACATAATTCTTCTATATTAAAAGTTC
TTACATTTATTCCACTTTTACAATAACCATCTATAAAAGTAAATTCTATATCAAAATCTT
CTAAATTTCTATCTTGTATTATCTTAATTAACTCTCTACCTTTCATTTTCTATTTCCTCC
TACATTATATTCCAATTTCCATAAGCCCAATGACCTAACACTGTTCCACATTGTTTGCAT
CTAACACTATATTCTTCTCTACATCCCCAATCTGTGTAATATTCATCATATTCTTCCATT
TCTTTGCTGCCACATTTATCACACTTTAAAGGTGTTCCATCACTAGATATATACCCCATA
TCAATACACCAATCTATATATTCATCAATAGATTTAAACATTATTATTCTACCTCCCGTA
TTCTACTCATTAAATTTCTACAATATTACAATGTTCTTGATCTAAAATATCTTTTAACTT
CTCAGTAGTTATGCTCAATTTAGATGCAATTCTATCTAATGCATTGGTTTTTATATATGG
ACAACCATATGGCGGTAATTTGGGTATTGAAATTTCACCTATATCATAACCAAAGCTACC
AGTTCCTCCAAATGCACCTTTTTTACACATATAAACTAAATAGCCATCTCTACCATCATT
CCCATACGTTCTTCCTGTTGTTAAAAATGGACAATCTTTACAATTTTTTATTTCAAAATC

TAATGTTACTTTCATTATTCATTCTCCTTATAAACCATTTTATATTCTCTAATTGCTATA
ACTCTTTTCATGTCAACAACTCTATCGTCTTCTTTAGAATATTCTCCTCCACAATCTAAC
AATATATCTTTTCCTATAAGTTCTTTTATTTTATCATATGGTAGTTCATCAGATATTGTT
AAATCACGTTTGTTTTCTATGGTTAATATATAACCATATCTACCATTCTTTTCTTGTGGT
GTACGTGTATCTGACATTCCATAGTATATAAAACCACAAATTGTGACAAGTCCTATCAAT
GTTACAATTTTATAAAATATATTATAAAATTTTTCTTTACTCATTATTACCCTCTTTAAT
TATTTAATTTATTTTTCTTAAAATGTTACTTTTATTTACATATAATTACTTCATCAAAAT
GTATTTTTATTTTACATCCACCATAATTTTCAAGAATATGTGGTTTATTTAACTCTCTTG
TCCATGTATAAAAAGTTTCTAACCATAAATCTCTGTATTGTTTAGAATTTAAATATATTT
CTTTTGGAAAATTCCTGTTTTCTTTATAAAATTTATTAATGATATTTATGCATTTTTGAG
AAATATTCATATCTACTTTTATATCCACTACAAATCAAATCCTTTCTTTATCATATTATA
AGTGGTTGATGTTAAACATCCATAATTACATTCTTGTTCTCTTATATAGTCACAACTAAA
TTTCAACATATCTTTAATTATTAATTTGTTGTATTTATTACAAACATTACATAATTCTAC
ATTATCATTTTCAATATTACATATTAAAAATCTATTGCTGCTTTTTGCATATCCTATTCT
TATTAATTCATCTACAATTAAAGTTTTCATATTTGGATGTTTTCTTAATATATATTTGAC
ATCTACATCGTCATATACACTTCTTCTTACTGCATAATAAGTGTCTTCAGATATTTTCAT
AAGTTTAAAAGTTCTTTCTGATTTCTCATCTAAATAAGATTGCTTAGGTGAGTAGTTAAA
GCATTCTCCTCCATTAAAGAATCCATATTCACTATCATAATATTTTTGTATGGTAATTTC
TTTAAGATTACACATTCCTTCTTTATTATTTTTACAAATATCTAGTTCACATTTTACTCT
CATAGCTTCCTCCTAAAACATATTCAATTAACTCATTGTTATTTATTCTGTAGTAACCTA
TATCAGCATAATCATATCCTTGTATAAAATTATCTCTCCATATCTTTTCTTTTTGATTTA
ATTCGACTAAATACCAAACTTTCCAATCTATATATTGATCTATAACCCTATTCATAATTC
TTTTGGTTTCATCATCAAATGATACACCCTCTATAAGTTTTACTCCAAATATATCTGTAC
TAGACCATGAAGCATAATGTAAATAACTATCTGGAATACATATACCATAAGTCCAACATT
CCATTTGATTATCAAATAATAATCTTTTCTTTTGCTTTATGTATTCACATTGTATGTAAA
ATAATATCTTTTGCAACTTTAAATTACTTATAGGTTTTTCTTTAGTTTCACAATAATTTA
AAACATATTCTGATACATCCAAAACATCATAAATCATTTTAAAACATCCCCCAATTAGGA
ATATATTTTAAATTATCTATATCATCTAAATTTATATAATTAATTATTCTAGTTGTTACC
CCATCTTGATTAACTTTTGAAATATCCTGAAACTTAACATTTACTTCCCATATGTTTAAA
TTGTTATCTATTTCTACATTAAAACCTATTATTCTGTTAAATTCCTTAATAAAGTTAGGA
GCATATTTACATATATTTGAGCATATTAAAAAATTAGTTTCTCTACTCATCTGTATTTCT
ACCTTGTTTTCATTAAAACCTAGTGCTGCTAACTTATAAGTTAACTTATTTAACTGTTCC
ATAACCTTATTATATTTTTCTTCATCTTTCATATTAATTACCTACTCTCTTATTGTTGAT
ACTAAATTATAAGTTGTTATAATAGTTATGACTGATGCCGAAATAGAATTACCTATAAAT
ACATTGTCTTTAGACCAAATTCCTAATATTAATGATAATACGCTTAATAACCACATAGCT
ATTACACCAATTATTGCTAATTTCATTGTTTTATCCTCCTAATTGTTCCATAATTCATAT
GGGATTTTATTTCCAACTCTTATAACGGCTATTTCCTTTTCTTTATTAGAATCTATATCA
TATCTATCTACTAATTTAGTATTAAAGCCATGTTCTTGATTATATTTAATTTCTGCTGTT
TGATTAAAAACTTCCACTGTTTCTCCGTCAACCAAATATTTCTTCAATCCTAGATCAGAA
GCTCCTCCACAAAAACCACCTGAAGCATATTCATTTTTTACTATGTAATACTTCCACATG
CTTTTAGGACTATACCAAGGTTTTTCAATAACTATATATCCTATTATTAATTTCCCAGTT
TCAACATCTCTAGCCATATGCCTATAGTCTTGTTGTTTATTCTCTTTGTCAAAAATTAGT
ATGTTCATGATTATCTCTCCTTCTATTTAATTAAAATTTCCAATTTATTGCTTATTTTTA
ATATTATTTAAGGCGTTTTTATATTCTTCGGTAAGTTTAATTGATATGTATATATGATTA
ATTATATCAATATCCTCATCAAAATATTTCTTTGCTTTATCAATTGCATCATCCACATTA
TCATATGAATATAATAAATTTAAATCATTGCCTCTTATATCATATTGAAACTCTATTTTT
TCATTCTTTTCTAAATCGACACATATTTTCATAACATCGCCAATATCTATGTTTAACTGT
TTGGAAATAGAACCAAATTGCAAATTATCAATTTTATTTTCCAAATATGCTTCTTTTATA
AAATCTTCTATCATCCAAATATTTTTATTGTCCAATATCTATCATTCCTTTCATTTATTA
TAAATATATTCTCTTTTATTTTCTAATCACTTCTTTCCCACTTTTTAAATCTTTAATTAT
CTTTTGATATAATTCTTTAGCCTTAATTTGTGTTTTAAATTTATATTCTATCTCTTTTAA
TCTATCTATTATATCTTCTTTTTGACTTATAAAGCCATTCAATATTTCTTTTTCTATTGG
TATTTCATTTTGAAAAATACTCTTTAAGCTGTTTATTCTGAATTCAATATTATTCATATC
TTCCCATAATTCTTTTCTTGATTTTTCATTTAACTTTACCATATGTATTATCACTTCTTT
CATTTATTGTAAGATTTATTTTATTTATAGGTAATTAGCTTTAGTTTAATATTAGCTAAA
TAATTATCTAAACTGAAACCAATTACCTAATATATCTAACAAGTTGGCAAGAACCACTCA

TTAATAAACTCTTCCTCAAACTCATCTCTAACCTTATGTCTTATCATAATTCCATCATTG
ACTTTTGCTATTACTATGTATTCTTTCTCTGCTTCAACATCTTCCATAATCCCACTTAAC
AAACATCTAAAATCATATCCATTCATGTGCTGTTTTAGATAAACAGATTCATTGTCATAG
ATGAACCTTTCTAAATTAAGTTCCTTATTCTTTGTCTTACTAAAGAATTCCTCTGGATCT
ATAAATATTTCATCATATATTTTAAAGTTTAAGTTTGTCATATGTATCTCCCTCTTCCAT
TGTTCTTATTTATACCGAACTTATGTTCGTATTCATTATACCTATTTTTTATTTTGTTTG
CAACTCTATCCAATAAATAAATTTTATAAAGCTAATGTTAATTCATACCAATCTTTTATT
ATTCCAATCTCTATCTTATGTATAATATCTTTTTCAAACTCTCTTCCTTTAAGTTTGACT
TTAGCAATAGTATCTTCTCTTTCTATTTTATCCTCTTTGAAAATCTCTCTATTATAAGCA
TTATTTTTATACCAGACTAAAATCTCATTTATAAAACCTTCTTTAATATCATCTGAATGT
AAGTATAAGTAATTCTTTAATTTATTTTTATTAAGCTCATGAGGGAATAACCCTCTAAGC
TCTTTGATTTCATTATTAATTATTCTCACCACACTTTATTAAATTTCTTACAAGTTCTAA
GACAGTCATTATACCTGTTCCTCCAGGAACTGGCGTGATATTTTTAAACAACGGATACAT
ATCTCTTGATATATCACCATATAAACCATCATCAAGTCTAATTATTCCTACATCTATTGC
TACTATTTTTGAAAAATCTTTAATTCTGCATTCACCAAAGAAATTTAAATCAAAATAATT
AGCTTGTCCTATTGCACTAATAAAAATATCTGAATTTCTTATATGTTCCACCAATACATC
TCTATCTGTTTTACTATTACAAATGGTTACCGTAGCACCTTTATTTATTAATAATTGTGC
TAGTGGTTTGCCTACTATATTACTTCTTCCTACTATAACTACATTTTTACCTTCAACATT
TATATTTTCATGTTCTAGGACTGTTATAATTCCTTTCGGAGTGCATGGAATAATTCCAGT
TTCATCTCCTATCATTAATTTACCTTTATTCACATTAGTAAATCCATCTATGTCTTTGTT
GGGATCTATAGCATTTATAACTTTTTCTTCACTTATGTGCTTAGGTAATGGTAGTTGTAC
CATTATTCCTGTTACACATTTAGTTTTATTTAACATATCTATTATATTTAATAATTCCTC
TTCAGATACACTCTCATCTAGTTTTATATGTTCGTAATCTATTCCTAATTCTTTGCATAA
TTTACATTTATTATTAACATATAAGTTACTAGCTTGATTATCTCCTACTTGGATAAAAAT
TCCTTTTATCTTGCTTAAATCCTTTTTCTTTAGCTCTTGTTTTATTTCTTCTCTTATCTT
TTTACAATCTATTATCATTAGTTGTTCTCTCCTTCTAATCTTTCATAATCTTCTTCCATA
CCATAGAATATAAGTTGTTTAGCATAAGGTAGTTTAGTTATTTCTTTGCAGAAACATTCT
TGCCATTCATATTTTAATCTATGATTCTTTCTTTGAATTATGATATTTCTTAATACTGCA
TAATTTAGATTTACTGTTCTAAGGTTTAAATAACTTTCTGAAAGTATTCTTTTAGCTGTA
ACTAATATTGAATCCTTTTCTTGTTGGGTTGTTGCTTTAAAATAATGATCTCTTAATACA
TTTAACTCTTTAATTAATTCATTTATTATTTTCCTCTCAAAATCACTATGCACTTCAAAC
ATATTAACTTTTATTTCGGTTTTCTTATCAAATAATTTATGCATTGTGCTTGTACTATTA
GCATTCGTTCCTATTTTATATGTATCAAACTCTGACCACCAATATCTAGGTGCGGTTATG
TTTAACCACACTTGAATTTGTCTCATAAATTTACAATGTTCTGTTCCTGCTCCTATTAAC
CTTTGAGCTAAATTCAAATCTTTTTCACCTATTACATCACCCTTTGTATCACTTAAACTC
CAGCTATTTAAGGGGTGTCTCATTCCAAACAATGCTTCGTCAATTCCACTAACTTTTAAT
ATTTCTACTTTCATATGTATTCCTCTCTATTCTTTGATTTATTTTTAAATATATGTTAAA
ATTCTCTTAACATCTACTATTATACTCTCATCGCAACAAATGTCAATCAATTATTTAATT
TATTTTAACATTTGTTATATTATTTACTAATTTTAATATTTCTAAGCCCTCTTTCATTTC
TCTAATATATTCATCCATACTTTCATTAAGATAACTTACCTCTACTAAATTATTATCTAT
GTAATATTCTTCTTTACCTCTATCATCATAAAATATGTATTTAATATTTGATCCGTTCAA
ATCATAAGTTATTTCTACCATATTAATCACCTTTTTATTTTTTATCCACACACTTAAACA
CCTTAAATCCAATATCTAAATTTTCTATTCCTTCTTTATCTTTATTCTCTTCCTTAATTT
TTTCTCCAGCTCTTCTAATTCTTTCTTTACCAATTTCACAAATATTTTTATAACCATTTT
TATACGCTTCACTTTTCTCATTACATAACTCAGGTAATTGAACCATTATAAACTTTCGAT
TACCATTATCCTCTGAATTAAGTTGCATAGTTGCATGAGCTGTAGTGGCTGATCCTGAAA
AAAAATCTAATATAACACAGTCATTATCTTCAATACTTATAATCCTTTTTATAAAATCTA
ATTGCTTTGGATTATCGAATGGTGTATTAAATTCTTTTAACATAAAATTATCTGGTCTTT
GATCATCTGACATTATTGACTTAGGTCTTTGCTTCATATTTTCTTTTAAATATCTTTTTA
TTCTTGGAAGACTTCCATCTTCATGAAAATGTATTCTATTATCCTTTATTAATGCATTTA
ACTTTTCTGTTGTGTATCTAAACCCCCCTGGTGGAATAGGATCATATTTATTAGTTAAAG
GATTAAATATTGAAGTATTATTGCCATTTTTAACACCTCCTAAATCTCCTTTTTGATAAA
TACCTCTTTCATCTATATACCAATAATTTGTAAAATCTATAAATCTAGGATATTTAGTTA
AATTCTTCAACTCATTTGTTATTTCTTCTTTATTTAATCCCATTTTTTTTAATTCATTAA
CCTTTTTTATATATTCTTCTATGTTATTTTTATCAACACTCCATTTATTATCACTATGTT
TCTTTATTAATATATTAATATTTTTAGAATAAATCAAACAATATTCATGACTTACCGATA

CAAAAAGGCTTTGATTTTTAGAACTATTAGTATTTCTTATTATATTGGCAATAAAATTTC
CTTCACCATAAATTTCATCACAAATTCTTTTTAAATTGCTAATTTCATTATCATCAATGC
TAATAAATATCACTCCATCATCAGCCAATAAATCTCTAGCAACCTTAAGCCTAGGATACA
TCATATTTAACCAATTTGTGTGTTTATCTTTTTTATTACCAAATTTATCATTGTATATAA
ATTTTTTGCCACTATTATATGGCGGATCTATGTAAATCATTTTAATCTTGCCCGTATAAT
CTTTTTTTAATAATTTCAAAATATCTAAATTATCACCCTCTATGTATAAATTTTCTGTGG
TATTTGGATTTTTAGATTCTTCTTTACAAAATATTAGTTGTTTATTAGTTGTTTTATCTA
CATCTAAAATAGAATTTGTCTTGCCTTTCCATATTAACTTAGGTGTATCATCTATTAATT
CTTCCATTTGTCTTATTTTATTTATATTCATATTATCTCCTTTATTTAATTATTTTATAT
TTCCTTAAAAGTATGATTTTAACTAAAATCTATTTTAGCATATGCAATTACTTCACTATT
TTTATTAGCAAATAGTATATATCCATTTTTAACTTCTTCATAAGTATATATTTTAAATTT
ACTATCTTTTGTATAATAATAATTCTTATCTATCCCTCCATTGTGTAGCTTATAAATATT
TTCTTTTTCTTCTGAATCATTATTATTTAAAATAATATTTGCTATTAAATCTCTTTTTAA
ATTATCTCTATATCTCAAATCATTATATGTAAAATAATTTCTAGTATATTTATTAATAAA
CACCTTTTCTACACCTTTAAAATCTATTCTTTGGTTTATTTGATTTATAGAAATATCACC
GCATCCAACAAGACAATTCTTAGGATCAATCTCGTGTTCTCTCATAATTACACCTCAATC
TTTTAAAATTAATATTTACCTTCTTCAATTCTATAACTAACTATATATTTAGAATTTATA
TTACAAAATTCCCCATCTATAAAAACTGAAATAACATCTTGTTCTTTTATTATATATTCA
AAATCACTTATCAACTTTGATTCATCAGAAAAGTATCCATATTCACCTTTTCTCCCCAAT
ATATCTATAAATTCTACAAAATATTTTGTGTTCATTCTACTCTACCTCCTTAATTAATTT
CATCATACAATGACTATGCCCAGAATATAATTCATCACCTATTTTACATAAATGCTGCTT
GCCTAATTCTACTTCTTCATGGCAATAATCACATAAATGTTCATCCCATTTATGTCTTTT
AATTTGTTTCTTTTTAGATTTATCTATAGCCTTGAAAAATCTATTTAATTCTTCTTTGTC
TAATTCTATATTTTTAAATATACTACTGGTTGCCATAATAACTACCTCCTTCTAACGTTC
CAACTTCCTTTAAATACCATTCATGTAGTTTATCAAGTTTTTTCTTTTCTTTTTTATTTA
ATGTATTTTGCCAAGATTTTGCTAAATAATTATTTAGCAATTTTGTATAGCTTATTATCA
TATCATTTTAACCTCTTTATCTAAACCAATTTCTCCATATTTTTGTTAATTTCATATTCT
TAAATCTTGATTGTTTTCCTTCACTTTTATTGAAACTATTTTCTTTATGGGGAATAAAAG
ACTCGTTATCAATCTCTTTATTAATAGGTGTTTCATTTTTCTTTTCTTTTATATCTTTAT
TATGTTTTATTATCTTTTTAACAACACCGTTACAAACTTTTTTGGCATGTTTCAGATCTT
CTTCATCTACAATTATTTCAACTATTTTATCTATGTATAATTCATTTTGCTTTTTATCAT
CATTATATTTGTATTTGACTCGATAATCTTTATTTTCTATTCTAATAGTTTCATTATCTC
TAAGCTCTAAATCTGTAATTATGGTTTTAAACTCTTTGTATTTTACAAAATTACGACTCC
TCCAAAACCATGTTCCCATAAAAGGTTTTGATATTGTCAATAAATTCTCATTTTGTTGTT
TCTTATAAAATCTAAAACGTCTTGATTCTCTTTGTACTGTTAATATTTTCTTAAAAATCT
CATCTTCAGTAGAATTTTTATCTAAAATATAACTTTTGTCATACCAATCATAAATATAAA
AGGAGCTTTCATATCCCATATGATAATATTCTTTAACCACTTGTCCATAAACATGATATT
GTATCATTTAAAATCATCCCCTTATTCTTTAAAAATAATAAAATCAATTAATATTGGTGA
TAATATAAAAACTATAAATATACCTATAATTATTTTAAACACACTATTATTTCACATCCT
ATTATTTAAAATTAATCATTTAATCAATGATATAAACATTATCATTTTTGCCATCTGAAT
CAAACTCTTCTTTCCATATTTCCTTATATTCTTGTAGCGTTGATATACCTTCAACATCAC
TCAATGTTAACTCACCTCCATTAAAAAAAATCCTTTTTATATATTTCTTTATCTTGATCT
AATTGTTGTAATTTTTCTATTAATTCTTTAACCTTCATTTACACTTCCTCCTAATTTAAC
AATGCAGTAATTCTTAAATTTTTGAATAAATGTTATGTTTTGCTTAATTACTTCTTTTCT
CATTTCTTTTTGGTATTTTTCAATATCTTCTAAAAGTTTTTTTGATTTTTCTTTATGTAT
TTCTTCAACTATTTTTGGCTTATCTAAATATATATTTATTCCTTTACTCCCACAATCAAT
AATTTCTTTAATGTGATATCTTTTGTTATTATAACTCATACAGTCTCCACATTTATATTG
AACACTAAGTCTTTCAAATTCATCTATACATTCTGTATTTAACGGTTTATATTCTACATT
CAATTCATATATATCATCCTTATTTATATCAAGCTTATCGAAAGAAGTTATCTCTTTTAC
CCTATAACAATATGGTATAAAATCCCTATCTATTATCTTATGAACACTATATTTTCTGCT
TACTACTTTTTGTAATACTCTAATTTTCACTTGTATCTACTCCTTTATTCCAGTTCCATT
ACAATATTTACATTTTACTTTCTTATCACTCCCTCTGATACTACCATTATAATCAACCAT
ACTTTGCATTGCTTTTAATCTTCCAGTACCCAAGCAAAAAGGACATATACTTTTATTTGC
AAAAGTATTTATTTTAATATTACTCATTATCTTTACACTCTCTTTCTATAACATCTTTTA
CAAAATAACACCACGGAGATTGTCTTCTCTTTAAAATATCATCTAATTCTATTAATACAT
CTTTATCAAAAGTATAGTCTTTATATTCCCAGCTTATAAGTTCTGAAAATAAGTCCCATT

CCTCATATCCATTATTATCAGCAATAATTTCATCTGCTTTTAAATCTATAAAATTAAATC
CTTCTATCCTTTCTTCAAAAATCCATTCCTTCTTCTTTTCTATTAATTCTATTAACTCAT
CTATATTACTAAATTGTCCTATATAACCTTTTGCTCCACCAAACATATCAGTGAAACTTT
TTACTCCGAAAACTAAAAAATCTTTTTTCATACTTTATAATACACCTTCTCTTTCTTCTA
TTTTAATATTACGAACATATGGCTGTCTTCTTAATAAATAATATCTATCATAAGCATCTT
CTTCATGTGTATATTTTTCTATTTGTGTATTAATTTTTGATTCATAATAACTTCTAAATC
CATAATCAATTATATATGTAATAACAAACTCTTTATTTTCTCTTGATTTTCTTCTTCTAA
GTTGTAAAACGGGAATTTCGTTATTCTTTCTTCTTTTGTTACATTCATTAAATTCTATAT
TTAAACTTGAAGTATTTATAGAAAAATAAGTTTGTTGTTCTCCATATTCTGTCATAGTCA
TAGCCGTTATTTCTTTTTTATTATCAAATTCTCCTTCTCTTATGACTATTTTTATAGATG
GTTTAGAATCACTTTCATATATCTTTCCTATGTTTTCTTTCTTTAATATTTGCCAAATCT
TCATATAAAACAACCTTTCTACCAATTATTAATATTCTATTTTTAATCATTTCTTTTATC
ATAACTAAAAAATACTGTATTATAATACTTATCAGATTTACAAATTTCGTTAAAAATATG
AAAATCATCATTATAACTTTGATTATATATTTCAAAACCATTCTTTCTACCTAATTCAAT
TATTGATGTTAACATTTTTATCCATGTATCGATATCTAATTCCATTTATATCTACTCCTT
TATTAATTATTTATTTACAAATAAACTCTTGATTTTTACCATATATTTCTATATAATTGA
GATATAAAATGATGGTGATACCTCAATGTAATTAATTTAAAAAAGTCTTTTCATTATCAC
TTTCATTTTATATAGGAGACTGATGTTCATATCAGTCTCTTTTTACTTATTTTTGAAATA
TTCTCCAATTTCTAACCAATTATTAAATCTTTCAAACTTATCACATTTTACATTATGAGG
TTGCGTAAATAATAATCCTTTCCCACTAAACTTTTCTAAATTATGGACACCATCGTCAAT
TAAATAATCACCTTTGAATATAGATTTATCACCAGTAAATATGTAATGTGATTTAGGTAT
AAAAGGAAAGTTTTCTTGTAACCAATCAAATTTAGCTTTAAAGCTCATTCTACTTGCCAT
TGCATCTGTTATAATATAAATATCATAATCTTCGCTTAATTCTTTTAATACTTCTTGAGA
ATGCGGTACAACCTTTAAATCTCTATAAAACTTATGAAAAGAAAGTATATCATAAATCTT
TTCTCCACAACAAGGTTTAACATATTGATGTATATTCCATGTTTTTATATCATCTACACA
TAATTTATCTCCATATAGTTTATTATATGTATCTATCCATTTTGGTAATAATTCTGCTAA
CACATCATCTTGATCTACCCCTATGATACCCTTTTTATTTCTTACCTCATTTTTAACTTC
TCTCATTTGCATAATCAACATCTCCTTTTATTAGTTAATTATTTAATTTATTTCAATATT
AATTAAAACTAACTTTTTTTATCTTCTTTCCCATATTTTAATTAAACAAAGAAAATCTTC
ATTAAATTTAAAAACTTTTGATATGCTTATACTACATACTGATCCAGTTATAGTCATATT
ATTTTTATAATTATCTAATGAATTGAAAACAACGCCATTATTATTTATTAACTTATTATT
AGAAATAAGCCTTCTCTCTCCATTGACTAATTCAACGACCATTCCATTTCTTAAATCTTC
TTTATTAAACATTTTATTATTCATAACTACTCCTCTAATAACTTCATTAAATCTTCCTCG
ACAAATATTTTAACACCATCTTTAATAGCTTTATCTTTTTTACTACTTTTACTAGAATTG
TTGGCAACTATTAAATAATCTAGTGATTTTTTGTATCCACTTTCAACTATGGCACCCAAT
TCTTCTAATTTACTTTTTAACTCACTTTTCTTTAATGTGAATTTTCCTGTTGGATAAACT
CTCATATCCTTTAATGTATTTTCTTTAGCTACAACTTCTATCTTTTCTTCTGTTACAAAG
TTTATTCCATCTATAAAACTATTAACTAGATTTATATTGTCTTTGTTGTGAAACCAATCT
ACTATGCTATTAGCAACTATTTCTCCACAATCCTTCATCTTTAATAAATCTTCTACTGTT
AAGAAGTTCATTTTCAATAAAACTTCATCAAGGTTATTGCCCTCTATTGTTGAAATAGGC
AATTCATTTACGTACTCTACTAATGTTTTTGCTGTTGTTTTACCTACATTTGGAATACCT
AAAGCATATATAAAATTCTCTAATTTACATTCCTTAGACTTTTCTATATTTTTAACTAGA
TTATTAAATGATTTAACGCCAAAACCTTCTAATCTTTTAATTTCTGTTTTATCTTGTAAT
TTATAAATATCTTCAATGCTACTTAAATATCCTTTTTCAATAAACCTTTCTATTGTCTTT
TCACTTAATCCTTCAATATTCATAGCATTTCTACTACAATAATGTTTTATCTTTTGAACT
AATTGAGCTTTACAATTAGGATTTTCACACATTAAGAACTCAGCATTATCACTTATTTTT
ATATTTGTCTCATGACCACATACTGGACATTTACTAGGTATTTCTAATGTGTCTGATTGA
GTTAGATTTTCTCTGATTTGAGGTATTATTTGATTTGCTTTATAAACTGTGATTTTGTCT
CCTAAACCTAGTTTAAATTTCTTCATTGTACTAACATTATGTAAAGTTGCTTTAGTTACT
TCCGTACCATCTAATTCTACAAGATTAAATATTGCAACTGGAGTTATTACACCAGTTCTT
CCTACTTGCCATTGTACTTCTTTTAATAATGTTTCTTCTTCCTCATCATAAAACTTAAAC
GCCATTGAATGATTCGGATGATGTGCTGTATTACCTAATGATTCCCCATATGATTTATCG
TCATAAGTAAATACTAATCCATCGATTGGAATTTGTTTTTTAGCAGCTAAACTTTTTAAT
TCTTCGATATCCTTATTAAGATTATTTACTATTTTATAATCGACTACATCAAACCCTTGT
TCTTCTAGCCATTTTAGTTCTTCTACTTTACTTATAAATTCTTTACCAAAGACGTTATAA
GATAAAAACTTTACCTTTCTCTTTGCACAAATTTTACTATCTAGCTGTCTTACGCTACCT

GAAACTAAATTTCTAGGATTCTTATATTTCCCATTTTTATTAATCTCATTAAAGGTATTA
TATGTTATTATAGATTCTCCTACTATATGTATTTTTCCTTTGTATTTAATCTTTTTGGGA
ACATTAATATAAGTTTTAACATTATGAGTAATATCTTCACCTATTTCTCCATTACCCCTT
GTGCTTCCTTCTATTAATTCACCATCTTCATATATTAAATCAGTAGTTAAACCATCTAAT
TTAAGCATTCCTACGGCTTTTTTATTACCAATAAAGCTATTTAATTCATCTATATCTTGA
GTTTTACCCAACGATCTTAAAATATATTCATGCTTAACTTTTTCTAATTTGCTTTTAACT
TCATATCCAACATTAACAGAAGGTGAGTTATTTAAAACTATTCCAGTTTCTTTTTCTAAT
TCTTTTAATCTATCAAGTTTAATATCCCATTCTTTATCACTCATAATAGTATTCGAAGTA
TTATAATACATATCACTAGCCTCATTCAATTCAGATATTAAGCTTTTCATTTCTTTTATC
TTATATTCACTTCTGTAACTTTCCATCTCATTCTTCCTCTCCTTTATTAATCTCCTTGGC
TCTTTCGCATTTTTTACCACAATGCATACAGCCATTTTTCTTCTTATATATGCAATCACA
AGTCATCAAATCAAAAGGACATACCTCTAAAACATCTCCTATCTTCTCTACTACATCAAG
TCTTGGAAATTTCTCTCTGTCGTTTTCTAATCTTGATATTTTAGTAATGCTAGTTCCAAC
TTCCTCAGCTAATTTTGATTGTGAATAATTTTTCATTTTTCTATACATCTTTATATTTTT
AAAACTGATTTTACAATTACCCATAATAAAACTCATCTCCTATATGAATTAAATTCACTT
TAATATTCTTTTTATTATATCATTATTTTTCGCAAAAAAATATGGTAATTTAAACCAAAA
TACATATATTTTAAAAATATTTTGTCGTAATTATTCGCATTTGGTAAATAAATCTTGCGT
ATAATTAAAATATGGTAACTGTCTTGCAAATCTACTTCCTTTTATCTTATACATAATTTC
TCCAAAATGACCTCTAAATATTATAAAATCTTCGTCATTAACTCTTTCAAATTTATCATT
AGGATAAATCTCTAAATGAGATAAAACTTGTTTCAAACTATACAACTCTTTCACTTTCAA
ATTTCTCCTTAAAATATTCATAAAACTCATTATTATTCTTAAATGTTAAATCAGCTTTAA
ATCCTCTTAACTCTTTTCCATCATGTTTCAATCTATATATAATATCTTTTATTTTATCTC
CTCTTTTAAACATTCTATAAGCTCTTTTAAAAGGATTTACATCTATATAAACTGTTACAA
TATTATATTTGTCTTTATTCTTCATGCTATCAATACCATGTCTATCAATAATGTAAAAAT
CTTTATTAATTATATCTTCTTCCAAAACATAATATCTGTTATTATTAATAATTGTTTTTG
CTATTGCTTTGTCAAATTCCTTGTCTGCTTGTTTATTATCAATAAATAGATGCGTACCTT
TTTCATTAGGTCTTCTCGCTCTAGTAGTCCTTGAAACCACTTTTTCCTTCTTAAACTCCT
TACAAAGTCTATCTACTGAATGATCTTTACCAGCAGAACTCTTAGCGACAACAAGAAATA
ATTCTTTTTTCATATGTAACACCCCCTTTATGCAGCTATCTCTAAAACTTTATTTAAATT
TTCTTTTTCTAATTGATTTATGTATATTCTTTTGTTTTTACCTAAGTTGTTATAAATATT
CTTCACTTGGTAATCTTCCATAGATACTATTATTTTTAAATTACCAAACATTCTTGCTTG
TACATTTTCTATGTTATCTGTCCATATCTCCTCCCCTAGAATAAAGTTTCTAGTTAATAA
CTTTCTAATCTCTAATATACTTTTGGTTTCGTTTCCTTTTACATATGTTCTATATTTATG
TATTATTCCTTTGTCTATTTTTAATAAATTAGTTCTCATAATAAAGCATCTCCTTATATG
TATTAATTATTTAATTTATTTAAACTTTCTGAGTGCAAATTTATTCATCATTTTTCTTTT
AACTTTACGCTCAGCTTTTCCTCTAAGGTATTGTTTAACCTCTTCATTACTCATTTCTTC
AAATGGTTTATGTATCATAATGTTCTCTCCCTCATATGCAATATATTTTTTATTTCTCTT
AACATCTATTAGTATAATACCAATTATACTAAATGTCAATTGAAACTTTAATTTATTTCA
ACTTTTTTTGTACTTTCCAACCATTATCGTATGTTAAATTTAATGTATCACAGCATTCAT
TAACTATAACTCTTGCATTATCTTCTAAAATATTACATCTTAAAATTGAATATTGATACT
GTTCACAAAAATCTATTAGCTCTTTTATTTTATTTATACTCTTCTGTGGATTATACATTA
TTTCTCACCTCTAATTTAATTAATACTTTGATTTATATTTAAAATATAAATCTGTAGGAA
GGATATTACTCTATCCTACATTTTTTAATCTCTTCAAAATCTTCTAATGCCTTATTTCTT
TCAGGATCATTATCTCCATAGTCTTCTCCGACAAAATAATAGCATAATACATTTTCTTTA
TTGTTATTTATCCCAACTAATATTATTTGATGATTGTCATTTAATCCTTCTAAACATTCT
ATAAAATCTTTAACTTCTTTGTTGATTGGATTATCATTATATTTAACTCTAAATTCTTTA
TATCTTTGTTTTAAAGAATCAATATAATCCCAATCTACCTCATCTACAAAATATTTATAT
ACTTTATCTAAATCTACTTTAAAGTCATACTGCATAAATTCACCTTCTTTCTTTAATACC
ATATCATATCTTTACTATAATTGTCTAAATCTATTTTATAATAGTCTTTATTAGCTGGAC
TTGGTATTATATATCTGGAATCTACAAATAAATTTTCATCCTCTTCTAAATCATAGCATA
AACTATATCTATATTCATTCGATGCTTCATCATATACTATACACAATACATTATAATCAT
ATCCATCACAAATAATACTAAATGGCAACTTATAATAGTTAGTGTTATAACTACTTGCCT
TTCTAATATGATATTGATTTAAAAACTCATCATTCAACTTTAAATACATTTTATCTACAC
CCCCTTTTTTAATAAATTAACTCAAATATCTCATTAGTATCGTTTATGTAAAAAATATCA
TTGGCATGATACTTAATATACTTTTCATATGTATCACATCCTTTAGGAAGTTTATATTTA
TCAAAATCTTTAAATGTTTCAAAACATAATCTTTTACATTGATCCTCTAAAGTTTCTCCG

TTTAATCTTTGTAATTTAATTTTTTTATTCATAATTATTATCCTTTCTTTTATTTTCAAC
ATTTTATAAAACTATCAATTTATAAAGTTAAACTTCCTCATGATAAGATCTTTCATTTAA
AAGTCTACCATCATCAACATCATAAATCTCACATTCTTTTTTTATGTAGGCAACTTACTA
TACAATCATCCTTACCTTTGAATTTCCTCATTTTTTAACCTATATAAATAAATAATGTTA
CAAAAAACAAAACTATTAATATGTAAATAATTCCCGCAACCAAACATCCAACTTTTATTG
TTATTATAGCTATTAATAAAATCACAATCATTAATAATATAAATAATATTACATCCATCA
TTACTCTCCATACATTTCACACATACAATTCTTTTCTACTAAATATAATATATCTTTTGG
TGAATCCTCTTCCCACCAATCAAGAAATAATGTGCATACATACAAGTCTTTACCTTCTAT
CTTTTCTCTATATTTACATCTGCTCATTAATATTCTCCTTACTTTAATTCTATAATATCT
TTTTCATTAACCCAAACTATATATTGACATACTTTTAATCCGTATAATATTTGATCATCT
TCCTTTTTTATTTTTGATATATTCGCTACAGTTCCCTTTGACACCTTTTCAAAATTACTA
ACTATAATATCCTTTTTGTAATTGTACCTTTACTCCAACTTTTAACATAATTATTCCTCCT
TAAAATTCAAATTCTTCAGTAAGTTGATTAAACTTACATCTTTCGCATATTTCATCACCG
TTTTCAAATAATTCAACTCTTAATCTTCCACAATTAATACACTCTTTATCTATGTAACCA
TAACATTTACCTTTTTCTTTATCGACCATTATTAACTCCTCCCTTTTAAAAGCAAGTGCT
TTAATTTCATAGAAACCATTACCAACATAGTCTGCTTCATAAAGATCATTTGCATTAAAA
CCATAACTTAATAATTCATATTTATCTTCAACTTTTTCAAAATCAATCCTTACTTTTGTT
CTACTTATATTTGTTCCTTTCTCTACAAAACCGAATTATTGGGGCTAAAATACTTCCTAT
TCCTAATGCGATAAAAATATTCGTTAGTATTTCATTGAAATTCATTTTATTCCTCCTCGT
AACATTCCCAACATAATACTCTATCTATATCTCCTGATCCACCCTCCTCTTCATTTAAAC
ATTCTACGTCTCCACATAAAAATACAACTTCTTCTCTATGAAAATTTTCTTTACATTTAT
CACATTTATACATAATAATTCTACTCTCCTCTATTCTTTAATTTATTTTTTATTTAAAAC
AAGCAATTTATAGTGTTATATTTTATCTATAGCTTGTTTAATAGCTGAATAAACCCCTAT
AGCATAACTCTCTTCCATATGGTCATAACCCCATATATCTTCTCCGACAACTAATTCTTT
TGCTTTAGATAAACAATCTCTTAAATCATCTTTTAATTCATCTCTTTTTCTATCATAATC
AGTATAAACACTCATGTTTAATCATCTCCTATTCTATTAATCCATCTTTGATTAATTCTA
CTATTACATCCAAATCACATAGTTGCAATAAGCCTGTTCTTTTAGCCATAGCTAATATTT
CAAATTTTCTAGTATGTATATTAACGACTAATCTAAATTCTGAACTATAACAACTTTTAA
ATTCATAATAGTAATTATTTTCTCCATAATAATGTTCTCCTTCTTCACAATTATCTGGAT
CTTCTATAAAACCATATTTCTCTAATTCTTTAAAATCTATATTGTCTTTTAACTTCATTA
TTTATCCCTCCTGTTTTATATATTTATCCTCATTATTAAATTCTATATCTATTTTTAATC
TAAGATATGCACAGAACAACATATTTTGTATTTGTAAATGTGAATACCCCTTATTGATCA
ATTCTTTATAAAAAGGATATATTGATTTTATAAATTTATCTACATCTCTGCTTGTCTCAT
CAGAAAATTGTTCTAATCCATTATTTATTTCTATAAAGTTTTCTTTCATATTATCTCCCT
CCCATTCTTTTGTTAATAATTATCCCAACAACCTTGAGAATAACCTAAATCATATACTCT
ATCCATTAATTTAACTCTCTTTATAGCTTTAGAATATGTATCTGCTGAAGTAAACTCTTT
ACCATTTCTTTCTATACCAAAATAATTATTACCTTTCTTTATAACATCAAAGTTCCTTCC
TTCAAAAATCATTTTTCTCATTTTATCACTTTAAAACACTTTTTCTATTCCTAACTCTCT
CATCTTATCAAATATTTCTTTTTTAGTTCCTATAAAAACTGTTGATTTTGTTTCTTCATT
TTTCAAAAACCATTCATCTTCATTAAAAAATGGTGAACCAATTTTCCAAACCGGATTTAT
CGTATAGGATATTGAACCTTTCCTATACAAAGCTAAAACATCAAATTTCATCTAAATATC
CTCTCTTTATATCTATTTTTCTATCACTTCTACTGACAATTTAATTCTACACTTGGCGTT
TCCAAAATCATATATACTGTCTGGAGTTATTGAATATAAGCCTCTATTTGATGTACTTCT
TTCATAGTCATAAGGTTTTCTTTTTATTATATTTATGAAAGTTTCTTTATCAACATCCCA
GCAAAAACTATAACAATCACTACTATTAATTCCTTCAAATTCTAAATCAATACTTTTAGT
CTTTAAATAGTCTTCCATTCTGCACACCCCTTAATTTTAAATAAAAATTCTATTTTATGA
AGTTATAAATCAAATTTCATTTCTATCTCTCCTTTTCTAAATTTTCATTTTGATATATTA
AAGTAGTGGCTGGAAAACTTATTTCCCCCACGTTACCTAAATTGTCTCTTTCTACCATTA
CTGGTATATACTCAATTTTATATGTATAACCCTTTTCTTTTCTTTTATCAAATAATTTTT
TTCTAGAAAAGAAATCCCTCATTAAATCTTTTTGTAATAACTTTAATGTAGATATTTTCA
TATTCTCCTCCTTAAAATTTCAAAATCTTCGTTCATTACTTTACCTCTATTTATTGATTT
TTTGAAATCTAGCAAACCAATAATATTCTCTGTCTTTATGCCATGATAAGGGATCATCGG
ATTTTCTTACTTGCCCGTCACATATCCATCCTTCTTTAATCATTTCTTCAACATGTGACA
TTCTCTCTTCTACACTTTCATATTTATACTCTTGAATTATAGTTTCTAATAGTTTTATTC
CTTTACTCATTTTTATCCTCCAATTTTAGCATTTTTAATTTATTCTTTCTATTTAGTTAT
TCTCTAATAAATCATCTTCATTTAATCCGCTATTTTTAAAGTCATTATATAATTCTTTTA

TTTCTTCTTGTGCCTTATCGAAATCAAACATACTAGAAGCGTGTAATTCAATGTTACATA
TTGCCTCCCCTATAGTTTCTAATCCACAATCCTTTCCTATTTTTATAGCTCTTTTTAATT
TCATAATATTATTCCTCCTAAAATTTAATTTACTTTAAATCTCCTCTTTTAAGCACTTCT
AAAAACTTACATAATAGAATATTTCTTCATTGTTGAAATCAATATCTATTCCTAATTCTT
TTAATTTATCTACATCTTCTTTAATGATTAATACTTGGTCATATTCTTTTTTATATTTGC
TCTTATATCTAAAATAGTTTTCTAAATCCCAGTTATTATAAGTATAAAATAACTCTTTTT
CTCTTATATCAGTTTCTCCACTCAATATAAAATAAAGATATTCATCTAAATCTAAACTAT
CTTTTATTTCTCTAATCTTTTTAACTTTTCTAATATATTGATCTAGCATTATATATCACT
CCATTTAATTAATCATCATAATACTCATCACCAAATTCTTCATAATCTGCAAATCCCCTA
TTATATCCTTGCTTAAAAGCCCAATCCATTGCATTTACTTTATTTATTGCTTCAAATTTA
GTCCTTGCTGGTGACACAACATTTTCAAAAATGCTATATTGAATTTCAAATCTCTCATTT
GATTTTCTAATAACGTTAAAATATTTACCTTCATATACAATCTCTAATTCCATATCTTCT
CCACCCCTTATTAAATTGCTACTGTAATTTTATACAATATTACATCTTTTATATTAGGAT
TTTTACTGTACTCCCAAATTTCTAACATTAAATTATCTCCTTCTTTTCTCTCACAACCTT
TAGAATATCTTCCTATTTTCTTTCCTTGAGATTGTAGTTCTTGTATTTTATTATCTAAAT
TTTTTTCATTGTAAATTTTCCATATTTTTTCTCTAGCTATTTTTAATCTTTTTTGATAAT
CTTTATATCTATTAAAACCCTTGATATAAGCTTCAATGCCCATTATTTCTCACCTCTATC
AAATCCACAACCACCACCAGTTATTAATCCTTTGCTTTCTATCCACTCTATATAATCCAT
TTAAGAATTCTTCTAATTCTTCATTAGTAAATTCTTTTCCATTTGCTTTGCTTATTGCTT
GATCTAAAATTATTGTTGTTTTATCTATTAAAGTTTTTTCAATATTCTTATCTTGAGATA
ATATGTATTCATATAGGCTTTGTATAACATATATATCTACATATCCTCCTAGAACACCAT
CGAAATTTTCTTCCAAAAACTTATTTTTATATTTCCAATCATTGAAAAAACTATCATCGC
TAATATCAATAAACTTTGTTCCCTTCCCTTTAAAAATTGCCAATTCCATACTTGTATAAT
CATATATATTTTCTAATGTTTTTCTAGGTTCACAATAATGTGATGAAGAAGCTTGAATTG
ACAATGTATAATCGCCTACTGATAAATGATTAAATAATCTATAACTACAACTGCTTTCTG
TTGTTTTTAATAATTCTCTAAAATCTATCATTTTTTACACCTCTTATATGTATACTATAT
GTATTTAATTCTAATTAAAATTTCCCATTTATTTATATTAAAAACATCTTCTTTTCTAAA
GTAGGAAGATTATAGACTACTTATAATCCGTATTTTACTACGTTTCTATTTAATTTTTAA
TTATGTACTGAAAATTATGCTTTTTGGAAGTTGTACTCACCTCCTATAGAGATACTATCT
TGAATATCTCTATAGGAGGGAATAGTCCCTCCATAATCAAGTTTGATTGTTTCTGACTTT
AATTCTTTTAGATAGACTATATATTTAACTATTTCTTCATCGTGTCTTTTGATGTGATTT
AATATACTTATTGTCCAATTTAACTTATACCTTTCTAATATTTCCACTAAATTATCTTCA
ATAAATTCTATCAATTGTGAATTATATTTATTAGACAACTTGGTAAAACAGGTTAACAAT
GTAATCTTATTTTCTAAACTATCACTAGATACAAAATATTTCTTTTTTATATCCTCTAAA
TTTTTTAACCTTTGTTGAATAACACTTTTTGATTCATTTTTAGCTATTGTTTTTTCAATC
AAAAAATTTTCCATAAAAAGCTCTTGACTAAACTCTTTATCTATATTACAACTAAGCTTT
TTTACTATAAAATTATCTAAATTATTCTTATTTTTTTTACTCTTATAGTCCCCCAATATT
TTAACGATAGCCATATTTTAACACACCCCTATTTTGTTGCTTTTTTTACTTATTTTTCTT
TTGTTACACCCTTTCAAAAAATCATTATGATTTAATCCATTAGCTTTACAAAAAGCTTCT
AAATCCTCCATAACAGCCTCTCTTTCTTCTATTAAACCTTCAAAGAAATTTTCTATATCT
ACTCCATTTTTGATCGCATATCTTATGTGTGAATCTATTCTTTCAATTTCATTAACTTTC
TCTTCTATTATTTCTAAATTCTTTTCTTCTATAGATAATAGATTTAAAGATAATGCCGCT
GCAACATTTATTTTCTTTCTATCTTTTGGGCTTATTTCCCCTATTTTTTCTTTTAATTTT
CTTTTATCTAAAACCCTAATTTGTTCTAACATTGCCATACTTTCTTTTTGTAAACCTATG
TATTCCCCGTCTAGTTTTACGTGTGTAGGAATCTTATTTTTGTTTACCTTTGTTGTTATT
GGTGCAATTATTGTAGTAGGGCTATATCTGTTTCCTTTGTTGTTTTGAATTATTACAACT
GGTCTCATCCCATATTGTTCGCAATCTTGAACTCCTTCTGGTTTTTCTCCAAGATTAGCA
TAAAATACATCTCCTCTTTGACAATTTTTAATCATTGTTTCCATTTAAAATCGCTCCTTT
TTATATTATTCTTTGTTGTATTAATCTCTTTATCTTATGTCTTTATTATACAATAGATAA
TATTATTTGTCAATCAATTCTTTAATTTATTTTAACTTTTATCTATCGTAAATTATATGT
AATAAATAAGCACCTACAAAAGAAGTAGATATTACCCCTAATAAATATATTATTGCAACT
CCTAATATCGCCACGCCTGTTGTTAATCTATCTATTATATTTTCTTTGATTTTATCTCTC
ATCAATTCCATATTTATCATCTCCTTTGATTATATATTAACATAAATATCACTATAAATC
AACCAATTATTTAATTTATTTTAACATTTTATAAAATAAAAAGACTGATAGGAATTTTAT
TTCCCATCAGTCCAATATTATTCTATTCCCATTTTCATCATAGTTATATTTCCCATATAT
AATATCATCATCATTTGTAATTTTTATATATAGATCTTTTAATTTTAAAGCTATATTGTA

AGATTTTTTTAACTCATATTCTTCTATGACTTCTATAAAATCTTTCATATCTAATTTGTT
CCATTTTTTAGTTCTTTTTATTTTTATAAGTGTATCTATTTCTGCATTTCTGAATAAATC
TTTGGCTGGTATATATTCCCTATTAGCTTCTGTGAAAAAGTCTATTATACCTTTTCTTAT
AGCCATCAAATCTATTACTTTACCTTTTGTAGATTTTAATATATACTCTGTGTCTATAAA
TTCTTCTTCAGATATTTTATGGTAAGCTTTTTTACGCATATATTCTTGCTTATCAGCTTG
AACTAAAACATCTAAAATTCTTTTGTCTATATTAATTTTCTTAACTACTTTAACATCTTC
TAAGTTATTTTCATCTCGATTTGTTACGTTAATTATATAGTTATCCAAATCTATATCTTG
CATTTTAAGATATCTTAATTCTTTATACTTAGATCCCTTTAATCCCAACCTTGCTAAAAG
TACAATTGCAAATTCATGCACCGTAATAATTTCTGCCCTATCTTTTATTTTATCCCATAA
AGCAAATAAATCATCTAAAGAAATATAGTTTTTTCTTATTTTTTCCTCGTTGATAATTGC
TATTTCTCTTGTGTTCCAGTTTATGCAAGGGTTATAGGTTGGGTTTAAACCTGTTTCTAA
TGCCCATTCTTCATAGCCGTTGATTAAGCTTTTTAATGAAGCTATTGTACTTAAATCTGT
TGTCATTCTTGTTTTTATTATGCTTAATGCTTCCATTTCATTAAACATCGCCAAATCTTT
ATCGTACATAACTTCAAGGTCATGTATGTGATTATTGAACATACTCCAATAATTTTGTTT
TGTTGTATCTGCGTACCCTTGTTCTTCTAAGAATTTTATTTTATTTTTTGAAAAAGTATC
TAAATTATTTATTTCTTTTAATGCTCTATTATAATCTACAATATAATTAGAAAACATAAA
TAACCCTCCTTTGAATTTTATATTAAATCTCTAAAGAAATTATAAATATCACTGATAATA
AAACTTTTATAATTTACTTTTAATTCTTTCATTTCATCTTTTAATATTATGGCGTTATTA
ATCACATTTTTAATTAATTCATACTCTTCAAGGTCATCTAATTTATTCTTTCGTAAATAA
CTCCATATTGCAAAATATCCTATCCAAATATTTTCACTTTTAAATATTTCCATTGTTTTG
CTATCTACTTTTTTATCTTCTAATATCTCAAATATAGTGTTTACAATTCTTACGTAATTT
TCAACACCAAAAATCATTATATTTCTACCTTCAATTTTTATTCTCTCATTTTTTTCTACA
TATCTTAATCCTTTTTCAAAAACTTCATAGTGGGTTAGTTTTCCCATTTCTATAAATTCA
TTTCTTGTCTTTGCTATTTGGTCATGTAAAATGCCTTTACTACCATTTACCTTCTTAATA
AAAGTGTTATAATTATTATCTTGTATAGCTTTTATATATTGTTTACTAGATACATTTGCT
AACATTTGTCTTCCAACGAATTTAGCAGCTCTTTCTGGTGTGATAATAGAAATATTTACA
GGGAAGCCTTTAAAACTTTTTTCTACATCATACCCCTCATTATATAACTCATAGATAGCA
GTATCTCTATGCCACCCATCAGTAAAGTCAACCACAGTATAATCTACTGCATCTATATCA
TAATTTATTCTTATTCTCAGTTGTCTTTTATTTTCATCATATTCTACGTTTGCTACTTTA
TCTTCTAAAAGTAAAATATTCAAACTTAATACGTCAGGAGGAAAATAATCTCCCTCTTTG
ATCATTTTTTTAATTCCTTCTACGTTTGGTCTATTCAATGTTATTTCTTTTCTTATTGCC
CCGTAGTTGGTTCTTGTTATAGTTGCTTCTCTTTGAGTAGCAAAATTATATCTTACTAAT
CTTTCTTTTCTAGCCCTATATTGTTCATGGGGTTGCCAATAGGTACAACAATAAAAATCA
TCACTATATTCATCTACATTGTCAAAAACTAAAACCATACTATCATCTTCTTTAAGATTA
ACATAAGTTTCATATGCTCCTAGTTCATTTGTATTAAAATAGCTGCTTGGTTTAAATTGT
TCTAAACCATAATTATATAACCCCTTTGCAATTGCTATTTGTTCATTTGTTTCCAAATCA
CTTATATTTAACCCGTTTTCTTCGTTCATTACTTCATTAAAAACAAAAGGGGCTAATTTT
TTCTTGATTAATTCATTTTTTACGGATCTATAAATTTTCTTTGTTTCATTAAAATCTATA
GATCCCATGTATTTATATACATCAGATTCAAACTTTTGAACTTTATTATTCATTTGTAAC
ACCTACCTTTTTATTTATTTTGTCCTTTTATGTCGTTTAATATGTTTTTATTATATCATT
TTATATAATTTAAATCAATTACATTTATTATTTTAAGATAGAACCCCACCACCACAATAA
GGTTCTATCTATTATCTATGATATATTAATTAAATTATATAAAACTTATGTATCCCCTCT
TTGCTATTTATACATAGCTCTCCAAACTCTAATAAACAAGTTAGATGTTTAATAATATTG
TCCACTGTTATTTCTAAAGTATCTAGTACAACTCGTACTGCATTTGGATATATAGTATAA
TCCTCTACGCAACCTACTTTAATATCTCCCCTTACATTTTTATAAATTACCTCCTCGTGT
AAATTTTCCTCCATGAATTCTTTAATTTTTTCCATCTTCTCCCATAACCTTTCCTTTTAA
TAATAATTTAAATTTAATTAACATTTATTGAACAAAATAAGTATAGCATTTTCTTTTTAT
TCAGACTATTGTTAAATTTAAAAATTATTCGCTTTTTTTCCACAAAATATTTATATTTAA
CATTTTATGTAATATATTTTAATTATTATTTTATTTTTATTTAATGACAAATTAAAAACA
TTTTTATAGTAACCTTCGAATATATTTTATTAAACACAGGTTGTGACATAAGTTTAAAAG
AAGTTTGTTATTTTAATATATCGTTAAGAAAAACGAGAAAGAGAAATAAAATTCTAGCCT
TCTTTTAAACTTATATTGATAATTAATTAGTTGCATTATCTTTTTTATTTTTTATATTCT
TTAAAACTTTTATAAATTTTCTTTCGCTCTTTCTTCTATTTTTATTCATATTTTTTCTCT
TTTCAGCTATCATAAAGTTGCATACCTTATTAAATTCTTTTAAAAAAGTATATTTATTTT
CAACATAATCAAATGTAATTATTTTAAATTTTCCAGTTGGAGTTATTATTCTCTTAACTA
ACTTTTGCTTTGCATTTACATTTAAAAGATATAAACAGCATACGTTTCCATCATTATCTA

CAAAGTTTGGAGATTTATTGAATATATCTTTGCTCGGTCTATTTATTATAGGAACTTCCT
TACATCCAACATTGAATACAGATTTTATTTCAGATAGCTCCATTAAAAATCTAAGCCCTA
GTGTTTGTAATAATTTTACTACAATATAAGAATCATATGATGCATTATGTAAATTTTCCT
CTACAAAATCCATATCTAGCATACTTGCTACTGTCTCTAAAGAAATAGGAAAATTTACAC
TAAAATATCTACTTAACCCCTTTTGCACATCTATATATTTTGCTTTGTATAAATGTTTAG
GTGGATTTTTACCAAAGTAATACATTAAATGTTTTTTAAATATCTGTTCATCATCACATC
CCCATGTTATTATTAAAGTTGCTTCTGATGAAGTTACAGACAGATCTTCTATAATTTTTT
TAGTGGCATACTTAAAATTAATTCCCTCTTTTTCTAATTTTTCCGTGGTTATTCCTGTTA
GTTTAGTTATTGACTTATGAATCTTTGTAGAAATATTTAAAGAACAGTAACTATTGGTTT
TGTTTATTATGTTAGCATTATCATCAATTGAATATCCAGCTATTTGTATAACTTCATTTT
TAAAACATTTTGCAAGACTTCTCTTTTTATCATAAACATGTCTTGGTAGAAGCATATTAA
ATTCCATATCTAGTACGATATATCTCATAAATCATCCCTCCTTATTTAATTTTCTATATC
TATATAGTATATGATTATGTAAATATAATTGCAAATTTTAAATAAAAGAAATATTTTATC
AAAATATTATTATCTATGTATTAATTCTTGTATTAATTCCACATCCATAAGTTCTTTATG
TTCGACATATTCAGGACTTTCAAATTTAACTGATAATTCTTCTTCAAAGTATTCTGTATG
TGGAACATTGGTTATACCACTAAATTGTAAGTGGTCGTTATTAACATTACATATAAAATA
AGCATTTTTACATTTTCTAGTTAAACAATCTCCATGAACTACTACCCCATTCATTCCTCT
AATCATCATGTTTAATAATAAGAAAGGAATACATCTATCACTTAACTCTTCTACTACGGC
GTATTGTAAATCTGGAGTATAATCAAAAGGCGAAAATTTTCTTCTATAATGATCCCAGTT
CTTAATCATTATTCCCCCTGTACCAGCACAACATTCATAATATAAACCATTATTTTCTTC
AACATCACTTATCTTTGTTAATAATGTTGCTACTGATTCTGGTGTAAAATCTTGTTTTTT
ATTTTTTCTATCTGCGTGTTCCTCTTGGAAATACTCATAGAACCAATCAAAATCTACATT
ATAATCGTTCGCTTCTAATAACTCTTTAAACATTTTTTCTCTTTTATCTTTATCATAAAG
GATCTTCATAATTTTTTCTGGTGCTTGATAACTATCCTCTACTCCAAATATTTTATTGTA
ATCTACCATCTTATTTATCTCCTCTCAACTTTAAAACTGACATTCTTAATAATTCTCTTT
TAAAATTTTCACTATCTCCCAAACTTTGTTTTGCATACTCCATCGCAAAGTCATTTAAGA
TTTTTTCTAGTTGATTATGTTTTTCTTCATAAAAACTCTCTCTTCTATAAATTTTTTCCT
CTAGCTTTTCAGGTATGTACTCAAATTCTCCTCCACCCATAGCACTATTTTTTCTCATAA
ATATCATCCTCTCTAATTTCCATAATGTTTAAATAAATTAAAGTTATTGTTGTTATATTA
TTATAATATCATGCCCAAAACATTAAGTCAATCAATTATTTAATTTATTTCAACATTTAT
TAAAATGTGCAATTTAAGAAGTTATACAAATAGATTTAAAAATAAAAAGCTGCCCTTAAT
AGAACAACTTTTATAACTCTTTAACACTCTTCTTCATAATCATAAGTAGCTAATACATTT
AAACCTGAAAAGCCAAATTCCTCTTTCAATACTTTCACCATAGTATAATTTAAAACCTCA
TTATACTTTTCTTTGCAAATCTCTTTTGCTTTATTACATATGTCAACAAACTCTTCTTTG
GTATATATCTTAGGATTGGTTAAATATTCATTATCCCAACCTACTCCATCTTCTAATCCT
TCTTCTTCAAACATCATGCTTAATTTATATACATTCATTTTACTTATCTCCTTTACCTAA
TTCATTTAAATCTATATTTACAGCCTTAGAACAGTCAATACATTCTATAATCTCAGCAAT
ATATTTTTTATTTGCTCTCTACAAACTCTTTTAAATCTTCAATACTTTCAAACTATTCGT
AAGTTAAATATCTATCAATATGTCTATAAGTTAATAATATTTTATTATCCATTTTAATAT
CACTCCTTAATTTTTTAATATATTTTATCTAAGTTGCTTTTATTTATCAAGCCTCATCTT
CTTGTAATACTAATTCATCACAATTACAACAATATATATTATCAAAAGAATCATCTATAT
TACTTTTATCTACATCATATATCATAGACAGATTTTCTCCATATTTATCAACCCTTTGTT
TTGCTGAAATACTTACATTTCTATAAAATTTAGATTTATTTCCACATTTAGAACATATTA
AACATTCCTCCATATAATCCGCCTCCTTTTAAAATTCGGTTTTTAATCTAAATTTTTAAA
ACTCTTTATTAATTTAGCCAGATCTTTCTCTTTAATAATATCTGTGCTTAAATCTAAAAT
AACTCTATCTAATTCTATATTTAACTTAATATTATTCTTTTTGCAAAGCATATTTAAAAC
AAATGCACTTGTTCTTTTATTACCATCTAAAAATATATGATTTTTAGTTATACTGAAAAC
TAAATGAGCTATTTTATCTTCTAACTCTGGATATAATTCTAATCCGAAAACTTCTTGATT
GCATCCTTCAATCGCACTTATTGCAAGATTTTCATCTTTAAAACCTACACCTACACCGCC
AAACATTGTAACTAATTTCTTATTAATCTCATATAAATCGTTAATAGTTATAAGATTTTT
TCTCATTACATGTCAGCCATCCTTTTATATAAATCAAAATTTTCATTGATTTCTGAGTCA
ATTTCTTCATTAGTAATTTTTCTAGTTTTATCAAGTTCTTTTTGAATATCAAAATTATAT
TTATACCCATATTCCTTTATAAAGTCTAAAACATCATTTAAACATATTTCTTTATTCATT
TAAATCACTTCCCATCTTTATATATAATTATATTTTAACATTTATCTACTCTTTAAGCTA
GATTTTTCTAACCTAAAGAGTAGATAAATGATTGATTTTATATACTTAAAAAATCTTGAA
TCACCACTATTTTACCGCTATCACCCTTTTCTTGAATAGCAATTGTTTTATCTATAGAGT

AATCCTCTGTACTATTACCTCTTACTCTTAATATAATTTCTGTATCATCACTATAACTTT
CTAATAAATCTCTTAATTCTCCAACTGTTGTTCTTGTCATTGTTAACTTCCTCCTTCAAT
AACTTATTATCTTTATTCTAAAACATTATACCTTTATTTAAAATACTTATTTTATAACTT
CTTCGACCTCATCTGGTGAAAATATCAACCTACCGTCGCATGTGTTTAATATGTAATATC
CTAAGCATCCTGATCCTATATCTTTGTGCTTTTCTAAATAGTTGCTATCTATTACACAAG
CTAATTTAAATATCTTTCCTTCTAATCCTGTTGCGAATTCTCTTACGCTTTCAGTATCTA
AAACTCTAAATTCTCTTTTCATTTTAAAACTCAACTCCTTCTTAATTCTTTAATTTATTT
AAACAAGCTTTATAAGTAAGAAACTTAAAGACTAAAGCTTTTATAGAGTTAAGATTTTAA
CTCTTAGATATGTATTGGATTTATCTCAAATCCCTTCTACAATTATTATTATACTCTTAT
TATATAAAATGTCAAGTAAAAATTTAATTTATTTTAACATTTTTATAAAATATCAGATTT
AATTAGTTTTGAGTTATACATAATATGTCAGTCCATTGTTCAACTTTAGAAAACTTATCA
AAAAGGTATTAATATTAATTGCCGTTTAAATGATAACTTTTCTATAGTGTAAGATCTACC
ATTCTGTGAACCAATTTCTTACTTTTAGACAATTTAAAGTGTAAAGGTATTACTTCTAGA
CAATTTTTAAAAATCTTTTATTAGAAAAACATACTAAAAAACTTATTTATACCTATATTA
TTATTTATATTGTATCTACATTAGACTTAAATATAAAATCTATCAAAAACAAATAACTAC
TAAGCTAAATTAATAATGGTATTTTTCTGTTAAGCAAGGATACGCAGCTTATAAAGAAAA
AAAGAGGTATAAAACCTAAGTTTTGTACCTCTAATAATACGTTATATCTTAAATAAATTA
ATTAAGTATGCTAGATCGTCAATGTTATTGCCTTTATAATAAATTCTAGGATTAACAATT
AAAAATTTCTTTTCATAATCTTCAATCATCATCATTGCCTTTTCATTATTGACAAAAATA
TTTAATAATTGGTTTTTAAACCTTGTAATGTTTTTCCCAGAATAACTATTGGTAAAATCT
CTTAATTCCTGGATCGTGAATGGCTCAACATCTTGCATAAGTTCACATTTAGGATTCTTG
CAAATTATATTATATTTATAATGTATAAATGGTAATAATTCTATAAATAAAGCTAATCTT
TTATGTTCTCTAGCTGTACTATTGTTATATAACTCTTGTATAGATTCTTTAAAGATCCTA
CTGTATTCTTGTTTATTATGTTTATCAACCTTACCAATAAAGCTTACTTTGTTGTTTATA
GATACTGTTTTGTCTTCATTAATTGTTATAAGTTTATTCTCCTCCAAAGTATTCTTTGTT
CTATAATACTCTTTTTGACTTAATTTTAATAAATCCATAAGATCACTCACCTTTATTAAT
TCATATTTGTTTTGTTTAACTCTTTTCATTAATCTGTTATCTCCATACTTTAGATAAGTA
CAAAGATATATAAATCTAAATATGTATTGTTTATCTAATTTACTAGGTAACTTTTTATAA
AAATTAAAATAAAAATGCCCTAAGTTATGTAATATATTATTCTCAAAATCACTTTTCTCT
CTAGTAAAATTAATTATATCTTTTATCTTTTCCTCTTCCTTAGCTGTTTTCACTGTATAG
GTTCTATCTAATATCTCTCCAGTCTCGTTGTTTATTAATGTACATTCTATCATTCTATAT
AACCATTCCTTTCTTAATATATCAATTCTCTTAATATTTGTTTATATACTTCTATGCCCT
GTATGTATTTATTTTTATATTCATAAGGTATTTGCCTTAATCTATTCTCTCGTTGTTCAA
TTTCAGATTCTACTTTATTAATTATATCTCTCCTATCTTTTCCAGATATTAAGGCTACTG
TTACACCTTGTTTAATAGATTCTACTACATACCCCTTACTAAACTTTTTAAACTTTACTA
AATACATTCAATCATTCCTTTCTAATATACCTTTATTTTGTTGTTTATAAGCTTATTGCT
GCAATGTAACCACCTCCTAATAGTTAATATTTATATAAACGCCTAAAAGGTGGCAAAATA
CCACCTAAAAAGCTATTATTGGTTAATTATTTAATTTATTTTAATATTATTGTTTTACTT
CTTTAAACCTAGATTTTATAAAAACAGAAGTTTCTTTCCCTAAATCTAATACATAATAAT
CCTCATTATTGTCTAATACTTTATATTTTTTGCCTTTCTTAATCCATCTTTCTACGCATT
TGTCATTAATACATTCCACTATCATATTTTATACCTCAATTCTCTTTAAAATTGTAAATT
TATATTCTATATAGATAAGTTTTCACCGTTGTTATAATTGTCTATATAAGTTTGTAGACA
ATTATCAAAGTCTTTTCTATTGTTAAAAATATAAGTATAATCGCCAAGTTCTGGAATACT
AAGTTCATATTTATTATTCTTATTATCTTCTATAGTCCACGCATTAGTAGAATATTCAGT
GTATATTTCATCTTTAGAAATTCTTACCTCGTCAGTTGATTCTGTTAATACTTTAGTAAT
TCTTAAAGGCTTTTTAACCATCTTATTTAAGTTGTTTGTATTCTCTATGTAAGTTGATAT
CATATTGTTTAATTCTTCTTTACTATCAGCTTTTAGATCCCAATCTCCCATGATTGAAGG
CGTAAACGTATATTCATTTGTAGAACTGTTTTCTAAGCTGTATGATCCATCTGAAAGCTC
TCTGTAAGTTTCTTTTCCTTCATCTAGCTTAATGTCTTCAGTTGACTCTGTAAGCACCTT
TACAAGTCTTACAGGTTGGTTATTACTTTGTTCTTGTTGTATTGCTAATGTAGGCTTTGT
ATTAGCCTTAACGTTTTGCATATGGTTGCTTATTAGCATTCCTATTATAATTCCTACCAC
TATTAATAAAGTTCCTGTTAATATATTTCTTGTTAATTTTAATTTATTCATTTTTATAAT
CTCCTTTGTTGTATTGTATTGGTTAAAGTTTAAACCTTAATTTCTCTTTAATTCTTTAAT
TTATTTTAAGATGTTATTGTAAGTATCCTAAAATCTTTGATTCTGAAGGAACACACATGA
ATACTAAGTTAAACTTCTTATTATAAGATCCTTCAGTCTTTTTAACTGTTCCAGTTGATT
CGTACACTACTGTACATTTTCCCTCATGTAACTCGTTAATGCTTTTTAAATTATCCATAT

TAAATTGACTCCTTTATATGTATATTTGAGGTTAACTTCCTCTTACAATATTATAATACC
ATCTAATTAAAGCTTTGTCGATCAAAACTTTAATTTATTTTAACATTTATTAAAAGTTAA
TATATATTAAATTAGAAAACCTTTCTACTTTACAATTATTAAATCTCTTAGACAATTGAT
CGTAAACTTTATAAGTCTTTCCTACAAAAGGTAATTCAACTACTACTGTGTTGTTTGTTA
AAGTTCTATAAATATCGCACTCTTTTATATTAAGATCCTTAACAGCTTCTTTAACCTTTA
ATAAGGCTTTGCAAGTTCTTTTCTTTGTTATTGGTTGTTGTTCAAGTATTCTTTCGAACT
CTAGTATATTTCTTTCAAATTCACTCATTTTCAACACTCCTCTTTTATTATTGGAAGTTT
AACCCCTCCTGACAATATTAATTATAACAGATTCTAAATTATAAATCAACCATTTATTTA
ATTTATTTCAACAATTAATATAATATTAAGTTATCTGTAAGTTTAATACATGTTTGTAGT
TGTTGTTTAATCTCTTCTACTTCTTCCAGATCTCCGTCTTCTTGGAATGTTTTTAAGTCG
TCTAACATATTAGTATAAGTATCAACAACGCATTTACTACCGTATTCCTTACCTATTAAA
GCTATAACCTTTTTAAACTCTTTTAGAGTTTTGCACTCTGTAAGAATCTTTTTAAAGCCG
TTTATACTTAAATGTTGTCCCATTCTTATATAATTAATGTTTATCTCTTGTAAATATGTA
TTAACAAACTTTCTAAAAGCTTTATAACTGTTATCACTTCTTAATTTATTAACTGCATCA
AAGAAGGTATCTTTGATAAATTGGTATAAACTTTCTGGATTTTTTAAGTCTTCTTTTTGG
TTTTCTATTTCTACAGTTTCAGTATTAAAAGTTATAGATTTTAAATCTTCTACTAAATCG
TCATATCCTTCATCACTTAAATAGTTGTATAGATTATCATTTAAAGTGATATTATTTTCT
ATTACAGTATTCTTTAAGAAACTATAATAGTTATCGTCTTTAATTTCCTCTTTGGTTTCA
CCAGATACTATTCTATCTGTTACATAATCGCAAACAAGATCAGTTAATAAATCATAATCA
GTATCATTATTATTTGTACTTATAGCTTCATGTTTAGCTTTAAAGAAAACTGTATCAGCA
TTATCAAAATAGTTTTTAGGATCATTGGCAACACTCGTAAAACTAATATCATCTAATTGT
GTCTTAATGCTGCTTTCTATATCGTGTAAGCCTTTTAATATACATATAGAATCACTTTCT
ACTATAGTTAATATACTGTCTTTTCTTCCTCTATACTTTCTATATGTTATTTGTAAAGCA
TTATCATATTGTGCGTATTGTACTAGCTGTATATCCTTTATAGTACATTTAATAATTTGG
CACATAGTAGATCCTAGAATGCTGCTATCACTTGCTACTATTGTGTATCTTTCGTTTAAT
TTAATGTTTTCTAGTTTAGCCATCTTGACCACTCCTTAATATGTATTTGGTTTATATCTT
CTCTACATTTATTATTATACTATAGAATAAGATATATTTCAATCATTTATTTAATTTATT
TTGACATTTATTGTTTATATGAATTAGCATAAATATTTAATAACATATTATCTCTATTAT
TTTTTATCTTGCATAAACATTTTACATATGAATTACAATTTGGTTTTATAATTAAAGTTT
CTTTACTTTTACACATAATTGCTATTGCATTATTTGAAAAATACTCTTTATTATCTAATT
TATCAATTAAAAAAGTATAATCTTTTAATAAATGTTTTGTAATTTCTGAATATTGTTTTT
CAGTTACTTTTATAATTTCATTTATTTTCAAATCCTCTTCGGCAAATTTTCCTTCTTTTA
TAATCTGATCGTATGTATGTTTCATTGTAAAATTACTTTCTTTTGGTATTAAAGTTTTTA
TAATTAAATTTTTATTAGAAGTATCTATTGTTTCTATGTTGTCAATAATATCTTCCTCTT
TTATTTCTTCATTAGCTTCTAATAAACTAATTTGTTCTACAAGTAAAATCAATGAGCTAA
CCAGATGACCAACTAACTTTATTTCATATCCTCTTATATATTCTAAAATATCATTTAATT
TATTTAATATCCAAACTTTCTTTGAAATTTTTATATTCTTTATCATATTAATAACTTTGT
ATGTTTCTTTTTCTAATATATCTCTTTTATCTTCTATACAATTTTCATCCATATATTTAA
GTTTGTTTATTATTTCTTTTCTTTTTTTTGTGATAGCTTTATTTCTATATTCTTCTTTTA
ATTTCACTAAGTCTTTATATGTATAATTATCTAAGAAATAATTTATAATAGCTTTTAAAC
TTTTGTTTGGTAAATTTTCTTCATTTCTAAATACAAATAATTCTTCACTGTATCCAGCTT
CATCTTTGACATATATACAATTGTTTTTACTATACATTTGTATATCTTCTACACCTTCTT
CTGAAGTTTCCTCATGATATAAGCTAAAGATCATTTTATTTTTTATAATTGTCTCTAAGT
ATTCTAATTCATTGTTTTTTAATATGTATTTTACCATGTTAACCACTCCCTAATTAATTT
AATCTCTATAATTATAATAACATATATACTTATAAATTTCAAGTAATTATTTAATTTATT
TTAACATTTTAATTATTTTTTAGAAGCATTTTTATATTAGATGTAGAACTGTAATAGTAT
AACATATTCCAGGTGTGTAGATAATTATATATAATAATGTAAGGATATCAAGTCGTGTTT
ATATTTCTTATATAATAAGAAAGTTTATAGTTTAGTTAGTGTTAGATTAGATTTTTTCTA
TCTTTTATAGATCATAAGAGTTCTTACATTATTATATATTATATAAGTGCTGCGTGGCTA
TAGGTTTATATGGTTGTGTAAGGTTATAAGAGTGTTAGAGTTGTTTAGTGTGTATTAATA
GTGTTTGTAAGTTGTTATAGCTCTATTGTAGTGTAATTAGATAGGTTAGAGTTGTTATTC
AGGTTTGGTTGTATTGGTGTTTGCATCTGTTTCTAGTGTTATGTGTTGTTATAAAGTCCT
AATTTCCGCCCTTTAACTTATGCACGAATTTTCATTTTAGGTCAAAAAAACGCCTATATA
AAAGGAAATTTATTCCAGTAATAACCACTCATATTTTCCTATATTTTACATTTATATATA
ATTCCAGTATGAGAGCAGAATAATTTTATTTTTATTCATTTTCCTAATTTTTACCAAATT
TTCTTCGCAAAAACTGTATTTTACGCAGAATAATAGCCATTTTATGGTATCTATAGAGTG

CAAGGTGGGGGTATTTTTACAATATTTAGGGGATTTACTGGAAGAAAATGGGAACTAACA
CTTCAACTTCCACACCAACTTTAAAAATTTTTACCTTAAATTCCACCTTTATTTATTAAC
ATTCTATTTACATATTATTCATACTTTATTCATAAATTACCTATCTTTATTCGTAATGCG
TTTCGTACAAACCTAGTTATATCAACGCTTAGAACCTATTTTGCTTTGCCCTATTTTTAC
TATTTTACCCTACTTTACACCCTTCAAACACGCATGAACACTAGCTTAGAACGAATTCGA
CTTAATCACAACTAAAACCAACTCTATTTTCACTCTATTAAACATAACTTTTTATTCGAT
ATTCTCTATCATTTTAAATACTCCACCAATACAGCTTACGAAACACTATAAATTTTATTA
CGAAGTATGTTTTAAAGCTATAAAAATGACAATAATTATAGTAAATAAGGAGGATTTACG
ATGAGCAAACGAATGTTTTTAGATAATTTTGATAATACAAATGGTTATTTATACTTTGGA
ACTCATGTATTTTCCATAGAATTGAGGCAATTAATACATATAGATCATGCAATAACACTA
GAATTAAGAAATAAATATCCCTTCCTGACTAATATTCAGATAGCTAAAATAATAAATAGC
CAGAATAAAATGAGTAATAATTAAATTTAGGTAAAAAAAGAGACTATATATCAATTTATA
TAGTCTTTAAATATTATTTAATAAGCTTAAATTGTAATTATAAAAACAAATCAAAATGAT
TAAAGTTATTATTAATAAAAGGTATCTTAATTATAAACCAATATTATAAATTTTATACAT
ACCTAATCCTATAAATCTGCATTTTTCATTAAATCTTCAATATCTTTTTTAGTAATTTTC
TTAATAGTTTTATTCTCAACATCTAAATAAATATTACTATATTTACTACCATTTCTACCT
TTGTATATATCAGTTTTGTTTATAATCTCAAAGTCATTATTAATTTTAGATAAAGCTTTT
TGAAGTTCTTTATTACCATTTTCATTGTCTACAAAAGTAATTCTAAGCTTTAACATATAT
TAACCCTCCTTAAAATAAAAGAGCTACAATAATTATGCAACTCTTAATGTAATAAATATA
ATGTTAATTAATCTAAATCAAAATCAAAATCAAATGCTGAATCATCAAGTTCTTCTATAA
CGCTTTTTCTTGATATAATAGGTTTTTGTACTTTTTGCACCTTTTCTTCTGAATCAATAT
CTGTATTAGTAGAAGAATACTTTGATTTAAAAATTACTTTTTGATTTATTGAAATACTAT
TTTTCTCTCTATCCTCAAGTTCTTTCTCTAACTCTCTGAAAATACCAGTAGGTATCTCAC
AACCACCTAATGCTATTAAATTTATTTCAGTAGATTTAGGATCTTCATTTTTATTAGATT
TATTTAAAGTAGAGTAAAAAGTATCTTTAGCTATCACACTATCAGTAATTTCTTTTAAAT
CATATCTATCTGTAATATTTGCACCACCATAAATACATATCATTTCATCTGGTAAAATAA
AAACACTTTTATCTCTAGCTGATCTTAAAGCTTCATCAAATAATAAATCTCTATCAGGTA
ATTCTAATATCATGCCATAGCCTTTAGCTGTTGTAATGTTTAAAAGGTCACTACTATCAA
TAGAACCAAATTTAATTGTATGGTTGGTCATAGTGTATTCTTTATCAATCATAGTTACAA
TTTCTTTGTTGATATCTAAAATATTTTTTCTCTTACCATTATAAGCAAATTTTATATCAT
TAAGGTATCCATCGTTATATAAACTAAGAACATCTTCAAAACATTGTATAGCATTAACTA
ATTGTTTGCTGCCTTCTTTTTTTGAAGGTATTACACCTACAACATTAATTATAATATGAG
GTAAAACCTTTTTTATTGTTTTTACCGTCTTCTTAAAAGAACCACTACCAGTTCCTCCAC
CCATACCAAGATAGATTAATATAGTTTTAAAATGGATAAAATCTTTAAAGAAATCCATTA
ATCTCATTTCATCATTTTCAGCAAATTCATTAGCTAATTCTCTATTACTACCAGAACCAT
CTGCACCATTAAATATAAAAGTATTTCCTTCCATTGCAAACTTTAAATTAGTCAAATCTC
CATAACTTGAATTGATATATGCTGTAGAATAAATATTTTTATATCTTTTCATTGATTCAG
CTAAGATATTTCCACATTGCCCCATTCCTAATGTTAATAAACTACTTCTATTCATTGATT
CTCACTCCTTTAATTTCATCTAAATAATTTAAACCTTTGTCTGTTATATAATAAGTAGTA
AGTTTTACTACTTTGACACCTTCTTTAATAAAACCTTCATTTTCTAAGACTTTTAAAGCT
CTTGAAACGGTTGAAGGACTTATTTGTTTTTCCTTTGGTTTATTTTCATTGCATTTATTT
AAAATAGCTTTCCTAGTCATACCTCTTATAGCTACAAGCCCTCTATTATTTTCTTTATCC
ACGATGTTTTTTAATACTATACATGCATTACTATTTAAATATTTCATAAGCTCACCTCCT
ATTTTTACTATTATAATCATATTTTATCACATAAAAGCATATAGTCAACAACTATTTTCC
ATTTTTATCACATAAAATCATTTATAATCATGCTTTTATTATAGTATAATGCAACAAAAA
AATATAAAAATAATAAAAAATGAATATAAAACTGCTTTAAATTCTAATATACTCTTATTA
ATTCATATTTAATCATAAATATTCATATAAATTCACTTTTAGTCATATTAAATCTTATAA
ATGCTATTTTTATGACTATATATGATTAAGATTGAACAAATAGGATTTAATATGAAAATT
AATTCAAGACAAGATTCTATCTTAATTTATTTAATTCAAGATATTGTATTATCTTAAATT
TTGTATTATAATTCAAGATAAGATTTTGAATAAGTAAGTAAAGTAGGAGGGGATTACTTT
GGGAAATAAAATAACCGATATATCTTTTAGATATGTACCTACTGATGAAGAATGCACTAT
AACTGGTGCTGAGATATCAAGAATTTTAAAATTAGATTCTCCACAAACTATATATGACTG
GGCAAATGCTTTTTCTGACTATTTATCTATAAAGAAAGTAGACGGTAGATGGAAATTTAG
TAAAAAATCACTAGATGAATTTAAGTTTATTCAATCATTAAGAAAAAAGAATTGGAGTTG
GAATCAAATTAGAGAACATATAAGCAAAAGAGGTTTCCAATTTTCACAATATGATTCAGG
ATTAATAGATCCTAATGATCCTATGGGATATGAAGCTTTAGCAATTCAAATCTCTATGAA

AAATGAATTAATGTTACAAAATTTCTTAAAAACTTTAAATCAGAGCTTAAATGTTAGAGA
TCAATTATTGTTAGAAGCTATAGATGAACAAATGAAAAACGGATTATCCAGTGTAGTTGA
ATTTGAAAATAGTAAGACTATAGACGAGTTAAAACAAACCAGAGAAGATTTAATGAAAAA
TCTTAAAACAACAGTAGTTAATTCTCAAGATAAAATTTTGGAAGAGATTAATGTTAACCA
AATAAAGGATTCAATTAAAGAAAATATTGATGGTAAAATAGAAAATTTATCAATAGATAT
AATAAAAAAACTAGAAGAACAGAACCAAGCCCAAGCAAAAACAATTGAAAACCTAACTAA
GCAATTAGAAAAAAGAGATACTGATATTTGCAATAGATTAAAAGAGTCTTTAGATAATCA
AAAAAATCTTCAGCAACAAGTTAATGAAAAGAAGGGATTGTTTAAAAGAATATTTGGTTA
ATGTGTTGTATAGTGTTAAATTATTCCTAGAAGGCTCTACAATCAATTTTAAATATTATG
TAATGTAAATTGTTGTCTAAAACTAAAACCTCTTAGAAAGTCATTTAAAGGCGTTCTGAG
AGGTTTGTTTATATATACAATATGATTAAATTAAATTTAGATAAAACTATTATACTATAT
TTCTTTTCATAAAACATTAAATTAACCTAAAAGAGACTAAATTGGGAGTTAAAAGGGACA
AAATTAGGAATGTAAATCATATTTCTTATAAAAAATCAAGAGTTTTCGACTAAAAAATGT
AAAATTATATATATTTTTAAGAGATATTCATATCACTATTCCTAAAATTGTCCCTTTTAA
AATGGTGTTCCCTAATTTTGTCCCCATTAGAACCTAAAATTGTCCCATTTCTTATTTATT
AAATTAAGTATTATATATATTATAAATATAAATTTATTATTATATTATATTAAATAATAA
TACTTTATATTTATATTTACCCATCAAAGAAATGGCTCTATATCTATTCTATAGCGATTT
ATTAGGGACAGAATTAGGATTGACAGGGGACAAAATTAGGTTTATAATGGGACAAAATTA
GGGAAAATGGGAGGGGATTATTATGAATAACAATAATTATTCTAATGCTATTTATAAATC
TAATAAACTTATCGAATCGTGTTATAATTTAACCACAGCGCAAAATAGAATAATTTATCT
AGCAATGACGAAATTAGAAAGTAGAATTTTAGAAAAAAACCTTAATATAAAACAAGTTGA
AGATTTGATTTATAGAAGTTCATTTGATTTAATAGAAATAGATGTTGACACTTACAAAAA
GACTTTTGGTATAAAAAGCAATAGTTTATATACCGAGTTAGAGAAGATTGCTACGGAGCT
TTATGAATCAGAGATTTTATATGTAGACTCCAAAGAAAATTTTGTAAGAAAGAGATGGGT
AATAACTTGTAGATATGACCATGACAAAAAAGGAATAGCACTACAATTTCATCCAGATTT
AATTTCAGACTTATTAATTTTTAAATCCGAATATACAAAAATGATTTTTGATGAATTTGC
CAGCAAAATAAAAAGAAAACATTCTTTTAGAACATATGAATTATGTAAACAATATGTAAA
TTATGGCTATAGAGATTTTTATGTTGATGATTATAGATTTAAATTAGATTTAGATGATGG
AGAGTATCCTGTTTTTAAAGATTTTAAAAAGAGAGTTATAGACACTTCCATTGATGAAAT
TAATAAATATACAGACTTACAAATCGAATACAAAGAATTAGAAAGAAAGAAAAGAGCTGT
AAATAAATTTAGATTTATCATAAAGAAAAGGGATTGTATGCAACTTAATATGTTTGATGA
CAACAAGCCAGCTATAAATAATACAGACGGAGAACATGTTGTTAATATGTTGTCACATAT
AATTAATAGACAAGTTAAACCTGGGGAAGCTAAAACAATATTATCTACAGCATTAACTGC
AATAGAAATTTATCCTGATTTAAAAGAGAGAAATTTAGGTGTTGTTGATTATATTAAAGA
GAAAATGATAGTGGTTAATCAATATATAACTTATAGCAAAAAGGAAATTAATTATATAGG
GGCTTTAATTATAGCCTTGAAAAACAATTGGCAAAATACAGAAGTTGACAATTTAGTGAA
TACTAATGTACAACTAAATAGTGATAGAGATTATAATTATGACAATTTAGAACAAATGCT
TTTAGGTAACATGGATTATGATCCTAATTCTTTATACAAATAATATAAGATAATTTATCG
ATATATTTTCTATAAACTCTTTAGACTGATTCTATTTTCAAGGATTAGTCTTTATTTTTT
TTAGCAAAAAACACTTATCGGTATTTGGTCTATTTTAATCATACGTATCGGTAAATCGTC
CATTCCATATCAATCAACAGATAAAATGCCGAGCATACATAACTAATCACAAAAATATCT
AAACATAATATTCTCAATGTCAACCACAACACACTTATACGCCTGTCTAAGAAGTTTTAT
AATTATTTAAGTATAATTTATCCTAAAGATTTTAATCTCCTCAGAACGCCTTTAAACAAT
AAATAAAACTATATGAGAAAAAAATAATACTTATCGGTAATTAATCCACCATATAAACAG
TCTATATAATTAAAACTTATAACAGATGATTTACCGATATAACTTTCAACGGTACTATGG
TCAAAAAATGAGCTATATTTTAAATTTAGAAGCTCAGACTTATAATTTATCAATTCAAGC
AAATAAATAAACAGAGTCTCATTTTTGCCACGAAACAATGCGACCTTAGAAGGGATGGAT
AAGACTTGAAAAACTAAAAAATTGAAATAAATTAAATAATTAGTTGAAATTATAAAAAAT
AAAGAGTAGAATAAAAAAGAACAAAACAAAAGCCAAAAAAGAAATAGCGTAGCGAACTCC
TCTCTAGACAAATTAGCGTTTAGCGTTTTTGGCTTGAGAGAGAATATTTAGTATTTTATT
ATATATTAGTATTTTATTACACCTCTGCGGGTATGGGTTTTTCCCATACTCAAATCTCAA
AAATCACAATCTGAGTATGGGTTTTTCCCATACTCGTTAAAATGACACATTATAAAAATA
TAAAGAAAGGGGAGATAAAATGAAGATTAATAAGGGGAGTAGTGTTATACCTAATGACCT
TATTATTGGTGATGAAAGTATATTGAACCAGTATGGAGAAAAAGCTTTTTTAATAATGTT
ATACATAAATATGCACAGAACTGGTTTTAATAAAGCTTATATATCTCTTGCAAATTGCAT
AGAAGAGACAGGATATAAACTGAATGATACTAAAGGTAAAACGAACGATCAATTTAAAGA

TGTATTGAAAATGCTAATAAAAGATAAGAAAATCACTACAAATACAAATAGATTTAAGTT
AAATGACTTAATAGTTTGTGAATTAGAAGAAATAAATAATAGCTTCTTTAAACTAGAACA
ATATCAATATGATTGGATTATGAAATCTAAAACAAAATCTAAGAAGATAAATTTATTGAA
ACTGTTTTGTTTAATAAAAGCTAGAATCTATAAAAGACAAAAAGATGAGGATATAAATGA
TGGATTATATGAGGTAGGTTTTCCTTCCTACAAGGATATAAAAAATAATTGTTCTATTAG
TGAGGGTAATATAAAAAAATATATTGATGAGTTAGTAAATTTAAAATTGATAAAATATGA
TAATTTAGGACAAGTACATAACACTATAACAGGAGAGATAAAAGAATCTCGTAATACATA
TGTAATATACAAAGATGGTTGGGAGAATGAATTAAATGGAGCAATGAGATTATATAAACA
AAAGTTAAAAGAAGAAGGTTGGGTAGTAGTTAAAAAGAAATAATAAAAAAATGTTGAAAT
AAATTAAATAAATGATTGACATTTTGAAATTAAAGGAGTAATATAATAAGTGTCATGAGG
AATAACATCAATTTAAAGACCAATATAAGCTTGTGTTAGATGTTTTATAGAATAAGAGGA
TAAATTATACTCTTAAAAATAAAATTGCTTACAAAGGCTTATAGAATAAAATAAAAGTAT
TGTTAAAATAAATTAAAGAAATGGAGAGAATAAAATGCAGAATATTAAAGTTTCAGAATT
AAAGAAACATCCAAAAAATGAGGAATTTTTTGATGATATTTATGGTGAAAAATGGGAGTC
ATTTATAGAATCAGTAAAAAGAAGAGGAATAGTTGAGCCAATAGTAGTTACGCAAGATTT
AATGATAGTAAGTGGACACCAAAGAGTGAGGGCTTGTGAAGAAATGGGGATATTAGAAAT
ACCATGTAGGATAACTCATTATCCTGATTATGATGAAAAATTAAACAAAACAAAAGAAGA
TATGATACTTGAAGATTTAATATCAACTAATATAATGCAAAGAGGTGTTGGAAATGTTAA
TCCTATGAAAATGGCAAGATGTGTACAAGAATTAGAAAGAATAAAAGGAATTAGACGAGG
GAGTGCTGGTAGATATAGAGGAAATCAAAATAAAAAATATAATCAATCAGAACTTGCTGA
AGATTTACATATGTCACGACAACAACTTCAAGATTATAAAAAATTAACAAATCTTATACC
TGAACTTCAACAAATGATTGAGAATGGTTCAATGAAAGCAACAGTAGGCTATAAAATATG
GGCTAGAATGTCCACAGAAGAACAAGAGAAGTTTTTTAATAATATTGGTCGTGAAAAAAT
TAAAACACTCACACAAAAGACTACAAAGGAATACATAGATAGGATAAAAAGTTTAAAAAA
AGGTGTTAAATTAAGTAGTGGGTCAAAAAGAAAATCTATATCAAAATCAATAGAAAATCA
TTTAATTGCAAGAAGTAAAGGGCATTGTGAAATTTGCGGGTATGGTGATTTAGATATGAT
AAGTTTATTAGAAAAACATCATATTAAACCAGTTTCAGAAGGTGGACAAGATGAATTAGA
AAATTTGATTATGATTTGTCCTAATTGCCATAAAACTATACATATTTTAAGAAATGAAAA
GGAAAATAGTATTAAAGACAACATATTAAAGCATTTAAATTCACATATAAACAGTAAAAT
GAAACTATACATATAAAGGAGAAATGACAATGAATAACAATTTACAAATATTTAAAAATG
AACAATTTGGACAAGTTAGGACTTTATCTATTGAAGATAAACCTTATTTTGTAGGAAAAG
ATATAGCGGAAATCTTAGGATATTCTAATCCAACTAAAGCGGTATCAATGCATTGTAAAA
ATGGAATTAAAACAAAAGTTCCTACAAAGAATAGAAGCGAACATAGTAAAGCAAGAAATG
CTCAAACAATGATTTTAATATCAGAAGATGATTTATATAAATTAATTCAAAAGTGCAAAA
CTAAATCTAAAATATATAAAGATAATCTTATTGAATGGCTGGTAAATGAATCTCTTATTA
GAGATAATGTTATGGTTTTAGAAACAAGAGATGAAATTGAATTTTTATATAAATTAGAAG
AAGCACTCAAATCTTTTAATATTAAAGGAATAAAACAATACAAAATACTTACATATAGAA
TTGATTATTATATACCTAAATTAAACATAGCAATAGAGTATGATGAAAATGATCATAAAC
AATATACATATGAACAACATGAATTGCGACAAAAACAAATTGAAAAAGAATTAGGATGTA
AATTTATAAGAGTTAGTGATAGTAAATCTGATGCTTACAATATTGGGGTTGTTATGAAAG
AAATAATAAAAAGAGAGGTAGCATAAAAATGAATGAATTAAAAATATTTAATAATAAAGA
TTTTGGAGAAATAAGGGTGATTAATAAAGAAGGAGAACCTTGGTTTGTAGGAAAAGATGT
AGCTGAAAAATTAGGTTATAAAAATTGTAGTAGAGATATAAATAGACACGTTGATGAAGA
AGATAGGCAAAACTACCAAAACGGTACTTTAGAAAGTAATAGAGGATTAATAATAATAAA
TGAAAGTGGACTTTATAGTTTAATACTTTCTAGCAAATTACCAAACGCAAAGAAATTTAA
AAGATGGGTTACATCGGAGGTTCTTCCTCAGATAAGGCAAACTGGAGGATATATACCTAC
AAAAGAAGAAGATAGTGAAGAAGATATAATGGCGAAAGCATTAGTAATAGCTCAAAAAAC
ACTTGAAAGAAAAAATGCAAGAATAAAGGAATTAGAACCAAAAGCTAAAGGATTTCAACA
ATTCATGGATACTAATAATACTTATTCATGGGATATAGTTGCTAAGAATTTAGGTGTAGG
TAGAAACACTATGTTATCTATATTAAGAGAAAATAAAATAATACAAACAGATGAATATTT
TGATAATAGTGGTAGAAAACATAGGGGAGAAAGACATAATGTTCCTTATCAAGCTTATAT
GAAATATTTTGATGTTAAATTTTTAATTAAAGGCGATAAAAGATATGCTAAAGTTTTAGT
TAAAGCAGAGGGACAAGAATATTTAAGAAGAAAATTAGTAAAGTTAGGATACATAAAAGA
AGCTGCATAATACATATTTACAAAATAAAAAATATAAATGAGAGAGGATAAAATACATAT
GGGCAGAATGAAATTTAATAGTCAACAAGAGAAAGTCATTAACTTTAAGAAAGGAGCAGT
TTGTGTTTTAGCTGCTGCTGGGAGTGGAAAGACGGCTTGTATAATAAATAGAATAAAGAG

ACTTGTAGAAGATGGTGTAAGTCAAGATAAAATATTAACAGTAACATTTACTAATAATTC
TGGAAGTGATTTAAGAAAGAAATTGAAAAATGAATGTTTAGAAAATGTTCAAGTAGGTAC
ATTCCATGCTATTTCGAAAAGAATATTAATGTCCGAGGGGATAGATGTATCTAAACAATT
ACCAACTTATGAAATAGAAAATATATTTAAAAGAATAGATAAAAAAGCTAAATGTAAAGA
AATAATGAGTTATATAGCCTTACAGAAAGTGTCTGGAAATAGTGTTATGGATATTGAGAG
TGATAGTGAGAGTTACACTGTAGAAGACCTAAGAACGTATTATAAGGCTTATGAAGATTA
TAAAAACTCTAAAAAAGCTTATGATTTTACAGATTGGATGTTACAAGCAATAAAAATATT
AAAATCTGAAAGAGGAAAATATTATACTTTTGATTATATATTAGTAGATGAACAACAAGA
TAATGATGTGGTACAAAATAGGTTAATAGATTTATTATGTCCTAGTGGAAATGTAATGGT
GGTCGGAGACGTGAGACAAAGTATTTATAGTTTCAAAGGCAGTTCACCAAAATTATTTAT
GAATTTTGATAAAAGATATGATAATGCAACAATATTAAACATGGATATTAACTATAGATC
TTGTAACAATATCGTGCAAGGAGCTAATAACTTTATTAAAAAGTATTTAGGGGATTTTAA
ATACTATTCAGATTCTATAGCTAATAATAAAAAAGATGGAAATATATCAAAGTTTTTCTC
AATTACAAAAGAAGAAGAAGGGGAAAGAATAGCTAATTTAGTAGCTAAAGATATAGAAAA
TGGTATGAAGCCAAGTGATATAGCAATATTATATAGACTTAATAAACAATCTTTTTACAT
AGAAAACGAATTAAAGAGTAATGGAATAGATTATCACATAGAAGCTAATAATAATTTCTT
CGAGAGAAAAGAAGTCAAAGCTATAGTATGTATGTTAAGGTTATTACAAGATCCAGAAGA
TGATGGAGCATATGAATATTTATACAAATTAAGATGTCATCCTTTTAGTTTCATGAGTAA
TAAATTATTAAGTGATATAGTTGATTTAGCTGCAAAGAAAGATATCTCATTGTTAGATGC
TTCAATGTATGTTAGGTCAGCAAAAACATATGAGAGAAAGAATTTAGATAGTTTTATGGA
TATATATGAGAGTTTATTATTACAAAAACAAAAAAGTAAAGATTTGTTAACCATAATTAA
TAACATAATTAAATTATTTAGATTACAACAATACATAGAGCAAAATTATGAAGGTGATGA
AATAGATGAAAGATTAGAGAGTTTAGAAGCTACTAAATCATTTGTAAGAAGTAATACGCT
TGAGAGCTTCCTTAGATTTGTATATACTTCAGAAACCACCAAAAAGAAAACTAAAAGTAA
TGAAGTGCAATTAATGACTATACATAAGAGTAAAGGATTAGAGTTTAAAAAGACATATGT
ACTTGTTAATGATAATGATTTTCCAAGTAAAAAAGCTTTGGAATCTGATAATTTAGATGA
AGAGGCGAGAATATTTTATGTTGGAGTTACTAGAGCTAAGGAAGATTTAGTTTTTAGTAC
CATAGGCAATAAGAGTTTATTTGTAGAACAATATTTTAATTAAAATGTTGAAATAAATTA
AATAAATGATTGATTTATTTTGGATTTAGTATTATAATAGTAGATGTAGAAGGGAGTGAC
AATTATGTCAATGAGTTTTAAATACACAACTTGTAAAAATGAAGAGTTTGATAGTTGGAA
TGGAAAATATATAACAGTCTATAATAGAGGAGCTATACATAAGGTTGTAACTAAAACAAA
AGAAGAGTGTGAAAAGATTTTAGAAGACTTTTATGATTATATCTCTTTACATGAACAATA
CTTTTGTACTGAAGGAATTGGAGAACCATATTATGACGATATGAAGGAAAAATGGATAGG
AGAATCTTACATATTTTTAAAAGATAAAGAAGAATTTGATTTTTATAAAAAACTATATAA
ACAATTTAAAGAAATGCAATGAAAAATATCAATATCTTAAAGGAGGTGAGATAATGAATT
TATTAGAATCTTATATAAATATATCAAAAACCAATAATGTTAAAATAAATGATATAAATA
ACATAAATCCAAAACAATTTGTTAATTGCATAGATAATATTCCTATAATATGTTATGAAG
TTAATTATGTATATACTACAGTAAGAGGAAATAAAAAGAATGGAAACAAATATTTCTTAT
TTAATTCTTTTAATCCTCAGATAAACATGAAAGAAAAATTAGATAAGTATATAGAAGATT
TTAATAAAGAACATCCTAACAGGAAACTGTTAAATGTAAAGTTTCTTAACAGCAAATGCT
TAGGATATATGACATTATAGTAGTCATATCACTTAAAGTTTCATTTGTATCAATCCTCGC
TTAGAGGTTGCATAGTCTTAATAATCATTAACCATTTTTATTAAGGCGAAACAAGATTGG
TTAAAGCGTAAAAAATTCACCACAATTTAATATTGGATTAATTAGTTTGATTGAGAAGGA
TCGTGATAATTGGTTAATTAAAGAAAATTTTATCCTTATCGAGTTTAGGTAATAAATTTG
ATAAGGAAGATGCGAAATGAAACCAATATTTAAAAAAGAAAAAGAATTTTTAGAAAGAAA
TTTAGGAATAGAATTGCCAATGGATTGTTGGAGACATGGAAGTAAAATTTATTTAAATGG
AGATAAAGATACAGTAATAATTGAATTTAAAGTTGAAAATCAAAAAATAAAAATCAAAAA
GAACAAAATAAAAGAAGTATTAGAGAAATATAATAATAAAACTATAAAAGAAGAAATCCA
AGAAAACAATGATAGATTAAATAATCTTATTGAAGAATCAATTAATAAAACCAAAGAATA
CATACTTAATCATCCAAATAATAATATAAGATTAAGCCATAGTGGTGGAAAAGATTCTGA
TGCAATGTGGTGGATATTACAAAAGGTTTTTAAAGACTTAAACATAAAAGATTATACTAT
AGACTTTTTTAATACCACTAATGATACAGCTCAAACATATTTGCATATAAAACAAGATTT
ACCACAAGATCATTTACAGATCAATAATCCTGAAAAGGGGTGGCATCAATGGTTAAAAGA
AGATAAAAATTATTACCTACCTTCAGTAATGGTTAGAAATTGTTGTAGCACATATAAAGA
AGGACAAGTTAAGAAGATATTAGATAAGAAAAAAGATTATTTGATATTTTTAGGAGCTAG
AAAGTACGAGAGTGCAAAAAGAAGTAAATATGATTGGGATTTAAATGAAGCTTGGTTAAA

ATCACATCCAGGAAAGAAATTAAATGTACCAGAGAATTGGTTAAGGTTTTTACCTATAGT
TAATTTCACCGATAAAGATGTTTGGTTATTAATACTTAGAGAAGGAATTAAGTATAATGA
ACAATATAATGTTGGGTTCAATAGATGCGGCTGTCTTCTATGTCCATATTCAAGTGATTA
TTCAGACTTATTAATAAAAGAAAACTATCCAAAACAATGGGAAAGATGGATGGACATAGT
AGAGAAAAATTATGATTTATATGATGTAGAGAGAAGGTTAAAATGGACAAAACATGAATA
TTGTGAATTAGGAAAATGGAAACAATCTACAAGCAAAGAATCCGAATACATAACTAAGAA
AGCCACTCCCGAAAGAATTAAGGCTTTAGCTGAATTAAAAGGATGTAGCGAAGAAATGGC
TATAAAATATTTTAAACAAGAATGTAAGTGTGGTAAGAAACTTAATCCTGATGAAATAGC
TATGTATTTAAAGATATATGGAAGATATGAAGGACAAGAAGATAATAGAATATATTTATG
TAAATCTTGTTTATGCGAAGAAATTGGAATAGATAAAAATACATATGCAGAAAAAGTAAA
AGAATTTAGAAATCAAGGTTGCAATTTATTTTAAGGAGGACAAAATTATGAAAATTTATT
ATGCTCATCATATGTGGAAGTATAATACAAAAGAAGAAAGAATGGAAATAGAGGCTATTA
AAAAGATATTTCCAAATTCTAATATAATAAATCCAAATGGTTCTGTTATTGAGACAGGAA
ATGAAGCTGAAGCTATGGAGCAATGTTTTAATTTTATAAGAGAATCGGATATTTTAATAT
TTACAACATTAAATAATAAAATTTTTGGAAGAGGTGTTTATGATGAGATATCTTTGGCAT
TGCAATTAGGAATGAAAGTATTTCTTTTAAAGGAAGATACCTTATTAAAAATAAATGATA
TAAATAGTATATGTGAAATAATAATTGATAAAACAAAATCTAATAGAGAGTATGCAAAAT
TATTGATATAAATTAAAGAATTAAGTTAAATTCGTGATTTTAAAGAGAATGAAATGGGAG
ATGAAATGTAAAATGACTAGAAGGAAAACACATGAGGAATTTGTTAGAGAAGTAAAAGAA
AGATACGGAGACGAATATGAAATTTTAGGGAAATATATTAATGCCCACTCTAAAATATTG
GTAAAACATAATTGTAGAGAATGTGGTTATTATAAATGGGAGATTGAACCCAATAATTTG
TTAACAGGTAGAGGATGTCCAATATGCAGTAAAGAAAAAAGAAAAAAGAAGCATATTGAA
TTTGTCAATGAAATATTGAATAAATATGGGATGAGTATACTATTTTGGGGAAATATGTAA
ATAGCCAAACAAAAATATTAGTTAAGCATAATTCAAGTAAATGTAATCATCATGAATGGG
AAGTTTTACCGAGTAGCGTGTTGAGAGGCTATGGTTGTCCAGTATGCGGAGGATTAATTG
CTAAGTTAGGTATCAACACCATCTGGGACACCGATAGATGGATGTGTGATCTCGGGGTTT
CTGAAGAAGATGCTAAGAGGTATAAAAGTAAAAGTCAAAAGAAAATAATAGTCAAATGTC
CAGATTGCGGCAGAGAAAAAAAGGTAGCATTAAATAAAATATATTATTATAAATCCATTT
TTTGTTCATGTAGGGATGGGAAATCATACCCTGAGAAATTCATTATGAATTTATTGGAGC
AATTAAATATTGATTTTGAAACAGAATATAAGCCACGATGGATTAATAATAAAAGATATG
ACTTTTATATTTCCGAATTAGTTATGATTATAGAAACTCATGGGAAACAACATTATTCAA
GTAAGACAACTTTTAAAAGTTGTGGAGGAAGGTCTTTTGAAGAAGAACAAATAAATGATA
AATATAAAAAAGAAATGGCACTAAAGAATGGAATTAAGCACTATATCGAGTTGGATTGTA
GGGAGAGTAATTTAGAATATATTAAAAACAACATATTAAATTCAGAGTTGAATAAACTGT
TTGACTTATCAAAAATTGATTGGACAAGTTGTGCGGAATTTGCTAATTCTAGCATAGTAA
AAAAAGCATGTGAATATTGGAACAATAGAAGTAAAGATGAGACAACAAGTGATGTTGGGA
AGATATTTAGTTTACACTATAGTACAATATTAAATTACCTTAAAAGAGGAACTAGGTTAG
GCTGGTGTAATTATAATCCTAAAGAAGAAAAGAAAAAGTGTGGTAGCAAATCGGGTAAAG
CGAGTGGAAAAAGAGTTGAGATACTAAAAAATGGAAAAAGTTTAGGGATATTTGAATCTT
GTCATGAATTAGAGAGACAAAGTGAAGAATTATTTAAAAAAGTGTTAAATTATAGTAAAA
TAGCTTCTGTTTGCAGAGGTGAAAGAAACACATATAAAGGTTATCAATTTAAATATATAG
AAAATGTAGCATAAATACATATTGTTTAAATAAATTAAATAATTGATATATAGAGGAGAA
TAACAAATGAGAGCAGAAAACACATACATATTAAATTTAGAATCAGCATACATATATAAA
GCACAATTAGAAGATGAAAAAATAGGATATAATACAAAGAAAAAGAAAAAAGATAAAGAT
GGAAAAGATACGAAAGAGTTTTTATTCACTAAAGATGTCTTATATTCAGCAACAATTCCT
TATAGTTTAGAAACTATAAGAGCTAATGAAAAATATCCTAATGAATGGAGAGAGGAGGAT
AATAAATATTACACGGATTTATTTGTTAATGTTAACTTTACAAAGCACTATAAAGTTGAT
GGGAAGAAAGAATTAGATGTTAAAAAGATAAGAAAACATCTTTATACAGAAGGATTTAAA
ATAAATGGAATAAAATATAAAATGTTTAAGAGAGGGGGAAGTAAAGCAAGAACCGCAAGT
GCTATATTTATAAAAGAGAAAATGTATAAAGACCTGTATGATAGATGTTTATTAGGATTA
GAGTTTCCTGTAGGAGAGTATTGTGATTTAACTTCTAAAAATGCTTATATATCTTTAATA
ATGTCTGGAATAATAGGAATAATTGATATAAAAAGAGAAGAAATTCTTATTATTGATGAT
GTAATGGGTAAGGAAATGAAGGTTAAGGCTTCTGTAACAGAGAGAAATGAAGATAATGAA
ATAGTAGTTAACCAGTATGAAGATTATCCAGTACAAAACAATATGACAGATGGACAAGGT
TTATTAGATGAATCTGTTTTTGAAAAAAATGAAATAATCAAAGGACATTCAGTAGCATTA
TTAAGAAATGACTTTACTAAGTGTGCAGCTTTTAATACTAAATTACAACAATATTATAAA

GAAAATAATATAACAAAAGTTTACGATATGTATAGAGGTTGGGTAGATAGTAAAGATATT
AAGTTAGTTATAACTCCTTCTAGTTGTAAATTCTTAAAGTTTACCAATAAATTTAATTCT
AAGAAAGAATGTTATTTACATTGGTGGTCACATATAGATTCAATGTTTGGTGTAGTTAAA
ACAGATCATATAGGTAATTATGGTTATGCCAATAGATTGTCTTATCAAATGATAAACTCA
TTAGAATTAAACTATGATGAAGTTAAAGAAATAGCTCAAGAAGAGATTAACTATATTAAA
ATGCTAAAGAATAATTGTTTAATTGATGGATGTAACAATTTAAGTAAAAAGCAAAAAGAA
AAAATAACAGAATTAAAAAATGATATGACATATTTCATGCATTACATAGGTAATAATAGT
CTTGAATTATCGTCAGGTGAAATGATAAGTGATTTATTATCTGTAAATAGTGATTATAGG
TTTACAAAGCAATTCAAAGATTATAAAAAAGAACAAATTAAAAACTACATAAAAGATGTT
AGAACTGGTAAGGTAAGAATAACTAATTCGTTATATTCTATATTATTTTCATGTCCATAC
ATTATGTTAAAACAAACCACACAAGAAGAGATGGTTACAGAAAGTATAAGTCATGGATGG
GAAGTATACTGCCCTAGATTTGATAATAATAAAGAGTTATGCATGATAAGAAATCCACAA
ATAAACAGTGGAAATATAGCTCATGTTACTAATACATATCATGATGAATATAAATGGTTT
AATTTAAGCGACTTTGTAGTTATACTTAATACATATGATGTAGATGTTATGAATAGACTT
CAAGGATGTGATTTTGATATAGATAGTGCTTTATTAATAGAACAACCTACAATAGTCTCT
AAGGCTAAAGAATGTATGGATAAATATTATACGCCTATTAATGCTATAAAAGGTAAGACG
GATTTAAAAAGAGATACACTTGAGGAATTAGCAGAATTAGATAATTATTTAGGACAATCT
ACTAGAACAATAGGACAAATAGTAAATAAAAGTGCTATATGTAATGCTTATATGTGGCAC
TATATAGCCAACAATGGAGATAAGGAACTTATACAAAAACTATATGATGCAAGTTCAATG
TTATCATCATTTTCTCAAATAGCTATTGATATGGCTAAAAAAAGCTTTATGGATAACAAT
GGTAAGAGAATGTCATTATTAATGGAGATGCAAAAAATAAATAAATGGAGAGTTAACGGC
AAGAATATATTAGATTTTGATAAAGAAACATTAAAAGATGAAACTGGAAAAGAAAAAATA
ATTAAGAAAATGATAGTACCTAAATTCTTTGGAGAGATAGCAGATAATAAGTTTAGAACA
TTAAAACATTTGGATTGTGGAATGGATTATTTACAAGATGTTATGGATAATGAGTTGGGA
AAAACTCTATCTACTAAATTAGTTGATATAAAAGACCTGTTACAAATTGGGAAAAACATA
GAAGGAGAAAGAAGTAGAAAAGAACATTTGGATAAAACATTAGATATAATAGTTAAATGT
AATAGAACAGTCAAATGGTGCAAAACACTTTCATGTAAAGAAAAATATGGGGAGAAGGCT
AGATATACAATTAGCAGAAATGCCAAAAAGAAAGCTATTGAAGAATTAAGAGACATAAAT
ATGTCCAATAAGACTTTTATGTTGATTCTTAAAAAAACTTTTGAAGTGTTACAAGAAGAT
ACAGTTAAAAAGTTTTCAGATATGGTATCTTTAGTCCTAACATTATTATATAATACTAAT
CAAAAATATTTTTTAAATTGCTTTAAAAGTAATAATATATTAGAAGATAAAGTATTAATT
CTCGATACAGAAGGAGTAGAAAATATATTTGGAGATACATATAAAATAATTAAAAAGTGT
AATAAAACAAAGGAAAATTCGTCAAAATAATTTTATATTAAAATCGTAACACCCTTCTAA
TCTAGTGATACCAACGGTTGTGGCGATTTAGCTAAGAACCTTATGAATGGGGTATTATGG
TAGAGTAAATAAAATTAAGTTTTTATTAATACTTCTACCGTGTGGTGAACGGAAAACTAC
ACTCCTTTATGTATTATAACATTTTATTCTCTCTTTAGTTTGGGTAGCTTCTCTCATGGC
TACCCTCATTAAAGAAAAATAAAAAATATAAATACATAGATAAATTAGTAATTAATAGCC
GTAAGGCAAAACATATTTTAAAATAAATTAAATAATTAAAAGAGAGAAAAGGAGAAATGT
AAAATGACAAAATTAGAATTAGTAAAAGGATTATCAGAAGAAACAGGATTAACTAGAAAA
GAGGTTGAAGAAAGATTATCTTTTATAGATAAAGCTGTAGAATTTTCAACTAAAGTACCA
GGAGTAAAGGTAAAATTAAGTAAATACTTTACAGTGGAAAGAAAGCATATTGAAGCAAAA
ACAGGAGAAATGACTAGAGTAAATGAAAACGGAGTTAAGGTCAAAGTACCATACAGTACA
GAAGCTCATGATGAATTATCTTTCAAAAAGACAACTGCTTCTAAAAGAGTTTAATACATA
TTAAAGTTAGGGTAGCTTAATGTTATCCTAACTAATTTTAAGGAGAGAATATTATGAGAT
TTAAATGTCAAACATATCATAAGTATGGAGAAGATGATAAATATCTAATAAATATTTACA
ATGACAATATGCCTTGTTTTTTAAACATTGAGGATTTAGAGGATAATTTAGGTGTCTATA
CTTCGTTAGAGGCTTTAATGGATGTTGAATGGTTAAAGAAACATAATATAGATTGGATAG
AAGAAGATGGATTAATAATACCTATTGGTTTAATATCTTCTACTTATAATGGTATTGTAA
ATTTAGAACAAGCTAAAATAATAAGAAACACTAGTTATTCTAATAATTTAAATGTTTATT
GTAGTAAAAGATATATATCTTTATTTCCAAAATATTTAAGAATGTTAAAAAGATTGGAAG
AAATAGAAGAGAAGTTTGAATCAGAAATAAAAGAATTTCAAAATTTATGAAATAAAGGAG
AGAAATAGTGGTGAAAACTGAAACAAAATATTATTGTGGACACTGTGGAAGAAAAATAGA
ATGTGGAGATACTTTTGCTATGATAGAAGAAACTCTATATACAATAGACAAGCATGGAGA
CTTATATAAAAGTCCAACAATAGATAATAAAGATGTTTTTTGTATAAATTGTCACTCTAA
AATCATGAAATTAGATGATAAATTTAATGAACTGTTAGAAAGTGCTGACGAATATTTAAA
AGTTAAAAAATAATTAATTAAATCAACAGTTTTATTTAAAAATAAATCATATAAAAGAGG

GATTATTTGTGAATAAAAGACAGATTAAAAAGTTAATGAAAAGTAATCAGATTAAATTTA
ACAAAATAAAACTAAATAAAGATGATGTTTTGGTAATAGATTTTGGAAAAGACTATTTTA
GTGATTCGTGTATGCAAAAAATATGCAGTTGGATTCATGATAGTATATTCCCAAAGAACA
AAATATTACCTTTACATGGAGGAATGTCTATAGGTGTAATAGAAAGAGGTGAATCAGGTG
GCAGTAAGAACTAACTCTATTAAATTTTCCAAAGCTTCTATATCTCAAAATGAAGAGGGA
GAATTTATTATAGAAGAAACTAAAAAAGATGAAATTATAGTAACCAACTTTACTAATGCA
TTATTAGAATTTGCTGGAGTAGATGGTTTAGAAATATCTATTGGTAAGAAATCTGAAACT
GTTTCAGAGGAATAATACATATTAAGGATTGGACTTATATTATATAAGCTGATACCAAGA
AAAATAACTAAATGTGTGCGTAAGTTATTTTACCATTATGGTTTACGTTTGTCGGTTTAA
AGTGTAATGCCATAAACTTCATTTTACCGTAATCCCACTAAGGCTATGAGTATTCAGGGA
TATTTATTCCTCTGTATTTATCCTCAAATAAGTGTCAGTATAATTTCATGAAGTACAAGT
GAAATGTGATAAGGATATAAACTGATTATCACTAAAAATCATATTGCCTTTAGCTAGGAT
GTTGTCCGACCGTAAGCTAAGAAAGCTAGATTATAGGTGGGTAGTTAAGAGTATGAGATG
AGTCTTATACTCTTTTTTATATTATGAATACATAGTTCAAAGGTAGAACGGTTGGCTGTT
AACCAACTAATGAGAGTTCGATTCTCTCTGTATTCGCCAAAATATTATTTATATATATAC
ACATTCCGCTACGTTGGAAAATGTATATATTTTTACAATTAACATTATAACAAAGTAAAA
TCAATAATTCAAGAGAGGAAAGATAAAAAATGGGAGAAAAAAAGATAAAAGCATGGGATT
TTTGCTGTAAAGCGTGCGGTAGAAAATTTTTAATGGAGAACTTAGAAAAGGATGGTTATG
TAGATACTATTAAGCTTGGAAAAGTAGTCTATTGTCCTTGCTGCGATAAAGAAGAATATA
TTCCAATGTAATTTTAACTAGGAGAGGATAAAATGGGAGAGATATTAAAGAGAAGAGATA
ATGAAAGTGATTTAGAATACACTAAAAGAATTGTTTATGCTAAATTGGTAGATAAAACTA
CCGATTTAGATTATTCAGAATTAAGTAATTTAATATATGATAAACCTTACAGCTCAGATG
TAGCAAGAAGAATGTTCTACGGAATGAGAAAAATTTTTGAACTTATGGATAAAGAAGGCG
TAAATTCTATAAGTGAGAATGAAATATTAAAGAAAATAGAAGAAAAATCATTAGAATTAG
ATTTACAAAGAAAGAAATTACAAGCAACCAAACTTGAACTAAATAGAAATCAAAGAATTA
AAAGTAGAAGAGAATTATTTTATGAAAATATAGGAGAGGAAATTGAGAGATTACCCCTTC
CAAAGTTTGAAGAAGTGCCTATTAAAAAAGTAAATGGTGAATATTTATTATGTTGGGCTG
ATTTGCATTATGGTGCTGATTTTATATCTGAAAACAATGAGTATTCAAGAGAAGAATGTA
AAACTAGAATGCAAAGATTAGCTTCAAGAGTTAAAAATATGTGCATAGAAAAAGGTATTA
ATAGCCTTCATGTTATAGGTCTTGGAGATGACATTCAAGGAATTCTTAGAATTTCTGATA
CTCAAATAAATGATGTACCCGTTATGCAATCAGTTGTTGAAGTTAGTAGGCTAATAGCTC
ATGTATTAAATGCAATTTCAAGTGTTTGTGATGTTACATATTATCATACTATGGCTAGCA
ATCATAGCCAAACTAGACCTTTAACTGCTAAACCAGACTTAATTAAAGAAGATTTAGAAT
TTGTTATAGGCAATTACATACATGATTTAGTAGAAAATAATGAAAGAATAGAAGTTAAAT
TATCAGAAAAAGATTATCATTCATTTAGTATAGCTAGTCAACAAATATTAGCTTTACATG
GACATCAAATAAAGAATATTAATAATGCAATTAAGGATTATTCAATGCAGCATAGAAAAT
TTTATGACATAGTTCTTATGGGGCATCTACATGGTGGTCAACAAATGAGTGTAGGGGAAA
GTGAAAATGGTAATAGTGAATTAGTAATAGTACCTTCTATAGTTGGAAGTGATCCATACT
CAGATACTTTAAAGAAAGGTTCTAAGTCTATGGCTAAGTTATTTAAATTAGAAGAAGGTA
ATGGAATAACTGAAAATTATACAATAGTTTTAAATTAGGAGAGAATAGATATGAATGAAA
AGTTGTTAAAAAAATTAGAAAAAATAATTAAAGAAAATTATGATAATGAAGCTTGTGGAT
ATACAGAAGAAAGGTCAGAGGGGAATTATAACGATGTATTCTCAGATGGTCAAGAAAATG
GTAGAAGTTGGTTAGCTTATGAAATAGGTTGTTTATTAGGGATGAAATTAGCAGAGCCAA
AAGAATCTGAATATAGTTGGGAATAATGAGGAGAATTAATATGAATATAGATTTTAAAGA
GGAAATTTTAGAAAACGAAGATATAACTATAGTTGGTAATTCAGAAATGATTAAACAATT
ATTATTTGATGTTACTAAAGATGTAGAATGTTTTGAATTAATTAGTAATGTTAGTATTTA
TTCTAAGTATTTATCTTTGACTAGATTAGAGAATACTTTATTTATAGAAGATGTTCAAAA
TGAAAAAGGTGATTTTATCTATATGGATACTGAAATGTTATGGATTGATGAAGAAGTTTT
AAATGAAGCTTACAAGAGAGGACAAGAAAGTGCATTTTTAGAATGTAGAAATATAGTTAG
TATATATTAAATACTTTAAATTTTGGATTTTATTTAAAATAATTAAAATAAAGTAAGAGG
TGTTGTTTTATGAAAATTACAGAAACCATAAAATCTTTAAAAGAAGAAATTGAAGATTTA
AAGAAACAATTAGAAGTTGTTGAAAGTGAAAGAGATCAGTTTAAAGAAATAGCTGATAGA
AATGCCATTAAGTTAATGAAATATGAAAATATATCTGAAAAAGATATTATAGAAAGATTA
AGAATGTACTAGGAGAGATGTATTATGAAAGAATTAAATACTATTCAAAAAAGAGAAAAG
TTAAATAAAGTATTTGCTTCTGGAGAAAAAGGATATGGTGGAGCATATCATCAATATCAT
ATTGAAGCTGTTGAAAAATATAATGGACTACCAGTATATACTACTACAATTAATTTTCAA

AAAGGGGCTAGAAAAGAAGAAAAATCTCAACATGGTGTAATTGATACTGATTTATTAGAG
ATAGTAAGAGATAGATTACATTGTTTTCAACAAGGTGAATTTACATCAGAATATAATTAT
CAAGCTTTAATCCATGTTGAAGAGGCTTTAATGTGGTTAAATAGAAGAGTCGAAGATAGA
ATTGAGAGAAATGTATTAGGTGAAAATAAAAAATAATAAAAATAAATGAATGGAAGTCTA
GGATTAATTTCTTAGGCTTCTCAATAATTATATATTGCAAAGATAATTATTAAGGTAATT
ATGCTTGGAGTATATGATAATACTTCCAATAAATAAAAAATGGAGTGTGAGTGTGTTATG
AATTGGAAAGAAGAATTATTAAACGCAAAGGAAGAAGTTGTTTATGTAAAATATAAACGA
GGTGAAGGAGCTACATACACAGTAATAGAAAAATTATTAAATACTGATAAAGACCTGTGT
GTATTATATTTAGGCAACTACTATATTGTAGAAGATATATTATCAAAATTATATAGTAAT
AAAGATAAGAGAATTGAAAGTTTATTATGTAGTATCGAAAACAGAGAAATTATAGTTAAT
TTAATAAATAATAAATGGCTAAAAATAGTTTGTACTAGATTGAAAAATGCAGATGAAAAC
TTTAAAAGAGGTTTAAGATTTCATATAGGTATCGCAGATAATTCAGATTTTAGTATGCCT
AAAATACTAAATCATTGTAAACAAAAAATAATAATAATGTCTGATGAAGACACAACATCT
TTTAGAATAATTAACTCTGATAAAGTTAAAGAAAAGCTAAATAAAGATAAAAATCAAGAA
TTATCTTTAACCCAAAGAATTAATAATTATAGAAAAAAGCTGTTTACCGAACTAGAGAAT
ATACCCATGAATGATAAAACAACTATGACAAGAGAAAGAATTATAGATATGATTAGAAGT
TTAGATCATATTGGAAGGTAGGTACTATTATGAAATTTATTAAAACAGCTTGGAGTATGA
TGGAGAAACCAACTAAAAGATTATTATTATCAGGATTAATATTGATGATAGGTTTCTTAT
TGGCTTTTATTAATGAATGGATATATGCTATTTGTTTATGTTTAGCATTAGCTAATGTAA
TACATACTGGTTGGAAATATATGTTTAAAGAAGAATGGGAAGAATTTAAGTTAAAAGTTA
AAGATAAAATAGAATCAGATAATATAAAACAATGATTTTAAAGAGAATGTAAAAAGATGA
GGTGTGTAAAATGTATAATACAAATATTACAGATAGATTAAATAAAGGTATTGATAGAGC
TAAAGAACTAAAATATAAAATTGTACATATAGAAATGAATAAAGATCAATTTAAAGATTT
ATTATCAGAAGAAAATGGAAATAGAACAAATTGGGTAGTAGCCTTAGCTGATTATAAAGG
TTATAAAATAATTGTTCGTGAAGACATGGAGAATCCAGAATGGGATAATAAAACACCTAT
AAAACTTATCATTAAATCAAGTAAAAAGAAAATTAAAGATGATATTACTAGAGAAATTAA
AAAATATATTGAATGTTAAATAATTTGAATTATAGGACACCTAATTAATAGGTGTCTTTT
ATTGAGATTATTTTTAGAAAGGAGGAATATCTATTGGCAAAAGATAAAGAAATATATTAT
TGTACTATGCAATTATCACCCAAATGTAAAAAAAACAATGGACTTTTAGATGAAAAGGAT
TTTTATTCAACAGCTAATGAAGAAATATTTCATAACGGTAGACTCTCAATCTGTAAACAC
TGTCTTAAAAAATTTGTTTATGAGGACAAAAAAATAAATTTAGATAAGTTTAAAAATATA
TTACAAATATATGATATTCCTTTCTATGAAAAAGAATGGAATGCTTCATTGAATGGTTCT
AAAGAGGTTTTAGGCTCTTATATGAGAATAGTTTATTTAAATTATAAAGACAAGCATTGG
AAAGATGGAGATATAACCGATAAAAAACTTATATATGATGAAAGCGACATAGGAAAATTA
TCTGAAAGAGAATTATTAAATAAATGGGGTTCTGGATTTTCATTGGATGAATTGCAATGG
TTAGAAAATAATTATTATAATTGGACTACAAATACAGATTGTAAAAAATTTAATATACAA
AAATTGGTTAAGCTCATATGTATTAAAGAATTAGATATTAGAATTGCTAGACAAAATGGA
AAGCCAACAGATAAACTAGAAAAATCATTGCTTGAATTAATGAATAATTCTAATTTAACA
CCAAAAACTATGAGTGCCATGAATGAAACTGATTCAGCTAAAAGATATGGGAAATGGTTG
GAAGATATAGAACAAAATGAACCAGCGGAGTATTTCAAAGATAAAAGTATATATGAAGAT
TTTGATGGTATAAAGGGTTATTTTGATAGGTTTATACTTAGACCATTAAAGAATTTGCTT
ACTAACACTAGAGAATTTGACCATGAATTCAATGTTGAAGATGGTGAAGAATAATGGCTA
GTTATAGTAATTATAAGAATAATCAACAAAAATATAAAAATAGCAACGATATGTCTAAGA
GACCTATGCAAGTTATAACATCTGAGGATCTAAATGAACAATTCAAAGAAAAACTGATAA
ATTGGACAACTTTTTATAGGAGAAACATTCATAGATTTGCAGAGCATTATTTAGGAATTC
AATTACATCTATATCAAAAAATAATGCTTTATTTAATGCATTTAAGTCCACTTGTTATTC
TATTATGTGCTAGAGGTATAGCTAAATCTTTTATAACAGCTTTATACGCATGTTGTGTTT
GTATATTATATCCAAACAGTAAAATATTAGCTACAGCTTTAACCAAGAAGCAAGGTGGAT
TATTAGTTACAGAGAAAATTCAAAAAGAATTGATGGTTATGTCTCCTAATTTACGGGGTG
AGATCAAAGATATTAAAACCTCGCAAAATGCAATAGAGGTAGTATTTCATAATGGTAGTT
CATTTATTGTTTCTACTGCCGATGATAAAGCTAGGGGATTGAGAAGTACATGCCTAATCA
TAGATGAATTTAGATTAGTTAAAAAATCCAATATCGATGCAATTTTATCGCCAACTGAAA
TAATAAGACAAGCACCTTATATTAAAAAATCCGAATACGCCCATTTAGCTGAAGAACCAA
GAGAGATATATCTTAGTTCTGCCTATTATAAAAGTTCTTGGATATGGCAATTTATAATAG
ATACAGTTAAAGATACATATAAAAAGAAAGCTATGATATTTGCTACAGATTATGCACTTA
CGCTAAAACATGGAATACGTACAAAAAATCAATTATTAAGAGAAAGAAAAAAATTAGATA

GTGTTACTTTTGATATGGAATATAAAAATTTAATGATTGGCGGAAGTGATAATCAATATT
ATACATTTGATTTATTATCTGAAGCTCAAACCATAGAAAAAGCATGGTATCCAAAAACAA
TAGAAGAGTATTTTGCTTCAAAAGATAACAATAAATATAAAAGATTTGGTAATATCCCAA
AACAAAAAGGTGAAGTAAGAATAGTATCTATGGATATAGCAATGTCAAAGTCAACAAAGA
CAACTAAAAACGATAATTCAGTTATTAAATGTATCAGAGGACTATTAGTGCAAGATCACT
ATGAAAGACAAGAAGTTTATACTGAAACATTTGAAGGTATAGATATAGATAGTCAAGCAA
TTAGAGTAAGACAATTAATGGAAGACTTTAGTGCAGATTATTTTGTTTTTGATGCAAGAA
CCTATGGAACAAACTTAACGGATAGTATGGCAAAGACTCTATATGATAAAGAGAGAGATA
CAGAATATCCTCCAATAAAAGTTTTTAATAATGAAAATTTGGCAGATAGATGTAAAAATC
CTAGTGCTGCACCTATAATGTGGGCATTCATAGGTAGCTCAAAGTCAAATCATCAAATGC
ACACAACAATGCTAGGTAGTTTAATGAATAAAAAATATAAAATGTTAATTTCTCATACAA
AATGTAGAGAAGCTTATTTAATGGATAAAAAAGAATATGAAGGTGGTTCTCCAGAATATA
GAGCGTGGCTTGAATTAGCTTATATGCAATCTGATTTAACGTTAAATGAAATGATAAATC
TTAAAAAGAATTATGTAGATGGAGGAAATATTAAATTAGAAGAACCATCAACTGGTACAA
AGGATAGATATGTTTGTGCAGCTATGGGAAATTTGTTTATACAAGAAGAGTTTGAAACCA
AATTAACAGCTAGAACTGTTACTTTTGATGAAGAAGATGATTATGTAATGTGGATATAGA
ATGGAGGTGAATTTTATGACAGTAATTAAAGAAGGTGAACCAGGATTCGGTTATGATTAT
GTGAATGATGGAATGCCAATTGCTTTTACGAGTGATTTGTTTACAGATGGTGTAACTAAT
GAAGTTAGTATGGATACTATTAAACAATGGTTAGCTAGTCCTCAAAAATATAAAAAAGAA
TTAGAGAAATATGCTATATATCAATACATAAGTAATGGAGATATATTCCAATTATTTGAT
TTAATGAGGATATTACCTAAATTAAATTATAGCATTAAAACATTAAAGCTAAATAATAAA
AATAATGAATATACTTTAGCTTGTAGAAGAGCTATGAAAGATATTAATCATAAAGAACTA
ACTAGAGATATACTATCTCAAACAATATCGTCTGGTACTTTATGTGGTTTATGGGTGGGT
GGTTCACCTAACAAAAAAGGCAAAAAGAATAAAATACAAGAAACTCCATATCCAATATTA
TTTAATAACTTAGAATGTTTCTTTCCAGCTAGAAGAAAAAGAGGTAAATGGGCTATATGG
TGTGATTTATCATATTTTGATGATATGGATGAAGATGATTATAAGCAAGGTTTATTTGAG
GAATTAAATCCTTATATAAATGAAAAAATGTTAAATGATTATGCTAATGATCCTAATTGT
AGATATATTGAGTTTCCTATTGAAAGAAGCATTTGCATTAGAACTCATGTTTTAAGAAGA
AATCAAAGATTTGGTTTACCTTGGAATACTCCAGCTATAAAAGATATTAAACATCAAGAA
AAATTAAAAAATCTTGAAAAAGTAGCTGCTAATAAAGTTATGAATGCAGTTAGTGTATTA
ACTATAGGTAATGATAAAAATCCTGAAGATTATGGATGGAAAGCAGTTGGTAAAGTAGTT
AGAAGTAATATTGCTAAAGATGTTAAAAGAGTTTTAAATACTAATAAAGATGGTGAAACT
TCTGTGGTTATATTGCCTGAGTTTTGTGATTTTGAACAAAAAGCACCTCAGACAAATGTA
TTAAATCCAGAGAAATTTCAATCTATTAATTCAGATATAGGGAATGACATAGGAATAGCT
AGAACTTTAACCAATGGTCAAGGTGGTAATTATGCTAGTGCTAATTTAAATCTTGAGATT
ATATATAATAGAATTTCTGAATTATTAGAGAGTATAGAAGTTGAAGTTTATAATAAGTTA
TTTCAGATAATATTACCTTCTGATATAGGACAAGACTACTATTTAGAGTATGAGAAAGGT
TATCCATTATCCACTACTGAAAAGGCTAAGATGTTTAAAGAATTACACATGTTAGGATAT
TCATTGAAGCCATTAGTTGAATTATTGGGAGAGGATTTTGATGACTATGTAGAAAATTCT
ATATATGAAATAGAAGAGATGGATTTAAGAGAGAAGATTAAACCTCCTATGTCTACTTAT
ACTATGAGTGGCGATGAAGAAGTTGGTAAACCAAAAGGAGAACAATCAAATAATGACTCA
ACTATAACAGTTGAAGAAAATGGTGGTAATAGTCAACCTAAACCTAGTGTTTAAGGTTGA
TTTTTTATATTAAAAAAAAATAATAAAGAAAGGGGTTGACAATAGTTGGCTAAAAAAAGA
TTTTATGAATTTAAAAATATAGCTAATAAAGATAGTGAACTTTATGTTTATGGTGAAATC
TGCGGTGGAGCTGACAAATGGGATGAATCTGATGTTACATTTAAAGATTTCAAAGATACA
TTAGAAAACATGACACAAGGTTCAACTTTAAATATGTATATTAATAGTCCAGGAGGTAGT
GTTTTTACTACTCAATCAATAATAGCAATGTTAAGACGTGCTAAAGAAAATGGTATTAAA
ATCAATGCTTACATAGATGGATTAGCTGCTTCATGTGGTTCATGGCTTCCAATGATAGCA
GATGAAATATTTGTATATCCTCAATCAATAATGATGATACATAAACCTTTATGTGGAGTA
TGGGGAAATGCAGATGAAATGAGAAAAGAAATAGAGGTTTTGGATAAAATTGAAAATGAT
GTAATAATACCTCTATATATGGAAAGAGCAAAAGAAGGGGTAACAGAAGACATACTTAGA
GAAAAAATGGCAAATGAAACTTGGCTAAGTGCTGACGAAATGCAAGAATTATTTAATGTT
ACTTTATTAGCAGATGAAAGACAGATAGCTTGTTGTGTAGATAAAGAAATCTTTAATAAA
TATGCTAATGTTCCAGAAGAACTATTAAAAATAGCAAATAAAGAAGATGATGGGGAAGAA
TCTCCTAAAGAACCAGAACAAGCTAAAAACCAAGCTACCAAAGAATTAGAAGATAAAATA
TCTAATTTAGAAGGTGAAGTAGGAAATCTTAAAAAAGAAAAAGAGGATTTAATTAAAAAT

AAAGAAGAAGTTTCAGAAAAATTAAATCAAGCTAATGAAAAAATTATAGCTTTAAATGAA
GAAATTACTACCATGAAACCTTTAGTTGATGAATATGAAAAAATTAAAAAAGAAGAAGAG
GTTAGATTAGAAGAAGATAAGTTAAAAGATTTAACTAATGAATATGAAAAGAAATTTAAT
TCGTTAGGAGCTTCTAAGAAGTTTAAATCAGAAGAAGTTCAAAATCTATTAAAAGAAGCT
GTAAAAGATTCTAATAAGAAAAATGAACTTAATTCTATTATAGTAAATCTAATTCAAATT
AAAGATGAGAACAAAGTAAAAACAAAATCTATAGATAATAGTGCTAATATGGATAATTTA
GTTCCTAATGATGAAAACGGTGCTAGTAAATATGGATTTAAATAATAAAAATAAATAAAA
GAAATAGAAAGCGAGGAATTTATAAATGGCAAGTGAAGTATTAAAAGCGTTAATAAATCA
AAAGGAAAAACATGTTGTTGGTTCATTAAACAACGCAGATATAAGAACAGTAAATCAAGG
AGCTATAGCAGAGGCAGATATAGATAACTATATGATAGTTGAACTTGGTTTTAATGAAGA
AGGAGAAAGAACATTTAAGCTATTATCAGATATAACTCATAAAGGTTATTTGGTAGCTTC
TCCTGAAAACTATATGAAAGAACTAGGAGAAAATATCAGCGGTTTCTTTAATGGAAAAGG
AGAAAGAGGAAGAATAGTGATACAAGATTTCGGAAAAAGATTTGAATGTTCTAATGTTGT
TGCATCTGATGGTTCATCAGCAATTAAAAATGGTATGGAAGCTTATTTTGATCCAGAAAA
GAAACAATTTATAGTAGATGTTAAAAAGAGTGATTCAAAGTTAGCAACTGCTGGTAATAA
ATATATAGTTGTAGATGTTGAAGGTAACACTTTAGGCGGACAACAAACAATAAGACTAGA
AGTGGCTATGTAATATAAAAAATAATAATAAGAAAGAGAGGAATTAATATATGCCAATAA
AAAGAATGACAATAGACGAAAAATTAAAGAACTTAGGAGTTAGAGTTTTTGAAAATAAAA
TGTTAATTCAAAAAATAGGAGAAGAAGAAGTAAATGTTGATGAAAAAGATTTCTTAGATA
TTTGCGAAAAAACATGGGGACATGGAATGCCTTCAACTGAAAACTTAGAAAGATTTAATA
AATTAATAGTGGAAACTGCGGAAACAATAGCAGAACCAAAAATAACAAAAATATTAAATC
TTTTATCACAATATAAAAAAGTTGATATAGATGCCGTAGTTATGTATGATATACCAAAAA
CAGCAAAGGGTAAAATTCTTTATGCTGCTAATGGTTCAGGAGTAGACTTAGTTAGAATAG
GTGCTGAAGATACTAAGAAAATAGCAAAAAGAACTACTTTTGCTTATGGTGCTTATTATG
AAATAACAAGTTTCTTAGCAGATCCAAAACAAGGATTCAAAGATGCTGTTACATTATTAG
CTAACGCTAAAATAGAAAAATTCTTTGAATTAATGTTTGCTTGTATGAAAGAATCTATTA
AGAATGCTGAAATACCAACTAATAACACTAAAGAAGGTTCAGGATTAAAATTAGCTGATT
TCCAAAAAGTTGAAAATACAATGATAAGATTAGGTGGTGGAAGACCATTATTTGTTGCTG
ATACTGCTTTAATTAATCATTTTGCAGACCAAATACCAACAACTCAAGCTGCATTATTAA
CAGACGAAGTTAAAGATATGTTAAGAGAAGATTTAGTACCTTCAAAAATTTCTAAATCAA
TAGCTATGACATTCCCTAATCCTTGGATTGATGATAAAAACTCTAAAGTTAAATTTAGTG
TAAAACAAGGATTTATGTTCCCATCAAATGCTAAAGGCAAACCTTTTGGTATTACTGAAT
ATGGTAACAGAAGAGAATATTCTAAGGTAGATACTGAAACAGAAAGAGTTGAACTTACTG
TTAAGTTTGATGCTGACGTTACATTATTAAATGGTAGATACTTAGGTGTAATAGAAGACG
ATTCAATAACACTTTAATTTAATTAAAGAGGTGGTTTAAAAGCCATCTCTTTTTATATTA
TTTATTTTTAAAGGAGAGGATATAAAAATGGAAGAACAAATAATTTTAGAAAGACATAGA
CCTACGCCATATGTAGTAAATTTTGAAAATAAAAGATATGAATGGTTAGGTGCTAAAAAT
GGAGTACCTAGTACAAAAAAAGTACCAAGGGAAGTGTATGATTTTATAGCAATGGGTTCA
TCAGCATTAGAAAGCGGAAAATTAGTGTTAAGCAAAAAAATGACTGATGATTTAGTAGAA
GAATTAAAAGAAGAAATTCCAGATATTGAAAAATATGAAGTTAATGCCCTAACAAAAGAA
GAAGTAGAAAAAATATTAAAAGGAAATTTAAAATCTATGGAAAAAGCATTTTCTGAAATA
GAGGATTTTGGAACAAAACAATTCATATACAAGGTTGCTAAAGAAATAAAAATAGAAAAT
TCAAATAAACAAAAAATGATTAAAGATTTAATAGGATCAGAATTATCAATAGAAGATTTA
TTTGCAGAAGAATAATAAGGTGGTGAATTAATGAATAATACTACCTATGATGAAATATGG
GAAACTTTTATAAGCGAATGTGGATATATTTTTAATGAAATACCTAAGAATGAAAAAATA
ATAAAAAATATGATTAGGAATTCTATTGCTAAATATAATCAATTAGCTATTAAATATGGA
ACCTTTAATGGCAATATTGTAAGCAATGATGAAACTGAAAAAATAAATACCAAACTTACA
GATATTGAGCTACTTATATTAGCCAATATTATAGCTTATAATTTTGCAAAATTTAAATAT
ACAGAATTTACAAGTTTATATAATGTTGTAGCTAAAGATTTAGGAATTAAAGATTATAAA
GCTCAATGTGTCGCAAGAGAAAGAACCATTAAGGAATTTAAAAAAACATATTTATCTCTA
ATTCAAGATAGTGATTTAGCTGAAAGGTTAGGTGATTAAATTGAAAACTTTTGATTATTA
TGATTTTTCACCTAACGAAGCAAAAATTAATAGTGGAAAACTTATCTTTGAACAACATAT
GTTAAAAGGTATTGAAGGCGAAGATGTTTTTATAAATGATGATAAGGAATCTACTAGAGT
TTTAATTCAAAATACTTTAAATACAATGAATGAAAATAAAGAAGAAAGAAGTATTCAGGT
AACTATGAATACAAAATTAAAAAGAGGAGATTACATTAAATATAAAGATAATAATGAAGA
TGTAACTTATCTAATTATTAGCAGAGTAGATAATCATAAAGCTTATTTAAAAGCTAAAAT

GAGATATTGTAATCAAACTTTAATCTGTGAAGGACAACCATATCCCATTAAATGTATCGC
AGACAACACAACTTATGGTACTAAGGGTGTCAAAGATAATAGTTATTATGAAGAAAGAGA
CGATCGTCTTAAAGTATGGGTGCAAAAAAATGAGTGGACAGATAGATATAAAGAAAATAT
GAGATTTATATTTGATAACAAAATTGTTTATGAAGTTACAAAAATAAATGGTGCTATATT
AGATGGATTATATATTATGGAGATGATACTTAGTACATTAAAACCTGGATTAGATAAGCC
TGAATTAAATATAGCTGAAAATGGAACTTTATTAGATGAGGTACAAGCAAATAATAAAGT
TGAAAAACAACAAATAAATGAAGAAAAAACAGAAGAAGATGTAGAGCCTTCTATGGATTT
AAATGTTAGTAAATTACATGGTGGAGAAAGTCTTAATATTAAAGTTCAACCAAAATCAGC
AGAATTAAAAGTAATTGGTGAAAACATAATTACTGAAAAGGTTGAAGATGGATTATATAA
GTTAACAGCTAAGGCAACTAAGAAACCTAATGTAGTTAAAGTATCTTTAATGTATAAGGA
TAAGGAAATAGTTAAGAAGGGAATTCTGATTTATTAGAAGGGAGGAGATGTTTTAGTGAG
TTATTATGCAGAACAATATAATAAAGATATTCCTCTTTCTAATAAAGCTACTATGAGTAT
GAATATAAAAAGATATTTAGTAGATAGAATCAATAACGATCAAGACATAGTAAGATATTG
CAGATATTTGACTAAAACACCTTTGTTGGAAATGGGAATTGATTATAAGGACAAAGTTGT
TGAACAACCTGATTTAGATTGTGGATTATTAAAACCTTTAAGTGAACATAGAGATAAAGA
TGGTAATTATACAGAATATACAGGTGTTGAAACTAGAGGGAAAATTCTTATTCCTTATGC
TTTTGATAGTAATTTAATGGTAAAAGAACAATTATTTATATTTGTATCAAATAGCTATGC
TGCTTTTAGTAATGTTTACAATACAGGAGAATACACATTTGATATAGTTATAACCTATAG
CCCCACATACAATGTTTTAGATCCATATGGAGAGGAAAGAAGTTTAATGATAGTAGACAG
AATATGCAGAATGTTTGATGATATGTACCCTGAAAAAAGTGAAGTTGCTGAAGATATAGG
TGATGTTCAATTAAACATTAGAGCTATAGAGGAAAGAAAAGTTGGTACTAATGGCACTAT
GGCTAGAGTAGTTAAAATTGTAGCTAAACCAATAACTAATAGGGAGTTGGTTAACCATGC
TTAAAGATTATTTTGGACAAGCTGATTATATAGAAGGTATTGGAGAAATATATCCTATTA
AAATAATAGAATATCAAGAATTTCAAGAATTAGCTCAAAGATATATAGCTATAGATAAAA
GAGCATTGGAAATAGAATTAAAAGAAAAACTTGATATGAGTACATTAATGCTAGTTTTAA
GTCAAATTAAAGCATATGAAATAGCTAATAATGATGATTATTTAGCATTAATGAATCCTG
ATGATTTAGAACAATATCAAAGATTAAAAGAAGCTGAATATAAACTTAATATTGATGATT
TTGTTAAAGTCTTAAAAATGGTTTTACATAAAGATGTTATTTATGATGAAGAAAATGTGA
AATTCAAAATACAAGATGAGAATGTTTTATTAGACAGAGAAATAAATGAAAGTAATTTCG
ATGATTTTAAAAATGTTGTAATGAGACAAAATTTATTATTTACACCTTTGTATTATGAAG
ATCCTATTTTACAAAGTATCTTAATGAGTTTAAGAGAAAACAAATCACAAGAAGATGGTT
CTCAATTTGATTTGGAAACAATATGTCAAGTTGTATCTAATGAAAAGAAAATACCACAAT
ATGAATTAGAAAATTTTACTTATTACATGATTATTGCTGATTATACAAGATTAAGTGTTA
TAGATAATCACGACTTAGGTAAACAAATACAATCAAGTGGTTTTGCTAATGAAGGTTTTA
AGATACCAAGAAGAGATGAAATTATAAATCTTTATAAACATCCAGAAAGTACGATGATTG
AGTTTAACTCCGAAGTTTATGACAAAGGGGACAAACTATAATTAAAAATAAATTAAATAA
ATGAACAAAAGGAGGAATTTATTAATGGCAAATGCAACTCAAAAAGATTTATTACAAGGG
TTAATTTCTACAACTACAGGAAATATAACTCTTATAGATCATGAAACAGGAATTCAATTA
TATTCTAAAACTTTACAAGATATAGGAATAGATATAAAAGAAGATAATAAAAAATTACAA
GGTGGTATAGGTAATCCGACTATATACTCATGGGGTGAAAATAGACAAATTGAAATCTCA
TTAGAAGATGCTACTGCTAAATTAGACTTTACAGCAGCTAAATTCGGACAAATGCTACAA
AAAGGTGAAGTTAAAGCTTTCCAAGAAGCACTTAAATATACTGCAAAGGGTGGTAAATTC
ACATTATTACAAGAACCAGCTAAAGGTGTTAAACCACAAATGTTTAATATGGCAACTGGT
GAGTTAATATCTGATGAACATGTTACTGTTTCAGGCAAAGAAGTAACTATATCAGAAGGT
GTAACTGATGGCGATGTTTATGTAAATGGTTACGATATAACAGTAACAGATGCTTTATAT
TTCGATATAGATGCTAGCAAATTTGCTAAGACATTTGAAGTCATCATATCAAATCCAGTT
GTTTTAAGACAAGCTAATGGAACATTCAAAACTACATATATAAGACAATATAGATTCCCA
AGTGGAAGACTAGAAGGTTCTATGAAAGATGATTTAAAATCTAAATCTGATGGTGGTAAA
GTTTCTTCTAAGATAGAAATATTAAAACCTGAGAATTCCGACACAATGGGTAGAATCATG
TTATTACCTTTAGAAACTTTTGGTAAATCTCAATTACAAGCTATGGGATTTTCAAAATAA
AAAATATAATTGGAGGTGAAATATCCTCCTTTCTTATTAGAGTAATTATTCTTATAAGAA
ATACTCTTAAATGATGATTATTGTAATAAGAAAAGTCATTAAATGAAGGATTTTATTTAA
ATTAAAAAATAGAAGGAGAGGATTGTAAGTGTTAGAAAATTTAATTGCAAGTGAATATTT
ATATGAGAATAAAGAATTTAAAATCAAAGTAAAAAATCTAACTGAGAGTGAAAAGTTAGA
GTGGTATAAAAGAATGGAAGAAATGAAAGAAGCTAACGATGGAGAACTAAATAATGAAGA
ATTACTTTACGATATGTATAGAGAATTAATTGTATGTGAAGGAGAAATGAATTTCAATGA

AATGTCTTTTGAGAATTTCTTAATATTTGTTAGTTCTGATTTTTTAACAGAAGTAGCCGA
AGATATAAATAGATGTATGACTACTTTATATACAAGCATAATGAAAACTGGATTATTAAA
TCAAAAGACTAAATTAGAAGAATTAGAAATGAATATGGCACTATTATTAGTTCAACAAAA
ACTTAGCGATCAATTAACTTTAATACAAGAACAAACAAAACAAGAAAGATGGGTTAAATC
TGAATTAAAGAGAATAAGAAATATAAAACCACATCCTGAATTAGATGAACTAAATAGAGT
TAGTAGATGGGCTATATTCAAAACTAAATGGAAATTAAATAGAGAAATAAGAAGAAAAGA
AAAATTACAACAAAAAGAGGGACAATAATGTTATGGATATTGATATAGATAACATAGAAG
ATTTAGAAGAATTTATTGATAAATTTAATAATAGTGATTTAGAAGAAGTTTCTTTATCTG
TTGGTGAAGATGTGGGAGATTTATTACACAAAAATATTAAAGAAATGGTTTATGAAGGGG
CGTTTCAACCTCATTATTATGACAGAAGAGGAGAAAATGGTGGTTTTGGAGATAGAAGAA
ATATAACTGTTACCCCTATTGGAAGTGGAGTAATCACGGTTGAAAATATAGCAAAGGGTA
ATGAAAACGAACCATATGCAGATGCTAAAGGTGAAAGATTAGATGAAATAATTGAATATG
GCGAAGATTATACATGGAATAGACAACCTAACGCCAGACCAGTGTTTGAATTTACTAAAG
AAGAATTGGAAAGTGGAATATTAGAAGATATTTATAAAAGAAAATTAAAAAATATTGGTT
ATGATATAGAATAAGCCATAATATTATATCCCTTTTTCTACAAGAATATACACTAGGTTT
ATAAACTTAACAATTTATTAACAACTTTGTCACTTTTTCAAGACAAAAAATGTTATAATT
AAGGTAAACAAGGAACAATTTAGCTAATGAAATTTCTTTAATTACATGAGTTTTACATGT
TATCATATAAGTAAATATATGGAAATGAGGGAAGTGAAACTTTGTGGTTATAAAGAAATG
GAATGGCAAAGCAAAATTGAAGGGGGGATATGACATGGCAACAATATCATTTATGAAAGA
ATCTGTTATAGATAACAAAGCTGCTGATATTTTAATATCTCAAATGGAAAGAGCTAAGAA
AATGCCAAAACATACATCTAATATAGATATAGAAGAAAACATGAGGGAAGGTAGAAGATT
ATTAGCAAACTTGTTATCCAAGCGTCAGAAATAATTAATACATCAGAAGAGTTGTTGAAA
GAGATACAAACAGATTTTAAATGTAATAGAGACGAAGACATAGAGCGATTTTTTAAAGTT
TCATTGAAAAGAATGGAATCTAGTGATAAATCTAGGACTTATATCATTGTCGATAAAGAA
GAACTTGAACAGAATGATAGAATTAAGGTTCTAGGATTTTATAGTTTAGCATCCCATAAT
TTCAATGTAAAAGAAGGAACTAGCAAGAGCAAAGTAAAGAAATTAGATGGTTTTGATAAA
AATGCAAAAAGTATTCAATGTTGGTTAATTGGTCAATTAGCAAAAAATGATAATTATGCT
GATGAAATTACTGGTAGTGAAATAATGGAATTTGCAGAAGAATCAATACTACTATTGCAT
AAACTTGCTGGTTTAAGGACTATATTAGTAGAATGCAAACATGAAGCGAAATTAATTAAA
TTCTATGAAAATCATGGTTTTGAATTTTTACAAGAATCGACAGTAGATGGATTAATACAG
ATGACCAAAACCGTTAATTCGTAAAGAACTTAGTATAATTGTTTACTAGGTTCTTTTTTA
TTTAGAATTAATTGTTAACATTGTGTAAAAGTGGTATAATTATCTTAATAAATAAGATAA
GGAGATGGATGTTGATTATGGAAACTTTAATAGATTTTGCTATAGTTATTGGTATCATAT
TAATTATATGTTTTTTGATAAGTAAAAGTACAGATAAAGATATTGCTAAAAGAAAAAAAG
AGGAAAAAGAAAATTATAAAAAACAAGGTTTAAACTTAGCGAATAGTTATTGGATTAAAC
TATATTCTGGATTTAGAGAATATGGAATAAAAGAAGATGTTAAATTAATGTTATTTAAAG
ATAGGATTATATTTAAAATAGACTATAAAGTAGAAAAAGAGATATTATTTAAAGATATAG
AAGATTATAGAATTCAAACAGAGTCTCAATTAATAGAGAAAGCTTCTTTAATGAAAGTAG
CTTGTTTTGGAGTTTTTGGTCTTGGTATGAAAGGAAAAGAAAAAGAATTAAATAAAGAAT
ATGTCTATATAAGAGCCATTTATGAAGGTGAACAAGCTAATATTGTATTTGATAGAGGTA
TAGGCGGAAATGAAGAAATTGTCCAAGAACTTCATAGATTAATAAAAGAATATAAAAATA
TAAATATAGATAAAAATGTTGTAATTGAATAATATATGTACTAAGAAGCTATTTACTAGC
TTCTTTTTTAATGTAATTATTTATTAATTTCTTTCTTATTATGAAATACTGGTTTTCTTT
CTGACCTATCTAATTCTATTAAATCAGCTAAATCACAATTAAAATAGTCGCATAAAATAT
CAATATGTTCTCTAACTATATGTTTATAGTTGTTACTGGCATAAGCACTTATTGTAGCTT
GACGAATTCCTGTTGCTTTAGATAATTCCTTTTGAGTCATTCTATGTTCGGCTAATAACA
TATGCAATTTCATTCTCATTAATATCACCTCAATTGTATTATAACTTATCTTAATAAAAA
AATATACGTTTTTTTGAATTTTAATCCGAAAAACGTATTGACTTATACGAAAATCGGATT
TATAATTAAACCATAGAAACAAATTAAAATAAAAAAATACATAATATGAGGTGGGTACAT
ATGATTAATTTTAAAATATATGATGAAATATTTATAGATCCAGAGGAATTCTTTAGTAAG
ACAAAGAATAAGGAACTTAATTTAGAAAGGTTCATCTATGACAATGAATCAGTTTATTTA
AAACAGCATATGAACGGATATGATTTTAGATGTTTATTATTAGGCATAAAAGAAGATGTT
GAAGCTGAGAAAGAATACATAACAGTTGCAAAAGTAAATGATGGAATTATGATAAGACAT
AAGGTTCGAGATGAGTTTGAGGATATGGAAATCTGTAATGAGTGGATGGTGAAATGTAAT
GCCTAAAAAGAAAACACATGAGGAGTTTATTAAAGAGATTAAAGATAAATATGGGAATGA
GTATGAAGTTTTAGGTGAATATAAGGGTAATAAAACAAAAATATTAGTCAGACATAATTG

TAAAGAATGTAATTATCATGATTGGAATATTACACCAGATAATTTATTAAGAGGAAATGG
ATGTCCAGTGTGTGGAAAAGAAATTAATAAAATAAACAATAAAGAGTTCCAAAGAATGTT
TAAAGAAAAATATGGAAATGAATTCACGTTACTTGATGAATATAGGAGTAGCAGAGATAA
AATGAGGATAAGGCATAATTGTGAATATTGTGGTAATCATATTAGAATTATGAGAGCTGA
TTCTATACTAAAAGAACTGAAATGCAGAGTTTGCGAAGAACTAGAGTATAATGAAAAAAC
TTTTAAAGAAAAACTTTCAAAGAAAGAAAGAGGCAGCTATGAATTAATAAGTGAATATAA
GAATAAAAATACAAAGGTTCTGATAAGACATAAGTGTGGCTATTCCTGGAAGGTTGAACC
AAGTGTAATTCTAGGCAATAGAGGATGTCCAAAATGTAACGGTGGTATTAAAAGCACACA
TGAAGAATTTATACAAAAAATTCAGAATAGGTTTAAAAATTCTTATTCTATATTGGGAAC
ATATGTTAATAATCATACTAAAATTAAAGTTAGGCACAATTGTAAGGAGTGTAAATTCAA
TGAGTGGGAAATAAAACCATCAAGTTTGTTAAATGGGGCTGGTTGTCCTGTATGTGCCAA
TCAAAAAGCGGTTCTAGGCATCAATACAATTTGGGATACAGATCGTTGGATGATTGATTT
AGGTGTATCAGAAGTGGATGCTAAGAAGTATACACGTTGCAGCGGACAAAAGATTACGGT
TAAATGTCCAGATTGTGGTAAGGAGAAGAAAAAACGAATAGCCAATATTTATAATGAAAT
ATCAATTTCTTGTTCTTGTGGAGACGGTAAGAGCTATCCAGAAAAATTCGTATTTAATTT
ACTTGAACAATTGGATGTAGAGTTCGAAACAGAATATAAACCTAGATGGATTGATAATAA
AAGATATGATTTTCATATTAAAGACAATAATTGTATCATTGAAACACATGGGATGCAACA
TTACGACAGTGGATTCGATAAAATTGGAGGTAGAGATTTAACTAAAGAACAACAAAATGA
TAAATTCAAGAAAGAAATAGCCTTAAAGAATGATATAAAGCACTATATAGAGTTGGATTG
TAGGGAGAGTAATATGGATTATATTAAGAACTCTATTCTTAATTCTGAATTAAATGATTT
ATTTGATTTAAGCAATGTAGATTGGAACAAATGTGCTGAATTTGCTAATTCTAATAGGGT
TAAAGAGGTATGCGAATATTGGAATACAAAGCAAGAAGATGAAACAACGACCGATTTAGA
AGATAAATTTAAATTAACTCGAACAACTATAATTAAATACCTTAAAAAGGGAACTAAATT
AGGGTGGTGTAATTATAATCCTAAAGAAGAAATGGAGAAATCAGGAAGAGCAATTGGTAA
GAAAGTTGAAATATTCAAAGATGGACAAAGCTTAGGTGTATTTAAGTCATGTGCGGAGTT
AGAAAGACAAAGTGAAAAATTATTTGGAGTTAAATTATTTAATCAATGTATTTCAGATAT
TTGCAATGGTAAACAAAAACACCATAAAGGTTTCACTTTTAAATATGTAGATTAGCAAAT
AGAGAGGGTTGATAAAATGCTTATAAGACTAACTAAAAATTATAACAATAAAAGAAATTC
AAGAATTTATAACAAAGGTGAAGTTTTAGAGTGTGAAAATCTCTACAATGGAATACTAAT
GATTAATAAGAGCAAAGATTATATATTAAGACAAGCTGTAGAAGTTGTTTGTTGTTAAGG
GGTTGATAAATCATGAAGATTATTGATGTAGATAATAATGAAGCAACACAATATTTACAT
GAGAAACTGGACATAGTAATTGAGAATGGATATATAAGCTTGGAAGATATAAGTATAATC
TTAGATAATCTTGCCACAAGTTGTAGTGATGAAGAAAGACAAAGAATTAAGGAAGAAGAT
GAAATTTATGAAGAAATGAAAAATAACTTTGGTAGATTTAATGAGGAGTGAAGAGTAATA
ATAATATTACCTTTATATAGCTTGGACATAAAGTGTTTTAAATTATTGACAATACAATTA
ATAATATATATAATTTAATTGTAAAGATAATTAATATCTTAGTCTCCGAAGTGTTACGGG
ACGTAAATGTAGAATATAACACTATAGCGTGTTTTATCCTCCGAGAGAACGGGGATTTAA
AATGTAGAATATTCTCATTAATTTTGTTTAGTCTCCGAAGTGTTACGGGACGTAAATGTA
GAATATAACACTATAACCCTTTAGAGGGTTATTTTTGTATTTAGAGGTAAATTATATGTC
TAAAATAAGAATATGTTCAATTAAACAAGAATTTTATGATTTATTTGATAATACTTGCGA
ATTAGAGAAAAATGATAAGAGACCATGTTTAATAGTTTTGAAGTTAAAGTATAAAGGTAA
GAAATATGATTTTGCAATTCCTTTTAGATCAAATATATATGGACAAGCAAAAAGGGAGGA
ATACTTTCCATTACCACCAAGAAAAACAACCAAAAAAGGTAAAAAACATGGTTTACATTA
TATAAAAATGTTCCCTATAATTAAAAGTTACATAAACTCATACTATTCAGAAAATAAAGA
TAATGAAAATGATATAAGAAAAATAGAAAAAAACTTTAAAAAAATAGTTGATGAGGCTCA
AGCATATCTTGAAAATTATGAAAATGGAACTAGGTATAATTATTGTGTTAATATAGATAA
AGTTATTAAAGTAATTAATAATAACTTTATTATTCAACAAGAAGTTGCATTTACATCTAT
TGAAAAAGAAAATAATAAATAGTACATATGAACTTAGTATAAATTGATTACTAAGTTCTT
TTTATGTAATAATGAGAAAGAGATAGAAGTTGCAGCAACAGAATTATAATAATTAAATAT
GAAAATAATGATAAAGAACTTAGATTTTAATATCTAGGTTCTTTTTTATGTAATAAAACG
GGGAAAGGGGGAATTATATGTCTTTAAATCTATCATTCGGTGGAAAGTTAAATAAAGATA
AGGTCTTACAACAACTTGAAAAAGATATTAAAAGTTTAAGTAAAAATTTAGGGGTTGAAT
TAGAAAACGTATCTTTAAAAGATGTTGATAAAGCTACAAGTCAGATACAGAAGCAAATAA
ATCAACTTAGTAAGAATATTAATTTAAACATAAGTAAAATTAGTTTAGGGAACAATGGAG
CTTTACAAGATATTCAAAAACAAATTAACTCAGTACTAAAAGGTGAAAAGATAAACCTTG
AAGTTAAGAATAATATAAAAGAAGTATCTAGTGAATTCGATGTTTTAGATAATAAACTTA

AAAATTCATTTTTAAAACAAGAGCAATACTTATCTCAATTAGAAAATAGACTTAACAAGA
TAAAAGAAAGAAGTATTACTCAACATACCAAGAATGAAAATTATGATAATTCAAGAGATT
TAAATTCTATACAAAATATACAAAATAGAATTAATGAAATCAAGGCTAAGAATATTAGAT
TATCTCAAGAGGAGAAGAATTTACTTCAGCAACAACTAATTGAACTGGATGCGTACATAA
AAAAAGAATCTAGTAAAAGTGATGAAATCAATAGAAGTACTAGATTTTTAACATCGCAAT
TGAGAAGTTTAGAATCCTTAAAAATAAGAGTTGATAATCGTGGTGGCGGTGATAAAACTA
AACAAAGTCAGTTATCCAGTGAACTTGAAAGACAGATTAACTCATATAAAAAACTAATAG
AAGAAAATAAAATATTAGGTAGTGTAGAAAGACAACGTATACAAAAAACTACTAACGATA
TGAAAGTTCAGACAAATGAATTAGTAAGATATCAATCTCAAATGAGTAATATCTTAGGAA
GAATGAAGGATTACTTTATTGGGGGAAGTGTTATTGCTCTATCTGTCGGAACTTTAAAGG
AAGCATTTAGTACAATACAAGAAGTTGATACGGCGTTAACTGATTTTCGTAAAGTTACAG
ACCTAACAAAAAATGAATATTCAGAGTTTGTTGATTTTGCAAATGAAAAAGCTAAAGAGT
TATCAGGTAGCACATCAGATTTTATTCAATCCACAGCAAACTTTATCCAAATGGGTTATC
GAAATGTTAATGAAGCCAAAAAGTTAGCCGAAGACAGCCAAATTTATATGAATGTCGGTG
AGTTAACACAAGAACAAAGTACAAAATCATTAATTACTATTTTAAAATCATTTAATTTAG
AAGCTTCAAAATCAACAGAGATACTTGATAAAACCAATCAGGTAGCAAACGACTTCCCTA
TCACTGTGGCTGGTTTAGCTGGAGGAATGTCTCGGGCGGGATCAGTTTTAAAGAATGCGT
CCAATGATATAGATCAGTCAATAGCATTATTGACAGCAGCCAACACTTCTATACAAGATC
CTATTCGTGTTTCAAATGGTTTAAAAAGTATAGCCCTCAACCTCGAAAAGGTTAAGATGA
AAGCTGGTAAGGCAACACCTAAGTTACAAGATATGATGATGACATTAACAAAAGGTAGGG
TAAGTTTAACAGATGTAAATGGAGAATTTAAATCAACCTATCAAATCATCAAAGATCTAG
GAACAGTTTGGAATGATGGTACACTAAAAGCTAATGAAAAAGCACTTATTTTACAAGAGG
TAGCTGGGAAACATCAGGCTAATGTATTGGCTTCTTTATTAAGTAATGCTAAAGATTTAG
ATAAAATTTATGAGGATAGTAAAAACTCTGCTGGTTCGGCTTATTTAGAGAACCAAAGGT
TCATGGATTCTATTAATGGTCGTTTAAATGCTTTGAAGGAGAATACTAAAGGGATTTTTC
TTGATTTGTCAAACACTGATTTTTTAAAAGGAATAATAAGTGGGATAAATAGAGGAGTTA
GTGCTTTAAGAGGATTAATTAAAGAATTTGGCGCGATTCCAAGCGTTATATCATTGGCTA
GTGCCAGTATGACAACTTTAAACACGCAATTTAGAAAAATGGCTATAGATAAAAATTTGT
TTGGCATTGGGAAAATTGAAGAATTATTTATTAGTAAACAATATGGATTAAACAATAAGA
TAAAAGAACAAAGAGAATTAATCAAAGAAATAAAATTAACAAAAAATGCGCAGTTAGGAC
TAAATAAGGCTACTCTTTCCTATGGAGTTAATTTGGGGAAAGTAAAGGGGAGTATAATTG
CAACAAAAGCAGAACTTGGATTATTAAGGGTTGCAGCTACAGCAGCTCAAGCAGCTATTT
CGTTTGGAGTTGGACTGGTAGTTTCATTTGCAATTGAAAAATTAGTGACTTTTATCGATA
AACTCCATACAACTAGAGAAGAATTAAAACAATTAAATACGGATTTTTTTAGTGCTTCAA
AACAGAGTAATGAAACAATTAGTAAAGCAGAAGAAAAATACTCAAAAATAAAAGAATTAC
AAAATCAGTTAGCAAATACAAAAAATGAACAAGAAAAAATAGAATTGCAATCCGAATTAA
ATAAACTTCAATCAGAGATGGTAACTCTATTGCCTCAAACTAAAAATGGATTGGACTCTC
AGAATAATGCTATTGCTAATAATAATGCTCTTATCGAAGAATCTATAAGATTAAAAAAAG
AAGAAAGAGAAGAATCGGCTAAAAAAATAGCACAAGAAAATAGTAAACATATGGATGTTT
TTGATAAATATTTTGAAAATAAAAATAATTATCAAAATATGTCTTCAAACCCTTCTACTG
GTTTAAACAAATTTCAAAAACTACTTTTAATGGGCACTGGGCATAATAGCGCTATTAAAA
AAGCTGATACACAAGAGGTTAATAAAGAACTTCAAGAACAATTAGCAACGATAACAAGTA
TTAAACAAGCTGTAGCTGATATGAAAGCTTCTAAAATGTCAGATATTGAAATTAAAGCAC
AGTTTGGTGGAGTTGATGTTTTTGAAAAAATTAGAGAATTTGATAATTCACTTCAACAAA
CTCAAACTACCGCTGATAACACTAAACCATCATTAGAAGGATTAACAAATCCACTTGATG
CAATTAAAGAAAGTGCAACATCAGCAGCAGATGAATTAGATAAATTAGGCTCAAAATTCA
ATAAACTTACTGGAAATATTGGCTTAGTACAATCAGCTATGTCAGAATTCCAAAAAACGG
GAACATTATCAGCAAAAACAGTAGGTTCTATGTTGAATTCTGGGGATACTAGAGTTATAT
CACTTTTGGCTGATAAAAATAAATTCATGGAGAATAGTATTGCTTTAGAAAAAACATTAA
GAACTGAAAGTGATGCTACATATAGACAAGCAATACAAAATTCGCAAAATTTATTGCAAC
AAGGGATAAATGATTTATCAAGTAAACAAGCTATTGAAACACAAGCTATGAATCATAGTG
TTGGTCTATCTAATCAAGAAACTCAAGCTAAAATGAATAACTATGCCAATGATACTAACG
CTCATGCAAATAGCGAAAGTACAAAGGTGACTAATGGTCAAAATGGAGCAGAGGCTAGAA
CTGAGGCTTCTTCACAAGAAACCAATAATAAAGCCGAACACTATAAAACTGATAATACTA
ACCACACCAATTTAGTTACAACCAAAAGTAAAGCAGCAGTAGATGGTGCTAACGCTATAC
AAGGGGCTAATAGTCAAATGGTAGGTAATATGGCTGGAGCATACAAAACTGATGTTAAGA