# Fimbrial systems in *Klebsiella*: An investigation of genetic variants of type 1 fimbriae

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Toyosi Aduke Obasanjo

Department of Infection, Immunity and Inflammation

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# Abstract

**Fimbrial systems in *Klebsiella*: An investigation of genetic variants of type 1 fimbriae**

**Toyosi Obasanjo**

*K. pneumoniae* associated genomic island KpGI-5 was found to possess a novel type 1-like gene cluster dubbed *fim2.* Despite extensive studies on *fim2*, certain issues regarding the operon remain unresolved.

In this project, the transcriptional analysis suggested controlled expression of *fim2* during bacterial growth phase. *In vivo* assay using *G. mellonella* killing assay did not show any statistically significant *in vivo* role for *fim2*, but suggested a contribution to immunogenic properties of the bacteria. To ascertain fimbriae forming capabilities of *fim2*, a hybrid fimbriae of *fim* and *fim2* was constructed such that *fimA* was replaced with *fim2A* on the *fim* on pJTOOL-7 plasmid. This plasmid was transformed into afimbriate *E. coli* HB101 (HB101/pFim-$HY_{A:2A}$). *fim2A* was also replaced with *fimA* on *fim2* (HB101/pFim2-$HY_{2A:A}$) as well as *fim2H* replaced with *fimH* (HB101/pFim-$HY_{H:2H}$). Transmission electron microscopy of HB101/pFim-$HY_{A:2A}$ and HB101/pFim2-$HY_{2A:A}$ showed evidence of fimbrial structures on the bacterial surface, confirming that *fim2A* bears similar function with *fimA* and hence, confers fimbrial forming capabilities. The replacement of adhesin *fimH* with *fim2H* did not show mannose sensitive agglutination of erythrocytes characteristic of *fimH.*

Other *Klebsiella* strains were screened for the presence of *fim* and *fim2* genes. Of the strains that tested positive for *fim2*, 3 unique strains sp15, sp25 and sp28 tested negative for typical *fim* genes. PCR mapping and sequence analysis done in this project showed that this operon bears high similarity to *fim* but contains higher synonymous nucleotide substitutions than normally found among *Klebsiella fim* and harbours a unique *fimK* homolog. Analyses suggest microevolution of *fim* to *fim3* in these strains. Ambiguity surrounding species classification was clarified using multiple locus sequence typing (MLST) method. *In vitro* assays and *Galleria* killing assay did not show statistical significant differences between *fim* in *K. pneumoniae* KR116 and *fim3* in sp28. This thesis reports first possibility of microevoution of *fim* gene cluster within *K. pneumoniae* and also confirms the fimbriae coding potential for novel *fim* variant *fim2*.

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**For Elizabeth and Memuna**

## Abbreviations

| | |
|---|---|
| λ | Lambda |
| µg | Microgram |
| µl | Microlitre |
| µM | Micromolar |
| aa | Amino acid |
| AB | Antibiotic resistance cassette |
| bp | Base pair |
| | Blood stream infection |
| c-di-GMP | Bis-(3'-5')-cyclic dimeric guanosine M monophosphate |
| $CaCl_2$ | Calcium chloride |
| cDNA | Chromosomal DNA |
| CPS | Capsular polysaccharide |
| DA | Downstream arm |
| PDE | phosphodiesterases |
| ESBLs | Extended spectrum beta lactamase |
| FRT | Flp recombinae target |
| g | Gram |
| GI | Genomic island |
| GP-RBCs | Guinea pig red blood cells |
| HPI | High pathogenicity island |
| IPTG | Isopropyl-β-D-thiogalactopyranoside |
| h | Hours |
| kb | Kilobase |
| l | Litre |
| LA | Lysogeny Agar |
| LB | Lysogeny broth |
| LF | Left flank |
| LPS | Lipid polysaccharide |
| M | Molar |
| MLST | Multi locus sequence typing |
| mg | Miligram |
| min | Minutes |
| ml | Millilitres |
| mM | Milimolar |
| ng | Nanogram |
| °C | Degree celsius |
| $OD_{XXXnm}$ | Optical density at XXXnm |
| ORF | Open reading frame |
| PCR | Polymerase chain reaction |
| PLA | Pyogenic liver abscess |
| qRT-PCR | Quantitative real time PCR |
| RNA | Ribonucleic acid |
| RF | Right Flank |
| SOE-PCR | Spliced overlap extension PCR |
| $T_m$ | Melting temperature |
| tRIP-PCR | tRNA interrogation for pathogenicity islands, prophages and genomic islands -PCR |
| U | Units |
| UA | Upstream arm |
| UTI | Urinary tract infection |

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# Chapter 1. Introduction

*Klebsiella* was first identified in 1882 by Anton von Frisch as a bacillus isolated from patients afflicted with rhinoscleroma, a bacterial infection that causes inflammation of the upper respiratory tract. The isolated bacteria were then named after a German microbiologist and pathologist, Edwin Klebs (Trevisan, 1887, Von Frisch, 1882).

In recent times, many physiological differences and similarities among varying members of the *Klebsiella* genus has been discovered. Some of these characteristics will be further described in this thesis. Generally, the members of *Klebsiella* genus are usually described as encapsulated non-motile Gram-negative bacteria (Montgomerie, 1979).

## 1.1 The *Klebsiella* species

### 1.1.1 Taxonomy

Historically, the classification of *Klebsiella,* like with other bacteria was based on certain features of pathogenesis and origins. With the advent of modern scientific methods and technology, several confusions have caused the reclassification of some of the species within this genus (Martínez, Martínez et al., 2010). The *Klebsiella* genus is however generally divided into eight taxonomic groups namely *K. pneumoniae, K. variicola, K. granulomatis,*

*K. oxytoca, K. terrigena*, *K. planticola*, *K. ornithinolytica* and *K. mobilis* (also considered as *Enterobacter aerogenes*) (Brisse, Grimont et al., 2006).

The species group *K. pneumoniae* is further divided into three subspecies known as *pneumoniae, ozanae* and *rhinoscleromatis* (Brisse, Grimont et al., 2006). While the biochemical patterns of *ozanae* and *rhinoscleromatis* allow for different designation of these subspecies, they cannot be separated from *K. pneumoniae* by DNA relatedness (Brenner, Steigerwalt et al., 1972) and hence, treated as a subspecies of *K. pneumoniae* (Brisse, Grimont et al., 2006).

*K. variicola* is the newest addition to the *Klebsiella* genus. These species were isolated from banana plants (Drancourt, Bollet et al., 2001) and phylogenetic analyses of *rpoB, gyrA, mdh, infB, phoE* and *nifH* genes from several putative *K. variicola* strains showed a tight cluster that supports a new lineage of species within the *Klebsiella* genus, but also bear a relationship to *K. pneumoniae* (Rosenblueth, Martínez et al., 2004). *K. granulomatis*, a recent addition to *Klebsiella* family, was proposed to have been transferred from *Calymmatobacterium* (Carter, Bowden et al., 1999), given its close relationship to *K. pneumoniae* (based on 16S ribosomal DNA sequence (rDNA)) and observed similarities in pathological traits of *C. granulomatis* to *K. pneumoniae subsp. rhinocleromatis* and *K. pneumoniae subsp. ozanae.* Notably, the close relation of *K. granulomatis* to *K. pneumoniae* has also given rise to the suggestion that *K. granulomatis* may be a subspecies within *K. pneumoniae* as seen with sequencing and phylogenetic analyses of several genes such as *rpoB* and *phoE* genes (Drancourt, Bollet et al., 2001, Carter, Bowden et al., 1999).

It has been suggested that some of the categorised *Klebsiell*a species should be transferred to other genuses. For example, *K. terrigena*, *K. planticola* and *K. ornithinolytica* should be moved to the genus *Raoultella* based on 16s rDNA sequence analyses. However, phylogenetic analyses of the 16s rDNA sequence did not give significant proof for monophyly of *Raoultella* (Drancourt, Bollet et al., 2001, Brisse, Grimont et al., 2006) and *rpoB* sequence analyses also could not group the putative *Raoutella* species into a single cluster (Drancourt, Bollet et al., 2001). *K. mobilis* has also been categorised as *Enterobacter aerogenes* (Drancourt, Bollet et al., 2001) but is usually included in the *Klebsiella* family based on relatedness of 16S rDNA (Drancourt, Bollet et al., 2001, Brisse, Grimont et al., 2006). DNA related studies have also showed a significant divergence between *K. oxytoca* and *K. pneumoniae* (Jain, Radsak et al., 1974, Brenner, Farmer et al., 1977) even though it was previously considered as a biogroup of *K. pneumoniae* (Edwards and Ewing, 1972, Brisse, Grimont et al., 2006).

### 1.1.2 Epidemiology

*Klebsiella* are ubiquitous in nature and are generally found in a range of hosts and habitats. Members of this genus are found in diverse environments such as water, soil, sewage and plants as well as colonising mucosal surfaces of mammalian cells of humans, horses and swine (Edberg, Piscitelli et al., 1986, Seidler, Knittel et al., 1975, Bagley, Seidler et al., 1978, Podschun and Ullmann, 1998). While carriage rates of *Klebsiella* by mammalian host varies

with different studies, infections caused by these species are usually found in gastrointestinal tract, urinary tract, blood, liver and a range of other tissues in both humans and animals (Van Aartsen, Stahlhut et al., 2012, Podschun and Ullmann, 1998). A survey of carriage rates estimated that 29 – 35 % of individuals carried *Klebsiella* spp. in their stool while a lesser rate of 3 – 4% was observed for throat carriage (3 – 4 %) (Davis and Matsen, 1974). These numbers slightly increased in hospitalised patients (Davis and Matsen, 1974) who were affected by both antibiotic therapy and length of hospital stay (Podschun and Ullmann, 1998, Pollack, Nieman et al., 1972).

*Klebsiella* species like other Gram negative bacteria have limited growth on the skin and are regarded as part of the transient micro flora(Kloos and Musselwhite, 1975, Rosebury, 1962). However, they are known to cause equine metritis, inflammation of the lining of the uterus and have been observed as a vital cause of bovine mastitis (inflammation of breast tissue) (Zadoks, Griffiths et al., 2011, Aartsen, 2011).

The extent of colonisation of human hosts by *Klebsiella* spp. can range from asymptomatic carriage to opportunistic infections. In some instances, *Klebsiella* are known to cause grave community infections such as pyogenic liver abscesses (PLA) and Friedlander's pneumoniae, caused by *K. pneumoniae* and often seen in chronic alcoholics (Carpenter, 1990).

Although PLAs are typically caused by *Escherichia coli* as well as a host of other bacteria and associated with underlying hepatobilary disorders, *K. pneumoniae* has emerged as a vital pathogen in eastern Asia since the 1990s as the major cause of PLAs (Peters and Craig, 2001, Rahimian, Wilson

et al., 2004). Between 1996 and 2004, there was increase in *K. pneumoniae* associated PLAs in Taiwan from 11.2 to 17.5 per 100,000 population (Tsai, Huang et al., 2008). Complications from *K. pneumoniae* associated PLAs can also lead to further health complications such as endopthalmitis and meningitis (Thomsen, Jepsen et al., 2007, Ko, Paterson et al., 2002). *K. pneumoniae* community acquired meningitis can also occur without association of previous PLAs (Ko, Paterson et al., 2002). There have been reports of *K. pneumoniae* associated PLA and meningitis in other parts of the world like Europe, Canada and USA (Giobbia, Scotton et al., 2003, Karama, Willermain et al., 2008, Nadasy, Domiati-Saad et al., 2007, Sobirk, Struve et al., 2010, Aartsen, 2011). Community acquired pneumoniae is another prevalent community acquired infection caused by *K. pneumoniae* in some parts of the world particularly in elderly people**.** In Malaysia and Japan, studies estimated the incidence rate of pneumonia caused by *K. pneumoniae* in elderly people at 15 to 40 %, which is as high as the incidence of infection caused by *Haemophilus influenza* (Podschun and Ullmann, 1998, Qureshi, 19 August 2014). Although these figures are different in various parts of the world, the mortality rates caused by complicating *K. pneumoniae* infections can be as high as 50% and can go up to 100 % with people who already suffer from alcoholism and bacteraemia (Qureshi, 19 August 2014). A mortality rate of 21 % was also reported with *K. oxytoca* (Qureshi, 19 August 2014). In other endemic and subtropical regions, rhinoscleroma infection is caused by *K. pneumoniae* subspecies *rhinoscleromatis.* It is a chronic granulomtous inflammatory process of the nasopharnx, usually unidentifiable at earlier stages due to low specificity of clinical signs but can be treated by long-term

antibiotic therapy (Hart and Rao, 2000). Another rare disease caused by *K. pneumoniae*, specifically its subspecies *ozanae* is chronic atrophic rhinitis.

Apart from community associated infections, *Klebsiella* spp. are known as opportunistic bacteria that readily colonise hosts with underlying disease and decreased immune response (Podschun and Ullmann, 1998). Hence, members of the *Klebsiella* genus are some of the major causes of nosocomial infections around the world.

*Klebsiella* spp. have been incriminated in 3 to 8 % of nosocomial infections and generally recognised at vital pathogens that colonise sites such as the urinary tract, lower respiratory tract, biliary tract, and surgical wound sites as well as blood stream (Qureshi, 19 August 2014, Podschun and Ullmann, 1998, Reacher, Shah et al., 2000). The range of clinical symptoms and syndromes include pneumoniae, diarrhea, bacteramia, upper respiratory and urinary tract infection, meningitis and others (Qureshi, 19 August 2014). The use of invasive devices and urinary cathethers are some of the factors that contribute to higher risks of nosocomial infections with *Klebsiella* spp. (Podschun and Ullmann, 1998). Also, underlying diseases such as diabetus mellitus, chronic alcoholism, chronic cardiac, renal or pulmonary problems together with the malignancy and age extremes are patient-specific factors that may also increse the risk of nosocomial *Klebsiella* infections (Marchaim, Zaidenstein et al., 2008, Meatherall, Gregson et al., 2009). Infection by *Klebsiella* has also been connected with ankylosing spondylitis. This is a chronic autoimmune inflammotory diease that affects spinal joints and large synovial joints and may be triggered by the molecular mimicry of *K.*

*pneumoniae* surface antigen K43 to human HLA-B27 antigens (Ogasawara, Kono et al., 1986). Reports suggest a correlation between *Klebsiella* infections and Crohn's disease, an auto-immune disease of the gastrointestinal tract (Ebringer, Rashid et al., 2007).

It is well known that most of the opportiunistic nosocomial infections associated with *Klebsiella* spp. are usually caused by two species in particular, *K. pneumoniae* and *K. oxytoca.* However, other species such as *K. planticola* and *K. terrigena* have also been isolated in nosocomial settings (De la Torre, Manuel García, Romero-Vivas et al., 1985, Hansen, Gottschau et al., 1998, Podschun, Fischer et al., 2000, Podschun and Ullmann, 1992b).

*K. oxytoca* has been incriminated in infections causing bacteraemia among infants, particularly premature babies and those in neonatal intensive care units. These species are also found among infants diagnosed with neonatal septicemia (Qureshi, 19 August 2014).

Epidemic outbreaks of nosocomial *Klebsiella* infections are very serious and particularly feared are epidemic outbreaks due to antibiotic resistant strains of *Klebsiella* which restricts treatment options and can also significantly increase mortality(Cosgrove and Carmeli, 2003, Tumbarello, Spanu et al., 2006). Several epidemics of highly resistant *K. pneumoniae* infections in neonatal intensive care units have been reported as a cause for concern (Cordero, Rau et al., 2004, Damjanova, Tóth et al., 2007, Randrianirina, Vedy et al., 2009).

Statistics reported by the Center for Disease Control and Prevention in

United States of America (USA) associated 8 % of endemic hospital infection and 3 % of epidemic outbreaks to *Klebsiella* spp. (Qureshi, 19 August 2014). According to other reports, *Klebsiella* is rated as the second most comon cause of BSI (blood infection) associated with Gram negative bacteria (Ronald, 2003, Yinnon, Butnaru et al., 1996). As mentioned earlier, a major cause of concern with epidemic hospital infections is the outbreak of multiresistant strains.

The carriage rates and development of many of these multi-resistant strains have been further advanced by extensive use of a broad-spectrum antibiotics in hospitalised patients (Qureshi, 19 August 2014). Many *Klebsiella* strains harbour β-lactamase genes within their chromosomes. These β-lactamase genes such as $bla_{SHV}$, $bla_{LEN}$ and $bla_{OXY}$ endow the *Klebsiella* strains with high resistance to aminopenicillin and carboxypenicillin antibiotics (Chaves, Ladona et al., 2001). However, it has been observed that 10 – 20 % of *K. oxytoca* strains possess mutations within their promoter sequences that causes an over expression of $bla_{OXY}$ which subsequently increases the level of resistance to carboxypenicillin antibiotics (Fournier, Lu et al., 1995).

Within the *Enterobactericae* family, the members of the *Klebsiella* genus have been recognised as the most frequent members to harbour ESBLs (extended spectrum beta lactamase). A study revealed that many of *Klebsiella* spp. isolated from patients harbour at least two β-lactamase genes (Paterson, Hujer et al., 2003).

Apart from ESBLs, *K. pneumonoiae* strains have also been found to genetically encode carbapenemases. These carbapenemases inactivate carboxy- and

aminopenicillins, cephalosporins and carbapenems which are typically used as a last resort in many clinical infections. Several types of these carbapenemases have been described such as metallo-β-lactamases (e.g NDM-1), expanded-spectrum oxacillinases (e.g. OXA-48) and clavulanic acid inhibited β-lactamases (e.g. KPC) (Nordmann, Cuzon et al., 2009).

Also, aminoglycoside-modifying enzymes have also been known to cause aminoglycoside resistance in *K. pneumoniae*. Some of these enzymes are usually encoded by genes like *aac(6')-lb* and *aacA43* that are carried on plasmids rather than on the core chromosome (Partridge, Thomas et al., 2011, Sarno, McGillivary et al., 2002). While mutations in the chromosomal genes *gyrA* and *parC* are known to regulate quinolone resistance in *K. pneumoniae* (Deguchi, Fukuoka et al., 1997), efflux pumps and DNA gyrase protection systems are mechanisms based on plasmid-encoded *bla* genes (Galani, Souli et al., 2010, Karah, Poirel et al., 2010).

Several measures have been recommended and put in place to prevent the spread of *K. pneumoniae* infections in hospitals. Since medical equipment and hands of medical staff could be reservoirs for *Klebsiella* spp., strict adherence to basic personal and environmental hygiene standards for the maintenance and cleaning of medical equipment as well as good hand washing practice all aid in the prevention of nosocomial *Klebsiella* infections (Ransjö, Good et al., 1992, Montgomerie, 1979, Goetz, Rihs et al., 1995). Also, regulation of antibiotic use in hospitals is another measure to control nosocomial *Klebsiella* infections (Qureshi, 19 August 2014).

## 1.2 Typing methods

From an epidemiological point of view, typing methods for discriminating between bacterial isolates belonging to the same species or clonal complex is a very important factor in the prevention and control of endemic and epidemic outbreaks. This is particularly relevant to bacterial isolates like *Klebsiella*, which are known to cause nosocomial outbreaks (Podschun and Ullmann, 1998, Brisse, Grimont et al., 2006). Several methods have been used to distinguish between *Klebsiella* isolates with varying degrees of success. Traditional methods include phenotypic based methods such as serotyping, bacteriophage typing and several others (Podschun and Ullmann, 1998). Recent developments in identifying similarities and relatedness of bacteria at a genotypic level have greatly improved molecular typing methods used to discriminate between bacteria types and subtypes (Brisse, Issenhuth-Jeanjean et al., 2004). This section will review and discuss some of the more widely used phenotypic and molecular typing techniques.

### 1.2.1 Phenotypic typing schemes

In other to identify *Klebsiella* spp., several traditional typing methods have been used over the years. These traditional typing systems are based on the phenotype such as biotype, serotype or phage typing.

The biotyping method of species identification is based on their differences in biochemical and culture tests. While several biochemical schemes were developed for identifying *Klebsiella* species (Cowan, Steel et al., 1960, Barrow

and Feltham, 2004, Rennie and Duncan, 1974, Fallon, 1973), commercial available systems such as the API 20E systems are mostly used for biotype analysis (Podschun, Heineken et al., 1986, Simoons-Smit, Verweij-van Vught et al., 1985a). This biotype method however has several limitations in its suitability as an epidemiological tool for identification. The more recent commercial available biotype systems were mostly developed for taxonomic identification and hence, the subspecies division within isolates is more difficult to obtain among isolates (Ayling-Smith and Pitt, 1990).

Serotyping, another phenotypic method, is based on the reaction of capsular antigens on the surface of bacteria to specific antiserum. The capsular polysaccharide (CPS) is one of the carbohydrate structures found on the surface of *Klebsiella* cells. *Klebsiella* spp. generally possesses a well-developed polysaccharide capsule that provides the mucoid appearance observed on colonies (Podschun and Ullmann, 1998). There are 82 capsule antigens characterised, of which 77 of these antigens form the basis on distinct serological types (Podschun and Ullmann, 1998, Wu, Huang et al., 2010). The O-antigens of this genus are molecules comprised of repeated carbohydrate subunits linked to the core antigen of its lipopolysaccharide (LPS) (Shankar-Sinha, Valencia et al., 2004). While 12 different types of the O-antigen has been characterised, O-serotyping is not used in *Klebsiella* identification because exposure and classification of these antigens can be hindered by the heat stable capsular material (Ørskov, 1954, Ørskov and Ørskov, 1984). The Quellung test is the traditional technique used in capsular serotyping. During this test, the CPS absorbs capsular specific antibodies

causes swelling of the capsule, which can be viewed under a microscope (Habib, Porter et al., 2014, Mizuta, Ohta et al., 1983). Although capsular serotyping in contrast to biotyping provides better reproducibility and better differentiation among most clinical isolates(Ayling-Smith and Pitt, 1990, Podschun and Ullmann, 1998), this method also has several drawbacks. One major limitation of serotyping is the vast occurrence of serological cross reactivity among the 77 capsule types, especially since cross activity is known to occur between one or more capsular types (Ayling-Smith and Pitt, 1990, Ørskov and Ørskov, 1984, Podschun and Ullmann, 1998). Also, the CPS can be a weak immunogen consequently providing weak immune response reactions, that give results difficult to interpret and susceptible to subjective interpretations (Podschun and Ullmann, 1998). Moreover, the lack of commercially available anti-capsule antisera for *Klebsiella* makes this technique quite costly.

Bacteriophage typing for *Klebsiella* was developed in the 1960s (Przondo-Hessek, 1966, Slopek, Przondo-Hessek et al., 1967). This method of typing is based susceptibility of *Klebsiella* strains to several phages that are maximally sensitive in distinguishing species among bacterial isolates and also been used to distinguish among capsular serotypes in *Klebsiella.* It is however mostly used together with serological testing (Gaston, Ayling-Smith et al., 1987, Podschun and Ullmann, 1998). Like other methods of phenotyping, bacteriophage typing has its drawbacks such as the lack of standardisation in methods as well as the limited availability of bacteriophages able to infect all the *Klebsiella* capsular types (Wareing, Tye et al., 1996).

Bacteriocin typing has been recommended by several authors for phenotypic typing of *Klebsiella* isolates (Bauerfeind, 1984, Buffenmyer, Rycheck et al., 1976, Hall, 1971, Podschun and Ullmann, 1998). Bacteriocins are protein toxins or complexes of toxic proteins produced by bacteria. These toxins inhibit the synthesis of protein and nucleic acids as well as uncouple electron transport from active transport of several ions and compounds (Konisky, 1982, Neville and Hudson, 1986). Bacterial isolates can be identified either by their sensitivity to bacteriocins produced by a number of strains or their ability to inhibit specific indicator strains (Podschun and Ullmann, 1998). Because many members of the *Klebsiella* family do not produce bacteriocins, this method of biotyping has become a choice technique for identifying isolates that belonging to *Klebsiella* genus (Podschun and Ullmann, 1998). However, more than 40 bacteriocins produced by *Klebsiella* species have been identified and divided into several groups based on their physiochemical and biological properties (Buffenmyer, Rycheck et al., 1976, Podschun and Ullmann, 1993). Due to the instability of bacteriocin preparation, the use of the bacteriocin typing has been modified so that bacteriocins are synthesised on an agar medium immediately before the strains to be typed are inoculated by a multipoint inoculator (Buffenmyer, Rycheck et al., 1976, Edmondson and Cooke, 1979, Hall, 1971, Podschun and Ullmann, 1998). This method has proven effective in testing clinical and environmental isolates belonging to *Klebsiella* (Bauernfeind, Petermuller et al., 1981, Bauernfeind, Rosenthal et al., 1993, Podschun and Ullmann, 1993, Podschun and Ullmann, 1998).

### 1.2.2 Molecular typing schemes and MLST

Molecular methods for epidemiological characterisation of bacterial species are based on the differences in their genomic DNA sequence and organisation (Van Belkum, Struelens et al., 2001). These methods have radically reformed the ability to discriminate among bacterial groups and subgroups. In recent years, many molecular methods for *Klebsiella* typing have been developed and used to compare isolates from varying time points and geographical locations (Brisse, Grimont et al., 2006). While some of these molecular methods are generally not standardised (Brisse, Grimont et al., 2006), the use of a particular molecular typing method is dependent on the epidemiological context and the scale of which the method is used (Sabat, Budimir et al., 2013). Some of the most commonly used molecular typing schemes include PFGE, MLST, AFLP (see below for details), some of which will be briefly discussed in this chapter.

Pulsed-field gel electrophoresis (PFGE) is a widely used method of molecular typing for many bacteria as well as *Klebsiella* (Lebessi, Dellagrammaticas et al., 2002, Hansen, Skov et al., 2002, Sechi, Spanu et al., 2001, Chang, Fang et al., 2000, Poh, Yap et al., 1993, Toldos, Ortiz et al., 1997, Brisse, Grimont et al., 2006). To perform PFGE, a purified genomic DNA extract is cut with a restriction enzyme that recognises infrequently occurring restriction sites in the bacterial genome (Brisse, Grimont et al., 2006, Sabat, Budimir et al., 2013). For *Klebsiella*, restriction endonuclease *Hae*III*, Xba*I and *Spe*I have been largely used in the epidemiological typing of clinical isolates of *Klebsiella* (Brisse, Grimont et al., 2006). DNA fragments obtained from

restriction digestion are separated by 'pulsed-field' electrophoresis on agarose gel. The direction of electric field passed across the agarose gel is changed repeatedly so that the separated DNA fragments form a pattern that is visible on the gel (Sabat, Budimir et al., 2013).

The use of PFGE for molecular typing has several advantages because it addresses a large portion of the investigated genome (> 90 %) with a high level of discrimination among isolates (Hansen, Skov et al., 2002). However, this method is labour intensive, time consuming and technically demanding and also prone to some subjectivity, making reproducibility and standardisation quite difficult (Vimont, Mnif et al., 2008, Sabat, Budimir et al., 2013). Moreover, while PFGE typing results should not be disturbed by smaller mobile non core DNA, single band differences have been observed in some bacteria that carry large plasmids (more than 50 kb) (Sabat, Budimir et al., 2013) limiting the power of this technique.

Multi locus sequence typing (MLST) is another method of molecular typing using nucleotide sequence of several gene loci to characterise genetic relationships among bacterial isolates (Enright and Spratt, 1999, Feil, Li et al., 2004, Maiden, Bygraves et al., 1998). This method involves the amplification and sequencing of several genes at specific loci within the bacterial genome, which include *rpoB, gapA, phoE, infB, tonB*, for *Klebsiella* strains (Diancourt, Passet et al., 2005, Wang, Li et al., 2013). For each locus, unique sequences (alleles) are given arbitrary numbers, which combine to give an allelic profile based on the identified alleles. A sequence type (ST) number is then assigned to each allelic profile (Diancourt, Passet et al., 2005, Sabat, Budimir et al.,

2013). A MLST method has been developed for *K. pneumoniae* and it has also been shown that MLST analysis can discriminate among epidemiologically unrelated isolates (Diancourt, Passet et al., 2005). The allelic sequences and ST profiles for *K. pneumoniae* can be accessed in a central Internet database (http://bigsdb.web.pasteur.fr/klebsiella/klebsiella) (Diancourt, Passet et al., 2005, Brisse, Fevre et al., 2009). The database also provides access to online software called eBURST used for investigation of the genetic relationships between bacterial isolates of each ST (Brisse, Fevre et al., 2009, Diancourt, Passet et al., 2005). An important benefit of the MLST scheme is that results obtained are usually reproducible and confers standardisation (Sabat, Budimir et al., 2013, Vimont, Mnif et al., 2008). However, this method may be costly depending on the number of isolates and loci to be investigated (Sabat, Budimir et al., 2013).

Amplified fragment length polymorphism (AFLP) was firstly initiated as a genetic tool for plant breeders (Vos, Hogers et al., 1995). This method of molecular typing is based on polymerase chain reaction (PCR) amplification of digested fragments of genomic DNA (Brisse, Grimont et al., 2006). In this method, extracted genomic DNA is cleaved with two restriction endonucleases while the adaptors are ligated to one of the sticky ends of the restriction fragments (Vos, Hogers et al., 1995). After, the digested fragments with adaptor ends are selectively amplified by PCR with oligonucleotides complementary to the sequences of the adaptor and restriction site (Sabat, Budimir et al., 2013). This technique enables precise co-amplification of around 50 to 100 of restriction fragments while fluorescent dye-labelled PCR

primers are typically used as in this process. This allows the fragments to be recognised after they have been distributed on an automated DNA sequencer by the size of the fragments (Mortimer and Arnold, 2001, Savelkoul, Aarts et al., 1999). The AFLP technique has been investigated for its ability to discriminate and profile sets of strains involved in a nosocomial outbreak of *Klebsiella* as well as for the recognition of *Klebsiella* species and phylogenetic relationships (Jonas, Spitzmüller et al., 2004). Though AFLP fragments are typically separated on polyacrylamide gels, a variation of this protocol using agarose gels instead was developed for *Klebsiella* (Van der Zee, Steer et al., 2003). Benefits of the AFLP method include immense discriminatory power and reproducibility of results as well as the flexibility in the sequence coverage of the genome (Brisse, Grimont et al., 2006). However, this method is limited by the fact that it is labour-intensive as an average analysis can take up to three days and can be costly as many of the reagents and systems utilised are expensive (Sabat, Budimir et al., 2013).

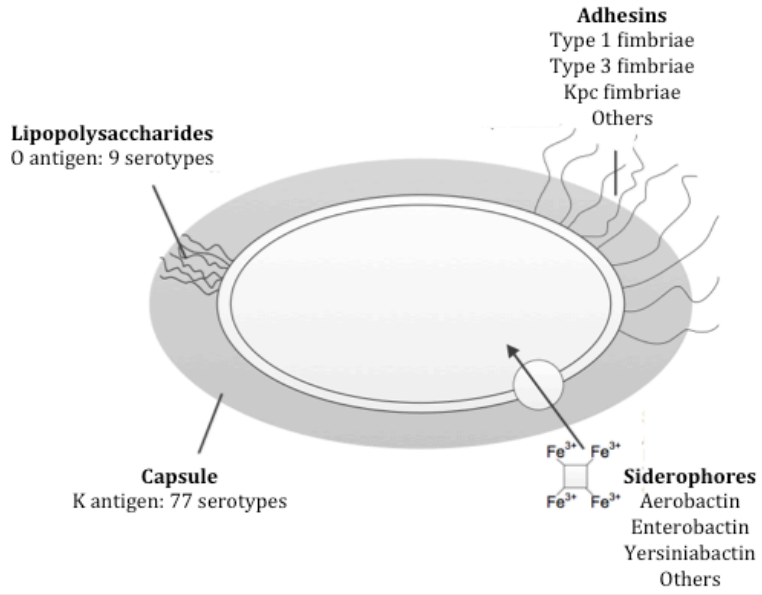
Other PCR based methods such as random amplified polymorphic DNA (RAPD), repeat-based PCR (rep-PCR) or enterobacterial repetitive inter-genic consensus sequence PCR (ERIC-PCR), are dependent on the amplification of unknown DNA fragments (Brisse, Grimont et al., 2006). These are applicable even without previous knowledge of the genome sequence and hence, very useful for quick examinations of the origins and course of clonal epidemic and has been applied to epidemiological studies of *Klebsiella* (Ben-Hamouda, Foulon et al., 2003, Shannon, Fung et al., 1998, Lhopital, Bonacorsi et al., 1997, Gori, Espinasse et al., 1996, Eisen, Russell et

al., 1995, Brisse, Grimont et al., 2006). However this method is also limited by low reproducibility and low level of standardisation, in spite of several efforts to standardise the RAPD technique(Van Belkum, Struelens et al., 2001, Vogel, Jones et al., 1999). The RAPD technique has also been applied in identification of divergent clones within *K. pneumoniae* and *K. oxytoca* (Brisse and Verhoef, 2001, Brisse, Grimont et al., 2006).

## 1.3 Virulence factors

*Klebsiella* strains possess a wide variety of phenotypic traits and tools that contribute to the pathogenesis of these bacteria. Several mechanisms have been identified by *in vitro* and *in vivo* models that show the interaction of these bacteria to host cells (Podschun and Ullmann, 1998). This section will discuss some of the well-described factors and properties that contribute to the pathogenicity of these strains such as siderophores, capsular polysaccharides, lipopolysaccharide and fimbrial adhesins (Figure 1-1).

**Figure 1-1. Schematic representation of *Klebsiella* virulence factors**




*Klebsiella* spp. harbour different factors and mechanisms that contribute to the virulence of these bacteria during host infection. Some of these factors are discussed in this chapter. Diagram is extracted from a schematic by Jon van Aartsen J., 2011 and based on a schematic by Podschun R. and Ullmann U., 1998.

### 1.3.1 Siderophores

For many bacterial species, iron is an essential nutrient for growth. It is an essential factor in the electron transport chain as well as several other cellular processes (Neilands, 1981). In most mammalian tissues, iron is sequestered by carrier molecules such as transferrin and lactoferrin or sequestered intracellular as haemoglobin or ferritin (Weinberg, 1978). This reduces the amount of iron readily available to bacteria and for these bacterial cells to strongly grow in host tissues, they must be able to acquire iron from the iron transport proteins contained within hosts. Different species have developed a wide range of secreted factors in order to acquire iron. In gram-negative bacteria, iron is bound to siderophores, which is then transported intracellularly through the bacterial outer membrane. Within the

bacteria cells, the iron is released from the siderophores by different mechanisms (Lawlor, O'connor et al., 2007). *Klebsiella* spp. along with other members of the Enterobactericae can encode several siderophores systems, three of which are most prevalent. The most common siderophore systems are enterobactin, aerobactin and yersinibactin all of which have high affinity for iron and can collect iron from host carrier molecules (Lawlor, O'connor et al., 2007, Koczura and Kaznowski, 2003, Perry and San Clemente, 1979). Enterobactin (also known as enterochelin) is present in many strains and is characterised as a catecholate siderophore (Neidhardt, Ingraham et al., 1996, Mokracka, Koczura et al., 2004, Raymond, Dertz et al., 2003, Visca, Filetici et al., 1991). Aerobactin is a hydroxamate siderophore and found in a smaller percentage of enterobacterial strains (Brock, Williams et al., 1991, Perry, Balbo et al., 1999). When aerobactin synthesis genes were heterlogously expressed in an aerobactin negative strain, an increased level of virulence was observed when the mutant was tested in a mouse peritonitis infection model (Nassif and Sansonetti, 1986). Yersiniabactin is a phenolate siderophore and is found among even fewer bacterial isolates. Although it was first described for *Yersinia* species, it has been found among some isolates of other members of the Enterobactericae and could have been acquired via horizontal transfer (Bach, Almeida et al., 2000). In *K. pneumoniae,* Yersiniabactin is coded for by the HPI island and related to mobile elements, such as ICEKp1 (Hsieh, Lin et al., 2008).

### 1.3.2 Capsular polysaccharide

*Klebsiella* strains usually form prominent outer capsules made up of complex acidic polysaccharides known to contribute to virulence of *Klebsiella* (Corsaro, De Castro et al., 2005, Toenniessen, 1914, Cryz, Furer et al., 1984, Baer and Ehrenworth, 1956). These polysaccharides consists of repeating subunits of sugar, four to six in number, as well as an uronic acid subunit (Corsaro, De Castro et al., 2005, Ørskov and Ørskov, 1984). The capsular material covers the bacteria cell surface with thick bundles of fibrilous structures arranged in extensive layers (Al-Hasani, Rajakumar et al., 2001). The capsule is responsible for the mucoid appearance of bacterial colonies on plates and provides protection for bacterial cells. It protects the cells from immunological responses (such as phagocytosis by polymorphonuclear leukocytes (Podschun and Ullmann, 1992a, Simoons-Smit, Verweij-van Vught et al., 1986) from hosts (Podschun and Ullmann, 1992a, Simoons-Smit, Verweij-van Vught et al., 1985b, Podschun, Penner et al., 1992, Simoons-Smit, Verweij-van Vught et al., 1986, Podschun and Ullmann, 1998) and also prevents death of the bacteria by bactericidal serum factors (Williams, Lambert et al., 1983). This property was further emphasised by a study that showed the rate of phagocytosis was inversely proportional to the mass of capsule generated and hence hindering the binding and internalisation of the bacteria by human monocyte-derived dendritic cells (Evrard, Balestrino et al., 2010). Studies have shown that a large concentration of *Klebsiella* capsular polysaccharide could cause immunological paralysis in mice (Batshon, Baer et al., 1963). The arrangement of the *K. pneumoniae cps* gene

clusters is similar to that of *E. coli* group 1 cps cluster (Rahn, Drummelsmith et al., 1999).

There are 77 antigenically different capsular serotypes that have been identified and included in the international K serotyping scheme (Ørskov and Ørskov, 1984). *Klebsiella* strains expressing the capsule types K1, K2, K4 and K5 are observed to be more virulent in murine models than those expressing other capsule types (Simoons-Smit, Verwey-van Vught et al., 1984) and are usually identified in severe *K. pneumoniae* infections in humans (Mizuta, Ohta et al., 1983, Simoons-Smit, Verwey-van Vught et al., 1984, Brisse, Grimont et al., 2006). In a mouse peritonitis model, the capsule types K1 and K2 were found to be particularly virulent compared to other serotypes (Kauffmann, 1949, Mizuta, Ohta et al., 1983). The K1 serotype is often found with *K. pneumoniae* strains isolated from pneumonia patients and PLA patients (Brisse, Fevre et al., 2009, Fang, Lai et al., 2007, Brisse, Grimont et al., 2006). Serotypes K2, K4 and K5 are often associated with community acquired pneumonia (Brisse, Grimont et al., 2006). It has also been observed that capsular serotypes such as K7 and K21a that confer low virulence may be associated with the mannose content of the CPS (Podschun and Ullmann, 1998). These capsular types contain mannose-α-2/3-mannose or L-rhamnose-α-2/3-L-rhamnose (Corsaro, De Castro et al., 2005, Podschun and Ullmann, 1998). These sequences can be identified by carbohydrate binding proteins such as lectins on the surface of the macrophages thereby leading to lectinophygocytosis (Athamna, Ofek et al., 1991). Lectinophagocytosis is described as phagocytosis based on detection between surface lectins on one

cell and surface carbohydrates on the opposite cell(Ofek, Goldhar et al., 1995). It has therefore been suggested that *Klebsiella* strains with capsules that do not bear mannose are less recognised by macrophages and are more affiliated with infectious disease (Podschun and Ullmann, 1998).

*Klebsiella* CPS is typically believed to mediate and affect virulence in these strains and has been investigated by several experiments. Although the virulence properties of *K. pneumoniae* CPS investigated in gastrointestinal murine model showed little or no significance in their effect on colonisation, the CPS was recognised as a virulence factor in mutagenesis experiments involving the respiratory and urinary tract murine models (Favre-Bonte, Licht et al., 1999, Maroncle, Balestrino et al., 2002). The CPS has also been identified as a vital factor for virulence of *K. pneumoniae* in murine pneumonia model (Cortes, Borrell et al., 2002). Other signature-tagged mutagenesis experiments have also shown that the capsular material can also affect *in vitro* biofilm formation on abiotic surfaces (Balestrino, Ghigo et al., 2008).

### 1.4.3 Lipopolysaccharide

Lipopolysaccharides are described as antigens expressed on the surface of bacterial cells and consist of lipid A, a core oligosaccharide and an O antigen side chain (Hansen, Mestre et al., 1999). Although nine O antigen types have been identified; O1, O2, O2ac, O3, O4, O5, O7, O8 and O12, the most frequently observed antigen type among *Klebsiella* isolates is O1 antigen. In

the case of serotype O1, O antigen is made of two disaccharides, D-galactan I [→3)-β-Galf-(1→3)-α-Galp-(1→] and D-galactan II [→3)-β-Galp-(1→3)-α-Galp-(1→] (Shankar-Sinha, Valencia et al., 2004). D-galactan II constitutes the distal end of the O chains (Shankar-Sinha, Valencia et al., 2004). The subunits are arranged into chains within the bacterial cytosol and consequently transferred to the outer membrane for linkage. Through an α-GlcN group, the assembled subunits are linked to the lipid A and core antigen components of LPS (Vinogradov, Frirdich et al., 2002).

Although the CPS is undisputedly recognised as a very important virulent factor in *Klebsiella*, the O antigen could account some of the variability in the severity of illness encountered in patients (Cryz, Mortimer et al., 1986, Fung, Hu et al., 2000). It has been shown that O antigens are capable of protecting *Klebsiella* from complement-mediated killing (Tomas, Benedi et al., 1986, Williams, Lambert et al., 1983). The effectiveness of this protection of the O antigen is however dependent on the chain length (Ciurana and Tomas, 1987). The O5 antigen was identified as a factor in the colonisation of urinary tract in murine models (Merino, Altarriba et al., 2000). This was further affirmed by studies, which showed that deleted mutants of several core polysaccharide synthesis genes exhibited lower virulence in the urinary tract of murine models (Struve, Forestier et al., 2003). Colonisation of the gastrointestinal tract was also debilitated in these knockout mutants (Struve, Forestier et al., 2003).

*K. pneumoniae* lipopolysaccharides are encoded by a 12-gene cluster known as the *waa,* and located between the *kbl* and *coaD* genes (Regue, Izquierdo et

al., 2005). The lipopolysaccharide cluster also appears well conserved between O serotypes (Regue, Climent et al., 2001).

### 1.4.4 Fimbriae and adhesins

The ability of *Klebsiella* to adhere to tissue surfaces in the host is an early and important step in the initiation and progression of the infection in patients. The adherence capabilities of many members of the Enterobacteriaceae family are mediated by different types of pili, which are also known as fimbriae. Pili can be defined as non-flagella, filamentous projections found on the bacterial surface. These structures are usually made up of polymeric globular protein subunits that form the tertiary structure of fimbriae. Several fimbrial types have been identified based on hemagglutination assay profiles and electron microscopy findings (11).

There have been several types of fimbriae identified among *Klebsiella* spp. Some fimbrial types such as type 1 fimbriae are found in all members of Enterobactericae (Klemm and Schembri, 2000), type 3 fimbriae is found in majority of *K. pneumoniae* isolates (Allen, Gerlach et al., 1991). Other fimbriae identified in *K. pneumoniae* include KPF-28 fimbriae. In addition, afimbrial adhesins such as CF29K have also been show to play a role in adherence (Wu, Huang et al., 2010).

One of the more recently characterised fimbriae types in *Klebsiella* was discovered within the genome of *K. pneumoniae* NTUH-K2044. Nine fimbrial gene clusters were identified coding for the following predicted fimbriae:

*Kpa, Kpb, Kpc, Kpd, Kpe, Kpf* and *Kpg* (Wu, Huang et al., 2010). Of these nine fimbrial gene clusters, *Kpc* was discovered as the most prevalent among *K. pneumoniae* serotypes involved in this study (Wu, Huang et al., 2010). The regulation of the Kpc fimbriae is regulated by an invertible DNA element known as *kpcS* (Wu, Huang et al., 2010). The *kpcS* element is 302 bp in length and flanked by an 11bp repeats. The Kpc fimbriae contain a major fimbrial subunit downstream of the *kpcS* switch that mediated the expression of the Kpc fimbriae major subunit. The *kpcS* switch is in turn mediated by recombinase KpcI that is also encoded within the fimbrial genes cluster (Clegg, Wilson et al., 2011, Wu, Huang et al., 2010).

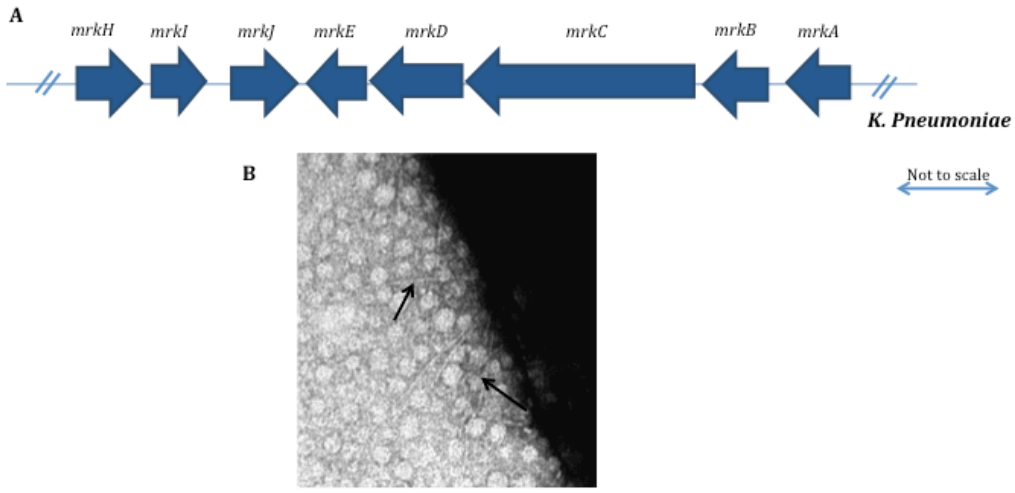
Another fimbriae gene cluster that is highly prevalent among *K. pneumoniae* is the type 3 *mrk* operon. This operon codes for fimbriae that were first identified in *K. pneumoniae* strains (Duguid, 1959) but also infrequently observed among *E. coli* strains. These pili are found to be 0.5 to 2 μm in length and bear a width of 2 – 4 nm (Ong, Beatson et al., 2010). The type 3 fimbriae were generally characterised with the ability to agglutinate tannic acid treated erythrocytes in a mannose resistant manner (Duguid, 1959, Thornley and Horne, 1962). The fimbriae were named after this phenotype, which was, described as the mannose-resistant, *Klebsiella*-like haemagglutination, hence *mrk* fimbriae (Duguid, 1959, Murphy and Clegg, 2012). However, further research has showed that the type 3 fimbriae are able to agglutinate sheep erythrocytes as well as yeast, also in a mannose resistant manner (Stahlhut, Struve et al., 2012).

These fimbriae play a vital role in mediating the formation of biofilms by

*K. pneumoniae* on biotic and abiotic surfaces, thereby contributing to the virulence of these bacteria (Murphy and Clegg, 2012, Rosen, Pinkner et al., 2008, Schroll, Barken et al., 2010). These fimbriae were originally depicted as organelles that attach to plant roots (Korhonen, Tarkka et al., 1983) but were later found with adherence capabilities to different cells in humans. These fimbriae are capable of adhering to endothelial cells, epithelial cells of the respiratory tract as well as the uroepithelial cells (Hornick, Allen et al., 1992, Tarkkanen, Virkola et al., 1997, Würker, Beuth et al., 1990).

The type 3 fimbriae are encoded for by several genes within a cluster in the chromosomal genome or carried on a plasmid (Sebghati, Korhonen et al., 1998, Gerlach, Allen et al., 1988). This gene cluster named *mrkABCDF* is comprised of five genes that code for the assembly components of the fimbrial appendages (Figure 1-2). The MrkB and MrkC are genetically and molecularly similar to the family of periplasmic chaperones and usher proteins respectively. This chaperone-usher pathway is well associated with the assembly of a wide variety of fimbrial systems (Morrissey, Leney et al., 2012, Allen, Phan et al., 2012, Thanassi, Saulino et al., 1998) and also responsible for the assembly of the fimbrial proteins coded for by *mrk* (Murphy and Clegg, 2012). Using this model, it is predicted that the fimbrial subunits are carried via the general secretory pathway to the periplasm. At the periplasm, the fimbrial subunit proteins form a complex with the type 3 chaperons, MrkB by donor-strand complementation (Murphy and Clegg, 2012).

**Figure 1-2. *K. pneumoniae* type 3 fimbrial cluster and structure**




**A)** Diagram shows an illustration type 3 operon encoded by *Klebsiella*. **B)** Picture showing associated *mrk* fimbrial structure seen in the work of Struve C. *et al*., 2009. *E. coli* HB101 transformed with a plasmid encoding *K. pneumoniae* type 3 fimbriae seen as thin 4- to 5-nm filamentous organelles (highlighted by black arrows) on the surface of the cell.

In this assembly pathway, fimbrial subunits are transported via the general secretory pathway to the periplasm where a chaperone, in the case of type 3 fimbriae encoded by *mrkB*, forms a complex by donor-strand complementation with the fimbrial subunit proteins. This complex is then transported to the scaffolding or usher protein, in the case of type 3 fimbriae, MrkC, found in the outer membrane of the cell. The assembly of the fimbriae is produced by the addition of the fimbrial subunits developing appendage (Rose, Welsh et al., 2008, Leney, Phan et al., 2011, Allen, Phan et al., 2012, Morrissey, Leney et al., 2012).

The type 3 fimbriae are made up of polymers of the major fimbrial subunit, MrkA while MrkD forms the fimbrial tip adhesin that determines the fimbrial binding specificity of the *mrk* operon (Murphy and Clegg, 2012). Although the function of MrkF may be unknown, experimental work has shown that this protein may play a role in the assembly of the type 3 fimbriae while part of

the structural component of the fimbriae (Huang, Liao et al., 2009).

In an experiment to identify factors mediating *mrk* regulation, it was shown that a gene adjacent to the known *mrk* gene cluster named *mrkJ,* encodes a functional cyclic diguanylate phosphodiesterase. The increase of cyclic-di-GMP results in an increase in the transcription of the major fimbrial subunit, *mrkA* and hence increases expression of the type 3 fimbriae on the cell surface (Johnson and Clegg, 2010). Further analysis of this region revealed two other genes (*mrkH* and *mrkI*) localised next to *mrkJ*. These genes were predicted to encode proteins that contain domains able to affect and mediate cyclic-di-GMP (Murphy and Clegg, 2012, Clegg, Wilson et al., 2011). Analysis of the MrkI shows that it belongs to a family of regulatory proteins described as containing a LuxR-like DNA-binding domain (Murphy and Clegg, 2012). The MrkH was observed to possess a PilZ domain, which has been shown to directly activate the transcription of *mrkA* promoter and in the presence of cylic-di-GMP, also binds to the regulatory region of *mrkA* (Wilksch, Yang et al., 2011).

#### 1.4.4.1 Cyclic di-GMP signalling

Bis-(3'-5')-cyclic dimeric guanosine monophosphate (cyclic-di-GMP) was first determined as a factor in the activation of membrane-bound cellulose synthase of *Gluconacetobacter xylinus* (Ross, Weinhouse et al., 1987). Since then, this molecule has been shown to be vital in the change of planktonic motile bacteria to stationary biofilm-associated bacteria. Moreover, it has

also been shown to mediate many of the virulence factors in several bacteria (Tamayo, Pratt et al., 2007). Cyclic-di-GMP synthesis is achieved by enzymes known as diguanylate cyclases. These enzymes consist of a conserved GGDEF amino acid domain and generate cyclic-di-GMP from two molecules of GTP. Other enzymes known as phosphodiesterases (PDEs) contain EAL domains which facilitate the breakdown of cyclic-di-GMP to 5′-phosphoguanylyl- (3′-5′)-guanosine, which is further degraded by nonspecific PDEs into two molecules of GMP (Romling and Simm, 2009). Several of the diguanylate cyclases and PDEs are merged to signal input domains, so that alterations in the intracellular concentration of cyclic-di-GMP can be controlled by intracellular or extracellular signals. Alterations in cyclic-di-GMP concentration are relayed to the cell by allosteric binding of effector molecules (Benach, Swaminathan et al., 2007, Sondermann, Shikuma et al., 2012). As mentioned earlier, MrkJ contains an EAL domain and has been shown to mediate type 3 fimbriae expression by affecting intracellular concentration of cylic-di-GMP (Johnson and Clegg, 2010). Apart of *mrk* gene cluster, the type 1 fimbriae gene cluster also contains an EAL-containing gene, *fimK* that was similarly predicted to control type 1 fimbriae expression via cyclic-di-GMP (Rosen, Pinkner et al., 2008, Aartsen, 2011).

## 1.4 Type 1 fimbriae

### 1.4.1 Type 1 pili and *fim* operon

Type 1 pili, also known as the common pili, are described as thin polymers, with a width of 5-7 nm as well as 0.2-2μm in length. These pili are able to adhere to mannose containing structures thereby enabling the attachment of these bacteria to epithelial cell surfaces (Klemm, 1986). These fimbriae are also characterised by a mannose sensitive ability to agglutinate guinea pig red blood cells (Struve, Bojer et al., 2008).

Type 1 fimbriae in enterobacteria are regarded as a vital virulence factor as they endow these pathogens with the ability to bind and attach to epithelial cells of the respiratory, urogenital and gastrointestinal tracts in hosts (Ofek and Beachey, 1978, Balish, Jensen et al., 1982, Venegas, Navas et al., 1995), a crucial step in infection (Balestrino, Haagensen et al., 2005).

The role of these fimbriae in the pathogenesis of several infections such as urinary tract infection (UTI), has mostly been observed and demonstrated with *E. coli,* but have also been demonstrated in *Klebsiella* species, particularly *K. pneumoniae* in animal models (Fader and Davis, 1980, Fader and Davis, 1982, Maayan, Ofek et al., 1985, Podschun and Ullmann, 1998). The phenotypic expression of the common pili on the surface of bacteria is phase variable. This means that the encoding bacteria from time to time can shift from a fimbriate state where by the cell surfaces expresses numerous pili, to a non-fimbriate state, where expression of pili is minimal (Klemm, 1986, Eisenstein, 1981). It was observed that the capacity of the fimbriae to

adhere to mucosal surfaces maybe directly correlated to the degree of fimbriation (Ofek and Beachey, 1978, Eisenstein, 1981) in contrast to phagocytosis resistance that is directly affected by a lack of fimbriation (Eisenstein, 1981). Studies using animal models showed greater binding of *K. pneumoniae* to tracheal and bladder epithelial cells during the ON phase of its type 1 fimbriae than during the OFF phase (Fader and Davis, 1980, Fader, Avots-Avotins et al., 1979, Rosen, Pinkner et al., 2008). Also, a population shift in favour of organisms expressing type 1 fimbriae was observed in *K. pneumoniae* infection studies using murine urinary tract models (Maayan, Ofek et al., 1985).

The phenotypic expression and regulation of the type 1 fimbriae in enterobacteria is encoded by the *fim* operon. The *fim* operon is known for encoding several genes that make up several units and sub-units of the type 1 pili in *Escherichia coli* and *Klebsiella spp.* In fact, type 1 pili found in *Klebsiella* are highly homologous to the *fim* gene cluster in *E. coli*, which is also prone to phase variability. Although the *fim* cluster in *Klebsiella* is not identical to the *Escherichia coli* type 1 fimbria gene cluster, an overall high degree of structural resemblance has been demonstrated as well a general genetic organisation similar to *fim* operon in *E. coli* (Struve, Bojer et al., 2008, Gerlach, Clegg et al., 1989, Clegg, Purcell et al., 1987, Purcell and Clegg, 1983). Within the *K. pneumoniae* cluster, homologues of nine genes present within *fim* in *E. coli* were seen. The similarities between the genetic organisation of the *fim* gene cluster in *Klebsiella* and *E. coli* suggest a similarity within the type 1 fimbrial structure and biosynthesis of the pili in these organisms

(Struve, Bojer et al., 2008). However, in spite of the similarities, there are few distinguishing factors. One of the major differentiating factors that distinguish the *Klebsiella fim* operon from that of *E. coli* is the *fimK* gene, which is unique to the *Klebsiella fim* cluster.

Each type 1 pilus is made up of distinct protein subunits with the major pilus subunit being present in approximately 300-500 copies per pilus. The main structural pilus subunit FimA oligomerises to a right-handed helical quaternary structure with about 3.4 subunits per turn, termed the pilus rod. In contrast to FimA, subunits FimF, FimG, and FimH, which form the tip fibrillum, are arranged linearly. A single copy of the adhesin FimH is found at the end of the tip fibrillum, and one or several copies of FimG and FimF connect FimH to the pilus rod (Gossert, Bettendorff et al., 2008, Hahn, Wild et al., 2002, Ghigo and Beloin, 2011). (Figure 1-4). It has been observed that deletion of the genes that encode the FimF or FimG subunits decreases the number or increases the length of pili displayed on the cell surface, respectively. This suggests that both subunits are involved in the mediation of pilus assembly (Knight and Bouckaert, 2009, Gossert, Bettendorff et al., 2008, Ghigo and Beloin, 2011).

**Figure 1-3. Type 1 fimbrial gene clusters and structures**

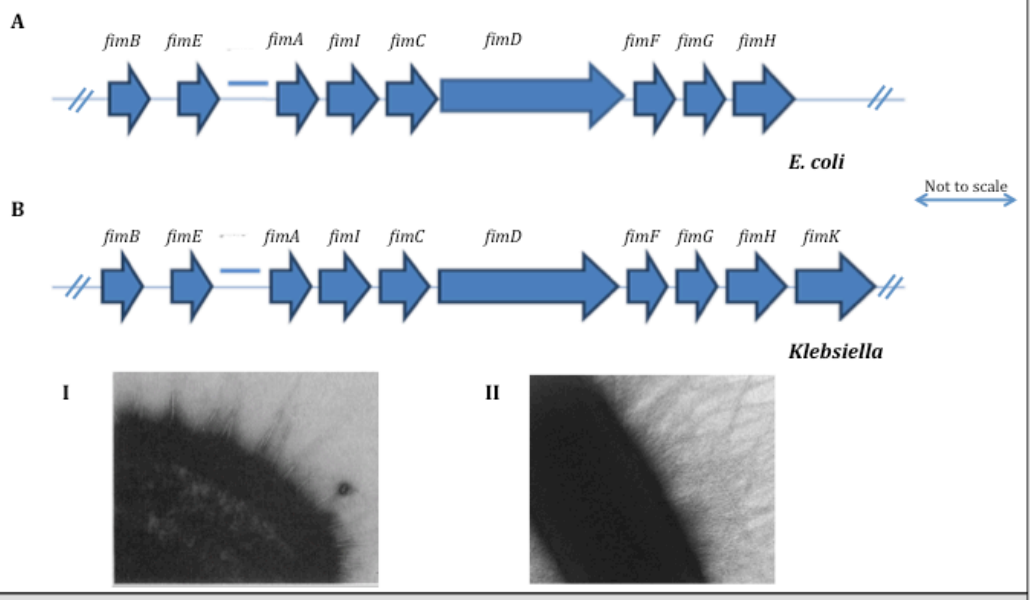



Diagram showing illustration of typical type 1 fimbriae operon in **A**) *E. coli* and **B**) *Klebsiella*. **I**) Electron micrographs show associated *E. coli* type 1 fimbriae as seen in Connell *et al.* **II**) Electron micrograph shows *Klebsiella* type 1 fimbriae over expressed in *E. coli* background as seen in Struve C., *et al*, 2009.

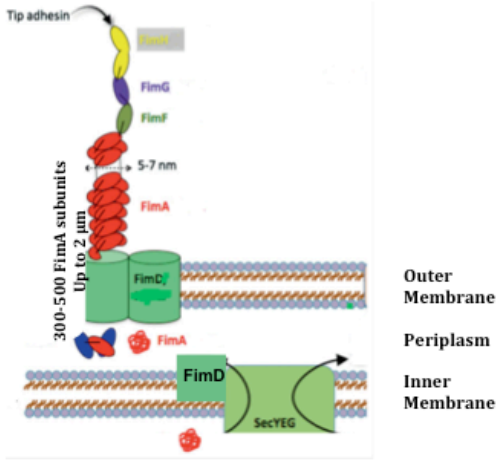
The assembly of the pili proteins that comprises the type 1 fimbriae is regulated by a common secretion pathway used in enterobacteria, usually used to produce adhesins which play a function in interactions between host and bacterial pathogen. This secretion pathway is called the chaperon-usher (CU) pathway (Clegg, Wilson et al., 2011). Following the definition and characterisation of this pathway in regulating the assembly of the P pilius and type 1 pili in *E. coli,* the CU pathway has been observed as the mode of assembly by many fimbrial types in several bacterial types. The CU pathway is notably characterised by the presence of two proteins (FimC and FimD), in which their genes are encoded within the *fim* gene cluster, that act as periplasmic protein chaperone and an outer membrane scaffolding protein

(Thanassi and Hultgren, 2000, Nuccio and Baumler, 2007, Clegg, Wilson et al., 2011).

Within the *fim* operon, several genes code for the structural subunits that make up filamentous fimbriae protruding on bacteria cell surface. However, some of the other proteins encoded by the genes within the *fim* operon do not make part of the structural fimbriae but are for mediation and assembly. The *fimC* and *fimD* genes within the *fim* gene cluster encode the periplasmic chaperone (FimC) and β-barrel forming membrane usher protein (FimD) respectively (Clegg, Wilson et al., 2011, Clegg, Purcell et al., 1987).

During pilius biogenesis, the pilin proteins are transported through the inner membrane by SecYEG and therefore have a cleavable signal sequence. Once in the periplasm, the pilin proteins are bound by the FimC chaperone, which aids in it's folding and prevents premature association with other pilin subunits. Upon contact with the FimD usher at the outer membrane, the chaperon-subunit complex dissociates releasing the pilin subunit protein so that the usher mediates its translocation to the cell surface (Clegg, Wilson et al., 2011, Ghigo and Beloin, 2011, Snyder, Champness et al., 2007). (Figure 1-4.)

**Figure 1-4. Schematic showing type 1 fimbrium assembly**




Schematic based on diagram by Ghigo, J and Beloin, 2011. Adhesins assembled via the CU are heteropolymers composed of a small number of different FimA subunits. A rigid fimbrial rod interacts with a flexible fibrillar tip ending in a single specific distal tip adhesin, FimH, which binds surface carbohydrates to host cells. During fimbriae assembly, subunits (pilins) are secreted into the periplasmic space via the general secretory pathway and bind to a specific chaperone that assists in protein folding and prevents premature assembly of the subunits. The pilin/chaperone complex is then delivered to the outer membrane usher, which serves as a platform for pilus assembly. The usher protein forms a pore in the outer membrane, allowing the passage of individual pilins.

### 1.4.2 Regulation of fimbrial expression

The production of surface fimbriae is highly dependent on the regulation of genes within the *fim* operon encoding the fimbrial subunits. In enterobacteria such as *E. coli* and *Klebsiella spp.*, expression of the genes that encode the type 1 fimbrial subunits is mediated via invertible DNA elements found within the *fim* gene cluster (Clegg, Wilson et al., 2011, Struve, Bojer et al., 2008). The invertible DNA element is known as *fimS* and is located before the *fimA* gene (5' to 3' direction), which codes for the major fimbrial subunit. Since the presence of surface pili is dependent on the assembly of the expressed pili subunits like FimA, the transcription and expression of the *fimA* gene plays a vital role in the control of pili expression in enterobacteria.

It has since been observed in *E. coli* that the transcription of the *fimA* is also dependent on the orientation of the *fimS* invertible segment. The *fimS*, also referred to as the *fim* switch, is flanked by two 9 bp inverted repeats and carries the *fimA* promoter sequence, which facilitates *fimA* transcription (Holden, Blomfield et al., 2007, Corcoran and Dorman, 2009, Clegg, Wilson et al., 2011). In one correct orientation of the *fim* switch, *fimA* transcription can occur, while in the other orientation, there is no *fimA* transcription. The ON and OFF inversion of the *fim* switch is in turn mediated by two fimbrial site-specific recombinases called FimB and FimE which are encoded by genes *fimB* and *fimE* also located on the *fim* operon. These recombinases act independently of each other and bind to regions close to and overlapping the 9 base pair repeats flanking the *fimS* invertible switch. FimE usually regulates the *fim* switch inversion from the ON orientation to the OFF orientation, while recombinase FimB is thought to mediate orientation of *fimS* in both orientations (Clegg, Wilson et al., 2011, Åberg, Shingler et al., 2008, Adiciptaningrum, Blomfield et al., 2009).

Bioinformatics analyses into the genetic and protein sequence of *fimK* have identified it as a 466aa diguanylate cyclase protein that possesses a signalling domain from 1 to 215 aa, and an EAL domain from 277 to 469 aa (Aartsen, 2011). Diguanylate cyclases (DGCs) have been found to modulate intracellular cylic-di-GMP levels and in turn have been identified as a vital bacterial signal that regulates the shift between planktonic and sessile lifestyles.

While Rosen *et al.* showed that expression of *fimK* acts to inhibit expression of type 1 fimbriae in *K. pneumoniae* cystitis isolate TOP52 (Rosen, Pinkner et al., 2008), recent study has shown that expression of *fimK* in *K. pneumoniae* CG43S3 increases the expression of the *fimA* by binding to invertible switch *fimS*. Though the exact function of *fimK* may differ from strain to strain, it is clear that *fimK* functions in mostly a regulatory capacity of the type 1 fimbriae.

## 1.5 *K. pneumoniae* genome structure and the involvement of mobile elements

The bacterial genome is made up of two parts: the core and the flexible genome (Medini, Donati et al., 2005). The core genome harbours genes that code for proteins necessary for cellular maintenance such as DNA replication machinery and ATPases. The genes contained in this part of the genome are typically shared by strains of the same species and usually stable within the bacteria (Hacker and Carniel, 2001, Mushegian and Koonin, 1996). The flexible part of the genome however can vary among strains of the same species as alterations can be caused by gene loss and/or acquisition within the genome. This plays a vital role in the evolution of species as the addition and even deletion of some gene encoding functions may facilitate the adaptation to new stimuli and environments (Dobrindt, Hochhut et al., 2004, Lawrence and Hendrickson, 2005, Aartsen, 2011).

Gene loss or acquisition in the flexible genome can occur on a small scale by

homologous recombination or mutations (Platt, Atherton et al., 1976, Lawrence and Hendrickson, 2005). On larger scale, DNA acquisition can occur by three different mechanisms: transformation, transduction and conjugation (Dobrindt and Hacker, 2001, Snyder, Champness et al., 2007).

Transformation occurs when DNA molecules from the surrounding environment are directly internalised by bacteria. Conjugation involves special pili structures that assist the transfer of DNA from one bacterial cell to another bacterial cell, which may be of different species. Transduction involves the bacteriophage-mediated transfer of DNA. This occurs when the DNA of the host bacterium is assimilated into the phage head during packaging, causing subsequent injection of the host cell DNA into a recipient cell. For all the three mechanisms, internalised DNA must undergo either site-specific or homologous recombination for chromosomal integration of the genetic element (Snyder, Champness et al., 2007).

Mobile genetic elements (MGEs) are DNA fragments that harbour genes encoding proteins that confer ability to facilitate intracellular and/or extracellular DNA mobility encouraging DNA translocation respectively within and between cells. The mobile elements are a major part of the flexible genome within bacteria and greatly contribute to horizontal gene transfer (Frost, Leplae et al., 2005).

MGEs occur in various forms such as plasmids, transposons, integrative conjugative elements (ICEs) and genomic islands (GIs). Within these elements, the composition and arrangement of the genes can occur in many various combinations and may encode proteins that may contribute to the

fitness or pathogenicity of the host bacteria (Frost, Leplae et al., 2005).

### 1.6.2 Genomic islands

Genomic islands are large chromosomal regions that have aberrant base composition compared to the whole genome, encode an integrase and insert at tRNA loci through the process of lateral transfer (Boyd, Almagro-Moreno et al., 2009).

Genomic islands can be very large and range from 10 – 200 kb in size while varying between different strains of the same species. Since they are horizontally transferred and acquired by bacteria cells, they do not form part of the core gene pool but in many situations increase bacterial fitness directly or indirectly (Van Aartsen, 2008). Genomic islands have been divided into several groups depending on their function so that resistance islands encode for antibiotic or heavy metals, metabolic islands confer the ability to utilise carbon and nitrogen sources, degradation islands endow the ability to break down novel compounds while pathogenecity islands are able to increase the ability to cause disease (Boyd, Almagro-Moreno et al., 2009).

Many strain-specific GIs and GI-like regions have been found among *K. pneumoniae* genomes (Ou, Chen et al., 2006, Ou, He et al., 2007, Van Aartsen, 2008, Chen, Ou et al., 2010, Zhang, Van Aartsen et al., 2011).

## 1.6 KpGI-5: a novel *K. pneumoniae* GI harbouring a putative fimbrial locus

The *fim2* operon was first discovered by Jon van Aartsen in our laboratory who also performed experiments aimed at the initial characterisation of this operon (Aartsen, 2011). This chapter will highlight and discuss some of the earlier investigations of *fim2* that lead to this current PhD project.

In the earlier work by Dr Jon van Aartsen, five tRNA gene (*tRNA*) insertion hotspots were screened for strain-specific DNA in sixteen clinical *K. pneumoniae* isolates. One of the strains isolated from the blood of a patient with neutropenic septicaemia, *K. pneumoniae* KR116, harboured an 'occupied' *met56 tRNA* locus in a tRIP-PCR screen using primers designed to specifically amplify across an empty *met56* hotspot. SGSP-PCR primer walking from the conserved *met56* UF produced 3 kb of a novel island upstream arm (UA) sequence. The novel island was called KpGI-5. The 3 kb of UA sequence from the island was analysed and no GenBank nucleotide homologs could be identified. However, this UA sequence possessed three ORFs that were similar to fimbrial subunits encoded on *K. pneumoniae fim* operon. These putative genes were also arranged in similar organisation to the *fim* (Figure 1-5). This putative operon carried on novel KpGI-5 island was called *fim2*. Experimental methods of island-tagging and fosmid-based marker rescue were used to capture and sequence the entire KpGI-5 island. Further sequencing revealed a complete novel KR116 *met56*-specific GI. The captured 14 kb island in its entirety was dubbed KpGI-5 (Van Aartsen, 2008, Van Aartsen, Stahlhut et al., 2012, Aartsen, 2011).

### 1.6.1 Putative *fim* variant –*fim2*

The *fim2* operon bears similar genetic organisation to the *K. pneumoniae fim* operon and contains homologs of all eight genes within *fim* (Van Aartsen, Stahlhut et al., 2012). Within the *fim2* operon is *fim2A*, which is predicted to code for a major fimbrial subunit (Fim2A). It also possesses putative minor fimbrial subunits (Fim2F, Fim2G, Fim2H) as well as homologs of the chaperon and usher proteins, Fim2C and Fim2D respectively. The close similarity between *fim* and *fim2* allows classification of the latter as a $\gamma_1$-type fimbrial system-encoding locus (Aartsen, 2011, Nuccio and Baumler, 2007). Sequence analysis of the eight putative *fim2* genes (ORFs) showed 60 – 92% identity to cognate *fim* proteins when translated and compared at amino acid level (Aartsen, 2011, Van Aartsen, Stahlhut et al., 2012).

However, differences between the *fim* and *fim2* clusters are seen by differences in transcriptional control. Within the *fim* cluster are genes encoding FimB and FimE recombinases that switch transcription on and off by the inversion of promoter harbouring *fimS*, localised upstream of *fimA* (Struve, Bojer et al., 2008, Aartsen, 2011). Within the KpGI- 5 island, no homologs of FimB, FimE or *fimS* were found suggesting that *fim2* expression is not mediated by *fimS* switch-like mechanism. Moreover, *fim2K* of the *fim2* cluster encodes only an EAL domain and does not possess a helix-turn-helix domain as seen in *fimK* in the *fim* operon (Van Aartsen, Stahlhut et al., 2012).

### 1.6.2 Sequence analysis and epidemiological profile of *fim2*

Following the screening of several *Klebsiella* isolates for *fim2K,* the KpGI-5 family was characterised as a group of related islands that possess a *fim2* operon. Of the 162 *Klebsiella* isolates screened, 21 strains, which were obtained from five different geographical locations, were confirmed as positive for *fim2K* gene and subsequently, the *fim2* operon. Due to the global spread of the KpGI-5-like islands, it was suggested that this locus is under positive selection and has been maintained within a subset of the *Klebsiella* population (Aartsen, 2011).

The *fim2*-positive strains were also screened for the presence of type 1 fimbriae and type 3 fimbriae by PCR screening of *fimH* and *mrkD* adhesion genes respectively. Majority of these *fim2*-positive strains were found to harbour these genes. However, similar faint amplicons were obtained during *fimH* PCR screening of *Klebsiella* sp25 and sp28. These amplicons were sequenced and though the *fimH* gene sequence was novel, sequence analysis done at that period showed that the predicted product was very similar to that of the related *K. pneumoniae* subsp. *rhinoscleromatis* ATCC 13884 strain (Aartsen, 2011).

Species identification of these *Klebsiella* strains (that also bear *fim2*) was also uncertain as previous biochemical tests and species-specific PCR test resulted in conflicting classification to either *K. pneumoniae* or *K. oxytoca* (Hansen, Gottschau et al., 1998, Aartsen, 2011).

### 1.6.3 *In vivo* and *in vitro* characterisation of *fim2*

Investigation of *fim2* expression showed that *fim2A, fim2H* and *fim2K* within the *fim2* cluster were readily expressed under standard *in vitro* conditions. Quantitative PCR experiments on *K. pneumoniae* KR2107 (a streptomycin-resistant derivative of KR116) cDNA library showed that *fim2A* was expressed approximately 30- and 90-fold less than *fimA* and *mrkA,* respectively, under laboratory growth conditions examined (Van Aartsen, Stahlhut et al., 2012).

The *fim2* locus from KR116 was also amplified by PCR and cloned into the high copy number vector pBluescript II KS+, the low copy number vector pWSK129 and IPTG inducible $P_{TRC}$-bearing vector pJTOOL-7 to produce pFim2-HCN, pFim2-LCN and pFim2-Ptrc, respectively. Each plasmid was individually transformed into the afimbriate *E. coli* strain HB101 and examined by electron microscopy in an attempt to visualise the putative Fim2 fimbriae. However, no definitive fimbriae were observed despite several induction methods. By contrast, HB101 bearing *fim* cloned into several plasmids produced visible fimbriae*. In vitro* adhesion assays using KR2107 and its isogenic *fim2* knockout mutant were performed to examine the *fim2* phenotype (Van Aartsen, Stahlhut et al., 2012).

In an experiment to determine if *fim2* influences the initial step of colonisation in gastrointestinal tract, strains KR2107 and KR2107*Δfim2* were mixed in equal quantities and monitored for thirteen days after being fed to mice. Results showed that no strain possessed an obvious competitive

advantage and both readily colonised intestinal cells in similar numbers. This suggested that *fim2* does not play a significant role in murine intestinal colonisation by *K. pneumoniae* (Van Aartsen, Stahlhut et al., 2012).

Murine models were also used to test the influence of *fim2* virulence in /on the respiratory tract and urinary tract. While data obtained from intranasal inoculation of KR2107 and KR2107*Δfim2* suggested no significant impact of *fim2* on pneumovirulence of *K. pneumoniae* in murine lung infection model, results obtained from transurethrally inoculated mice with 1:1 mixture of the KR2107 and *fim2* mutant suggested that *fim2* may exert a potential but subtle influence on the urovirulence of *K. pneumoniae* (Aartsen, 2011).

Influence of *fim2* deletion on human epithelial cells was examined using human HCT-8 ileocecal and human 5637 bladder epithelial cell lines to investigate adherence capability of *fim2* to intestine and bladder respectively. However, no significant difference in wildtype and *fim2* mutant strain was detected in *in vitro* tissue cultures (Aartsen, 2011).

Apart from epithelial cells and cell lines, *K. pneumoniae* is known to colonise abiotic surfaces such as urinary catheters and tracheal tubes (Barnhart and Chapman, 2006, Brisse, Fevre et al., 2009). Since surface-expressed structures are known to play a role in biofilm formation as seen with other fimbriae such as *mrk*, (Murphy and Clegg, 2012, Sebghati, Korhonen et al., 1998), the ability of KR2107 and its isogenic *fim2* mutants to form biofilms was investigated. Nonetheless, absence of *fim2* did not show any effect on *K. pneumoniae* biofilm formation under several laboratory conditions tested (Aartsen, 2011).

To detect any influence on biofilm formation that may have been masked by low expression of *fim2* or physical hindrance of fimbrial function by *K. pneumoniae* capsule (Brisse, Issenhuth-Jeanjean et al., 2004), *fim2* was cloned into a plasmid and over-expressed in *E. coli* HB101. It was observed that the strain bearing an over expressed *fim2* (*E. coli* HB101/pFim2-Ptrc) resulted in marginally denser biofilm than HB101 carrying an empty vector (HB101/pJTOOL-7), albeit on polyvinyl chloride wells which were not statistically significant ($P$ = 0.464). (Aartsen, 2011, Van Aartsen, Stahlhut et al., 2012).

Interestingly, a growth reduction was observed in *E. coli* HB101/pFim2-Ptrc when *fim2* expression is induced with IPTG. This growth inhibition also showed a dose-response relationship with IPTG induction concentrations and was not seen in other control strains HB101 and HB101/pJTOOL-7 (Aartsen, 2011, Van Aartsen, Stahlhut et al., 2012). These findings obtained from the *in vivo* and *in vitro* studies of *fim2* suggest a possible role for *fim2* in *Klebsiella* virulence.

## 1.7 Aims and objectives

As discussed earlier in this chapter, fimbrial systems play a vital role in pathogenesis and infection by *Klebsiella*. In spite of extensive work carried out to investigate the role in colonisation and infection played by *fim2*, a novel type 1 fimbrial variant discovered within KpGI-5 island, results previously obtained provided only minimal insights into the *fim2* phenotype

(Aartsen, 2011). Moreover, it is unclear whether the presence of the *fim2* operon can lead to the assembly of a complete fimbrial structure on the bacterial surface, as no fimbriae were observed on the surface of *E. coli* HB101/pFim-Ptrc even under IPTG induction conditions. This may be one of the reasons why previous *fim2* experiments did not show any significant phenotype for this operon. These ambiguities and uncertainties surrounding *fim2* are the basis of the first part of the investigation in this PhD project.

Extending from the investigation of the *fim2* was the discovery of distinct *Klebsiella* strains that harbour a *fim2* operon but lacked a characteric PCR profile supportive of possession of the *fim* operon coding for *K. pneumoniae* typical type 1 Fim fimbriae. Following the observation of a novel *fimH*-related nucleotide sequence among these atypical *Klebsiella* strains, certain experiments were done to investigate whether this new *fimH*-like gene was part of a third paralogous *fim*-like operon, or if the associated operon is an ortholog of the *K. pneumoniae* type 1 fimbrial operon found in a novel and/or as-yet unsequenced *K. pneumoniae* subspecies or indeed a distinct *Klebsiella* species. Investigation of these aspects comprises the basis of the second part of this PhD project.

In summary, this project aimed to pursue the following:

1. Further analyse the *in vitro* expression profiles of *fim2* under a range of conditions and, additionally, seek to obtain *in vivo* expression data based on use of a *Galleria mellonella* infection model.
2. Investigate the fimbriae-forming potential of *fim2* by constructing hybrid *fim-fim2* operons to examine if one or more of the putative

*fim2*-encoded proteins could assemble to form visualisable fimbriae when hybrid operons were transformed into *E. coli* HB101 and/or whether bacteria carrying these hybrid *fim*/*fim2* operons exhibited altered phenotypes by *in vitro* biofilm formation and growth curve assays.

3. Investigate the putative novel variant *fim* operon, now termed *fim3*, by PCR mapping and amplicon sequencing to obtain the complete sequence of the *fim3* operon and its flanking regions for detailed bioinformatics analyses. In parallel, further molecular species identification analysis of *fim3*-bearing *Klebsiella* strains was to be performed.
4. Characterise the putative *fim3* operon by generation of isogenic mutants and heterologous expression in *E. coli* HB101 to examine its potential contributions to fimbrium and/or biofilm formation, and *in vivo* infection utilising the *G. mellonella* killing model.

# Chapter 2. Materials and methods

## 2.1 Bacterial strains and plasmids

The *Klebsiella* strains used in this project were originally obtained as clinical isolates from Cartsen Struve (Denmark) as well as the Leicester Royal Infirmary and were stored in brain heart infusion broth with 30 % glycerol at 20°C/-80°C. Prior to their use in experiments, bacteria were routinely streaked out onto lysogeny agar (LA) plates and incubated at 37°C overnight. Single colonies were picked and cultured overnight in lysogeny broth (LB) at 37°C and 200rpm, unless otherwise specified.

When needed, *E. coli* growth media was supplemented with 100 μg/ml ampicillin, 30 μg/ml chloramphenicol, 10 μg/ml tetracycline or 30 μg/ml apramycin. When needed, *K. pneumoniae* strains were grown in media supplemented with 50 μg/ml kanamycin, 30 μg/ml chloramphenicol, 15 μg/ml tetracycline, 50 μg/ml streptomycin or 50 μg/ml apramycin, unless otherwise specified. Bacterial strains used and created in this work are listed in Table1. Plasmids used and constructed in this work are listed in Table 2.

**Table 2-1. Bacterial strains used in this study**

| Strain | Description | Source/ Reference |
|---|---|---|
| ***E. coli* strains** | | |
| DH5α | F- φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(rK-mK+) *phoA supE*44 λ- *thi*-1 *gyrA96 relA1* | (Hanahan, 1983) |
| HB101 | F- *mcrB mrr hsdS20*(rB-mB-) *recA13 leuB6 ara*-14 *proA2 lacY1 galK2 xyl*-5 *mtl*-1 *rpsL20*($Sm^R$) *glnV44* λ- | (Boyer and Roulland-dussoix, 1969) |
| CC118λpir | Δ(*are-leu*) *araD* Δ*lacX74 galE galK phoA20 thi*-1 *rpsE rpoB argE*(Am) *recA1* λpir | (Herrero, De Lorenzo et al., 1990) |
| S17-λpir | F' *thi pro hsdR*$^-$ *recA*::RP4-2-Tc::Mu Km::Tn7 λ*pir* | (Simon, Priefer et al., 1983) |
| | | |
| ***Klebsiella* strains** | | |
| MGH75878 | *K. pneumoniae* sputum isolate, sequenced | Washington University |
| sp15 | *K. pneumoniae* blood stream infection isolate, Copenhagen, Denmark | (Hansen, Gottschau et al., 1998) |
| sp25 | *K. pneumoniae* blood stream infection isolate, Copenhagen, Denmark | (Hansen, Gottschau et al., 1998) |
| sp28 | *K. pneumoniae* blood stream infection isolate, Copenhagen, Denmark | (Hansen, Gottschau et al., 1998) |
| KR116 | *K. pneumoniae* blood stream infection isolate, UHL-LRI | (Aartsen, 2011) |

| | | |
|---|---|---|
| KR2107 | Spontaneous streptomycin-resistant derivative of KR116 | (Aartsen, 2011) |
| KR2152 | KR2107 with insertion/deletion mutations of *fim* and *fim2;* Δ*fim2*::kan Δ*fim*::tet | (Aartsen, 2011) |
| KR2305 | KR2107 with insertion/deletion mutations of *fim* and *mrk;* Δ*fim*::tet Δ*mrk*C3091::kan | (Aartsen, 2011) |
| sp28*Δfim3* | sp28 with insertion/deletion mutations of *fim3*; *Δfim3::*kan | This study |
| KR116*Δfim* | KR116 with an insertion/deletion mutation of *fimK*; Δ*fimK*::gen | (Aartsen, 2011) |
| sp28*ΔmrkΔfim3* | sp28 with insertion/deletion mutations of *fim3*; *Δfim3::*kan, *Δmrk*::FRT | This study |
| sp28*Δfim3K* | sp28 with insertion/deletion mutations of *fim3*; *Δfim3K::*gent | This study |
| sp28*ΔmrkΔfim3K* | sp28 with insertion/deletion mutations of *fim3*; *Δfim3K::*gent, *Δmrk*::FRT | This study |

**Table 2-2 Plasmids used and constructed in this study**

| Plasmid | Description | Resistance | Reference |
|---|---|---|---|
| pKOBEG-Apra | Lambda Red expression plasmid, $P_{BAD}$ promoter | *Apr* | (Chaveroche, Ghigo et al., 2000) |
| pGEM-T easy | HCN cloning vector; $P_{LAC}$*lacZα* | *Amp* | Fermentas |
| pTRC99a | Expression vector with $P_{TRC}$ promoter and $lacI^Q$ | *Amp* | (Amann, Ochs et al., 1988) |
| pFLP2 | FLP recombinase-expressing plasmid | *Amp* | (Hoang, Karkhoff-Schweizer et al., 1998) |
| pJTOOL-4a | pGEM-T easy with the pUCP26 tetracycline resistance cassette flanked by FRT sites | *Amp, Tet* | (Aartsen, 2011) |
| pJTOOL-3 | Lambda *pir*-based suicide vector with expanded multiple cloning site. Derived from pDS132 | *Cml* | (Aartsen, 2011) |
| pJTOOL-7 | pTRC99a-based expression vector with a NotI site added between the NcoI and EcoRI restriction sites | *Amp* | (Aartsen, 2011) |
| pFim-Ptrc | *fim* operon (*fimA* to *fimK*) from *K. pneumoniae* C3091 amplified using primers PR1268 and Amp PR1221 (8.1 kb) and cloned into the NotI/SbfI site of pJTOOL-7 | *Amp* | (Aartsen, 2011) |
| pFim2-Ptrc | *fim2* operon (*fim2A* to *fim2K*) from *K. pneumoniae* KR116 amplified using primers PR1222 Amp and PR1224 (7.8 kb) and cloned into the NotI/SbfI site of pJTOOL-7 | *Amp* | (Aartsen, 2011) |

| | | | |
|---|---|---|---|
| pFim-$SC_{A:SC}$ | *fimA* on pFim-Ptrc is replaced by unit containing antibiotic cassette *cat* and *sacB* gene unit. | *Amp* | This study |
| pFim2-$SC_{2A:SC}$ | *fim2A* on pFim2-Ptrc is replaced by unit containing antibiotic cassette *cat* and *sacB* gene unit. | *Amp* | This study |
| pFim-$HY_{A:2A}$ | *fimA* on pFim-Ptrc is replaced with *fim2A* amplified and cloned from pFim2-Ptrc | *Amp* | This study |
| pFim2-$HY_{2A:A}$ | *fim2A* on pFim2-Ptrc is replaced with *fimA* amplified and cloned from pFim-Ptrc | *Amp* | This study |
| pFim-$HY_{H:2H}$ | *fimH* on pFim-Ptrc is replaced with *fim2H* amplified and cloned from pFim2-Ptrc | *Amp* | This study |
| $pHYA_{H:2H}$ | *fimH* on pFim-$HY_{A:2A}$ is replaced with *fim2H* amplified and cloned from pFim2-Ptrc | *Amp* | This study |

## 2.2 DNA based methods

### 2.2.1 Polymerase chain reaction

Many of the PCR products and amplicons obtained for use in this work was generated by GoTaq DNA polymerase (Promega). For long-range PCR products, amplicons were obtained by using Phire Hot Start DNA polymerase (Finnzymes). KOD Hot Start DNA polymerase (Novagen) was used when high fidelity amplification of short- or long-range PCR products was required. All enzymes were used according to manufacturer's instructions. The cycling conditions for each enzyme type are shown in Table 1A-C. Cycling conditions were altered according to primer melting temperature (Tm) and predicted amplicon size.

**Table 2-3. PCR cycling conditions of polymerases used in this work**

**A. GoTaq DNA polymerase reaction**

| PCR step | Temperature (°C) | Time | Number of cycles |
|---|---|---|---|
| **Initial denaturation** | 95 | 2 minutes | 1 cycle |
| **Denaturation** | 95 | 30 seconds | |
| **Annealing** | Lowest Tm | 30 seconds | 25 – 30 cycles |
| **Extension** | 72 | 1 minute/kb | |
| **Final extension** | 72 | 5 minutes | 1 cycle |

**B. Phire Hot Start DNA polymerase reaction**

| PCR step | Temperature (°C) | Time | Number of cycles |
|---|---|---|---|
| **Initial denaturation** | 98 | 10 seconds | 1 cycle |
| **Denaturation** | 98 | 5 seconds | |
| **Annealing** | Lowest Tm | 5 seconds | 25 – 30 cycles |
| **Extension** | 72 | 20 seconds/kb | |
| **Final extension** | 72 | 5 minutes | 1 cycles |

**C. KOD Hot Start DNA polymerase reaction**

| PCR step | Temperature (°C) | Time | Number of cycles |
|---|---|---|---|
| **Initial denaturation** | 95 | 10 seconds | 1 cycle |
| **Denaturation** | 95 | 20 seconds | 25 – 30 cycles |
| **Annealing** | Lowest Tm | 10 seconds | |
| **Extension** | 72 | 20 seconds/kb | |
| **Final extension** | 72 | 5 minutes | 1 cycles |

### 2.2.2 Primer design and synthesis

Primers were designed using Primer3 and synthesised by Sigma-Aldrich. All primers were dissolved in n$H_2O$ at 10 pmol/µl and stored at 20ºC.

### 2.2.3 Colony PCR

A single bacterial colony was resuspended in 35 µl of n$H_2O$ and heated at 100ºC for 5 minutes. The resuspended cells were then vortexed and re-heated at 100°C for 5 minutes. Cell debris was then pelleted at 12 000 xg for 1 min following another round of vortex and 1-2 µl of the supernatant was used as template in PCR.

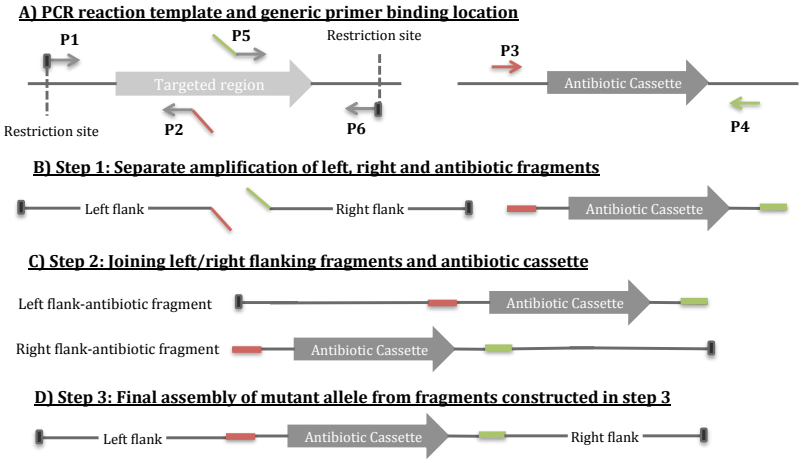
### 2.2.4 Spliced overlap extension-PCR: mutant allele construction

Mutant alleles were composed of a left homologous flank (LF), an antibiotic resistance cassette (AB) and a right homologous flank (RF). Flanks were homologous to sequences upstream and downstream of the region targeted

for deletion and ranged from 600 bp to 800 bp in length. To simplify the construction of mutant alleles, Splice overlap extension-PCR (SOE-PCR) was utilised such that DNA molecules could be recombined at specific junctions, regardless of the nucleotide sequence or restriction sites present (Horton, Hunt et al., 1989)Firstly, several primer pairs P1/P2, P3/P4, and P5/P6 were used to separately amplify PCR fragments corresponding to the LF, AB and RF, respectively. All fragments were then extracted and gel purified, as described below. Primers P2 and P5 were constructed such that their ends contained ~20 bp of nucleotide sequence complementary to the AB fragment primers P3 and P4, respectively. When products amplified using these primers are mixed, denatured and annealed in SOE-PCR, the complementary fragment ends anneal and act as primers for subsequent DNA polymerase extension reactions, producing a spliced product where two fragments have been merged. This phenomenon was utilised in step 2 (Figure 2-1).

Briefly, 10 ng of LF fragment was mixed with 10 ng of AB fragment and PCR amplified using primers P1 and P4, producing the LF-AB cassette. PCR cycling conditions were as mentioned earlier, but primers were excluded from the initial reaction mixture and added during the extension phase of the 5th cycle. In a similar manner, the RF-AB cassette was produced using primer pair P3/P6 and fragments RF and AB. The LF-AB and RF-AB fragments were then gel purified and used as template for step 3. Finally, 10 ng of both the LF-AB and RF-AB fragments were mixed and subjected to amplification using primer pairs P1 and P6, producing an SOE-PCR assembled mutant allele.

**Fig 2-1. Schematic of SOE-PCR for creating mutant alleles**




Spliced overlap extension for creating mutants SOE-PCR consists of three separate stages. (**A** and **B)** Initially, fragments corresponding to the left and right flank of the region targeted for homologous recombination and a selectable antibiotic cassette are amplified separately using PCR. The green line and red line on internal primers P2 and P5 correspond to 5' overlaps where the sequence corresponds to the antibiotic cassette amplifying primers P3 and P4, respectively. The internal primers (P2 and P5) have overlaps to the primers used to amplify the antibiotic cassette (P3 and P4) (**C**) The left and right flank fragments are spliced to the antibiotic cassette to create the 'left flank-antibiotic' and 'right flank-antibiotic' fragments. **(D**) In a final SOE-PCR reaction, the 'left flank-antibiotic' and 'right flank-antibiotic' fragments are coupled to produce the desired mutant allele**.** Diagram and legend extracted from Jon van Aartsen, 2011.

### 2.2.5 Genomic DNA and plasmid extraction

To begin DNA extraction, 5ml overnight cultures of the bacteria strains were inoculated by picking single colonies and incubated for ~12-16 hours at 37°C with vigorous shaking at 200rpm. The genomic extraction protocol was done as instructed in the ArchivePure DNA Purification System for Gram-negative bacterial culture. Plasmid DNA was routinely isolated from 5 ml overnight cultures using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich) according to manufacturer's protocol.

### 2.2.6 Gel electrophoresis, DNA purification and DNA sequencing

Gel electrophoresis for observation of DNA fragments was performed in agarose gels made from 1x TAE buffer containing 0.5 µg/ml ethidium

bromide. Agarose concentration varied from 0.7 % to 1.0 % depending on the DNA fragment size analysed. GeneRulerTM (Fermentas) were used as DNA standards. When necessary, DNA fragments were excised from agarose gels with minimal ultraviolet light exposure using a clean scalpel and extracted using the YORBIO Gel/PCR DNA purification kit (Geneflow) according to manufacturer's instructions. Image analysis was done using GelEval 1.21b Frogdance software. GATC Biotech was used in sequencing of PCR amplicons and plasmid inserts.

### 2.2.7 Restriction enzyme digestion of DNA

Restriction enzymes were obtained from Promega and NEB were utilised according to manufacturer's protocols. The reaction volume was routinely between 20 – 50 µl and was altered according to the enzyme chosen and DNA amount. When two enzymes were required for digestion, either the reaction was set up in a compatible buffer or was performed sequentially. When reactions were required for downstream applications, enzymes were heat-inactivated according to the manufacturer's instructions.

### 2.2.8 Ligation of DNA fragments

Ligation reactions were done in a final volume of 10 – 20 µl containing 3 U of T4 DNA ligase (Promega) and 1 – 2 µl of 10 x ligation buffers. The reaction was incubated first at room temperature for 60 min and then overnight at 15ºC, and subsequently transformed into *E. coli*.

## 2.3. RNA based methods

### 2.3.1 RNA extraction and cDNA library construction

Total RNA was prepared from *Klebsiella* and *E. coli* strains grown in LB at 37°C, 200 rpm using the Norgen Total RNA Purification Kit. The strains were typically grown for 16 h except in experiments otherwise stated. The Ambion TURBO DNA-free kit was used to remove residual DNA from RNA preparations and a cDNA library was synthesised using the QuantiTect Reverse Transcription kit (Qiagen) as recommended by the manufacturer. An identical reaction without reverse transcriptase was also done to check for DNA contamination.

### 2.3.2 Transcriptional analysis of fimbrial gene clusters *fim, fim2, fim3* and *mrk* gene clusters

Each cDNA library and its reverse transcriptase negative counterpart were analysed for transcripts corresponding to the *fim*, *fim3, mrk* and/or *fim2* gene clusters, depending on the experiment.

Regions corresponding to genes *fimA*, and *fim2A* were PCR amplified using primer pairs PR1601/PR1602 (233 bp) and PR1607/PR1608 (221 bp), respectively. Region corresponding to *mrkA* was PCR amplified using primer pairs PR1622/ PR1623 (211 bp). Regions corresponding to genes *fim3A*, *fim3H* and *fim3K* were PCR amplified using primers pairs PR2749/PR2750 (226 bp), PR2748/PR1604 (221 bp), and PR2740/PR2741 (222bp), respectively. Internal transcription control *rpoD* was amplified using primers PR1704 and 1705.

### 2.3.3 qRT-PCR analysis of *fim, fim2* and *mrk* gene clusters

For *in vitro* samples 2 µl of a $10^{-1}$ dilution of the cDNA library and its reverse transcriptase negative control were used for quantitative real-time PCR (qRT-PCR). qRT-PCR reactions (20 µl) were set up using SensiMix Plus SYBR (Bioline) according to manufacturer's instructions. For *rpoD* (PR1701/PR1702; 164 bp) and *fimA* (PR1703/PR1704; 157 bp), *fim2A* (PR1705/PR1706; 165 bp), *mrkA* (PR1707/PR1708; 155 bp) and *fim3A* (PR2751/PR2750**;** 171 bp**),** 10 pmol of each primer was added to the reaction mix. Cycling conditions consisted of 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 20 s. Each qRT-PCR reaction was performed in triplicate. qRT-PCR reactions were performed using the Qiagen Rotor Gene 6000. The relative quantification of *mrk*, *fim* and *fim2 and fim3* transcripts versus *rpoD* was determined according to the Pfaffl equation (Pfaffl, 2001).

## 2.4. Genetic manipulation

### 2.4.1 Preparation and transformation of chemically competent bacteria cells

Chemical competent (heat shock competent) cells were constructed using the following method. A single colony of *E. coli* DH5α, CC118**-**λpir and S17-λpir was inoculated in 5 ml of LB broth o/n at 37°C and 200 rpm. The overnight culture (500 µl) was used to inoculate 1 L of sterile fresh LB broth. The cells were then grown at 37°C with shaking at 200 rpm until they reached an $OD_{600nm}$ of 0.3 to 0.4. The cells were harvested by centrifugation at 3,000 xg for 10 minutes at 4°C. The supernatant was removed and the bacterial pellets were then gently re-suspended in 50 ml of ice cold 100 mM $MgCl_2$ (Sigma,

UK) and incubated for 10 mins on ice. The cells were then centrifuged again at 3,000 ×g for 10 mins at 4°C. The supernatant was then discarded and the cells gently re-suspended in 50 ml of ice cold 100 mM $CaCl_2$ (Sigma, UK) and incubated for 10 mins on ice. The cells were then centrifuged at 3,000 xg for 10 minss at 4°C, the supernatant removed and the cells re-suspended in 4 ml of ice cold, sterile 100 mM $CaCl_2$ (Sigma, UK) in 15 % (w/v) glycerol (Fisher, UK). Cells were frozen down in 50 µl aliquots and stored at – 80°C.

For heat shock chemical transformation, the plasmid DNA or the ligation mixture (1 – 4 µl), was added to a 50 µl aliquot of thawed chemical competent *E. coli* cells. The cells and DNA / ligation were mixed gently and then incubated on ice for 20 mins. The heat shock was performed by placing the cells in heat block at 42°C for 45 s, then immediately returned to ice, and incubated for 2 mins. Fresh 950 µl of Super Optimal broth with Catabolite repression (SOC) broth was added and the mixture was incubated at 37°C with 200 rpm for 1.5 h. Transformed cells were then plated onto LA plates containing the appropriate antibiotic and were incubated overnight at 37°C.

### 2.4.2 Preparation and transformation of electrocompetent bacteria cells

The method used for preparing electro-competent *E. coli* cells was as follows. A single colony was inoculated into a 5 ml LB broth, incubated overnight at 37°C and 200 rpm (no antibiotic was added). In the next day, 1 ml of the overnight culture was used to inoculate 100 ml of pre-warmed sterile fresh LB broth (dilution 1:100). The cells were then grown at 37°C with shaking at 200 rpm until they reached an $OD_{600nm}$ of 0.5 to 0.6. The cells were then

chilled on ice for 10 min. The cells were then harvested by centrifugation for 10 min at 4,000 rpm and 4°C. The supernatant was removed carefully and the bacterial cell pellet was then gently re-suspended in 50 ml of ice-cold 10 % glycerol and repeated centrifugation at 4,000 rpm for 10 min at 4°C. The supernatant was then discarded and the cells gently re-suspended the in 20 ml of ice-cold 10 % glycerol and repeat centrifugation at 4,000 rpm for 10 min at 4°C. The supernatant was then again discarded and the cells gently re-suspended the in 10 ml of ice-cold 10 % glycerol and repeat centrifugation at 4,000 rpm for 10 min at 4°C. Finally, the supernatant was then again discarded and the cells gently re-suspended the in 1 ml of ice-cold 10 % glycerol and repeat centrifugation at 4,000 rpm for 10 min at 4°C. Cells were frozen down in 50 µl aliquots at – 80°C (~20 aliquots). In total, there are three 10 % glycerol washes to get rid of salt. The cells were thus concentrated ~100 fold compared to the density in the initial growth media. All steps were done on ice.

To carry out the electroporation, 50 µl aliquot of electro-competent cells was taken from the – 80°C freezer and thawed on ice. The plasmid DNA was added to cells and mixed gently. The cells/plasmid mix then transferred to a pre-chilled electroporation cuvette (0.2 cm gap) (Geneflow, UK). The cells were electroporated in a Genepulser II system (Biorad, UK) with following settings 25 µF, 200 Ω, 2.5 kV. Immediately, after the application of the pulse, 950 µl of sterile SOC medium was added to the cuvette and the contents transferred to 1.5 ml eppendorff tube. The cells were incubated for 1.5 h at 37°C and 200 rpm to allow recovery and then plated onto LA plates containing appropriate antibiotics and incubated overnight at 37°C.

### 2.4.3 Lambda red recombination based allelic exchange

Lambda Red recombination-based allelic exchange exploits the increased rates of recombination observed when the phage λ Red recombinase system (γ, β, exo) is overexpressed in bacteria(Datsenko and Wanner, 2000). When linear DNA fragments homologous in sequence to the bacterial chromosome (such as SOE-PCR generated mutant alleles) are transformed, recombination can take place and chromosomal regions can be disrupted and/or deleted (Mushegian and Koonin, 1996, Chaveroche, Ghigo et al., 2000, Datsenko and Wanner, 2000).

Initially, plasmid pKOBEG-Apra (Chaveroche, Ghigo et al., 2000) was transformed into *Klebsiella* or *E. coli* strain targeted for mutagenesis and isolated on LA supplemented with apramycin and any other antibiotics necessary for selecting target strain. Transformation plates were also grown at 30°C. The presence of pKOBEG-Apra was confirmed by colony PCR using primers specific for the aparamycin cassete on the plasmid.

A single colony was inoculated in 5 ml of LB plus apramycin and grown overnight at 30ºC, 200 rpm. This culture was inoculated at a ratio of 1:100 (e.g. 500 µl to 50 ml) into fresh LB supplemented with apramycin and 0.2 % arabinose. Any additional antibiotic needed for the transformed strain was also included. The culture was grown at 30ºC to an $OD_{600nm}$ of 1.0, after which electro-competent cells were prepared and transformed with 0.5 -1.0 µg of SOE-PCR generated mutant allele. After non-selective outgrowth for 1 h at 37ºC, the transformation mixture was plated onto LA containing appropriate antibiotics and incubated at 37°C.

## 2.5 Other methods

### 2.5.1 Microtitre plate- based biofilm assay

Overnight cultures for each test strain, with 3 biological repeats from 3 single colonies were grown in 5ml LB. After 12 h of growth, the overnight cultures were diluted to 1:100 in 1ml LB rich media and mixed by vortexing. For each of the biological repeats, one single column and 8 individual wells of a NUNC 96 well polysterene plate were inoculated with 200 µl of culture. The plates were then incubated for 24 h at 37°C. After the elapsed time, the media is then discarded into a waste container containing 1 % Microsol solution. The plates were then immersed into distilled water gently until the wells were filled. The water was then discarded into a waste container and the plate tapped on absorbent surface to dry. A 0.1 % (v/v) Crystal Violet solution (125µl) was added to each well, which was then incubated at room temperature for 10 minutes. Crystal Violet was then discarded from plates and was then washed twice in two different water baths as previously described. Plates were initially dried on absorbent surface and allowed to air dry completely overnight at room temperature. Once completely dry, 200 µl of 95 % ethanol was added to all wells and incubated at room temperature for 15 mins. Absorbance was then read at 595 nm on 96 well plate spectrometer.

### 2.5.2 *Galleria mellonella* infection and killing assay

An overnight culture of the bacteria (100 µl ) was added to 5 ml of fresh LB for 4 hours. The 5ml culture was then pelleted by centrifugation and cells washed with 10 mM magnesium sulphate ($MgSO_4$). Pelleted cells were then

diluted with 10 mM $MgSO_4$ to $OD_{600nm}$ of approximately 2.00. The resuspension was then serially diluted to appropriate inoculum dilution. *G. mellonella* caterpillars were stored at 4°C and used within 7 days of shipment. Before inoculation, caterpillars were taken out of 4°C and allowed to warm up to room temperature for at least one hour. The caterpillars were picked randomly and swabbed with 70 % ethanol to sterilize the body surface before injection. A syringe was used to inject 10 µl aliquots of the inoclum into the hemocoel of each caterpillar via the last left proleg. The inoculum (100 µl) was also plated on LB agar to confirm the bacterial numbers injected into the worms. After injection, the caterpillars were put in clean petri dishes and incubated at 37°C. Two control groups were used in the *G. mellonella* killing assay. One control group of 5 worms were inoculated with just 10mM $MgSO_4$ to monitor killing due to physical trauma and a second control group containing 5 worms were not injected at all. Each experimental group contained 10 worms and the numbers of dead and live and caterpillars were recorded every 12 h. Caterpillars were considered dead if there was no response to physical touch.

For recovery of bacteria within each larva, each larva was repeatedly washed with and homogenized in 2 ml of 10mM $MgSO_4$. Homogenates were serially diluted plated on nutrient agar plates containing streptomycin 250 µg/ml or kanamycin 50 µg/ml, depending on bacterial strain.

### 2.5.3 Agglutination assay

Agglutination assays were performed using guinea pig red blood cells (GP-RBCs). GP-RBCs were stored at 4°C as 20 % solutions in Alsever's (Matrix

Biologicals). When required, a 5 % suspension of GP-RBCs was freshly prepared by diluting the 20 % stock solution 1 in 3 in PBS. Briefly, agglutination assays were performed on microscope slides using 20 µl of overnight bacterial culture (37°C, 200 rpm) and 20 µl of blood suspension. The microscope slide was tipped back and forth and observed for signs of agglutination for up to 20 mins. To test for mannose-sensitivity, 30 µl of 10 % (w/v) mannose was added to the agglutination reaction.

### 2.5.4 Transmission electron microscopy

A 5 µl volume of sample was applied to a hydrophilic Formvar-carbon coated copper grid (Agar Scientific) and allowed to adsorb for 5 mins. After wicking excess liquid, the grid was washed once using distilled deionised water and then stained for 15 s with 5 µl of 1 % uranyl acetate (pH 4.5). Transmission electron microscopy was carried out on a JEOL JEM-1400 microscope at 80 kV by Stefan Hyman at the Centre for Core Biotechnology Services, University of Leicester.

### 2.5.5 Data analysis and statistics

The survival curves were analysed using Kaplan-meir tests. The competitive index (CI) was calculated by dividing the ratio of *fim2*-positive to *fim2*-negative bacteria recovered from infected galleria larvae. Biofilm data were analysed using the non-parametric Kruskal-Wallis test and Dunn's posthoc analysis. *P* values less than 0.05 were considered statistically significant.

### 2.5.6 Bioinformatics

A variety of programs were used to perform various sequence analyses. These programs and their functions are listed in Table 2-4.

**Table 2-4. Bioinformatics programs used in this work**

| Name | Function | Reference |
|---|---|---|
| **BLAST** | Searches DNA and protein database for similar DNA and protein sequences (BLASTn and BLASTp, respectively). Variations of this program also translate DNA in six frames and searches protein databases (BLASTx) or database of nucleotide sequences translated in six frames (tBLASTx). | (Altschul, Madden et al., 1997) |
| **Pfam** | Database of protein families | (Bateman, Coin et al., 2004) |
| **PROSITE** | Database of protein domains, families and functional sites | (Hulo, Bairoch et al., 2006) |
| **Clustal Omega** | Visual multiple sequence (nucleotide and protein) alignment tool | (Sievers, Wilm et al., 2011, Goujon, McWilliam et al., 2010) |
| **Phylogeny fr** | Online tool for phylogenetic analysis | (Dereeper, Guignon et al., 2008) |

| | | |
|---|---|---|
| **NEBCutter** | Online tool to identify enzyme restriction sites in nucleotide sequences | (Vincze, Posfai et al., 2003) |
| ***In silico* PCR** | Online program to perform *in silico* PCR | (Bikandi, San Millan et al., 2004) |
| **Oligo Calc** | Oligonucleotide analysis | (Kibbe, 2007) |
| **Primer3** | Primer design | (Rozen and Skaletsky, 1999) |

# Chapter 3: Further characterisation of *fim2*, an operon coding for a predicted type 1 fimbrial system

## 3.1 Introduction

The discovery of the *fim2* operon resulted from an earlier PhD research project by Dr Jon van Aartsen on tRNA gene (*tRNA*) hotspots prone to insertion of genomic islands (GIs) in the *K. pneumoniae* genome. The tRNA loci usually act as insertion sites for mobile elements since they are highly conserved within bacteria and allow for a broader range of movement (Hacker and Kaper, 2000, Schmidt and Hensel, 2004).

As part of this study, five *tRNA* insertion hotspots in sixteen clinical *K. pneumoniae* isolates were screened for strain-specific DNA employing a technique called tRIP-PCR (Van Aartsen, 2008). Among these strains, *K. pneumoniae* KR116 that was isolated from the blood of a patient with pneumonia and neutropenic septicaemia was found to possess an 'occupied' *met56* tRNA locus. The tRIP-PCR technique utilises specific primers designed to amplify across the *met56* tRNA locus, such that a failed attempt to obtain an amplicon suggests an insertion at this tRNA locus. Subsequently, single genome specific primer based walking from the conserved met56 upstream flank revealed approximately 3 kb of a novel island sequence, which encoded some putative fimbrial genes. To capture the entire island harboured by this strain, the known tRNA-proximal arm of the island was tagged with a kanamycin resistance cassette via allelic exchange. A fosmid library of the tagged strain was then generated and kanamycin resistance cassette-bearing inserts were isolated by marker rescue technique. End sequencing of

overlapping fosmids, pJFos-1 and pJFos-4 also contained regions of the UF and DF of the *met56* tRNA locus, suggesting that together both fosmids span the entire strain-specific region. Further sequencing revealed a novel KR116 *met56*-specific GI. The captured 14 kb island in its entirety was dubbed KpGI-5 (Van Aartsen, 2008).

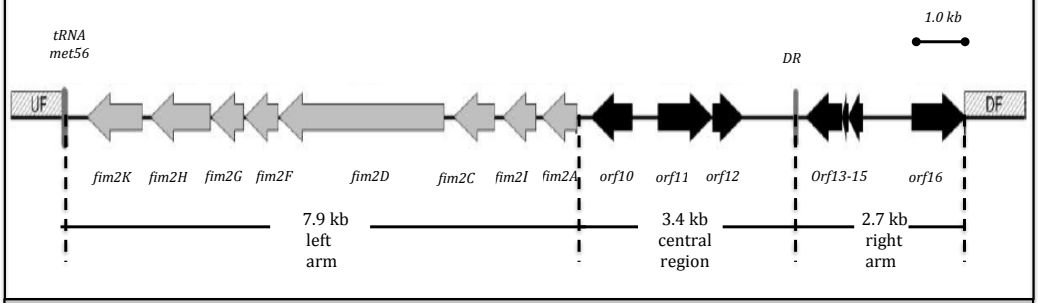
The ORFs within the KpGI-5 genomic island sequence were identified and BLAST analyses were performed for putative gene annotation. For descriptive purposes, the KpGI-5 genomic island is segmented into three different arms, a 2.7 kb right arm, a central 3.4 kb region and a 7.9 kb left arm. The 2.7 kb segment mapping to the right arm of the KpGI-5 was 90% similar to a region immediately downstream of *met56* in *K. pneumoniae* Kp342 and was predicted to encode two hypothetical proteins (Orf14 and Orf15), a metallo-β-lactamase family protein (Orf16) and a putative GCN5-related N-acetyltransferase (Orf13). The nucleotide sequence of a 3.4 kb central region did not match any GenBank entries and coded for three novel proteins; Orf10, Orf11 and Orf12 (Van Aartsen, Stahlhut et al., 2012) (Figure 3-1).

The 7.9 kb left arm of KpGI-5 possessed a novel eight-gene cluster that showed sequence similarity and syntenic arrangement to the chromosomally-encoded *fim* operons of *Citrobacter koseri* ATCC BAA-895 (~60 %) and *K. pneumoniae* C3091(~51 %). This cluster of genes was called *fim2*. This operon encoded homologs of all structural and biosynthesis-associated components of the well-characterised *K. pneumoniae* type 1

fimbrial system. These components include a major fimbrial subunit (named Fim2A), three minor fimbrial subunits (Fim2F, Fim2G and Fim2H), and a chaperone (Fim2C) and usher (Fim2D) (Struve, Bojer et al., 2008). (See Figure 3-1.)

Downstream of the *fim2H is fim2K, which* encodes a FimK homolog that possesses a matching EAL domain but not the FimK-equivalent N-terminal helix-turn-helix domain. Amino acid sequence similarities between the cognate *fim2* and *fim* products varied from 60 to 92 %. Moreover, there were no observed homologs of *K. pneumoniae fimB*, *fimE* or *fimS*-like invertible switch. All of the clinical *K. pneumoniae* isolates positively screened for possession of *fim2* were also confirmed by PCR to possess the species-conserved *fim* by screening for *fimH* (Van Aartsen, Stahlhut et al., 2012).

**Figure 3-1. Schematic showing features of KpGI-5 island in *K. pneumoniae* KR116**




Genetic organisation of KpGI-5 shown lying between the species-conserved upstream flank (UF) and downstream flank (DF) sequences. The eight putative fimbrial genes are labelled *fim2A*– *fim2K* as annotated from BLASTp similarities for predicted ORFs. The direct repeat (DR) sequences associated with KpGI-5 is 46 bp in length and comprises the 3' end of *met56.* Diagram based on schematic by Jon van Aartsen *et al*. (320).

In order to investigate *fim2* function several genetic constructs and different bioassay techniques were utilised. While RT-PCR confirmed the expression of

the *fim2* in *K. pneumoniae* KR116, deletion of *fim2* in this strain did not show any effect on infection or adherence in murine models (Van Aartsen, Stahlhut et al., 2012).

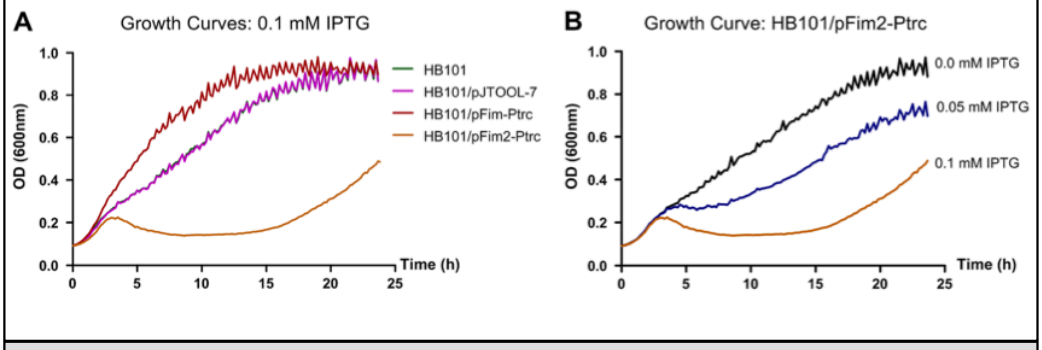
The *fim2* locus was also cloned under an IPTG inducible promoter on plasmid pJTOOL-7 into an afrimbriate *E. coli* HB101. This genetic construct was named *E. coli* HB101/pFim2-Ptrc and for comparison, *fim* operon was also cloned under the same promoter on the same vector and also transformed into *E. coli* HB101. This *fim* based construct was known as *E. coli/*pFim-Ptrc (Van Aartsen, Stahlhut et al., 2012).

The afimbriate nature of *E. coli* HB101 allowed for the examination of specific-fimbrial assembly in isolation of other host-encoded fimbriae and also permitted the visualisation of fimbrial structure while identifying specific phenotypic traits of these fimbriae. Moreover, heterologous expression of fimbrial operons in *E. coli* overcomes any potential physical hindrance and/or visual shielding resulting from the presence of the *K. pneumoniae* capsule, as has previously been seen with type 1 fimbriae in (Schembri, Blom et al., 2005, Struve and Krogfelt, 2003).

With these genetic constructs, it was observed that over expression of *fim2* cloned in pJTOOL-7 could contribute to biofilm formation ($P < 0.001$). However, no visualisation of fimbriae was observed in *E. coli/*pFim2-ptrc*.* This was not the case with *fim* operon expressed in *E. coli* as abundant number of pili was observed (Van Aartsen, Stahlhut et al., 2012).

Notably, despite the lack of visible fimbriation in *E. coli* HB101/pFim2-Ptrc induced with IPTG, major induction-associated growth retardation was observed (Figure 3-2A). HB101/pFim2-Ptrc growth inhibition showed a dose–response relationship to IPTG concentration and this was not observed with the control strains HB101 and HB101/pJTOOL-7 (Figure 3-2B). By contrast, over-expression of *fim* appeared to improve the growth rate of HB101/pFim-Ptrc but had no effect on final cell densities as compared to other strains (Van Aartsen, Stahlhut et al., 2012).

**Figure 3-2. Previous results showing IPTG induction of HB101/pFim2-Ptrc causes a major growth reduction**




**A)** Growth curves for HB101, HB101/pJTOOL-7 (empty vector), HB101/pFim-Ptrc and HB101/pFim2-Ptrc. The growth curves for HB101 and HB101/pJTOOL-7 are largely superimposed as these are very similar. **(B)** Growth curves for HB101/pFim2-Ptrc grown for 24 h in LB broth containing 100 µg/ml ampicillin supplemented with 0.0 mM, 0.05 mM or 0.1 mM IPTG. Results graph from Jon van Aartsen *et al.* (320).

Part of this PhD project was to further identify phenotypic characteristics associated with *fim2* in addition to examining its influence on *fim2*-harbouring *K. pneumoniae* strains by testing the virulence of these strains in a *G. mellonella* killing assay. In addition, this project further investigated some of the conundrums surrounding *fim2* and its ability, or lack thereof, to promote fimbriation.

## 3.2 *In vitro* expression of *fim2* in *K. pneumoniae*

While previous research on *fim2* confirmed its expression under both *in vitro* and *in vivo* conditions(Van Aartsen, Stahlhut et al., 2012), it was speculated that comparison between *fim2* expression and that of other fimbrial systems could shed more insight into understanding this operon. The expression of *fim2* was previously tested under various laboratory growth conditions such as varying temperatures of 25°C and 37°C, as well as different growth mediums such as LB medium, RPMI 1640 ('physiological' medium), Artificial urine media (AUM) and M9 (minimal) medium (Van Aartsen, Stahlhut et al., 2012). While these experiments show highest *fim2* expression at 37°C in LB and M9 medium, other conditions such as growth phase which may affect its expression were not tested. Several studies have shown that bacterial growth phase can have an effect on fimbrial expression. For instance, recent research on type 1 fimbriae of uropathogenic *E. coli* showed the repression of type 1 fimbriae by specialised signal transduction system CRP-cAMP, occurs during the logarithmic phase and is increased during the stationary phase. This is consistent with adaptation to stress by promotion of biofilm formation or entry into host cells via involvement of type 1 fimbriae (Müller, Åberg et al., 2009). In *Salmonella enterica* serovar Enteriditis, it was reported that the *sef* operon, which codes for SEF14 fimbriae showed optimal expression during late exponential growth phase and was repressed during stationary phase (Edwards, Matlock et al., 2001).

To further investigate expression of *fim2* in this project, RNA was isolated from strains KR2107 and its isogenic deletion mutant, KR2305. Overnight

cultures of the bacteria strains were grown in LB at 37°C, 200 rpm following a 1:100 dilution. Cells were harvested at 3 h and 16 h and cDNA libraries were constructed using random primer based RT-PCR. PCR analysis of the cDNA libraries detected transcripts corresponding to the fimbrial genes being screened for, while reverse transcriptase-free mock cDNA libraries did not yield any products, confirming the absence of DNA carryover.

### 3.2.1 *fim2* is expressed at a lower level than *fim* and *mrk*

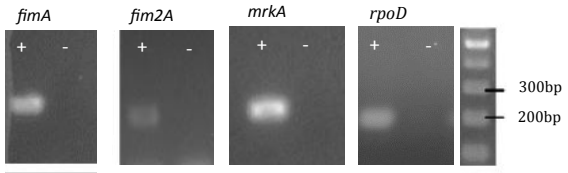
In order to examine the relative expression of the putative *fim2* operon in comparison to those of the *fim* and *mrk* fimbrial operons under normal laboratory conditions, RT-PCR was performed on the *K. pneumoniae* KR2107. This strain is a *fim2*-harbouring spontaneous streptomycin resistant derivative of KR116. Transcripts corresponding to *fimA* were used to test for expression of *fim* operon since FimA comprises the major subunit of the fimbrial structure, just as *mrkA* transcripts were used to test for expression of *mrk* operon as MrkA comprises the major subunit of the type 3 pili. *fim2A* was assayed as an expression marker for the *fim2* operon and *rpoD* was assayed to normalise transcription levels. The results indicated that while all three fimbrial operons are expressed under normal laboratory conditions, *fim2* is consistently expressed at a lower level than *fim* or *mrk* (Figure 3-3). Quantitative image analysis by GelEval was used to measure the optical densities of each gel band, such that brighter white and more intense bands are closer to values of 0.000 and a complete black background is given an optical intensity of 1.000. The utilisation of GelEval allows for a method of quantification of agarose gel electrophoresis results. GelEval analysis

quantified the reduced expression of *fim2A* compared to *fimA* (as well as *rpoD*) to be approximately 1.2 fold less. *mrkA* was found to be more expressed than *fimA* (as well as *rpoD* ) at about 1.5 fold.

Quantitative real time PCR (qRT-PCR) was also done to measure the level of expression of these genes. As seen with RT-PCR results, *mrkA* is more expressed than *fimA* which is in turn more expressed than *fim2A*. While the values may differ, the patterns and levels of expression among these genes are concurrent with results from RT-PCR and gel analysis. The qRT-PCR results show that relative expression of *fimA* was about 1.5 fold higher than *fim2A* expression while *mrkA* was seen to be 4 fold higher.

**Fig 3-3. *fim2* is expressed at a lower level than *fim* or *mrk***

**A**

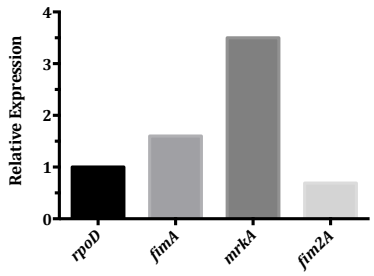



Key + cDNA - no reverse transcriptase control

**B**

| Gene | Optical density |
|---|---|
| *fimA* | 0.543 |
| *fim2A* | 0.744 |
| *mrkA* | 0.368 |
| *rpoD* | 0.592 |

**C**




**A)** cDNA extracted from KR116 was used to compare the relative expression of *fimA, fim2A* and *fim3A* under the same *in vitro* conditions. *rpoD* was amplified as an internal normalising control. **B)** Table showing average optical densities obtained for each gene following RT-PCR and visualisation on gel. **C)** cDNA extracted from KR116 was also used in quantitative RT-PCR of the aforementioned genes. Relative expression of each gene was compared to *rpoD.*

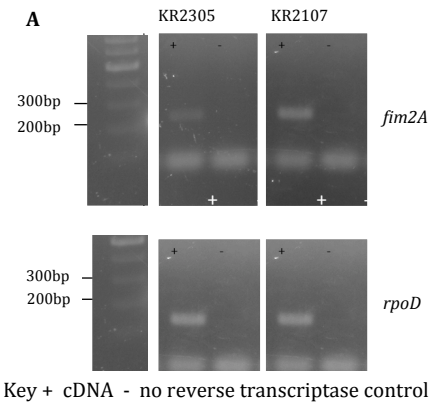
### 3.2.2 Expression of *fim2* is not masked by preferred fimbrial systems such as *mrk* and *fim*

Several other characterised fimbrial systems are expressed in *fim2* harbouring *K. pneumoniae* strain KR116 (Van Aartsen, Stahlhut et al., 2012). Previous research into fimbriae systems has indicated cross-talk regulation such that the increased expression of one type of fimbriae may affect the expression of another type in a particular environment or stimulus (Sjöström, Balsalobre et al., 2009, Snyder, Haugen et al., 2005). In *E. coli*, during coordinate regulation of type 1 and type P fimbriae, a regulator for type P fimbriae expression known as PapB hinders FimB-assisted recombination and thereby negatively affects type 1 fimbriae expression (Xia, Gally et al., 2000).

To investigate whether the expression of these other fimbrial operons may have an increased effect on the expression of *fim2,* cDNA libraries were generated from *K. pneumoniae* KR2107 and its isogenic dual *fim* and *mrk* deleted mutant KR2305. Expression of *fim2A* in both bacteria was compared following amplification of the *fim2A* transcripts collected at mid log phase. Results (Figure 3-4) showed less expression in isogenic mutant KR2305, showing that absence of *fim* and *mrk* expression does not increase the expression of *fim2* under normal laboratory conditions as postulated. GelEval was also used to analyse the intensity of the amplified bands. GelEval analysis estimated similar values for the *rpoD* expression in both strains while *fim2A* expression was slightly increased in the absence of *fim* and *mrk* in the strain. However, quantitative RT-PCR analysis confirmed that *fim2A* expression,

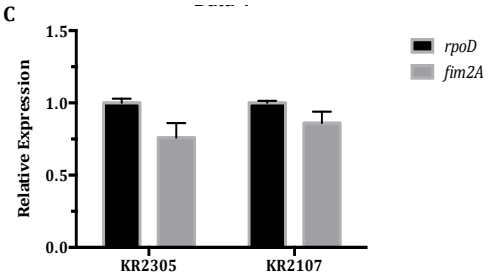
when compared to internal *rpoD* control, is not significantly more expressed in the absence of the fimbrial operons *mrk* and *fim.* (Figure 3-4).

**Figure 3-4. *fim2* expression is not masked by *fim* or *mrk***




B

| Gene | Optical Density | |
|---|---|---|
| | KR2305 | KR2107 |
| *fim2A* | 0.722 | 0.692 |
| *rpoD* | 0.624 | 0.622 |




**A)** KR116 streptomycin derivative KR2107 and its isogenic double *fim mrk* knockout KR2305 were tested for *fim2A* transcripts obtained from cDNA libraries grown under the same *in vitro* conditions. **B)** Table showing optical densities of RT-PCR results as estimated by GelEval. **C)**qRT-PCR analysis was also done on *fim2A* cDNA generated from both strains. Relative expression of each gene was compared to *rpoD* confirming that loss of *fim* and *mrk* does not affect the expression of *fim2.*

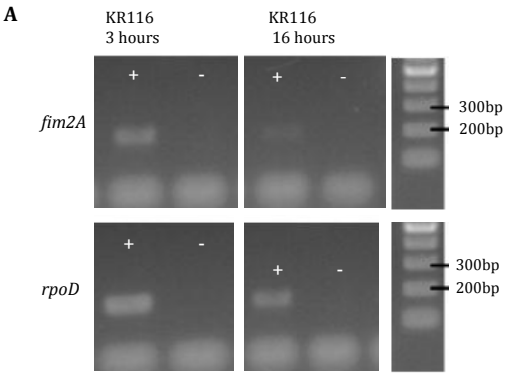
### 3.2.3 Expression of *fim2* maybe growth phase dependent

To explore other characteristics in the *fim2* expression profile, the expression level of the *fim2* in *Klebsiella* strains KR2107 at different time points in the growth of the bacteria were analysed. cDNA libraries were collected at mid log phase correlating to a time point of three hours after inoculation and late stationary phase correlating to time point of 16 hours after inoculation. *fim2A* was amplified from cDNA generated at these time points, while *rpoD* amplification served as an internal normalising control. Results showed more

transcripts could be detected and amplified at 3 hours than at 16 hours after inoculation (Figure 3-5).

**Fig 3-5. *fim2* expression may be growth phase dependent**

**A**




Key + cDNA - no reverse transcriptase control

**B**

| Gene | Optical Density | |
|---|---|---|
| | 3 hours | 16 hours |
| *fim2A* | 0.679 | 0.715 |
| *rpoD* | 0.626 | 0.654 |

**A)** *fim2A* was employed as a target gene to measure comparative expression of *fim2* operon in KR116 at different phases of growth. Expression *fim2A* is seen to be lower at 16 hours than at 3 hours when compared to *rpoD* at respective time points . **B)** Table showing optical densities of RT-PCR results as estimated by GelEval.

## 3.3 *Galleria mellonella* as a model to study virulence of *fim* and *fim2* mutants in *K. pneumoniae*

### 3.3.1 Introduction

In *K. pneumoniae*, *in vivo* bioassays can be used to determine the extent of virulence of strains often employ murine models in these experiments. However, the use of *Galleria mellonella*, a caterpillar of the greater wax moth, as a model system to study bacterial pathogenicity and therapeutics has proved effective in bacteria like *Acinetobacter baumani* (Peleg, Jara et al., 2009). In addition, the *G. mellonella* model has been used effectively as a model to observe pathogenesis and regulation of the *mrf* gene cluster in

*Photorhabdus temperata* K122, which encodes the mannose-resistant fimbriae of *P. temperata* as well as its recombinase regulators (Meslet-Cladiere, Pimenta et al., 2004). With this model, expression of several genes within this cluster was determined post-*Galleria* infection and before the death of the larvae. *G. mellonella* has also been exploited to investigate host-pathogen interactions for a range of bacterial species including *Pseudomonas aeruginosa.* (Jander, Rahme et al., 2000, Miyata, Casey et al., 2003). Moreover, a strong correlation between virulence as determined in *G. mellonella* and that assayed by mammalian models has been established (Jander, Rahme et al., 2000, Mylonakis, Moreno et al., 2005).

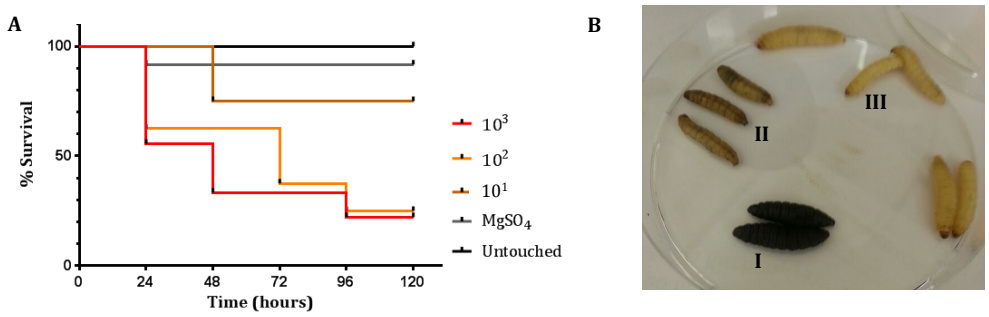
The *G. mellonella* system confers several advantages over several bioassay systems. Apart from its lack of ethical issues and economic viability of these larvae, *G. mellonella* caterpillars can be maintained at temperatures of 37°C and are therefore well suited to investigate human pathogens. Secondly, the infection inoculum can be precisely administered through an injection into the body of the caterpillar. Thirdly, *G. mellonella* caterpillars have both humoral and cellular immune response pathways regulated by antimicrobial peptides and phagocytic cells (haemocytes), respectively, enabling an assessment of host responses. Finally, the *G. mellonella* infection model is also amenable to antibiotic treatment, and hence the efficacy of antimicrobial agents can be assessed (Peleg, Jara et al., 2009).

### 3.3.2 Pilot experiments to determine and optimise methods and bacterial load

In order to employ the use of *G. mellonella* larva as a model to determine and compare level of virulence among *Klebsiella* species and strains, preliminary experiments were carried out to determine and optimise the appropriate inoculum methods and estimated bacterial load for infecting the larvae with *K. pneumoniae*. The appropriate inoculum containing estimated number of bacteria should not cause up to 50 % death within 24 hours and should show gradual killing with longer incubation. Preliminary experiments were performed with KR2107, a streptomycin resistant derivative of *K. pneumoniae* KR116. During the pilot killing assay, each group consisting of ten larvae were injected with different dilutions of KR2107 suspension, from $10^1$ to $10^8$, in a serial 1:100 dilutions. Each group of larvae injected with estimated to $10^5$ to $10^8$ of bacteria had more than 50% in the group dead within 24 hours thereby being unsuitable dilutions for *G. mellonella* killing assay in this project. It was also observed that there was no further killing of larvae by bacteria after 5 days of infection since the same number of dead and surviving larvae remained from day 5 to 8 (results not shown). After two independent experimental repeats, it was observed that bacterial inoculum estimated at approximately $1 \times 10^3$ (± 1.83) and $1 \times 10^2$ (± 1.54) killed less than 50 % of larvae within 24 hours making this inoculum size suitable for the killing assay (Figure 3-6). The inoculation of *G. mellonella* with KR116 also resulted in killing of caterpillars in a time- and dose-dependent manner in a process that included melanisation of the dying larvae. These results also demonstrate a very high level of virulence from *K. pneumoniae* KR116, as an

inoculum carrying as few as 100 to 150 bacteria was able to kill more than 30 % of larvae. Statistical analysis using Log rank tests confirms that survival curves represent inoculum dilutions of approximately $1 \times 10^3$ and $1 \times 10^2$ significantly different, where P values were estimated at 0.0012 and 0.0024 respectively. Lower bacteria inoculum at $\approx 10^1$ was found to be not statistically significant (P = 0.365). Therefore, inoculum sizes of approximately $1 \times 10^3$ and $1 \times 10^2$ were chosen as the ideal bacterial load for the *K. pneumoniae* killing assay.

**Figure 3-6. Pilot experiment to determine inoculum dilutions and optimise assay methods**




**A)** *G. mellonella* killing by *K.. pneumoniae* KR2107 is dependent on the inoculum. For infection assay, inoculum dilutions of $1x10^3$ (± 1.83) CFU and $1x10^2$ (± 1.54) CFU were suitable estimated number of bacteria inoculum as these dilutions did not cause up to 50 % death within 24 hours and showed gradual killing with longer incubation period. **B)** Melanisation of *G. mellonella* is part of the infection process with *K. pneumoniae* and highly pigmented black larvae are dead . The image was taken 48 h after an experimental infection with $10^2$ CFU/larva of *K. pneumoniae* KR2107. **I** (Dead and completely melanised larvae), **II** (Alive infected larvae in the process of melanisation and **III** (No pigmentation in uninfected larvae).

### 3.3.3 Investigation into *fim* and *fim2* virulence using *G. mellonella* killing assay

After establishing appropriate method and dilution for *G. mellonella* inoculation, a killing assay was performed with *K. pneumoniae* strains KR2107 and its isogenic dual *fim* and *fim2* deleted mutant, KR2152. Each

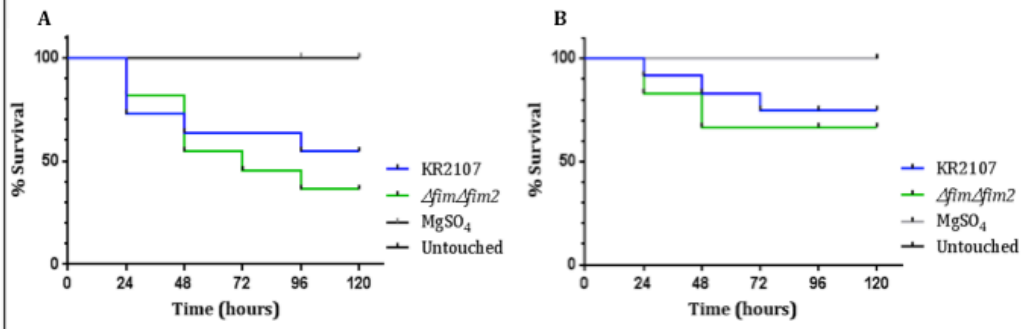
group of 10 independent larvae was injected with either wildtype or isogenic dual mutants. Two different inoculums were also used in this assay. With both inocula, the isogenic mutant KR2152 showed significantly more killing than wildtype KR2107, showing that it was more virulent to *G. mellonella*, at least as assessed by this assay (Figure 3-7)

Infection of larvae with $1 \times 10^3$ (± 0.72) of wildtype KR2107 resulted in 40 % of death by the 5$^{th}$ day. Infection by the *fimfim2* knockout derivative resulted in death of 70 % of larvae by day 5. These results were obtained following an aggregate of three independent experiments. When compared to the control group, survival curves for the wildtype and mutant were significant whereby P values were estimated as 0.0043 and 0.0036 respectively. Although, the KR2107 and mutant survival curves were found to not statistically different (P = 0.020), the pattern of virulence observed with the two strains was consistent (Figure 3-7).

The lower infection dose of $1\times10^2$ (±0.31) of KR2107 bacterial cells resulted in 30% total population death by the 5$^{th}$ day, which was again was lower than the 40% total population death caused by the *fimfim2* mutant. Both curves were statistically distinguished from the control group, where P values for wildtype and mutant were 0.0026 and 0.0041 respectively using Log rank test. Although the difference between the KR2107 and mutant curve were not statistically different (P = 0.0248), the experiment was repeated twice and the same pattern was observed where KR2152 killed a greater proportion of larvae than the wildtype KR2107. This confirms the mutant

KR2152 strain to be more virulent than the wildtype parent strain (Figure 3-7).

**Figure 3-7. *G. mellonella* killing assay to determine effect of *fim* and *fim2* on virulence of *K. pneumoniae***



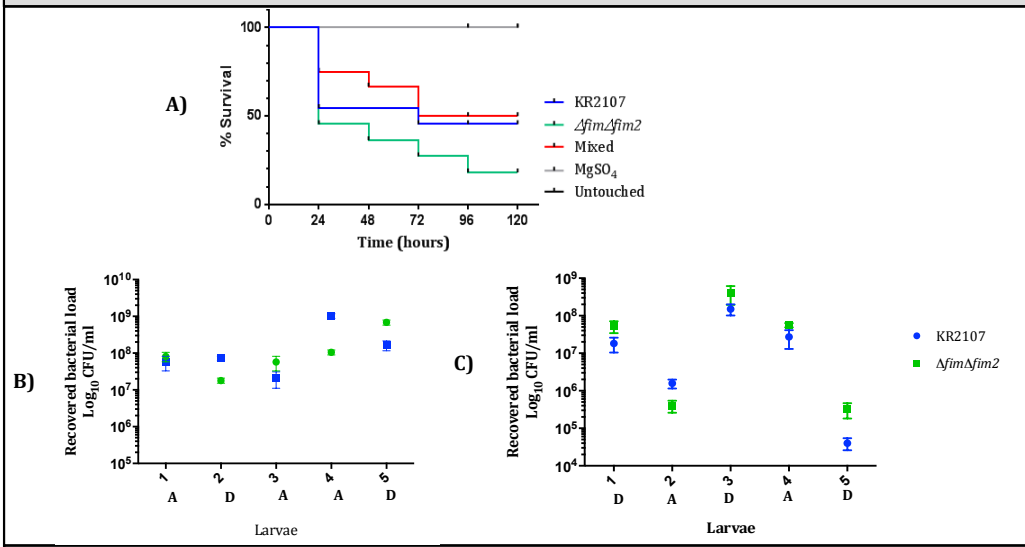

**A)** For infection assay, larvae were injected with $1\times10^3$ (±0.72) of KR2107 parental strain or its isogenic *ΔfimΔfim2* knockout mutant, KR2152 and incubated at 37°C for 5 days. **B)** Larvae were injected with $1\times10^2$ (±0.31) of KR2107 parental strain or its isogenic *ΔfimΔfim2* knockout mutant, KR2152 and incubated at 37°C for 5 days. For negative controls, some caterpillars were injected with comparable volumes of $MgSO_4$ and some were untouched with injection. Results show more killings among larvae infected with double knockout mutant than parent strain KR2107. Differences between KR2107 and KR2152 curve were not significantly different from each other (P = 0.020 at higher inoclum concentration [A], P = 0.0248 at lower inoculum concentration {B]), however significantly different from controls (P < 0.05 for wild type and mutant compared to control group at both inoculum concentration).

### 3.3.4 Relative fitness of wildtype and *ΔfimΔfim2* mutant by *in vivo* competition

As part of the investigation of *G. mellonella* as a model of infection, it was speculated that the more virulent strain would out compete the less virulent strain during the course of a mixed *in vivo* infection experiment. Hence an *in vivo* competition experiment to determine the relative fitness of wildtype and Δ*fim*Δ*fim2* mutant was done. The *G. mellonella* infection assay was performed as previously described with 10 larvae in experimental group and three independent experimental repeats. KR2107 and its isogenic derivative KR2152 were treated separately and then wildtype and mutant bacteria were mixed in a 1:1 ratio, and serially diluted yield inocula carrying $1\times10^3$ (±0.32). In this *G. mellonella* killing assay, the same pattern was observed

where KR2152 showed more killing of larvae at 80 % total population death than wildtype KR2107 within 4 days of incubation. Survival curves of single infection with wildtype and mutant were statistically significant ($P < 0.05$ in both cases). Infection with the mixed population of wildtype and mutant bacteria resulted in higher survival rates than with either wildtype or mutant alone, though the difference with the wildtype strain was only marginal and not significant by the Log rank test (Figure 3-8A).

**Figure 3-8. Relative fitness of wild type and *ΔfimΔfim2* mutant by *in vivo* competition**




**A)** For the competitive infection assay, larvae were injected with $1\times10^3$ (±0.72) of KR2107 parental strain and its isogenic *ΔfimΔfim2* knockout mutant, KR2152 mixed at 1:1 ratio and incubated at 37°C. Results show that the double mutant is more virulent than single wild type or mixed infection. Following infection with mixed *K. pneumoniae* populations, dilutions of whole-larva lysates were plated on selective nutrient agar, and colony counts were determined after **B)** 24 hours and **C)** 96 hours. Error bars show standard deviation of mean colony counts. D (dead at time of homogenisation) A (alive at time of homogenisation)

Following head-to-head *in vivo* competition of the wildtype and mutant, several larvae were injected with $1\times10^3$ (±0.72) CFU of 1:1 mixed KR2107 and KR2152 suspensions.

These larvae were then incubated at 37°C. After incubation for 24 h, five randomly selected caterpillars were each homogenised individually and plated on selective media to ascertain the proportion of wildtype and mutant within each larvae. This process was also repeated following a 96 h incubation period of inoculated larvae.

The phenotype and CFU counts of wildtype and mutant within each larva were noted. This was done to determine winning population and fitter strain by virtue of its numbers within the larvae, so as to confirm the hypothesis that the more virulent strain KR2152 would out populate the wildtype strain KR2107 in an *in vivo* competition.

As seen with previous killing assay (Figure 3-8), the dual mutant strain may be more virulent to the larvae then the parent wildtype strain. With the mixed competition assay it was observed at day 1, that two out of five galleria had significantly higher counts of wildtype, another two had a much higher count of the mutant strain while one *Galleria* showed little variation of the number of wildtype and mutant population. Of the two larvae that were pigmented and dead as a result, larvae two had a higher proportion of wildtype than mutant while larvae 4 showed a higher proportion of the mutant instead. The remaining three larvae alive at point of homogenisation showed one caterpillar with almost similar proportions of wildtype and mutant strain, one caterpillar with higher amount of wildtype and one with a higher amount of mutant strain. Based on the results obtained following 24 h post inoculation, the more virulent and fitter strain between KR2107 and KR2152 is not observable. However, the logarithmic increase of the bacteria

concentrations of each strain injected into the galleria caterpillars should also be noted. When compared to single infection killing assay, the lack of emerging winner comes as no surprise as percentage of death caused by single infection of wildtype and mutant at 24 h is similar. A significant change in percentage death among larvae reduces and occurs from the second day (Figure 3-8B).

At day 4, the results suggest the mutant derivative may be fitter *in vivo* than the wildtype as seen with the higher proportion of mutant colonies estimated in four out of the five larvae, three which were dead and one which was still alive and melanised at time of homogenisation. The only larvae with a higher proportion of wildtype to mutant was still alive and less pigmented at the time of homogenisation suggesting that the infection process in that caterpillar was still at early stages. These observations and results imply that dual mutant strain may be the winner of the mixed competition within *G. mellonella*. Figure 3-8C. Although the differences in the proportion of wildtype and mutant within the infected populations could be considered as only marginally different in some of the larvae, the average competitive index (CI) value of wildtype to mutant populations suggests that mutant strain is the winner of the competitive relative fitness in *G. mellonella*. The competitive index is a quantitative measure of fitness in bacteria such that relative fitness was calculated as the ratio of the number of wildtype to the number of mutants recovered from all the larvae homogenised at a specific time period. The overall CI value for the recovered bacteria at 96 h was estimated at 0.772, where a value less than 1 implicates the winning mutant

population. The overall CI value for the recovered bacteria at 96 h was also found to be significantly different from mean CI value for bacteria recovered at 24 h, suggesting that a winning population is more likely observed later than 24 h.

### 3.3.5 Discussion

The results obtained from *G. mellonella* killing assay demonstrated that the isogenic *fim fim2* double knockout mutant is more virulent to the larvae than the parent *K. pneumoniae* KR2107 strain. As adhesion properties are known to be a vital virulence factor in *Klebsiella* and the first step in the initiation and progression of infection (Clegg, Wilson et al., 2011, Pizarro-Cerda and Cossart, 2006, Struve, Bojer et al., 2008), it would have been expected that the widtype strain would be more virulent than the deleted fimbriae mutant as the wildtype strain would have higher adherence capabilities to host cells than the knockout mutant strain. However, the results suggest that the immunogenic cost of fimbriae may be more important than the adherence benefits of the type I pili. Previous research studies have demonstrated that the type 1 fimbriae in *K. pneumoniae* as well as *E.coli* type 1 fimbriae are definitive immunogens (Lida, Mizunoe et al., 2001, Witkowska, Mieszała et al., 2005).

Since the *G. mellonella* larvae contains humoral and cellular immune response pathways regulated by antimicrobial peptides and phagocytic cells respectively, it is likely that the *G. mellenolla* immune system is able to detect the wildtype bacteria cells and attack them more than they are able to attack

the less fimbriated mutants which are more likely evade detection and continue to infect the larvae.

It should be noted that survival pattern of mixed population is quite similar to that of single wildtype infection. While mixed infection assay shows a slightly higher survival of larvae, P values for the difference between mixed infection and single infection was quite high ($P = 0.198$). Therefore, the slight increase in survival of mixed infection may due to random events. However, it is clear that the de-fimbriated *K. pneumoniae* mutant causes more death in *G. mellonella* larvae than single infection of the wildtype or mixed infection of wildtype and mutant, providing a strong argument for the immunogenic cost of the fimbriae.

Results of *in vivo* competition between KR2107 and KR2152 suggest that the KR2152 mutant is fitter has seen in the higher proportion of mutant colonies after the $4^{th}$ day of infection. While these results support the suggested hypothesis, several experimental factors will need to be examined in greater detail and the experiments further optimised before any firm conclusions can be drawn. In this project few larvae were homogenised and plated due to time and experimental constraints. A larger sample population could provide a more robust and statistically significant results.

Also while larvae lysates were obtained at specific time points in this work, it is possible that difference between the growth and fitness of the mixed infection of wildtype and mutant strains is better observed at the point of

high melanisation of the larvae which usually has varying times dependent on each larvae.

In the event of application of *G. mellonella* killing assay to other *K. pneumoniae* strains, other variations of the killing assay experiments should also be considered when dealing with other strains. Different *K. pneumoniae* have different level of virulence and may require a variation in method such as need different concentration of inoculum used to infect the larvae.

## 3.4 Determining the fimbriae forming potential of *fim2* operon by creating hybrid *fim-fim2* operon in *E. coli*

### 3.4.1 Background and introduction

In order to elucidate the potential of *fim2* to code for the synthesis of fimbriae, an experiment to construct hybrid operons containing both a mixture of *fim* and *fim2* genes was initiated in this PhD project. *T*he *fim2* operon was previously cloned into IPTG inducible plasmid pJTOOL-7 and transformed into *E. coli* HB101*.* In previous work done by Jon van Aartsen, it was observed that *fim2* might contribute to biofilm formation when heterologously expressed in *E. coli* HB101. However, no visualisation of fimbriae was observed in *E. coli* HB101/pFim2-Ptrc, named for the HB101 strain *fim2*. This was not the case with the *fim* operon cloned under the same

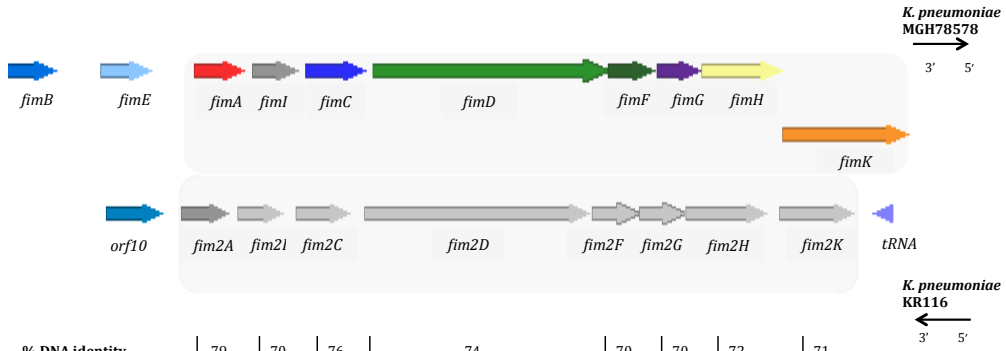
conditions, HB101/pFim-Ptrc, which was able to produce abundant and visible fimbriae. In addition, supplementation with IPTG to induce expression of *fim2* appeared to slow the growth rate *of E. coli* HB101 while the opposite effect was observed with HB101/pFim-Ptrc (Van Aartsen, Stahlhut et al., 2012).

Due to these unanswered questions concerning *fim2*, namely the lack of associated visible fimbriae and its negative effect of growth when expressed *in E. coli*, it was hypothesised that one or more *fim2* gene might account for these observed phenotypes. If so, the creation of a hybrid *fim/fim2* operon may help to shed more insights into the phenotype and function of *fim2*.

### 3.4.2 Sequence comparison of *fim* and *fim2*

Before proceeding with the hybrid creation, comparative sequence analysis of *K. pneumoniae fim* and *fim2* operons was explored (Figure 3-9.) This comparative analysis was done to identify which *fim* genes and *fim2* homologs are best suited for trans-complementation in the creation of the *fim-fim2* hybrid operon. Genetic sequence of the *fim* operon is based on referenced *K. pneumoniae* strain MGH57857 while *fim2* was sequenced from *K. pneumoniae* KR116.

**Figure 3-9. Sequence comparison between *fim* (A-K) and *fim2* (2A-2K) as cloned into pJTOOL-7**




| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **% DNA identity Blastn** | 79 | 70 | 76 | 74 | 70 | 70 | 72 | 71 |
| **% Amino acid similarity Blastp** | 92 | 74 | 91 | 84 | 70 | 68 | 61 | 54 |
| **Gene size (fim/fim2)** | 549/ 549 | 528/ 531 | 687/ 630 | 2613/2559 | 531/ 528 | 501/ 504 | 906/ 927 | 858/ 1413 |

Level of nucleotide variation between *fim* and *fim2* ranged from 70% to 79% similarity while variation at the amino acid level ranged from an identity of 61% to 92%. Sequence comparison showed *fimA* and the corresponding *fim2A* to be most similar at nucleotide and amino acid levels and highest dissimilar gene (apart from *fimK* and *fim2K*) is the adhesin coding for *fimH* and the *fim2H* homolog. Therefore, the genes chosen for hybrid creation were *fimA* and *fimH*, (with the highest and lowest level of similarity) and complemented with the corresponding *fim2* homologs.

Through comparative sequence analysis, it was observed that the level of nucleotide variation between *fim* and *fim2* was spread among the genes ranging from 70% to 79% similarity. At the amino acid level, the level of variation between *fim* and *fim2* proteins was seen to be more diverse, ranging from an amino acid identity of 61% to 92% (Figure 3-9). In depth comparison of the genes showed that variation at amino acid level between *fim* and *fim2* was usually found within first five to twelve amino acids at the N-terminal ends of matching proteins (data not shown). This level of variation at amino acid level is also spread among the fimbrial proteins.

Sequence comparison also showed that *fimA* and its corresponding *fim2A* have the highest nucleotide similarity at (79%) as well as highest level of identity at the amino acid level (92%). The most variant gene at nucleotide

level (apart from *fimK* and *fim2K* which may function as regulatory proteins) is the adhesin coding *fimH* and its *fim2* homologue, *fim2H*. As such the *fim* and *fim2* homologues chosen for construction of *fim-fim2* hybrids were identified as *fimA* and *fim2A* as well as *fimH* and *fim2H*, which showed highest and lowest levels of similarity, respectively. Moreover, FimA and FimH are known to comprise vital structural subunits of the pilus. The FimA protein forms the major subunit of the pilus while FimH forms the adhesion unit of the pilus, which attaches to epithelial surfaces and is also most prone to pathoadaptive evolution (Stahlhut, Chattopadhyay et al., 2009).

Previous research on the creation of a hybrid *K. pneumoniae/E. coli fim* operon showed that FimA may be the most important fimbrial component in determining the sugar-binding specificities of the fimbriae, not FimH as previously assumed(Madison, Ofek et al., 1994). The creation of *fim-fim2* hybrid is also significant as this method can be applied to other genes in these operons. This may in turn, shed more insight on *fim2* function and structure and also provide more understanding of the *K. pneumoniae fim* operon.

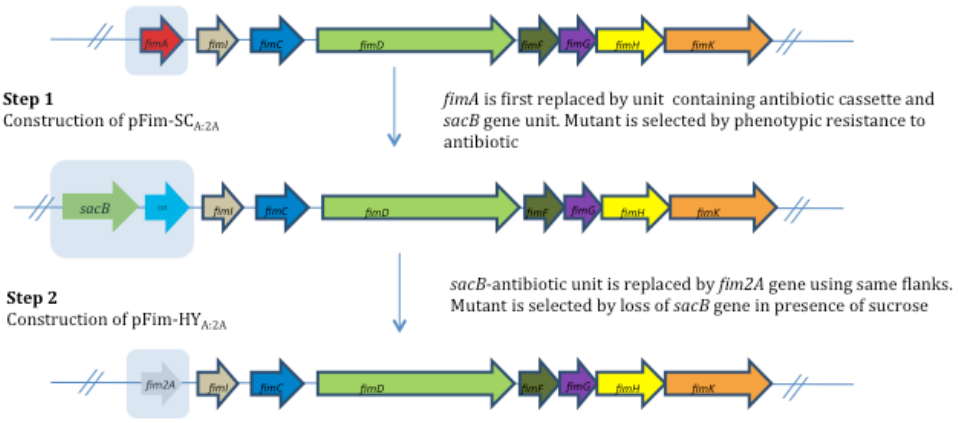
## 3.4.3 Creation of hybrid fimbriae

### 3.4.3.1 Creation of hybrid *fim-fim2* operon via lambda red based method

Lambda Red recombination-based allelic exchange is dependent on the increased rates of recombination seen when the phage λ Red recombinase system is overexpressed in bacteria. Three phage-derived lambda Red proteins are vital for carrying out double stranded DNA recombination, (*γ, β, exo*). *γ* prevents the degradation of linear dsDNA nucleases while lambda exonuclease (*exo)* degrades dsDNA in a 5′ to 3′ manner and *β,* binds to the single-stranded regions produced by *exo* and promotes recombination by promoting annealing to the homologous genomic target site (Mosberg, Lajoie et al., 2010).

To create a hybrid *fim-fim2* operon cloned into the pJTOOL-7 expression vector, the lambda Red allelic exchange method was employed to swap the target gene with a *cat-sacB* unit amplified from the plasmid pJTOOL-3; the former encodes chloramphenicol resistance and the latter, a levansucrase precursor, respectively (Figure 3-10). This precursor converts the available sucrose to levan, which is lethal to Gram-negative bacteria thereby providing a method of counter selection. Hence, the target gene on the expression plasmid was replaced with a required *cat-sacB* unit previously amplified from the plasmid backbone of pJTOOL-3. The *cat-sacB* cassette was then replaced via sucrose counter selection with new gene of interest.

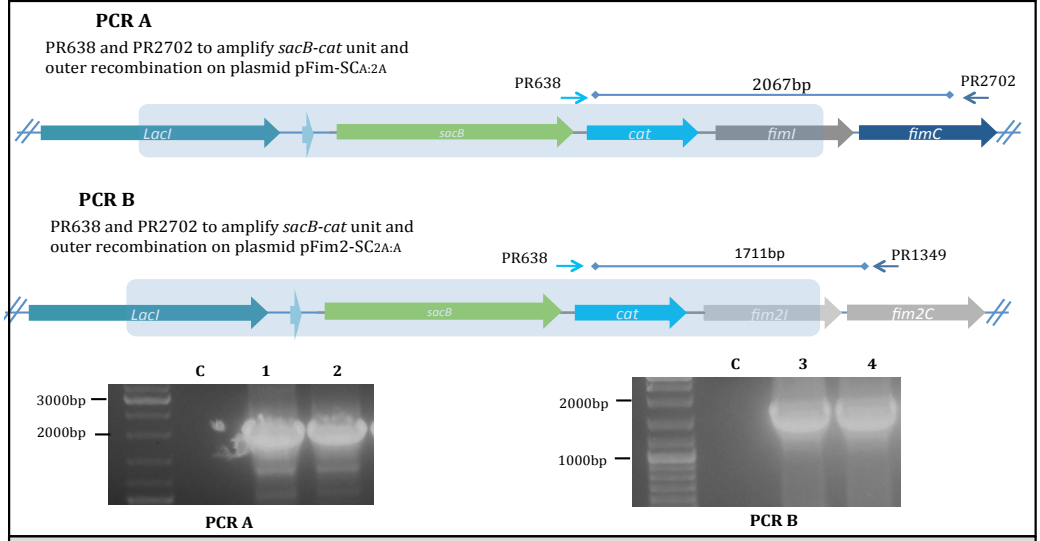
**Figure 3-10. Creation of hybrid *fim-fim2* coding fimbriae via Lambda red recombination**




Schematic representation of hybrid *fim-fim2* on such that *fimA* is replaced with *fim2A* on *fim* operon in HB101/ pFim-Ptrc to form HB101/pFim-HY$_{A:2A}$. Lambda red recombination enhances homologous recombination between identical left and right flanks. Region of recombination is highlighted in blue. Same method and principles was applied to *fim2* transcomplementation.

Creation of *fim-fim2* hybrid began with the replacement of *fimA* with *fim2A* on the *fim* operon, and replacement of *fim2A* with *fimA* on the *fim2* operon. The intermediate plasmids, pFim-SC bearing the *cat-sacB* cassette in place of *fimA* and *fim2A* were created and confirmed by PCR shown in (Figure 3-11).

**Figure 3-11. PCR confirmation showing creation of intermediate plasmid where *fimA* and *fim2A* have been replaced with *cat-sacB* unit**




Mutants were confirmed by patching and PCR screening across *cat* and region outside recombination. 2 colonies were screened to confirm replacement of *fimA* with *sacB-cat* on pFim-Ptrc, (PCR A, colonies 1 and 2) and 2 other colonies were screened to confirm replacement of *fim2* with *sacB-cat* on pFim2-Ptrc (PCR B, colonies 3 and 4).

However, the second step in replacing *cat-sacB* unit with the *fimA* and *fim2A* proved unsuccessful. While colonies obtained showed the desired phenotypic traits such as growth in sucrose and ampicillin and sensitivity to chloramphenicol, PCR confirmation of these colonies here inconclusive and proved negative for the desired gene replacement (PCR results are not shown).

### 3.4.3.2 Efficiency of lambda red system to create hybrid *fim-fim2*

The lambda Red recombination technique is widely utilised in many recombination experiments (Huang, Lam et al., 2014, Lesic and Rahme, 2008, Murphy and Campellone, 2003). While the efficiency of this system may differ according to factors such as choice of bacteria strain and plasmids, method of transformation, competency of cells may influence this efficiency of this system, the efficiency of this method in the creation of the hybrid *fim-*

*fim2* operon was not certain and was suggested as a potential cause in the unsuccessful hybrid creation. This was analysed with a mathematical estimation of the efficiency of this system in this experiment. To quantify the efficiency of the lambda Red recombination, equal volume of transformation was plated onto non-selective ampicillin (selects for plasmid background) and selective chloramphenicol (selects for plasmid carrying *cat* through recombination) plates separately. The ratio of number of bacterial colonies that harboured the required intermediate plasmids was estimated by the number of colonies on chloramphenicol plates divided by the number of colonies on ampicillin. This ratio was calculated as approximately 1 in 100, which may be regarded as a sufficient level of efficiency for the recombination experiments that had been performed.

The plasmid used in this experiment, pJTOOL-7 is regarded as a medium copy number vector. While the previous efficiency calculation provides an estimate of how many colonies could be successfully transformed, the proportion of plasmids that have undergone recombination is unknown as several plasmids (parent and recombined) could be carried within the bacteria cells. In some other studies, it was shown that re-transformation of bacterial with a much lower concentration of extracted plasmids by transformation were more likely to produce colonies that carried one plasmid per cell than cells transformed by electroporation (Goldsmith, Kiss et al., 2007). In order to estimate proportion of plasmids that have undergone allelic exchange in one transformation (assuming one plasmid is transformed into one cell that develops into a colony), plasmids were extracted from

transformation suspension and re-transformed into fresh competent *E. coli* cells and selected as previously described. In this experiment ratio of plasmids transformed was determined as approximately 1/300. Moreover, a proportion of the colonies grown on ampicillin were patched unto chloramphenicol plates. Out of 450-patched colonies, only 2 were able to grow on chloramphenicol. These suggest that the lack of success in *fim-fim2* hybrid creation may be as due to other factors and not necessarily the efficiency of the lambda red system in this experiment.

#### 3.4.3.3 Discussion

While the efficiency of the lambda red method may not be the cause of failed trans-complementation in this experiment, the creation of the intermediate pFim-SC plasmids suggest the issue lies with the counter selection of intermediate plasmid and replacement genes with *sacB*, which produced many false positive transformants. Although *sacB* gene has been widely used as a selective and counter selective tool in genetic modifications (Li, Thomason et al., 2013, Reyrat, Pelicic et al., 1998, Li, Heine et al., 2013), other researches have highlighted several issues with *sacB* gene. Other bacterial mutagenesis based researches have observed counter-selection inefficiency with the use of *sacB* commonly producing false positives as observed in this project (Raschdorf, Plitzko et al., 2014, Wang, Bian et al., 2014). It has been suggested that the observation of a high proportion of false positive colonies during *sacB* counter-selection is likely due to the rapid spontaneous gene inactivation in the presence of selective pressure and unstable *sacB* expression (Raschdorf, Plitzko et al., 2014, Scheffel, Gardes et al., 2008). It is

therefore likely that the failure of hybrid *fim-fim2* creation in this project is as a result of the complexities of *sacB* gene expression. Moreover, the presence of many false positive results requires laborious and cumbersome replica platings and PCR tests which is not effective and resourceful.

While the use of *sacB* gene as a counter-selection tool may pose possible underlying issues, previous work on positive and negative selection in *E. coli* showed that the *tetA-sacB* unit was 4 times more effective than *cat-sacB* for selection and counter-selection, possibly because *sacB* expression was higher in *tetA-sacB* cassette. Moreover, the added toxicity of the *tetA* gene in the presence of fusaric acid added to the sucrose medium allows for stronger counter selection (Li, Thomason et al., 2013). Therefore, the use of *cat* as a selection tool may have also reduced the efficiency of the markerless hybrid creation by lambda Red recombination.

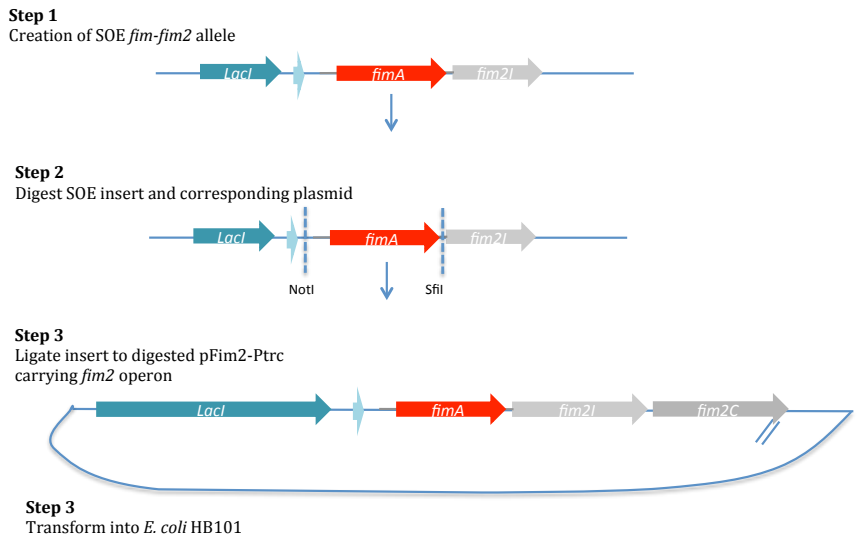
Apart from the *sacB* gene, other experimental factors could affect the creation of hybrids and the efficiency of the lambda red method in this work. For instance, the age of competent cells used in transformation was identified as a major factor in experiment. While freshly made competent cells were used for transformation efficiency estimation, repetitions of transformation in order to create several hybrids meant that some competent cells had to be made in bulk and stored for later use. It was observed that the ratio of bacteria colonies that carried recombinant plasmids dropped from 1/100 (as previously estimated) to about 1/200 with cells that were about 2 and half weeks old.

### 3.4.4 Creation of hybrid *fim-fim2* by digestion and ligation of SOE allele and plasmid

As discussed earlier, the lambda red recombination techniques was unsuccessful in the creating the hybrid *fim-fim2* and therefore a different approach was employed instead. In this method, linear DNA allele containing the mutant gene of interest is created with left and right flanks containing restriction sites that are also present on the target plasmid.

This allele is created by spliced overlap extension PCR so that the "gene of interest" is flanked on the left and right by sequences identical to that of the target plasmid. The restriction sites are chosen based on the vector of interest such that digestion completely cuts out the "target gene". Any extra flanks digested outside the "target sequence" is replaced with flanks from the SOE product. The created SOE allele and the target plasmid are then digested with the restriction endonucleases. Following the extraction and purification of the digested DNA, the allele and digested plasmid are ligated overnight and transformed into *E. coli* DH5α. The obtained colonies are screened by PCR for the new mutant gene in the correct position and plasmid extracted from a positive tested colony is re-transformed into *E. coli* HB101. (Figure 3-12).

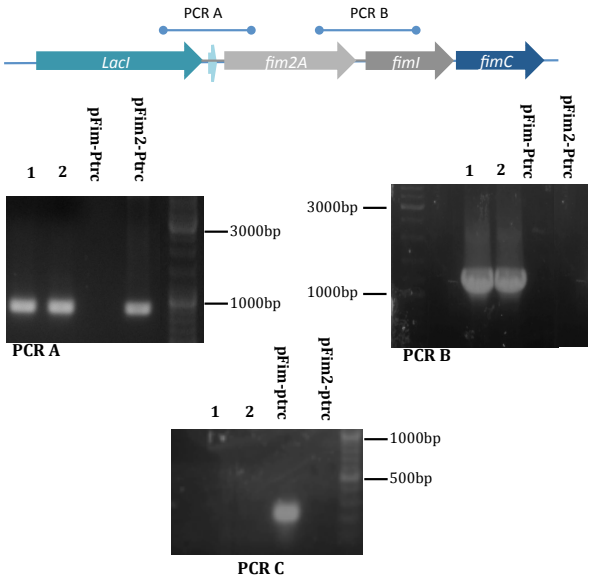
**Figure 3-12 Creation of hybrid *fim-fim2* coding fimbriae via ligation**




A schematic representation of method used to construct *fim-fim2* hybrids using creation of HB101/pFim2-$HY_{2A:A}$ as an example. SOE construct contains left and right homologues flanks flanks as well as restriction sites that are in exact match to plasmid pFim2-Ptrc. Digested allele and plasmid are ligated and electro transformed into competent HB101.

To confirm the creation of the hybrid operon, PCR screening was utilized to amplify loss or presence of target and new genes respectively. PCR amplification of replacement genes and flanks further away from cloning region also confirmed the creation of intended hybrid *fim-fim2*. Following sequence comparisons of *fim* and *fim2* operons, *fimA* was chosen as a suitable gene to be replaced *fim2A,* while *fim2A* in the *fim2* operon is replaced with *fimA*. The plasmid carrying hybrid *fim* operon with *fimA* replaced by *fim2A* is dubbed pFim-$HY_{A:2A}$ while plasmid carrying hybrid *fim2* operon with *fim2A* replaced by *fimA* is dubbed pFim2-$HY_{2A:A}$ (Figure 13A-B).

**Figure 3-13A. Confirmation of hybrid *fim-fim2* operon – pFim-HY$_{A:2A}$**



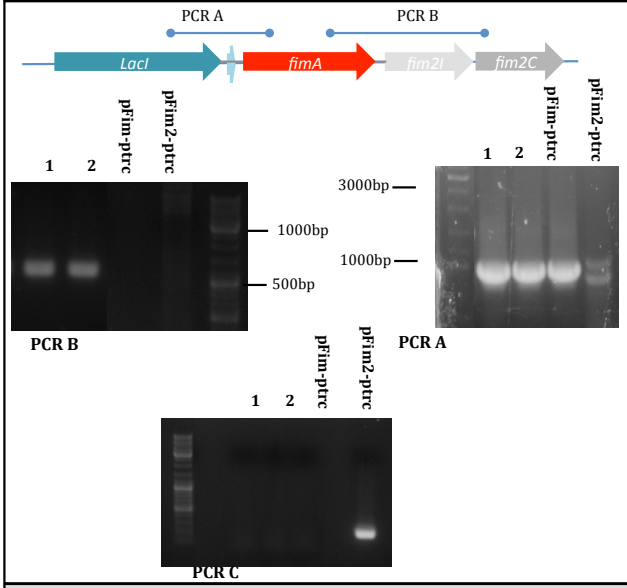

**PCR A**
-Primers PR2789 and PR1608 (0.8311kb)
-Confirms the correct genomic location of *fim2A*

**PCR B**
-Primers PR1607 and PR2702 (1.14kb)
-Confirms the correct genomic location of *fim2A*

**PCR C**
-Primers PR1704 and PR1705 (0.221kb)
-Confirms the loss of *fimA* in pFim-Ptrc

Colonies 1 and 2 putatively bearing pFim-HY$_{A:2A}$ were screened using PCR to confirm their expected results. As expected, HB101/pFim-HY$_{A:2A}$ possessed the mutant *fim2A* gene in the correct genomic location as seen in PCR A and PCR B. Moreover, the native *fimA* gene was lost as seen in PCR C. Although no negative controls (without template) have been shown, these were performed and were negative.

**Figure 3-13B. Confirmation of hybrid *fim-fim2* plasmids – pFim2-HY$_{2A:A}$**




**PCR A**
-Primers PR2789 and PR1704 (0.853kb)
-Confirms the correct genomic location of *fim2A*

**PCR B**
Primers PR1703 and PR1349 (1.294kb)
-Confirms the correct genomic location of *fimA*

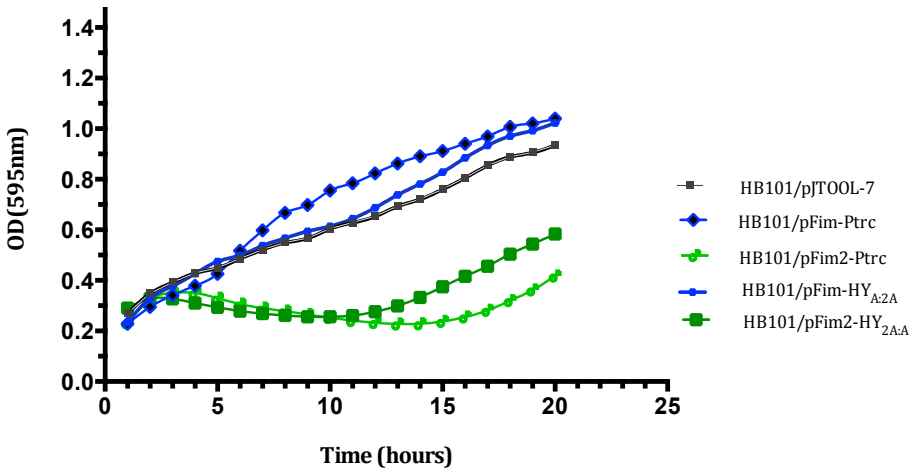
**PCR C**
-Primers PR1607 and PR1608 (0.233kb)
-Confirms the loss of *fim2A* in pFim2-ptrc

Colonies 1 and 2 putatively bearing pFim2-HY$_{2A:A}$ were screened using PCR to confirm their expected results. As expected, HB101/pFim-HY$_{2A:A}$ possessed the mutant *fimA* gene in the correct genomic location as seen in PCR A and PCR B. Moreover, the native *fim2A* gene was lost as seen in PCR C. Although no negative controls (without template) have been shown, these were performed and were negative.

### 3.4.5 Effect of IPTG-induction of hybrid *fim-fim2* on growth of HB101

As previously mentioned, previous investigation of HB101/pFim-Ptrc and HB101/pFim2-Ptrc showed that IPTG induction of pFim2-Ptrc resulted in induction-associated growth reduction in HB101, which was the opposite result with induction of pFim-Ptrc. As the first gene at the start of transcription during the induced expression of *fim2*, it is possible that the expression of *fim2A* within *fim2* operon is a factor to the growth retardation of HB101. The hybrid fimbriae clones HB101/pFim-$HY_{A:2A}$ and HB101/pFim2-$HY_{2A:A}$ together with original plasmids were analysed in a growth curve following IPTG induction. Results (Figure 3-14) showed a similar increase in HB101 growth rate following the over expression of pFim-Ptrc and pFim-$HY_{A:2A}$. Induction of pFim2-$HY_{2A:A}$ showed similar growth reduction as observed when pFim2-Ptrc is expressed in HB101 (Figure 3-14).

**Figure 3-14. IPTG-induction of hybrids fimbriae on growth of HB101**

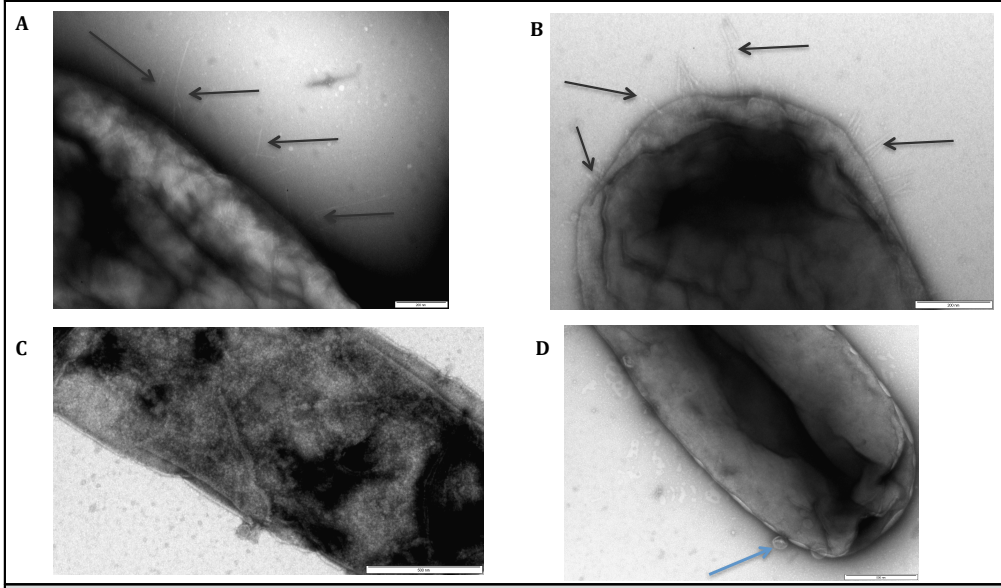



Growth curves for HB101/pJTOOL-7 (empty vector), HB101/pFim-Ptrc, HB101/pFim2-Ptrc, HB101/pFim-$HY_{A:2A}$ and HB101/pFim2-$HY_{2A:A}$. Growth curves for HB101 and transformants were grown for 24 h in LB broth containing 100 µg/ml ampicillin supplemented with or 0.1 mM IPTG.

### 3.4.6 Transmission Electron Microscopy of hybrid fimbriae

Hybrid fimbriae constructs HB101/pFim-$HY_{A:2A}$ and HB101/pFim2-$HY_{2A:A}$ were examined by transmission electron microscopy in order to visualise any potential fimbriae produced by these hybrid clones while HB101/pFim-Ptrc and HB101/pFim2-Ptrc were used as positive and negative controls respectively in this assay. The fimbrial operons cloned under the IPTG-inducible $P_{TRC}$ promoter had their expression induced with 0.1mM IPTG. As expected, HB101/pFim-Ptrc produced visible fimbriae, which was similar to fimbriae produced by hybrid HB101/pFim-$HY_{A:2A}$ (Figure 3-15A-B). Hybrid clone HB101/pFim2-$HY_{2A:A}$ also behaved similar to control HB101/pFim2-Ptrc by not producing distinct visible fimbriae (Figure 3-15 C-D).

**Figure 3-15. Visualisation of hybrid *fim-fim2* clones by electron microscopy**

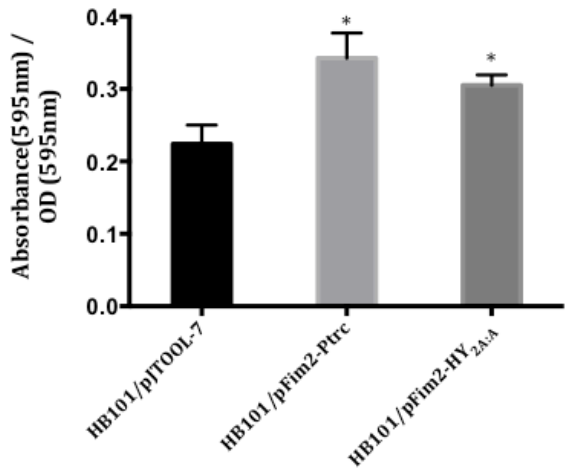



Transmission electron micrographs of (**A**) HB101/pFim-Ptrc, (**B**) HB101/pFim-$HY_{A:2A}$ (**C**) HB101/pFim2-Ptrc and (**D**) HB101/pFim2-$HY_{2A:A}$. Arrows highlight fimbrial structures observed in pFim-Ptrc and pFim-$HY_{A:2A}$. No fimbriation was observed in pFim2-Ptrc and pFim2-$HY_{2A:A}$ except for 'blobs' observed on surface of some of the cells (highlighted with blue arrow).

### 3.4.7 Effect of IPTG induced expression of hybrids on biofilm formation

It was previously observed that pFim2-Ptrc induction in HB101 might contribute to increased biofilm formation(Van Aartsen, Stahlhut et al., 2012) under conditions of 48 h growth at 37°C (Van Aartsen, Stahlhut et al., 2012). To examine if the biofilm contribution is applicable to the hybrid pFim2-$HY_{2A:A}$ clones, a biofilm assay was done under the same conditions. Results showed that cells harbouring pFim2-Ptrc or pFim2-$HY_{2A:A}$ exhibited similar enhanced biofilm-forming abilities as compared to the vector only control (Figure 3-16).

**Figure 3-16. Effect of IPTG induced expression of hybrids on biofilm formation**




Biofilm formation assay results shown were normalized to take account of pre-wash total cell numbers based on OD595 readings performed at 48 h, just prior to washing off non-surface adherent cells and crystal violet staining. Data shown in all cases represent means and standard deviations of three biological replicates, each assayed in eight wells (n = 24). An asterisk indicates a highly significant difference ($P < 0.0005$) from HB101/ pJTOOL-7. Statistical analyses were performed using the non-parametric Kruskal-Wallis test.

### 3.4.8 Characterisation of mannose binding specificities of *fim* and *fim2* hybrids

The hemagglutinating activities of the *E. coli* HB101 transformants were examined using fresh guinea pig erythrocytes to check for a mannose sensitive agglutination as seen in *fim* operon (Struve, Bojer et al., 2008). The induction of pFim-HY conferred agglutination phenotype while pFim2-$HY_{2A:A}$ did not show any agglutination of guinea pig red blood cells as seen in pFim2-Ptrc (Table 3-1).

In order to obtain further insight into the boundaries and possibilities of fimbriae phenotype of *fim2* and *fim-fim2* hybrids, heamaglutination assay was repeated on other variations of the *fim-fim2* hybrids. Since the *fimH* is known to be subunit at the tip of the fimbriae responsible for heamaglutination, a *fimH:fim2H* hybrid was created to observe if the *fim2H* phenotype may produce similar agglutination in *fim*. As previously mentioned, sequence comparisons of *fim* and *fim2* showed lowest level of similarity (apart from *fimK* and *fim2K*) between *fimH* and *fim2H.*

These other *fim-fim2* hybrids were also created using same method. In these hybrids, *fimH* on pFim-Ptrc was replaced with *fim2H* and resulting plasmid was named pFim-$HY_{H:2H}$. In addition, *fimH* on pFim-$HY_{A:2A}$ was also replaced with *fim2H* and resulting hybrid was named $pHYA_{H:2H}$ (Table 3-1).

**Table 3-1 Agglutination patterns of bacterial strains**

| Strain | GP-RBCs | GP-RBCs + mannose |
|---|---|---|
| HB101/pFim-Ptrc | + | - |
| HB101/pFim2-Ptrc | - | - |
| HB101/pFim-$HY_{A:2A}$ | + | - |
| HB101/pFim2-$HY_{2A:A}$ | - | - |
| HB101/pFim-$HY_{H:2H}$ | - | - |
| HB101/p$HYA_{H:2H}$ | - | - |

In this table, **+** shows a positive agglutination of guinea pig red blood cells by transformed *E. coli,* while **–** shows a lack of agglutination of guinea pig erythrocytes by transformed strains.

It was speculated that *fim2H* might have similar adhesion properties to *fimH* and cause agglutination of guinea pig erythrocytes. This hypothesis was disproved by lack of haeammaglutination of GP-RBCs when blood is mixed with HB101/pFim-$HY_{H:2H}$ and HB101/p$HYA_{H:2H}$.

### 3.4.9 Discussion

The adhesion and colonisation to host cells is frequently mediated by a diverse range of afimbrial and fimbrial adhesins, each thought to be specific for certain tissue types (Pizarro-Cerda and Cossart, 2006). Many *K. pneumoniae* strains are able to produce type 1 fimbriae (Podschun, Pietsch et al., 2001, Struve and Krogfelt, 2004, Podschun and Sahly, 1991), which are associated with mannose-sensitive agglutination of GP-RBCs. This phenotype is caused by the interaction of adhesion subunit FimH with terminally exposed mannose residues in N-linked oligosaccharides on cell surface (Stahlhut, Tchesnokova et al., 2009).

Previously it has been shown that the mono- and tri-mannose residues in FimH are highly conserved (Stahlhut, Tchesnokova et al., 2009) and although *E. coli* HB101 heterologously expressing the *fim* operon could mediate GP-RBC agglutination, it was shown that HB101 expressing the *fim2* operon could not(Van Aartsen, Stahlhut et al., 2012). As well as its inability to mediate GP-RBC agglutination, HB101/pFim2-Ptrc did not produce any visualisable fimbriae.

In this study, the flexibility of *fim* and *fim2* structures by systematic exchange in either direction of genes coding for the structural components of the operons was explored. It was observed that that *fimA* and its *fim2* homolog *fim2A* are functionally interchangeable. HB101 heterologously expressing hybrid *fim* based operon, fim-$HY_{A:2A}$ produced similar phenotype to HB101 expressing *fim.* Both operons when heterologously expressed in HB101 appeared to enhance its growth rate and were both visible under an electron microscope. The growth retardation observed in hybrid *fim2* based operon, *fim2-*$HY_{2A:A}$ is similar to the growth defect caused by expression of *fim2* and demonstrates that *fim2* operon may have potential to form fimbriae as *fim2A* is able to produce visualisable fimbriae in *fim-*$HY_{A:2A}$.

The hybrid clone HB101/pFim2-$HY_{2A:A}$ also behaved similarly to control HB101/pFim2-Ptrc by not producing distinct visible fimbriae. However, the IPTG dependent induction of *fim2* and *fim2*-$HY_{2A:A}$ appears to have a strange and unexpected effect on some of the HB101 cells. While a few HB101/pFim2-$HY_{2A:A}$ cells showed rare potential fimbrial-like structures as

previously observed (Van Aartsen, Stahlhut et al., 2012) "blobs" found at the outer membrane of the HB101 cells carrying pFim2-$HY_{2A:A}$ were much more consistently observed. See blue arrow in Figure 3-16D. By contrast, HB101/pFim2-$HY_{2A:A}$ cells were observed to be completely bald with no IPTG induction. Moreover, these "blobs" were infrequently observed in HB101/pFim2-Ptrc cells under IPTG induction. While it is unclear exactly what these "blobs" are, its is obvious that the IPTG induction of pFim2-HY has an effect on HB101 cells which may be some form of increased exocytocis of accumulated DNA proteins or substances within the cells (Figure 3-16C-D).

While heamagglutination experiments confirmed previous results of *fimA* and *fim2A* (Table 3-1), hybrid *fim* operon expressing *fim2H* in place of *fimH* did not produce mannose-sensitive agglutination of GP-RBCs. Based on a previously noted absence of visualised Fim2 in HB101/pFim2-Ptrc despite several induction methods and replacement of *fimA* with *fim2A*, hybrid replacement of *fim2H* with *fimH* was not constructed. Another hybrid type 1 fimbriae was genetically created by co-expressing genes encoding the fimbrial shaft *fim2A* from *fim2* and genes encoding minor subunit adhesion *fim2H*. Due to the inability of the *fim2H* hybrids to cause agglutination in GP-RBCs, it was now demonstrated that *fimH* and *fim2H* do not exhibit similar phenotypic functions and characteristics, in contrast to the functional inter-changeability of *fimA* and *fim2A*. The specific binding properties of Fim2H, the putative Fim2 adhesin, is yet be identified but it is unlikely to bind mannose residues as it possesses only four out of 14 mono- and tri-mannose

binding residues present in FimH (Aartsen, 2011, Van Aartsen, Stahlhut et al., 2012).

## 3.5 Future work

While several experiments and analysis were done in order to further characterise *fim2*, more studies is needed to resolve the *fim2* question.

Firstly, it is vital to determine other conditions that could increase the transcription and expression of *fim2*. This can be done by transcriptional analysis using qRT-PCR techniques or a standard β-galactosidase activity assay using *lac* operon fused with *fim2*. These techniques can be used to evaluate which other *in vitro* conditions such as pH, varying temperature, NaCl concentration, oxygen concentration, glucose concentration and others.

Analysis of fimbrial expression under several conditions *in vivo* could also be used in understanding expression of *fim2*. While an example of this was previously done (Aartsen, 2011), other studies evaluating on and off expression of *fim* in *K. pneumoniae* KR116 hinted that fimbrial switch is found in either both ON and OFF orientations or mostly OFF orientations within *G. mellonella* larvae (Hussein, ) Expression analysis of *fim2* following their infection of *G. mellonella* larvae could also be useful. RT-PCR of *fim2* genes collected from bacteria obtained from homogenisation of infected larvae would be useful in building an expression profile of *fim2*. Also, *in vivo* competition experiments with *G. mellonella* larvae could be repeated with much larger numbers of larvae and experimental repeats which would

improve statistical analyses of winning population during infection of *G. mellonella.*

In this chapter, the hybrid *fim-fim2* fimbriae that were created demonstrated the potential of *fim2* to form fimbrial structures. Due to time constraints, experimental assays that could be used to evaluate these hybrids were limited. However, further investigation of hybrid in HB101/pHYA$_{H:2H}$ (HB101 bearing *fim* replaced with *fim2A* and *fim2H*) would be interesting to observe. Experimental assays performed in this project such as growth assay, biofilm formation assay, transmission electron microscopy as well as heamagglutination assays using other substrates such as horse RBCs, sheep RBCs, yeast and others could prove valuable in understanding *fim2* function.

Following the success of *fim-fim2* hybrid creation in this thesis, other *fim* genes and their *fim2* homologs could be targeted for hybrid *fim-fim2* creation. Interesting targets would be genes coding for chaperon-usher system in these operons. As seen in this project, *fim2A* and *fimA* bear similar sequence and function, lack of fimbriation of *fim2* may be due to the assembly process. Moreover, some of the *fim2* harbouring strains have been found to contain IS-element within their potential usher genes (Aartsen, 2011), which may affect the assembly process. Also, immune gold labelling may be a more powerful tool in viualisation of hybrid fimbriae so as to observe structural differences of fimbriae formed by *fim*, *fim2 or fim-fim2* hybrids.

# Chapter 4. Mapping of novel *fim3* operon and identification of *fim3*-habouring *Klebsiella* strains

## 4.1 Introduction

Following the discovery of *fim2* in *K. pneumoniae* KR116, an investigation was done into the prevalence of *fim2* within the *Klebsiella* genus (Van Aartsen, Stahlhut et al., 2012). This involved PCR screening for specific *fim2* genes as well as confirming the presence of a typical type 1 *fim* operon normally detected in most *Klebsiella* species. Although the information on the genetic composition and physical characteristics of fimbrial systems in some other *Klebsiella* species is limited, it is generally assumed that among the most clinically prevalent *Klebsiella* species such as *K. pneumoniae* and *K. oxytoca,* core genes of the *fim* operon would be conserved. Recent sequencing of the complete *K. oxytoca* genome validates this assumption (Liao, Lin et al., 2012, Shin, Kim et al., 2012).

Hence, PCR screening of *Klebsiella* isolates for *fim* and *fim2* was also followed by tests to confirm the identity of the isolate as *K. pneumoniae* or some other *Klebsiella* species. Screening for *fim2* positive isolates was done using primers known to amplify the following *fim2* genes: *fim2K, fim2D, fim2A, fim2H* and *fim2C.* Screening for *fim* was done using primers to amplify *fimB, fimK* and *fimH*. Biochemical tests of the strains together with PCR amplification of specific genes were techniques used for species identification (Aartsen, 2011).

Out of 21 strains positive for *fim2*, three clinical *Klebsiella* isolates were unique (Aartsen, 2011). These 3 strains sp15, sp25 and sp28 did not generate any amplicons during PCR screening for *fimB* and *fimK.* A very faint band for *fimH* was observed following various methods to optimise the PCR reaction. The faint amplicon was sequenced revealing an allele of *fimH* containing several nucleotide substitutions. This along with the negative results for *fimB* and *fimK* lead to two suggestions, either that these strains are of a different *Klebsiella* species that are still unsequenced and uncharacterised or that a new *fim*-like operon may have been discovered which was dubbed *fim3*. This is the aim of this section of my PhD project.

This chapter details on how this novel variant of *fim* was mapped and sequenced and also discusses sequence analysis and genetic profile of the *fim3* operon in *Klebsiella*. In addition, this chapter reports on the species identification of the *fim3*-bearing *Klebsiella* strains sp15, sp25 and sp28 and explores issues concerning species identification in the *Klebsiella* genus.

## 4.2 PCR mapping of *fim3* operon

In order to map and sequence the postulated entire *fim3* operon that was harboured by *Klebsiella* strains sp15, sp25 and sp28, PCR screening for the presence or absence of other *fim* genes was done. Analysis of the previously obtained *fim3H*-related sequence data showed that while there are several nucleotide substitutions, the *fim3H* sequence bears a 95% level of similarity the typical *fimH* sequence. Therefore, a different set of primers that bind

specifically to conserved regions of *fimH* and *fim3H* would be expected to amplify both *fimH* and *fim3H* (Figure 4-1).

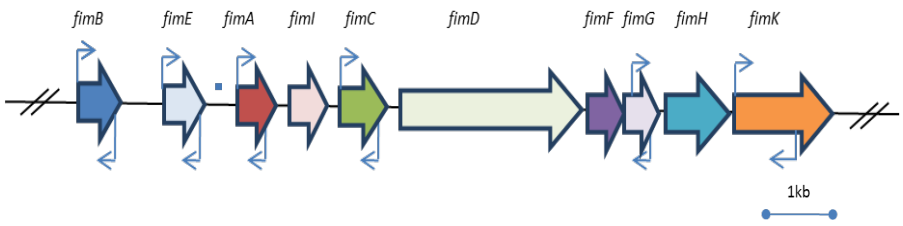
**Figure 4-1. Sequence alignment of *fim3H* and *fimH***


Multiple sequence alignment of *fim3H* from *K. pneumoniae* sp28 and *fimH* from *K. pneumoniae* MGH57857. Sequence alignment shows several nucleotide variations of these two genes, including an insertion of 3 nucleotides (amino acid – Leucine) at the leader peptide domain region of *fimH/fim3H.*

Primers used in screening for other *fim* genes were designed following multiple sequence alignment of all *Klebsiella fim* sequences in NCBI database. Alignment of these sequences showed conserved regions within *fim* genes and primers were designed to bind to these regions. Genomic sequences of DNA flanking *Klebsiella fim* operon were also aligned so as to determine if *fim3* lies within the same locus as the *fim* or found in a different context.

Figure 4-2 shows a schematic position of primers designed to amplify the *fim* operon using *K. pneumoniae* strain MGH78578 as a reference template. The table (Table 4-1) shows a summary of results obtained from PCR screening for typical *fim* genes among the three unique *Klebsiella* isolates.

**Figure 4-2. Primers designed for screening *fim* genes**




Schematic showing primers used to detect the presence or absence of typical *fim* genes within unique *Klebsiella* strains sp15, sp25 and sp28. The primers were designed following multiple sequence alignment of all available sequences of *fim* gens within the *Klebsiella* genus.

**Table 4-1: PCR screening results**

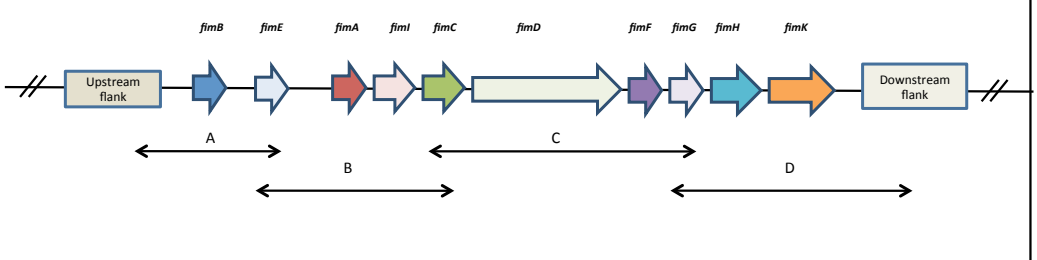
| ***fim* gene** | **sp15** | **sp25** | **sp28** | **MGH78578** |
|---|---|---|---|---|
| ***fimB*** | - | - | - | + |
| ***fimE*** | + | + | + | + |
| ***fimA*** | + | + | + | + |
| ***fimS*** | + | + | + | + |
| ***fimC*** | + | + | + | + |
| ***fimG*** | + | + | + | + |
| ***fimK*** | - | - | - | + |

As seen in table above, the results from PCR screening for *fim* genes among these *Klebsiella* strains are positive. Therefore, it was hypothesised that these *fim* genes, which may also be part of the *fim3* operon, are likely arranged in a similar syntenic orientation within these three strains, as one would observe in a typical *fim* operon of a *K. pneumoniae* strain. The genetic location of *fim3* operon was confirmed to be in the same locus as that of *fim* by PCR amplification using one primer specific to *fim3H* and another primer designed from multiple alignment of downstream flanking sequence of the *fim* operon (Result not shown).

Therefore, larger fragments of this operon were amplified using the previously designed primers, which were then sent for sequencing. The putative *fim3* operon was divided into four segments and sequenced to obtain overlapping sequence read from each fragment. Figure 4-3A shows a schematic of fragments used in PCR mapping of *fim3* operon using the *fim* sequence from *K. pneumonmiae* MGH78578 as a reference.

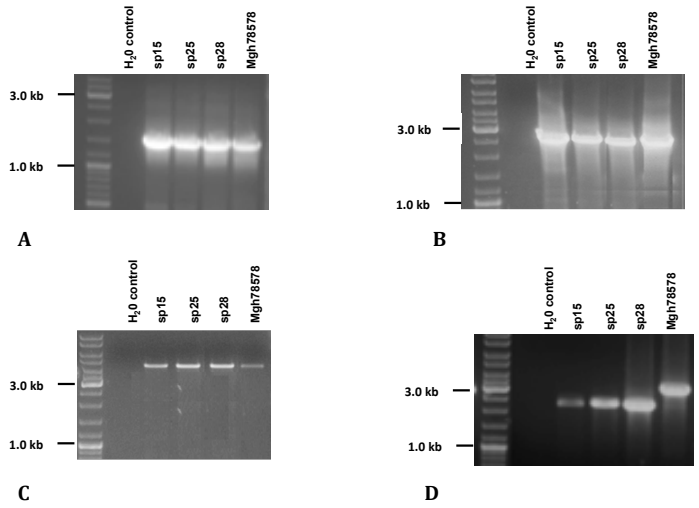
Based on the *fim* operon template, PCR fragment A was amplified from 120bp upstream of the *fim* locus to *fimE* while fragment B amplifies across *fimE* to *fimC*. Fragment C cuts across chaperon coding gene *fimC* to fimbriae structure subunit coding gene *fimG* while fragment D amplifies from *fimG* to 200bp downstream of the *fim* locus. Results obtained from PCR fragment A to C showed same size bands as in reference strain *K. pneumoniae* MGH78578 harbouring only *fim* operon. A notable difference was observed in PCR fragment D where obtained amplicons where estimated to be about 610 nucleotides shorter in size across the tested *fim3* strains (Figure 4-3B and Table 4-2).

**Figure 4-3A. Amplified fractions of putative *fim3* operon used in mapping**




Schematic diagram showing fractions of putative *fim3* to be amplified from *Klebsiella* strains sp15, sp25 and sp28 for sequencing and mapping of the operon. Overlapping sequences were read from each fragment using primers previously designed for *fim* screening.

**Figure 4-3B. Amplified fractions of putative *fim3* operon used in mapping**




Pictures of gel results from fragmented PCR amplifications. Fractions of putative *fim3* operon were amplified to be used for sequencing using *fim* operon *in K.pneumoniae* Mgh78578 as template and positive control. Figure 4.3A shows which gel result pictures of the putative operon corresponds to amplified fraction of putative *fim3* operon.

**Table 4-2: Mapping of putative *fim3* operon showing sizes of amplified fragments**

| PCR Fragment | Amplified region | sp15 | sp25 | sp28 | MGH78578 |
|---|---|---|---|---|---|
| **A** | Upstream flank - *fimE* | ~1.55kb | ~1.55kb | ~1.55kb | 1558bp |
| **B** | *fimE* - *fimC* | ~2.8kb | ~2.8kb | ~2.75kb | 2776bp |
| C | *fimC* - *fimG* | ~4.3kb | ~4.3kb | ~4.3kb | 4302bp |
| D | *fimG* – Downstream flank | ~2.25kb | ~2.25kb | ~2.25kb | 2755bp |

Analysis of the obtained sequences obtained from amplicons in Figure 4-3B and seen in Table 4-2 showed the presence of many of the *fim* related genes in the same order and approximately the same size including the inverted 9bp repeats that control the *fim* switch. However, a notable difference is observed in the presence of a truncated *fimK* type gene within the *fim3* operon.

## 4.3 Sequence analysis of *fim3* operon

Since all three *Klebsiella* strains showed identical results from PCR mapping, *K. pneumoniae* strain sp28 was arbitrally chosen as the reference strain for further investigation of *fim3*.

Sequence analysis of the *fim3* operon shows a high level of similarity to the *fim* operon in content, organisation and context but only 87 – 96 % DNA similarity based on analysis of matching pairs of genes. By contrast, all known *K. pneumoniae fim* sequences exhibit near identity at a DNA level, with the figures for individual genes ranging from 99 – 100 %. The presence of several nucleotide substitutions within *fim3* genes in comparison to *fim* was predicted to result in failed annealing by several primers, which were previously used to test for the presence of *fim* genes, thus explaining earlier PCR results.

The entire *fim3* operon showed highest nucleotide similarity of 93% to that of *K. variicola* At-22. n analysis of individual *fim3* genes showed that all of these genes (except for *fim3B, fim3A* and *fim3H)* to *fim* homologs in *K. variicola* At-22 and *K. pneumoniae* 342. The table below shows closest BLASTn homologs of *fim3* genes.

**Table 4-3: Closest BLASTn homologs of sequenced *fim3* genes in *Klebsiella* sp28**

| | | Closest BLASTn homolog | | |
|---|---|---|---|---|
| **Gene** | **Gene size (bp)** | **Percentage identity** | **Organism** | **E-value** |
| ***fim3B*** | 606 | 90 | *K. pneumoniae* KCTC 2242 | 0.0 |
| ***fim3E*** | 566 | 91 | *K. variicola* At-22 / *K. pneumoniae* 342 | 0.0 |
| ***fim3A*** | 549 | 93 | *Klebsiella pneumoniae* KCTC 2242 | 0.0 |
| ***fim3I*** | 528 | 93 | *Klebsiella variicola* At-22 *K. pneumoniae* 342/ | 0.0 |
| ***fim3C*** | 686 | 94 | *K. variicola* At-22/ *K. pneumoniae* 342 | 0.0 |
| ***fim3F*** | 530 | 94 | *K. variicola* At-22/ *K. pneumoniae* 342 | 0.0 |
| ***fim3G*** | 500 | 96 | *K. variicola* At-22/ *K. pneumoniae* 342 | 0.0 |
| ***fim3H*** | 905 | 95 | *K. pneumoniae* isolate 3861 *fimH* | 0.0 |
| ***fim3K*** | 646 | 87 | *K. variicola* At-22/ *K. pneumoniae* 342 | 0.0 |

Further comparative analysis of the *fim* operon distributed among annotated *Klebsiella* genome in the database highlighted a very high level of similarity among *Klebsiella pneumoniae* strains. At a nucleotide level, the *fim* genes show about a 99% identity when aligned with each other. *K. variicola* along with *K. pneumoniae* 342 were observed to be more diverse from other *K. pneumoniae* strains, but more closely related to each other.

In order to show the level of similarity of various type 1 *fim* across several strains and species, *fim* sequences of several strains were each compared to *fim* operon in *K. pneumoniae* MGH78578 using BLASTn. The resulting percentage value for similarity was recorded for each *fim* gene and recorded

in Table 4-4. This table produced a simple quantitative and qualitative matrix that showed level of similarity between the *fim* genes of several species. While most of the *K. pneumoniae* strains produced almost identical values that concur with high similarity to the reference strain, strains *K. pneumoniae* 342 and *K. variicola* At-22 while almost identical to each other showed lower similarity to the reference *K. pneumoniae* MGH5785 *fim*. The two recently sequenced *K. oxytoca* strains were also identical to each other and more dissimilar to the reference strain. Other bacteria belonging to other genus such as *Citrobacter rodentium* and *E. coli* produced even more dissimilar values. The *Klebsiella* strain sp28 was included in the table matrix and while sp28 did not show perfect identity to any of the known strains or groups, it was seen to be slightly more distinct when compared to the *K. pneumoniae* strains but more similar when compared to *K. oxytoca* or other species included.

**Table 4-4: Percentage identity of *fim* genes from selected bacterial strains compared to those of *K. pneumoniae* MGH78578**

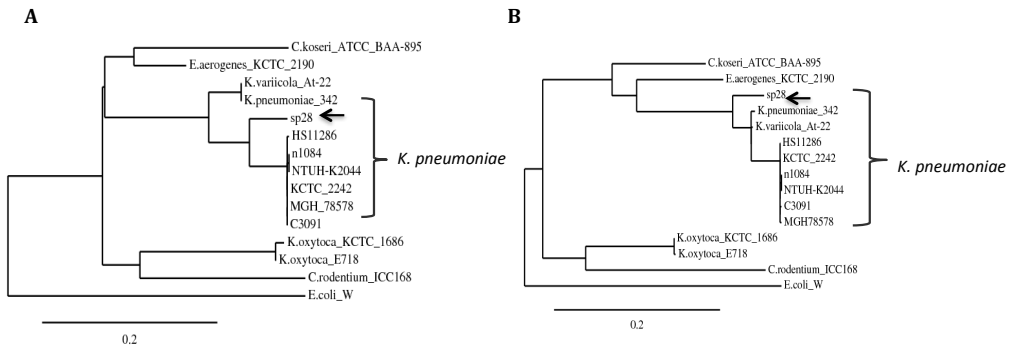
| | *Klebsiella* sp28 | *K. pneumoniae* NTUH-2044 | *K. pneumonia e* KCTC | *K. pneumoniae* HS111 | *K. pneumoniae* 1084 | *K. pneumoniae* 342 | *K. Variicola* At-22 | *K .oxytoca* kctc | *K. oxytoca* E718 | *Enterobacter aerogenes* | *Citbacter koseri* | *Citrobacter rodentium* | *E. coli* W |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *fimB* | 90 | 99 | 99 | 99 | 99 | 92 | 92 | N/A | N/A | 78 | N/A | 77 | 84 |
| *fimE* | 89 | 99 | 99 | 99 | 99 | 88 | 88 | N/A | N/A | 82 | N/A | 77 | 75 |
| *fimA* | 93 | 99 | 100 | 99 | 99 | 91 | 91 | 81 | 81 | 83 | 81 | 79 | 76 |
| *fimI* | 92 | 99 | 99 | 99 | 99 | 91 | 91 | 90 | 90 | 76 | 74 | 76 | 72 |
| *fimC* | 91 | 99 | 99 | 99 | 99 | 95 | 95 | 97 | 97 | 84 | 82 | 79 | 83 |
| *fimD* | 93 | 99 | 99 | 99 | 99 | 94 | 95 | 82 | 82 | 86 | 93 | 86 | 73 |
| *fimF* | 94 | 99 | 99 | 99 | 99 | 93 | 93 | 79 | 79 | 76 | 75 | 75 | 70 |
| *fimG* | 95 | 99 | 100 | 100 | 99 | 97 | 97 | 83 | 83 | 80 | 76 | 79 | 75 |
| *fimH* | 95 | 99 | 99 | 99 | 99 | 95 | 95 | 79 | 79 | N/A | N/A | N/A | 75 |
| *fimK* | 86 | 99 | 99 | 99 | 99 | 93 | 93 | 80 | 80 | 71 | 79 | 68 | N/A |

N/A (not applicable as gene homolog is absent)

The result from this analysis is somewhat concordant with phylogenetic analyses of *fim* genes done in this PhD project. Phylogenetic analysis of some of the core structural *fim* genes and its *fim3* counterpart were done to show the close relationship between *fim3* and *fim*. Phylogenetic analysis of *fim3* in relation to *fim* was done for all the genes within this cluster except for *fim3B, fim3E* and *fim3K*. Analysis of all these genes showed similar grouping of *fim3*

within *K. pneumoniae.* Figure 4-4 shows phylogenetic analysis of *fim3A* and *fim3C.*

**Figure 4-4. Phylogenetic analysis of *fim3* genes**




Phylogenetic trees of *fim3A* and *fimA* genes (A) and *fim3C* and *fimC* genes (B) of sp28 and other known species containing type 1 *fim* with *E. coli* as an out-group. *fim3* genes from sp28 are seen as a different branch within *K. pneumoniae* clade. Highlighted with direction arrow.

The phylogenetic analyses include multiple sequence alignment by MUSCLE and genetic distance calculated by Maximum Likelihood (ML). A well-defined group supported by high bootstrap values, was formed in the dendrogram recreated with these data. The dendograms in Figure 4-4 show relationship between *fim* and *fim3* genes and confirms *fim3* as a different branch within same cluster of *fim* from *K. pneumonaiae* (and *K. variicola*) strains. Phylogenetic analysis also confirms a higher similarity between *K. pneumoniae* 342 and *K. variicola* At-22*.* These phylogenetic trees also show the relatedness between *fim* operons of the annotated *K. pneumoniae* strains and *K. variicola,* also suggesting a closer relationship between *K. pneumoniae* 342 and *K. variicola At-22.*

While the high DNA similarity between matching *fim* and *fim3* genes suggests a similar function, amino acid sequence comparison show that some of the nucleotide difference (SNPs) result in non-synonymous mutations potentially impacting on the functional properties of the corresponding proteins. Figure 4-5 shows genetic sequence alignment of *fim3A* and *fimA*, as well as amino acid alignment of FimA and Fim3A.

**Figure 4-5. Sequence alignment of encoding fimbrial protein in *K. pneumoniae* sp28, 342 and MGH78578**


Sequence alignment shows that some of SNPs in *fim3A* code for synonymous mutations. Except for amino acid mutation at A107S (highlighted with directional arrow), nucleotide and amino acid variations present in *fim3A* are found in MGH78578 or 342. This pattern is also observed across the other *fim3* genes.

To evaluate the proportions of synonymous and non-synonymous mutations between *fim3* genes and *fim* genes, an average of all pairwise comparisons of the $d_N/d_S$ ratio between each *fim3* and *fim* gene was calculated. Pairwise comparisons between *fim* sequences from different strain populations were ignored. Sequence data was collected from 12 to 20 *K. pneumoniae* reference

strains in the NCBI database depending on amount of sequence data available for each gene. The ratio of the rate of non-synonymous mutations to the rate of silent mutations ($d_N/d_S$) is frequently used to evaluate the nature of selective pressure in molecular sequences(Stahlhut, Chattopadhyay et al., 2009). This $d_N$ and $d_S$ are usually calculated per replacement or silent site respectively, hence taking into account that random mutations generate more non-synonymous changes than synonymous mutations due to the structure of the genetic code. Therefore, when evaluating the $d_N/d_S$ ratio, neutral evolution is indicated when $d_N/d_S \cong 1$. A negative selection is signified when $d_N/d_S < 1$, while positive selection is shown when $d_N/d_S > 1$.

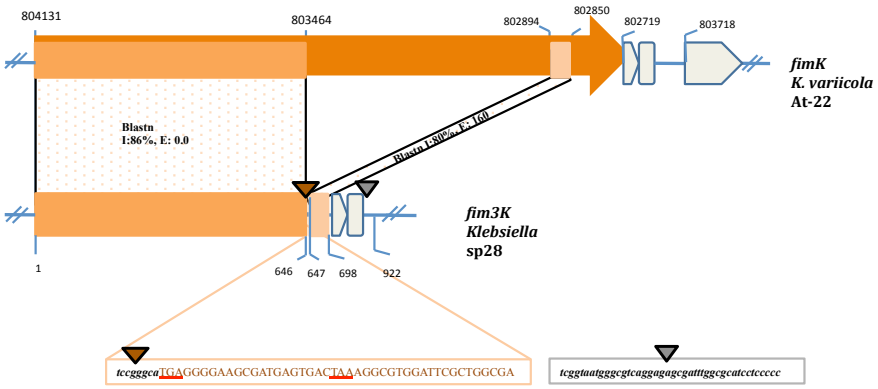
**Table 4-5: $d_N/d_S$ of *fim3* genes compared to *fim* genes**

| Gene | $d_N$ | $d_S$ | $d_N/d_S$ |
|---|---|---|---|
| *fim3B* | 0.0391 | 0.4799 | 0.0815 |
| *fim3E* | 0.0770 | 0.4108 | 0.1874 |
| *fim3A* | 0.0074 | 0.2843 | 0.0260 |
| *fim3I* | 0.0151 | 0.2828 | 0.0533 |
| *fim3C* | 0.0330 | 0.3656 | 0.0903 |
| *fim3D* | 0.0081 | 0.2161 | 0.0375 |
| *fim3F* | 0.2023 | 0.4731 | 0.4276 |
| *fim3G* | 0.0054 | 0.1757 | 0.0031 |
| *fim3H* | 0.0846 | 0.3734 | 0.2266 |

This observation suggests that *fim3* is a recently emerged variant of the *fim* operon most likely as a result of selection. This may be the first example of molecular adaptation of entire *fim* operon in *Klebsiella*. Evidence of molecular adaptation and evolution of the *fim* operon 'into' *fim3* is more apparent with regards to the comparison of *fimK* and *fim3K*. Although *fim3* shows a high level of similarity to *fim*, what makes *fim3* a distinct variant of *fim*, apart from the nucleotide substitutions observed in most of its genes, is the truncated *fimK*-like gene located at the distal end of *fim3* known as *fim3K.*

The pseudogene *fim3K* observed in the *fim3* operon appears to be truncated at the 3' end and has lost 766 nucleotides when compared to an intact *fimK* gene. The 5' 646 bp of *fim3K* is followed by 8 bp insertion that results in a frame shift and the generation of downstream premature stop codons. The 646nt sequence also shows highest similarity to *fimK* gene in *K. variicola* at 86% identity forming the lowest percentage identity among *fim3* genes to *fim* genes.

**Figure 4-6. Schematic representation of *fim3K* sequence analysis**




Schematic showing truncated *fim3K* compared to a typical *fimK* gene in *K. variicola* At-22 (highest blast identity). *fim3K* is truncated at 3' and maybe evidence of a pseudogene in *fim* type operon. The first portion of sequence (646bp) followed by 8bp insertion sequence ( ▼ ) that causes frame shift and false stop codons( underlined in red). A short genetic sequence (80% similar to the 5' portion of *fimK)* follows to the 224bp downstream flanking sequence of *fim3* operon, which also contains another insertion sequence ( ▼ ).

The 8 bp inserted sequence is followed by a shorter 46 nucleotide sequence that maps to 3' end of the *fimK* gene within *K. variicola* at an identity of 80%. It should be noted that this 46 nucletide sequence does not map to any gene in any other known *Klebsiella* strain except for a in *K. variicola*, which has highest percentage similarity to *K. pneumoniae 342* at 99% compared to other *Klebsiella* strains. The implications of the high homology of *K. pneumoniae* 342 and *K. variicola fim* operons would be discussed later in this chapter.

## 4.4 Identification of *Klebsiella* sp. harbouring *fim3* operon by MLST and phylogenetic analysis

While mapping the putative *fim3* operon in *Klebsiella* strains, it was suggested that the variation observed in *fim3* as compared to *fim* may be because *fim3* is harboured by a different species of *Klebsiella*, not *K. pneumoniae* as previously thought. As seen in Table 4-4, several members of the Enterobactericae family have a type 1 *fim* operon that may be very similar to *K. pneumoniae* as seen with *K. variicola* In order to show that the *fim* variant *fim3* is harboured by *K. pneumoniae* strains, several typing methods were employed for species identification.

Previous methods to speciate *Klebsiella* strains sp15, sp25 and sp28 produced inconclusive results (Aartsen, 2011). These methods include biochemical identification, which identified these strains as *K. pneumoniae*. However, certain limitations of biochemical testing made these results less than 100 % reliable (see Introduction for details). Therefore other molecular based methods were utilised to classify these strains.

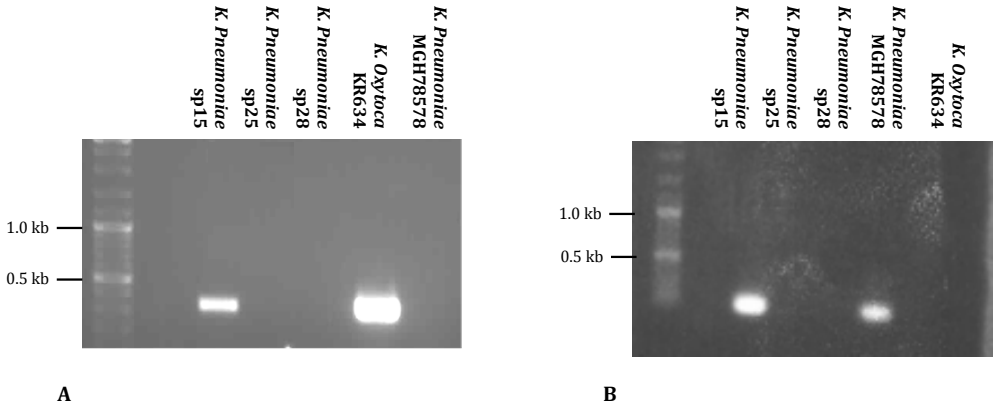
Species-specific PCR amplification assays were used to determine whether these strains were *K. pneumoniae* or *K. oxytoca* as seen before. To detect *K. pneumoniae* positive strains, universal primers based on 16S–23S rDNA internal transcribed spacer (ITS) of *K. pneumoniae* was utilised in this PCR detection assay (Liu, Liu et al., 2008). To test for *K. oxytoca* among the strains,

PCR amplification of the polygalacturonase (*pehX*) gene was employed. This method was based on the pectate degradation capability of *K. oxytoca*, which distinguishes it from other *Klebsiella* species (Kovtunovych, Lytvynenko et al., 2003).

The *K. pneumoniae*-specific 16S-23S rDNA ITS PCR assay yielded a positive band with sp15, but was negative for sp25 and sp28. Using the primers specific for *pheX* in *K. oxytoca,* a positive band was observed in sp15 but also found negative in sp25 and sp28 (Figure 4-7). PCR positive controls for sp25 and sp28 using primers to amplify housekeeping gene *tonB* confirmed that PCR results were not false negatives (results not shown).

According to these PCR assays, sp15 would have been classed as both *K. pneumoniae* and *K. oxytoca*, a notional impossibility, while sp25 and sp28 belonged to neither species. Therefore other methods of species identification were employed including sequencing and analysis of 16S rDNA and *rpoB* genes of these unknown strains.

**Figure 4-7. PCR identification of *Klebsiella* strains**



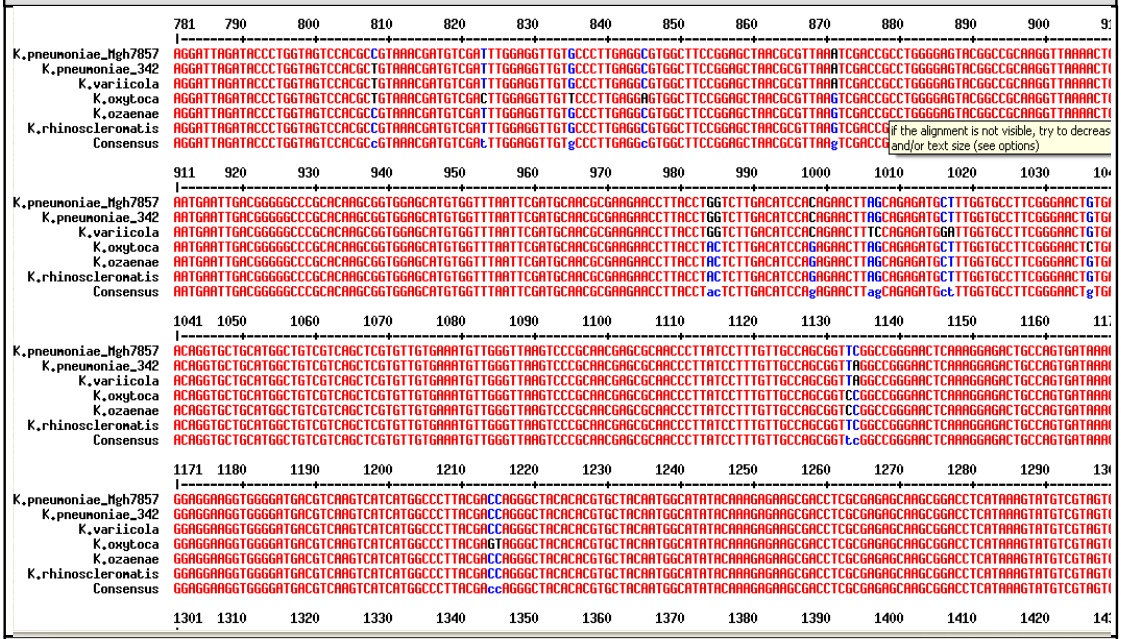

Gel picture showing species identification of *Klebsiella* strains. Picture A shows species identification for *K. oxytoca* using primers that bind to *pheX* gene and picture B shows species identification for *K. pneumoniae* using primers that are specific to its 16S-23S rDNA ITS region.

Obtained sequences showed high level of similarity between sp15 and sp25 and blasts hits showed high similarity with *K. pneumoniae* as well as other *Klebsiella* species which were not annotated. Phylogenetic comparison of full-length 16s rDNA sequences from *Klebsiella* species showed that close and very clustered relationship among all *Klebsiella* species and was not powerful enough to differentiate between enormous *Klebsiella* data and speciate the unknown *fim3* strains. However, there was a close relationship noted between *K. variicola* and *K. pneumoniae* 342 (Figure 4-8).

Although sequence data of 16S rDNA of these strains suggests a strong relationship with *K. pneumoniae*, other issues with the use of 16s rDNA made results obtained from its analysis unconvincing. This is due to certain properties of the 16S rDNA sequence, such as the presence of multiple 16S rDNA genes within genome, which are prone to recombination(Hashimoto, Stevenson et al., 2003). Moreover 16S rDNA is highly conserved in the

genome, it is unable to distinguish among *Klebsiella* species due to its highly conserved nature in this genus. Alignment of several copies of this gene from several *Klebsiella* species shows how conserved this gene is and several single nucleotide polymorphisms within some species are also found in other species.

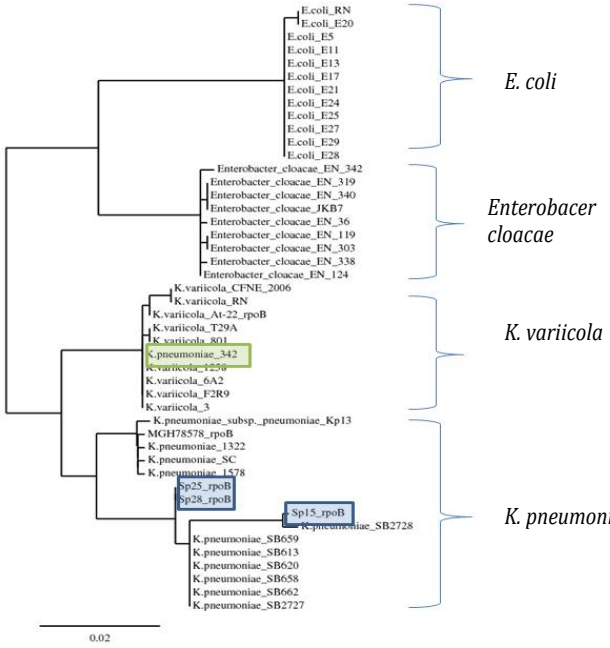
**Figure 4-8. Sequence alignment of 16s rDNA from *Klebsiella* species**




Sequence alignment of 16srDNA from 2 *K. pneumoniae* strains MGH78578 and 342 along with strains belonging to K. *variicola, K. oxytoca, K. ozanae, K. pneumoniae* subspecies. rhinoscleromatis. Only a portion of the whole alignment is shown. The high level of conservation and SNPs that are not unique to specific species makes this gene unsuitable for *Klebsiella* species identification.

Sequence data of the *rpoB* gene of putative *fim3* strains showed that alignment of *rpoB* sequence from sp25 and sp28 were identical, but still homologous to sp15 *rpoB* sequence. Phylogenetic analysis of *rpoB* sequences show that sp15, sp25 and sp28 cluster among *K. pneumoniae* strains (Figure 4-9).

**Figure 4-9. Phylogenetic analysis of *rpoB* sequences for species identification of sp15, sp25 and sp28**

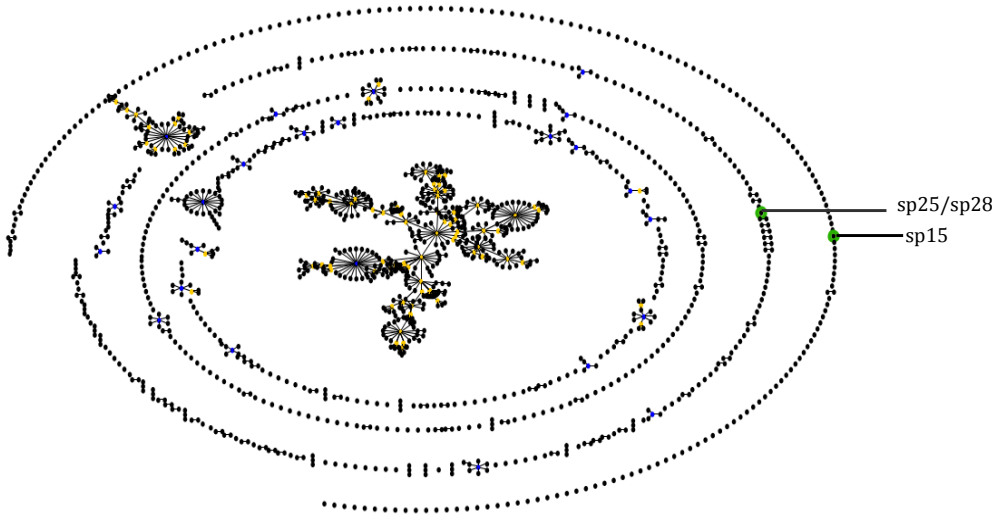



Phylogenetic tree showing relationship of sp15, sp25, and sp28 (highlighted in blue) to other strains. These *fim3* harbouring strains form a different branch within *K. pneumoniae* clade. *K. pneumoniae* 342 (highlighted in green) groups under *K. variicola* clade.

To further classify these strains as *K. pneumoniae* isolates, multi sequence locus typing (MLST) of these strains was done. Amplification and sequencing of seven housekeeping *rpoB, gapA, mdh, pgi, phoE, infB and tonB* genes for each *K. pneumoniae* strain is checked against the MLST database for *K. pneumoniae* which contains the allelic profiles of *K. pneumoniae* isolates with several and distinct sequence type (ST). In this MLST database, a different allele number is given to each distinct sequence within a locus, and a distinct sequence type (ST) number is given to each distinct combination of alleles (23). Each of the housekeeping genes within several loci was amplified and sequenced.

MLST analysis conducted was also done to determine the extent of genotypic diversity among the *K. pneumoniae* isolates in comparison to other *K. pneumoniae* strains. Among the three strains examined, 2 unique STs were identified. *K. pneumoniae* strains sp25 and sp28 showed identical genetic sequence of alleles used in MLST. However combination of these alleles was previously unrecorded in the *K. pneumoniae* MLST database for any previous isolates. Hence, a new ST number is to be assigned to a combination of these alleles. *K. pneumoniae* sp15 had different alleles from sp25 and sp28 at all loci and harboured different combination of these alleles unrecorded in the MLST database. Hence, sp15 would also be assigned a different ST. The 2 STs generated in this data set were singletons that do not belong to any clonal group or sub-group complex.

**Figure 4-10. eBURST analysis of sp15, sp25 and sp28**




Comparative eBURST analysis showing the clonal assignment of the STs assigned to sp15, sp25 and sp28 compared to that of the STs in the entire *K. pneumoniae* MLST isolate database. 2 new STs were identified for sp15 and sp25/sp28 highlighted. The new STs identified based on novel combination of allelic profile form singletons (not part of any clonal group) as seen with many STs in the *K. pneumoniae* database.

## 4.5 Discussion

### 4.5.1 Mapping of the *fim3* operon

In this work, polymerase chain reaction and direct sequencing of generated amplicons was done to identify the harboured *fim*–type operon in certain *Klebsiella* isolates. While several studies have implemented several approaches for mapping large unknown sequences including marker rescue and Southern hybridisation gene probing (Jogler, Lin et al., 2009, Rajakumar, Sasakawa et al., 1997), this approach of mapping a region >10kb was shown to be simple and possibly more effective compared to other previous attempts. Since each sequence obtained from each read is approximately about 800bp long, primers were designed for sequencing within each targeted fragment of the operon. These primers were designed after obtaining each read of sequence so that the primers and sequence obtained walks down the operon fragment. This sequence was very valuable in walking across the *fim3* operon.

It is vital to note that next generation sequencing methods such as pyrosequencing, Illumina Solexa and ABI SOLiD can now be used to accurately sequence whole bacterial genomes. However, while costs of these sequencing methods may be higher than the traditional sequencing methods, the costs of these sequencing methods are slowly reducing which may in turn cause traditional sequencing to become redundant (Metzker, 2009).

Moreover sequencing approach utilised in this work was viable based on the observed similarities between the *fim* and *fim* variant, *fim3* operon. In a

different situation where unknown regions or novel mobile genetic elements are involved, a different approach to sequencing would have to be employed.

### 4.5.2 Sequence analysis of the *fim* variant operon, *fim3*

The *fim3* operon is a novel variant of the type 1 *fim,* found in *K. pneumoniae* isolates isolated from blood of patients with bacteraemia. While the sequence and synteny of genes within *fim3* bears high similarity to *fim*, comparison of other *fim* sequences from other *K. pneumoniae* strains show a higher level of nucleotide similarity to each other than to the *fim3*. Several evolutionary forces are generally known as culprits in fixed nucleotide changes, polymorphism and diversity in bacterial genome namely mutation, genetic drift and natural selection (Kuo, Moran et al., 2009). Other studies have pointed to frequent horizontal transfer between bacterial clones and random genetic drift/recombination has causes of molecular mutations in bacteria (Stahlhut, Chattopadhyay et al., 2009).

In this work, the emergence of *fim3* was evaluated as either a random or selective process. The $d_N/d_S$ ratio calculated by pairwise comparison of *fim3* genes to *fim* suggests a negative (purifying) selection, which occurs to purge out deleterious mutations. Although several evolutionary forces may act on different genes at different times, the formation and maintenance of operons in bacteria have been shown to be facilitated by environmental selection and other evolutionary forces (Bratlie, Johansen et al., 2010, Nunez, Romero et al., 2013). As seen in *fimH* diversity pattern (Stahlhut, Chattopadhyay et al., 2009), $d_N/d_S$ ratio for pairwise comparison of *fim3H* and *fimH* is less than 1

which is also observed for all the *fim3* genes except for *fim3K* .

It is known that DNA changes are probably more harmful than beneficial and that negative selection plays a vital role in maintaining the stability of biological via removal of deleterious mutations. Hence, negative selection is sometimes called a purifying selection. The purifying selection ensures that functional gene features (like the *fim*) are fixed in a population and conserved over time (Loewe, 2008).

Moreover, the genome size reduction in *fim3K* points to a selective pressure that may have lead to the process of gene loss or development of a pseudogene. Evolutionary biologists have shown that deletion events in bacterial genome are as a result of selection, which is most likely, beneficial in a particular environment and may prove deleterious in others (Lee and Marx, 2012). Moreover selection driven gene loss has also been observed in bacteria (Koskiniemi, Sun et al., 2012), particularly in strains that specialise in specific environments (Cramer, Klockgether et al., 2011, Smith, Buckley et al., 2006, Ernst, Ichikawa et al., 2003). This may explain why the *fim3* isolates were strains cultured from sepsis patients most likely under similar treatments in the same period (Stahlhut, Chattopadhyay et al., 2009). Therefore it is possible that selection may have lead to an evolution of a typical *fim* to the *fim3* variant in these *K. pneumoniae* sepsis isolates.

Phylogenetic analyses of *fim3* genes with known *fim* genes in other strains supports the evolution of *fim3* within *K. pneumoniae fim* clade. Dendograms observed from these phylogenetic analyses shows a similar pattern to dendograms of *rpoB* genes used in speciation of the *fim3* harbouring

*Klebsiella* strains, such that *fim3A* forms a different and recent branch within *K. pneumoniae fim*. Likewise in phylogenetic analyses of *rpoB* genes, *Klebsiella* strains sp25 and sp28 form a different and recent branch within *K. pneumoniae* group. While phylogenetic analyses of the whole *fim3* operon in comparison with fim may have produced more robust results, phylogeny was limited to genes due to the limitations of bioinformatics programs in dealing with large data size. Moreover, higher variation in intergenic regions in these operons may not have produced accurate results has intergenic regions are more prone to random recombination and mutations (Ferreira, Borges et al., 2012, Hughes and Friedman, 2004).

In *K. pneumoniae*, previous research has showed that while the *fimH* alleles exhibit relatively low nucleotide and structural diversity and are prone to frequent horizontal-transfer events between different bacterial clones (Stahlhut, Chattopadhyay et al., 2009). In addition, the *K. pneumoniae* FimH protein is targeted by adaptive point mutations, which include a single amino acid deletion from the signal peptide that could affect the length of the fimbrial rod by affecting FimH translocation into the periplasm(Stahlhut, Chattopadhyay et al., 2009). In *fim3H* however, an amino acid insertion was observed in the signal peptide that may also affect fimbral rod of Fim3H. In the same study of *K. pneumoniae fimH* alleles, another FimH mutation (S62A) was observed in a uropathogenic clone of *K. pneumoniae*. This mutation is identical to one found in a virulent uropathogenic strain of *E. coli,* which suggests that the some of the FimH mutations may be pathoadaptive in nature (Stahlhut, Chattopadhyay et al., 2009). This particular study suggests

variation among only 90% of *K. pneumoniae* isolates which includes strains used in this project such as *K. pneumoniae* sp15. *K. pneumoniae* sp25 and sp28, which were dropped out of this study because *fimH* could not be detected by primers used to amplify this gene. Nonetheless, this study provided an early observation of microevolution within *fimH* genes depicted by high level of allelic variation of *fimH* gene among *K. pneumoniae* isolates. The present study of *fim3* an allelic variant of *fim* shows allelic variation in other type 1 *fim* genes and micro-evolution of these genes within this operon. Although allelic variation of *fimH* has also been observed in Enterobactericae species like *Salmonella enterica* and *E. coli* (Dreux, Denizot et al., 2013, Dwyer, Newton et al., 2011, Guo, Cao et al., 2009), the observation of *fim3* shows the first allelic variation of the entire *fim* operon within Enterobactericae.

The effect of the molecular evolution of *fim* to *fim3* in these *Klebsiella* strains is mostly observed in the truncation of the *fim3K* gene when compared with *fimK*. The *fim3K* gene almost resembles a pseudogene of *fimK* that has lost 714 bp at its 3' end. At the 5' end, 646bp map to the helix-turn-helix domain of the *fimK* gene. Although a truncated allele of *fimK*, *fim2K* has been previously described in *fim2* operon(Van Aartsen, Stahlhut et al., 2012), the deletion observed in *fim3K* is unique because the deletion within this gene eradicates the presence of the EAL domain in the formative protein structure. Bioinformatic analyses into the genetic and protein sequence of *fimK* have identified it as a 469 aa diguanylate cyclases protein that possesses a helix-turn-helix (HTH) domain from 1 to 215 aa, and an EAL domain from 216 to

447 aa. Diguanylate cyclases (DGCs) have been found to modulate intracellular cyclic-di-GMP levels and in turn have been identifies as a vital bacterial signal that regulates the shift between planktonic and sessile lifestyles (Rosen, Pinkner et al., 2008).

Further bioinformatic analysis of *fimK* , *fim2K* and cognate EAL domains suggests that these two proteins have a potential phosphodiesterase (PDE) activity and therefore be active within the bacterial genome. Previously, 10 amino acid residues within the EAL domain had been previously determined for c-di-GMP hydrolysis and potential PDE activity (Aartsen, 2011). The protein sequences of these of these genes *fimK*, and *fim2K* were aligned with other confirmed EAL domain proteins in other bacteria, where it was observed that the *fimK* genes contained 9 of all the previously annotated amino acid residues required for PDE activity. The 10$^{th}$ amino acid residue may not be necessary for activity, as amino acid substitution at this position as been found in other EAL proteins with confirmed PDE activity as seen in *K. pneumoniae* Blrp1 (Barends, Hartmann et al., 2009). These analyses along with other experimental work that the 10 conserved residues may not be required to form a hydrolytically active EAL domain and that FimK and Fim2K may be active PDEs (Rosen, Pinkner et al., 2008).

Although the EAL domain was thought to be the functional domain of the *fimK,* recent studies showed that it is the HTH domain of the *fimK* that binds to *fimS* and may regulate fimbrial expression in *K. pneumoniae* (Wang, Huang et al., 2013). Therefore, the remnant of the HTH domain of *fim3K* may be due

to the fact that a streamlined process of retaining only functional part of the gene provided that *fim3K* is still function in *K. pneumoniae.*

Phylogenetic analyses of the *fim3* genes suggest this deletion of EAL domain in *fim3K* as a recent evolution in these bacteria though the cause and mechanism of this deletion is still unknown.

Another observation made in this work is the level of similarity to *fim* operon in *K. variicola* At-22 and *K. pneumoniae* 342. Further comparative analysis of the *fim* operon distributed among annotated *Klebsiella* genome in the database highlights a very high level of similarity among *Klebsiella pneumoniae* strains. At a nucleotide level, the genes within these operons show about a 99% identity when aligned with each other. *K. variicola* along with *K. pneumoniae* 342 were observed to be more diverse from other *K. pneumoniae* strains, but more closely related to each other. These analyses suggests that speciation of *K. pneumoniae* 342 with ABI system may need to be further investigated with other methods of species annotation so as to be more confident with its classification as *K. pneumoniae* strain. However, *K. pneumoniae* 342 is a nitrogen-fixing strain isolated from stem tissue of *Zea mays* (Palus, Borneman et al., 1996)**,** while *K. variicola* At-22, also a nitrogen fixing strain, was isolated from fungus gardens of leaf-cutter ant colonies (Rosenblueth, Martínez et al., 2004). The adaptation to terrestrial environment, as seen in their nitrogen-fixing phenotype, may also explain close homology of their *fim* operons and possibly other genes and operons yet to be investigated and compared within their genome. Since many of the other *K. pneumoniae* strains have been isolated from human patients, their

close homology to each other may be as result of their adhesion and adaption to a human environment. This pathoadaptive nature of enteric bacteria is particularly important and has been documented in *fim* genes in *Klebsiella* (Stahlhut, Chattopadhyay et al., 2009).

### 4.5.3 Identification of *Klebsiella* sp. harbouring *fim3* operon

Despite previous biochemical and PCR analysis to determine speciation among *Klebsiella* species, it is clear that molecular analyses are stronger methods of identifying species rather than biological methods. However, some molecular methods involving specific genes are not strong enough to differentiate among Enterobactericae species. For instance, phylogenetic comparison of 16s rDNA from *Klebsiella* species showed close and very clustered relationship among all *Klebsiella* species and was not powerful enough to differentiate between enormous *Klebsiella* data and speciate the unknown *fim3* strains. Also, some results obtained from 16S rDNA analysis may not be entirely convincing. This is due to certain properties of the 16S rDNA sequence, such as the presence of multiple 16S rDNA genes within genome, which are prone to recombination (Hashimoto, Stevenson et al., 2003). The use of molecular typing methods such as MLST and phylogenetic analyses were very important in the species identification of bacterial clones particularly in the event of bacterial adaption and evolution.

## 4.6 Future work

Although the discovery of *fim3* and the differences between *fim3* and *fim* were discussed in this chapter, phenotypic similarities and differences of *fim3* to *fim* are discussed in detail in Chapter 5. However, potential aspects for future work include screening of a much larger clinical and environmental catalogue of *Klebsiella* strains for *fim3* using primers specific to *fim3K*, which is characteristic of the *fim3* operon. Screening among *K. pneumoniae* strains isolated, from several geographical locations and hosts environments, for other variations in the *fim* could also be useful in understanding the evolutionary relationship between fimbrial systems and pathogen habitat. This may also be useful and applicable to other known virulence systems that affected by the bacterial environment.

Also, screening within known *fim3* strains, sp15, sp25 and sp28 for allelic variations within other known fimbrial operons such as the type 3 *mrk.* Allelic variations in genes and operons encoding virulence factors may shed light into the pathoadptation of *K. pneumoniae* in clinical strains.

# Chapter 5: *In vitro* and *in vivo* characterisation of *fim3*

## 5.1 Introduction

In the previous chapter, *fim3* was introduced and described as a potentially evolved variant of the *fim* in *K. pneumoniae*. Owing to its high level of similarity to the *fim* operon (despite several genetic and amino acid variations across the operon), it was proposed that the *fim3* operon would bear a similar structure and function to *fim*. To investigate this hypothesis, *in vitro* and *in vivo* role of *fim3* was examined while comparing its pattern of effect to that of *fim*.

Several genetic constructs were used to examine the similarities and differences between the *fim* and *fim3* operons. Firstly, a *fim3* knockout was created in *K. pneumoniae* sp28 p28 double *fim* and *mrk* knockouts were also created. The *mrk* deletion mutants were created based on the suggestion that the expression of *mrk* type 3 operon which is well expressed in sp28 may reduce and conceal the expression of *fim3* as seen in other work with type1 fimbriae in *K. pneumoniae* (Wang, Huang et al., 2013). In addition, isogenic sp28 strains containing single *fim3K* and double *mrk* and *fim3K* knockouts were created to observe the effect of *fim3K* on *fim* expression. For comparison between the *fim* and *fim3* operon, *fim, fimK* and *mrk* mutants previously constructed in *K. pneumonaie* KR116 was also included in some experiments with the sp28 knockout strains. KR116 was suited for comparison with sp28 as it contains *fim* in the same locus as *fim3* in sp28.

KR116 also harbours a *fim2* operon within the KpGI-5 island and was isolated as a clinical specimen from a patients blood.

In parallel, *fim3* operon was cloned into IPTG inducible plasmid pJTOOL-7 and expressed in *E. coli* HB101 as described in chapter 3 of this thesis. Apart from the reasons mentioned for exploring fimbriae operons in afrimbriate *E. coli* HB101, cloning *fim3* into same background as *fim* (pFim-ptrc) allows for direct comparison between the two operons to examine for any significant difference in phenotypic effects of these gene clusters.

In this chapter, sp28 and its isogenic mutants were analysed *in vitro* using agglutination assays, biofilm assays and *in vivo* using a *G. mellonella* killing assay. Characterisation of the *fim3* expression profile was analysed by examining generated cDNA libraries with RT-PCR techniques and examining the *fim* genes under laboratory conditions. In addition, *E. coli* HB101 cells heterologously expressing the *fim3* operon together with HB101/pFim-ptrc were be analysed using agglutination assays, biofilm assays, growth curves and cell adhesion assays. Transmission electron microscopy of the heterelogously-expressed *fim3* in *E. coli* HB101 was done to observe any fimbrial structures as previously seen in *E. coli* HB101/pFim-Ptrc.

## 5.2 *fim3* bears similar phenotypic traits with *fim*

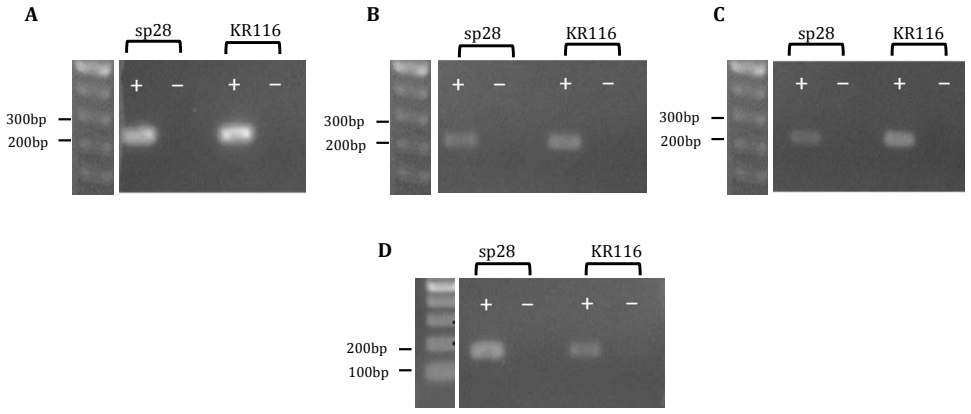
It was thought that *fim3* would behave similarly to the *fim* operon. Since, the *fim* operon is characterised by several *in vitro* phenotypic traits, experiments to validate the similarity of expression and phenotypic pattern of *fim* and

*fim3* was done. The *fim3* harbouring strain sp28 was compared to its isogenic *fim3* knockout mutant as several experiments have previously been used in type 1 fimbriae assay. Strain KR116 and its *fim* knockout mutant were compared to sp28 and its *fim3* knockout to show effect similarity between absence of *fim* and absence of *fim3* in *K. pneumoniae*.

### 5.2.1 Transcriptional analysis of *fim3* and *fim*

In this work, expression of *fim3* genes was investigated while comparing its expression patterns to homologues *fim* genes in *K. pneumoniae* KR116. Hence, RNA was isolated from sp28 that had been cultured in LB medium for 16 h (37°C, 200 rpm) and a cDNA library was constructed using random primer-based RT-PCR. This process was also repeated for *K. pneumoniae* strain KR116. PCR analysis of these cDNA libraries detected transcripts corresponding to several *fim3* genes in sp28 and the corresponding *fim* genes in KR116, while reverse transcriptase-free cDNA libraries did not yield an products, confirming the absence of DNA carryover. RT-PCR was done to detect *fim3A*, *fim3H*, and *fim3K* transcripts in sp28. In KR116, *fimA, fimH* and *fimK* were compared respectively in the RT-PCR analysis. Housekeeping gene *rpoD* was used as an internal control for this analysis. Results conformed the expression of *fim3* putative core genes as seen in *fim.* However *fim3H* was expressed at a lower level than all the other *fim3* genes analysed (Figure 5-1).

**Figure 5-1. Transcriptional analysis of *fim3***




Key + cDNA - no reverse transcriptase control

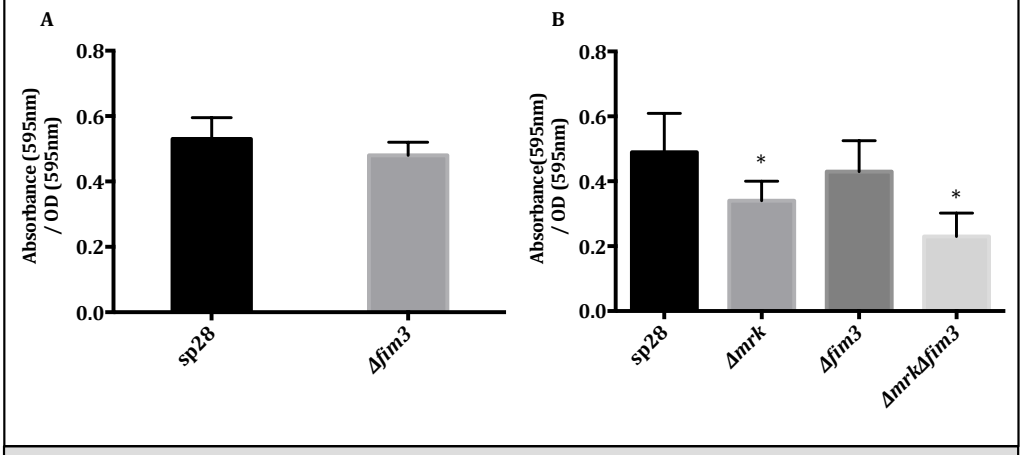
cDNA and mock cDNA (-) libraries generated from RNA extracted from *K. pneumoniae* sp28 were used to (LB, 16h, 200rpm, 37°C) was used to investigated the expression of *fim3*. Transcripts corresponding to (**A**) *fim3A*, (**B**) *fim3H* and (**C**) *fim3K* were detected and comparable to (**D**) transcriptional control *rpoD.* For comparison with *fim*, transcripts corresponding to (**A**) *fimA*, (**B**) *fimH* and (**C**) *fimK* were amplified from *K. pneumonia*e KR116. *rpoD* control was also done with KR116 **(D**).

### 5.2.2 Effect of *fim3* deletion on biofilm formation in sp28

The ability of *Klebsiella* to form biofilms on medical devices such catheters plays a vital role in development of many nosocomial infections (Schroll, Barken et al., 2010). Since surface components affect biofilm formation in *K. pneumoniae*, the effect of the potential *fim3* fimbriae on biofilm was assessed. In this experiment sp28 parent strain together with its isogenic *fim3* knockout mutant *Δfim3* was grown on polystyrene surface under static conditions for 24 hours at 37°C. Included in this experiment for comparisons was KR116 and its isogenic mutant *Δfim.* It was observed that absence of *fim3* in sp28, and absence of *fim* in KR116 did not show any significant effect in biofilm abilities of their respective strain (Figure 5-2A). As previously mentioned the presence of *mrk* type 3 fimbriae may mask some of the effects of type 1 fimbriae. To investigate if this holds true for *fim3* in sp28, a biofilm assay was repeated with isogenic *Δmrk* mutant and *ΔmrkΔfim3* mutants

included in this assay. Results showed that double deletion of *mrk* and *fim3* negatively influenced biofilm formation in sp28, more than single deletions of either *fim* or *mrk* (Figure 5-2B). This suggests that *fim3* may contribute to biofilm formation in strain sp28.

**Figure 5-2. Effect of *fim3* on biofilm formation**



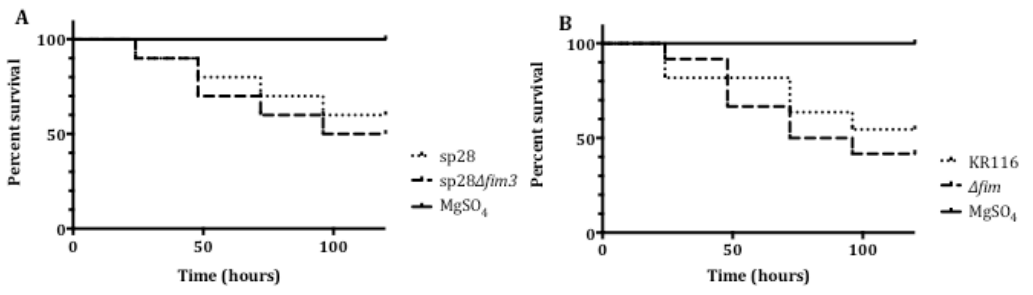

**(A**) Assay results show that single deletion of *fim3* in *K. pneumoniae* sp28 does not affects biofilm formation which is also seen in the deletion of *fim* in *K. pneumoniae* KR116. **(B)** Deletion of *mrk* in sp28 show that *fim3* may contribute to biofilm formation in this strain. An asterisk indicates a significant difference ($P < 0.005$) from wildtype sp28. Statistical analyses were performed using the non-parametric Kruskal Walis test.

### 5.2.3 Investigation into the *in vivo* role of *fim3* using *G. mellonella* model of infection

As previously mentioned in chapter 3, *G. mellonella* larvae may be used as a model to determine levels of virulence in *K. pneumoniae*. To investigate the effect on *fim3* on virulence of sp28, a *G. mellonella* killing assay was carried out with equal number of larvae injected with wildtype strain and isogenic *Δfim3* strains separately. Larvae were inoculated with approximately $1\times10^3$ CFU/ml (±0.07) of each strain and incubated at 37°C. Every 24 hours the

number of deaths in each was recorded and the infection was monitored for 5 days at which point the experiment was terminated. Infection of larvae with mutant *Δfim3* strain proved to be more virulent than wildtype, albeit by very little and difference between both curves (wildtype and mutant) were not significant. This result is however similar to the observed higher level of virulence in *fim* deleted mutants in *K. pneumoniae* KR116 (Figure 5-3).

**Figure 5-3. *In vivo* assay showing effect of *fim3* and *fim* deletion in sp28 and KR116 respectively**




*G. mellonella* killing assay was used to examine the of deletion of *fim3* on *K. pneumoniae* sp28 virulence in larvae, while comparing separately to *fim* in *K. pneumoniae* KR116. Larvae are injected with ≈ $1 \times 10^3$ CFU/ml of sp28, KR116 and their isogenic *fim3* or *fim* knockout mutants. **(A)** Results show that loss of *fim3* is more virulent in sp28 as seen with loss of *fim* in KR116 (**B**).

### 5.2.4 Investigating haemaglutination capacity of *fim3*

Most *K. pneumoniae* isolates express type 1 and type 3 fimbriae, which is characterized by mediation of mannose-sensitive agglutination of treated red blood cells and yeast cells, respectively (Stahlhut, Struve et al., 2012). It was shown that the detection of type 1 fimbriae expression in *K. pneumoniae* was feasible only by use of guinea pig erythrocytes (Stahlhut, Struve et al., 2012). To examine if *fim3* operon in sp28 would form fimbriae and adhesion with similar structure to *fim* operon, heamaglutination assay was carried out. The wildtype sp28 strain and its *Δfim3* mutant, together with KR116 and its *Δfim*

mutant were included in this assay. It was observed that sp28 was able to agglutinate GP-RBCs in a mannose sensitive manner (albeit slowly) and loss of *fim3* did not agglutinate GP-RBCs (data not shown).

## 5.3 Effect of truncated *fim3K* on expression of the *fim3* operon

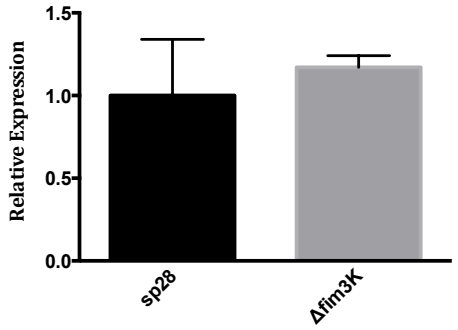
Based on the analysis of *fim3* sequence previously discussed in chapter 4, it is likely that the *fim3* operon is variant of *fim*. It was hence speculated that most of the *fim3* genes would bear similar function to their *fim* counterparts. However, the truncation of *fim3K* raise questions on its potential regulatory function in *fim3* expression, as *fimK* ha been observed to affect *fim* expression in several *K. pneumoniae* strains (Wang, Huang et al., 2013, Rosen, Pinkner et al., 2008). In order to understand the function of *fim3K* and how the truncation in this gene may have developed, experiments to examine the gene expression and its effect on *fim3* were carried out.

### 5.3.1 Effect of *fim3K* deletion on expression of *fim3A*

To investigate ifthe *fim3K* gene affects the transcriptional expression of putative major fimbrial subunit gene *fim3A*, a qRT-PCR assay was done. For this assay, transcripts corresponding to *fim3A* were tested by PCR from wildtype sp28 and its isogenic mutant *Δfim3K* which were grown for 16 hours at 37°C. Results did not show any significant difference in the relative

expression, hence suggesting that *fim3K* may not have a direct effect on expression of *fim3A* at level of transcription (Figure 5-4).

**Figure 5-4. Effect of *fim3K* on *fim3A* expression**



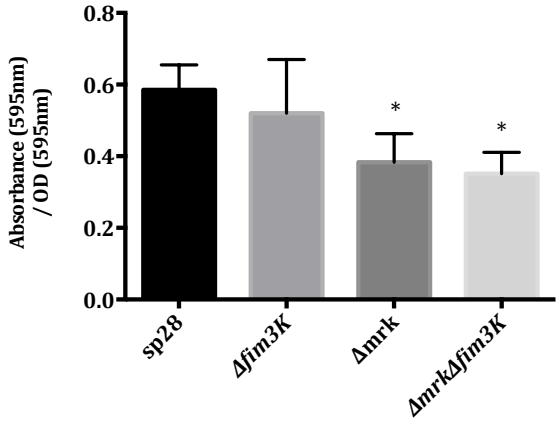

cDNA libraries made from *K. pneumoniae* sp28 and its isogenic *fim3K* knockout strain were used to determine the expression of *fim3A*. Analysis of *fim3A* expression using qRT-PCR did not show any significant increase in expression.

### 5.3.2 Effect of *fim3K* on biofilm formation

Biofilm formation in *K. pneumoniae* may be facilitated and affected by interactions between the polysaccharide capsule, type 1 fimbriae and type 3 fimbriae, together with other known and unknown factors (Schroll, Barken et al., 2010, Struve, Bojer et al., 2009, Struve and Krogfelt, 2003).

Previous reports suggest that the deletion of *fimK* in *K. pneumoniae* Top52 encourages biofilm formation (Rosen, Pinkner et al., 2008). Since the changes in biofilm formation were observed in the absence of *mrk,* the effect of *fim3K* on biofilm formation was done with the sp28*Δmrk* and isogenic double mutant sp28*ΔmrkΔfim3K*. However, this assay did not show any significant effect of *fim3K* deletion on biofilm formation (Figure 5-5).

**Figure 5-5. Effect of *fim3K* deletion on biofilm formation in sp28**




Biofilm formation assay results shown were normalized to take account of pre-wash total cell numbers based on OD595 readings performed at 24 h, just prior to washing off non-surface adherent cells and crystal violet staining. Data shown in all cases represent means and standard deviations of three biological replicates, each assayed in eight wells (n = 24). An asterisk indicates a highly significant difference ($P < 0.0005$) from wild type sp28. Statistical analyses were performed using the non-parametric Kruskal-Wallis test.

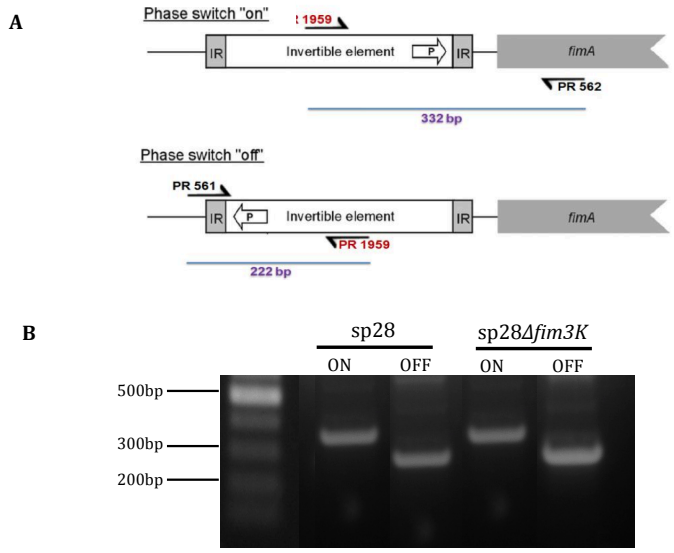
### 5.3.3 Effect of *fim3K* on phase variability of *fim3*

The transcription of *fim* operon is mediated by the orientation of an invertible DNA segment, *fimS* (Clegg, Wilson et al., 2011, Struve, Bojer et al., 2008). This *fim* switch along with the flanking 9bp direct repeats was present in *fim3* sequence. Previous reports have speculated that helix turn helix domain of *fimK* may regulate *fim* expression by binding to *fimS* and facilitating the on-phase orientation of the *fim* switch. Since *fim3K* consists mostly of a helix-turn-helix domain, an experiment was done to determine if the deletion of *fim3K* could affect the orientation of present of the *fim3* operon.

In this experiment, wildtype sp28 and its isogenic mutant *Δfim3K,* along with KR116 wildtype and *ΔfimK* were grown for 16 hours at 37°C after which

genomic DNA was extracted. An ON-PCR and OFF-PCR assay was done on 10ng of genomic DNA using primers that produced 322bp band for the ON-phase and a 222bp band for the OFF-phase. Figure 5-6 shows a schematic illustration of *fimS* switch orientation PCR assay. Results from this assay also showed while both orientations easily detected under shaking conditions in sp28, deletion of *fim3K* did appear to favour any particular orientation (Figure 5.6).

**Figure 5-6. Effect of *fim3K* deletion on phase variation of *fim3***




(**A**) Schematic illustration of *fimS* phase orientation PCR- based assay (excerpt from Hemin Hussein MSc). (**B**) Detection of *fim3* phase orientation in *K. pneumoniae* sp28 and its isogenic mutant sp28*Δfim3K* under laboratory conditions of 37°C and 200rpm. Results did not show any observable difference of phase variation between wildtype and mutant.

## 5.4 Effect of *fim3* heterelogously expressed in *E. coli* HB101

The *E. coli* HB101 strain has been extensively used to investigate *K. pneumoniae* and *E. coli* type 1 and type 3 fimbrial systems (Struve, Bojer et al., 2008, Struve, Bojer et al., 2009, Klemm, Jørgensen et al., 1985). In of this,

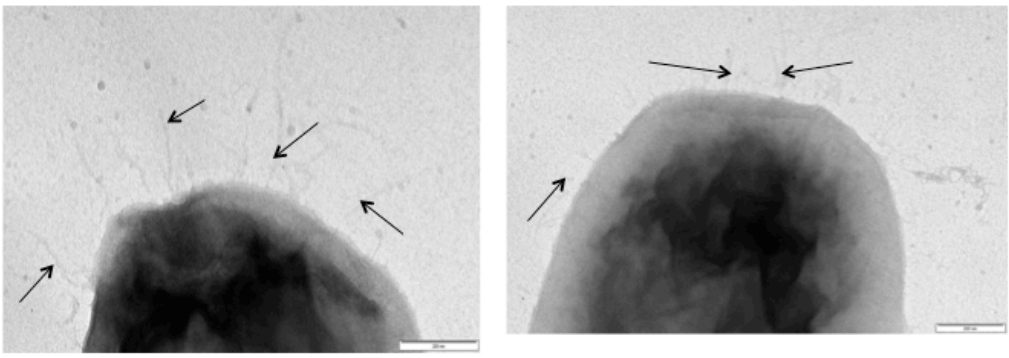
the HB101 strain was used to further the *fim2* operon. The advantage of studying fimbrial operons and structures cloned into this strain is based on the afrimbriate nature of the bacteria, which allows specific examination of a fimbriae without distractions from other host-encoded fimbriae. This has enabled the visualistion of fimbrial structure as well as phenotypic characteristics of the associated fimbriae. Moreover, the heterologous expression avoids potential physical hindrance on fimbrial function by the *K. pneumoniae* capsule that had previously been observed for type 1 fimbriae (Klemm, Jørgensen et al., 1985).

In order to validate the phenotypic expression of the *fim3* operon, *fim3* was cloned into IPTG-inducible plasmid pJTOOL-7 and transformed into the HB101 strain. The expression of *fim3* fimbriae was investigated by the transmission electron microscopy to visualise the associated *fim3* fimbriae. A haemaglutination assay was performed on this clone as previously described in materials and methods. Other experiments such as growth curves and biofilm formation assays were also done to observe if *fim3* could affect HB101 phenotype as previously seen with *fim* cloned into HB101 carried on IPTG-inducible plasmid pJTOL-7, HB101/pFim-Ptrc in chapter 3. The *fim3* operon cloned into HB101 (also carried on pJTOOL-7) was dubbed HB101/pFim3-Ptrc

### 5.4.1 Transmission electron microscopy of HB101 heterologously expressing *fim3*

In order to visualise *fim3*-associated fimbriae, transmission electron microscopy was done on HB101/pFim3-Ptrc cells. Overnight culture of this strain was re-innoculated with 1:100 dilutions in LB media and *fim3* expression was induced with 0.1mM IPTG. Negative staining of the cells show visualisable *fim3* fimbriae on the surface of the cells (Figure 5-7).

**Fig 5-7. Electron microscopy of E. coli HB101 expressing *fim3***



Transmission electron micrographs of two independent cells of *E. coli* HB101/pFim3-Ptrc. Arrows highlight fimbrial structures observed in HB101/pFim3-Ptrc.

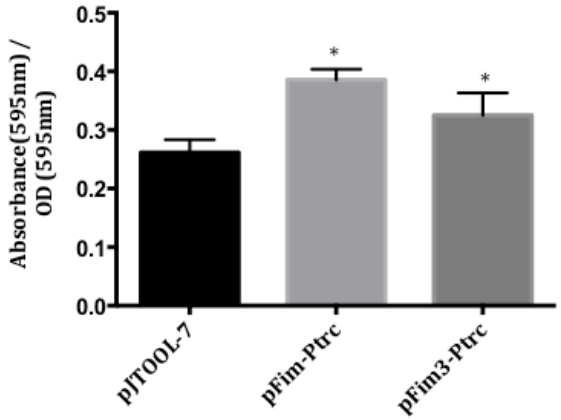
### 5.4.2 Haemaglutination of GP-RBCs by HB101 expressing *fim3*

To confirm the role of *fim3*-mediated agglutination, *E. coli* HB101/pFim3-Ptrc was examined for the ability to agglutinate GP-RBCs as previously described in Chapter 3. Similar to the *fim*-bearing control plasmid, pFim-Ptrc, the *fim3* plasmid was able to confer agglutination of GP-RBCs. The addition of 10% mannose affected the heamaglutination properties of pFim3-Ptrc showing a mannose sensitive agglutination of GP-RBCs (data not shown).

### 5.4.3 Expression of *fim3* may affect biofilm formation

In order to observe a potential and more significant contribution to biofilm that may have been masked by presence of other surface fimbriae or proteins as well as capsule-related physical hindrance of fimbrial function fimbrial function, the *fim3* operon was expressed from pFim3-Ptrc by inducing its expression by adding 0.1mM IPTG to the *E. coli* growth medium for 24 hours. Results showed that when compared to HB101 carrying the empty pJTOOL-7 vector, HB101/pFim3-Ptrc exhibited a higher biofilm formation, which was statistically significant (P <0.011). The biofilm observed from HB101/pFim3-Ptrc is also marginally similar to that of HB101/pFim-Ptrc, confirming similarity in properties of the two fimbriae (Figure 5-8).

**Figure 5-8. Biofilm formation assay**

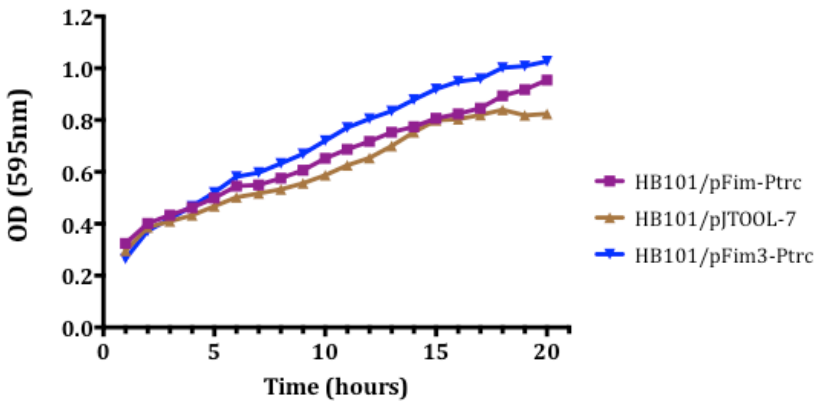



Biofilm formation assay results shown. Data shown in all cases represent means and standard deviations of three biological replicates, each assayed in eight wells (n = 24). An asterisk indicates a highly significant difference ($P < 0.005$) from wild type sp28. Statistical analyses were performed using the non-parametric Kruskal-Wallis test.

### 5.4.4 Effect of induced expression of *fim3* on growth HB101

As previously discussed in earlier sections of this chapter, the induction of *fim* operon in *E. coli* HB101 resulted in enhanced growth of *E. coli* HB101 (Van Aartsen, Stahlhut et al., 2012). To observe if *fim3* also shares this phenotype with *fim*, HB101/pFim3-Ptrc was also grown for 24 hours with IPTG induction of *fim3.* Like *fim, fim3* also appeared to increase the growth of HB101 when compared to *E. coli* bearing empty plasmid, albeit only a slight increase (Figure 5-9).

**Figure 5-9. Effect of *fim3* expression on *E. coli* HB101 growth**




Growth curves for HB101/pJTOOL-7 (empty vector), HB101/pFim-Ptrc and HB101/pFim3-Ptrc. Growth curves for strains were measured very 15 minutes while growing for 24 h in LB broth containing 100 µg/ml ampicillin supplemented with 0.1 mM IPTG

## 5.5 Discussion

The importance of the type 1 fimbriae in *K. pneumoniae* is evident as an initial step for colinisation of host cells by the bacteria and therefore a vital virulence factor for these species. The importance of the type 1 fimbriae is also seen noted in its consistent presence among many *K. pneumoniae* strains

(Podschun, Pietsch et al., 2001, Struve and Krogfelt, 2004) even in the presence of other fimbriae that may or may not contribute to adhesive properties of *K. pneumoniae* strains (Clegg, Wilson et al., 2011, Ghigo and Beloin, 2011). Although the presence of other chaperon-usher dependent fimbriae has suggested a potential functional redundancy of this fimbriae family (Ghigo and Beloin, 2011), the observation of *fim3*, a newly emerged variant of *fim* among the *K. pneumoniae* species verifies the importance of this system within the bacteria.

Due to high DNA and amino acid similarity between *fim3* and *fim* gene clusters, it was hypothesized that *fim3* would bear similar phenotype to *fim*. This was observed with isogenic *fim3* deleted mutants in sp28. In some other experimental analysis, KR116 and its isogenic *fim* deleted mutants were included. Because the *fim3* operon is located in the same locus as the typical *fim* operon would have been, comparison of *fim* and *fim3* was impossible within the same strain. Therefore, phenotypic effect of *fim3* deletion in sp28 was compared to the phenotypic effect of fim deletion in KR116, a clinical *K. pneumoniae* isolate which also harbours a *fim2* operon. Transcriptional analysis confirmed the transcriptional expression of several *fim3* genes including *fim3A,* which would code for major fimbriae subunit like *fimA*. *In vitro* and *in vivo* analysis showed the same pattern of effect of *fim3* deletion in sp28 and *fim* deletion in KR116.

Agglutination experiments of GP-RBCs also confirmed the similar characteristics of *fim* in KR116 and *fim3* in sp28. The haemaglutination abilities of *fim* and *fim3* were also confirmed with the heterologous

expression of *fim3* in *E. coli,* as was done with *fim.* While heterologous expression of *fim3* in *E. coli* enhanced growth rates and biofilm formation as seen with *fim* in *E. coli*, electron microscopy of these clones showed visualisable *fim3* fimbriae as seen with *fim*. These results verifies the hypothesis that *fim3* bears same function as *fim* and moreover, *fim3* is most likely an evolved version of the *fim* in *K. pneumoniae.*

As discussed in the previous chapter, the presence of several synonymous mutations within many of the *fim3* genes hint the possibility of selective pressures and molecular adaptation of these genes. There are several examples of molecular adaptation in bacteria many as a response to certain stimulus and environment (Solanki and Gupta, 2013, Ibraheem and Ndimba, 2013, Haiko, Laakkonen et al., 2011). Among the Enterobactericae, they are several examples of adaptation of bacteria to host environment by changes in DNA (Toft and Andersson, 2010). Natural variation of *fimH* in *E. coli* has been shown to be related to host adaptation and affect adhesion to the host (Sokurenko, Chesnokova et al., 1998). Among *Salmonella enterica*, several allelic variants of FimH adhesin confer differential bacterial binding to different types of mammalian cells (Guo, Cao et al., 2009).

While several experiments in this work concurs with the theory that *fim3* originally emerged from *fim*, it is not clear why this is the case. It would have been expected that bacteria with *fim3* would show more virulence and contribute significantly more to known phenotypic traits associated with *fim*. However, none of the in this study experiments showed any major difference between *fim3* and *fim* in experiments with the harbouring *Klebsiella* strain.

This is most likely due to the fact that the operons are carried by different strains and are not directly comparable. While experiments with *E. coli* expressing either *fim* or *fim3* also showed similarities in their phenotype, experiments done with these clones may not be sufficient to deduce which is more virulent. In many cases, virulence between strains is analysed by head to head competition of isogenic strains (Wang, Huang et al., 2013, Struve, Bojer et al., 2008, Rosen, Pinkner et al., 2008, Gaddy, Arivett et al., 2012). Although the HB101/pFim-Ptrc and HB101/pFim3-Ptrc strains share identical background, the subject of analysis, the *fim* and *fim3* operon, are carried heterologously on a plasmid that needs to be maintained with resistance to the presence of its background antibiotic. Moreover, the plasmid in which *fim* and *fim3* were cloned requires an induction with IPTG, so as to as facilitate the expression of either operon. These factors associated with the heterologous expression of *fim3* and *fim* outside their native strains affects the utilization of several assays that can be used in a head to head competition or comparison of *fim3* and *fim*.

Despite the lack of head to head competition between *fim* and *fim3*, it is interesting to note that *fim3* strains have only been found present in 3 *K. pneumoniae* isolates which all bear *fim2* and were isolated from a sepsis patient in 1991 (Stahlhut, Chattopadhyay et al., 2009). The *fim2* operon encoded in *K. pneumoniae* genome is part of a genomic island, which is usually characterized by horizontal gene transfer and insertion at a tRNA locus (Van Aartsen, 2008, Boyd, Almagro-Moreno et al., 2009). Other works have demonstrated and elucidated on the impact of horizontal gene transfer

on molecular adaptation in core genomes (Stahlhut, Chattopadhyay et al., 2009, Ricard, McEwan et al., 2006, Wiedenbeck and Cohan, 2011). This may explain the evolution of *fim3* from *fim* among *K. pneumoniae* isolates with *fim2* operon, which is haboured on KpGI-5 island laterally transferred into the *K. pneumoniae* genome. It is also possible that the sepsis host patients that carried these strains provided a unique environment via drugs, hospitalization etc. that created a selective pressure and environment for adaptation of these bacteria, which also may have resulted in molecular evolution of the *fim*. This, however, may be difficult to analyse as information on the patient information is harder to retrieve. Moreover, the environment that facilitated the molecular adaptation of *fim3* harbouring strains may be different prior to infection.

A distinctive feature of *fim3* is the truncated *fim3K* gene at the distal end of the operon. While bioinformatics analyses of this gene did show any known complete protein structure similar to this gene, the *fim3K* sequence is highly similar to the helix-turn-helix subunit of FimK but has lost the EAL subunit. The truncation of this gene suggested a form of gene decay and lack of function. However, RT-PCR of this gene showed that despite the truncation of this gene, *fim3K* is still transcribed within the bacteria. Further analyses to detect the effect of presence or loss of the *fim3K* gene on the *fim3* operon were unsuccessful. *In vitro* analysis of the loss of *fim3K* did not show any observable effect on the *fim3* phenotype suggesting a potential lack of function of *fim3K*.

The lack of observable effect of *fim3K* may be as a result of direct loss of

function. Although there are some examples of pseudogenes that are still functional (either in their original or specialized capacity) (Lerat and Ochman, 2005), there other examples were pseudogenes have completely lost their function with significant changes in gene sequence and protein formation (Lerat and Ochman, 2005, Feng, Chen et al., 2011, Kuo and Ochman, 2010). While there are several suggested ways in which pseudogenes might have come about, a predominant theory of "use it or lose it" (Cooper, 2014) may be applicable in the observed decay of *fim3K* gene. The exact function of *fimK* has been complex and different function has been annotated to this gene depending on *K. pneumoniae* strain. In *K. pneumoniae* Top52, the *fimK* gene was observed as a regulator that inhibits the expression of *fim* possibly through the EAL domain (Rosen, Pinkner et al., 2008). Recently however, *fimK* was observed to increase *fim* expression most likely by binding to *fimS* and increasing the expression of *fimA* encoding the major fimbrial subunit (Wang, Huang et al., 2013). Also, biofilm analysis of several *K. pneumoniae* strains in this lab showed that while some strains are affected by deletion of *fim3K,* others do not appear to show any significant difference with loss of over expression of this gene (Aartsen, 2011). These discrepancies may explain a purifying selection that lead to the truncation in *fim3K* such that a non-essential gene is discarded as several DNA processes such as replication are time-consuming and expensive in respect to use of energy (Lee and Marx, 2012). The molecular evolution of fimbrial systems and its impact of phenotype and virulence would be an interesting theme for future studies.

# Chapter 6: Conclusion

It was previously proposed, following the initial discovery and analysis of *fim2* that this operon may contribute to the virulence of the host *K. pneumoniae* strain as seen with *in vivo* studies of this operon. In spite of the previous and extensive study of *fim2,* a definite observation of *fim2* fimbriae structure and phenotype was not seen. Within this thesis however, creation of hybrid *fim-fim2* fimbriae shows the fimbriae forming potential of the *fim2* operon. The replacement of potential major adhesin *fimA* with *fim2A* was able to produce visualisable fimbriae, which was further analysed in other experiments. Due to the high level of similarity between *fimA* and *fim2A* observed along with the *in vitro* and *in vivo* analysis of *fim2*, it is very likely that *fim2* forms fimbriae for bacterial host attachment under specific conditions. Further investigation on the emergence of *fim2* and experimental anlaysis of *fim2* may provide more understanding of this *fim* variant, which may prove a potential target for antivirulence therapies. These antivirulence therapies, unlike traditional antibiotics, endeavour to disturb virulence properties of bacteria without directly affecting the bacterial viability (Cegelski, Marshall et al., 2008). Traditional antibiotics are known to inadvertently lead to antibiotic resistance of these bacteria, increasing their level of virulence (Alanis, 2005). Antivirulence therapies aim to wane the selective and evolutionary pressure on bacteria to develop resistance and also aim to minimise perturbations of the host microbiota (Cegelski, Marshall et al., 2008, Sintim, Smith et al., 2010). Type 1 fimbriae as well as P pili in *E. coli* have been selected targets for the development of antivirulence

molecules called pilicides (Åberg and Almqvist, 2007). These pilicides function by binding to the conserved regions of the fimbrial chaperon proteins, thereby disrupting the assembly of the fimbrial structure. Since a potential of *fim2* fimbriation has been observed in this project, antivirulence therapies targeting *E. coli* fimbriae structures may be applicable to closely related *K. pneumoniae* type 1 fimbriae and subsequently a putative Fim2 fimbriae (Åberg and Almqvist, 2007).

This thesis describes the first example of microevolution within the type 1 *fim* gene cluster of *Klebsiella* by PCR mapping and sequence analysis of *fim3.* This operon found within certain *K. pneumoniae* strains that possess fim gene homologs with higher non-synonymous changes than has ever been reported for *K. pneumoniae fim.* Moreover, the presence of truncated *fimK* homolog in *fim3* (*fim3K*) suggests selective pressure on these genes. While *in vitro* and in *vivo* analysis of *fim3* shows many phenotypic and functional similarities between *fim* and *fim3*, the emergence of *fim3* in *K. pneumoniae* has a very important impact on the topic of host adaptation in bacteria.

Within *K. pneumoniae* fimbrial systems, pathoadaptive mutations have been observed by single point mutations shown to increase the virulence of the bacterial strain (Stahlhut, Chattopadhyay et al., 2009, Kisiela, Chattopadhyay et al., 2012). Horizontal gene transfers have also been observed to add to virulence of *K. pneumoniae* (Frost, Leplae et al., 2005) and even assist adaptation of the bacteria to certain environments (De la Cruz and Davies, 2000, Ochman, Lawrence et al., 2000). Although genome loss and

degradation has been seen to contribute to the evolution of some bacteria (Day, Fernandez et al., 2001, Kuo and Ochman, 2010) and proposed in analysis fimbrial gene clusters among *Salmonella* species (Yue, Rankin et al., 2012), *fim3K* shows the first example of gene degradation in *K. pneumoniae fim*, potentially as a result of adaptive evolution to certain host and environmental pressures. Understanding how and why *fim3* may have developed from *fim* could shed more insight into the bacterial genetics and evolution.

Further investigation of pathoadaptation and microevolution of virulence factors such as fimbriae in these bacteria may be applicable to the development of therapeutics for combating bacterial infections.

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# Appendix 1. Culture media

### Artificial urine media (AUM)

*USE: Growth medium for bacterial cultures*

This complex medium was prepared as described by Brooks and Keevil (39).

### Lysogeny broth (LB) and agar (LA)

*USE: Standard liquid (LB) and solid growth (LA) medium for bacterial cultures*

Lysogeny broth (LB) was made by dissolving 4 g of tryptone, 2 g of yeast extract and 2 g of NaCl in $dH_2O$ to a final volume of 400 ml. LB agar (LA) was prepared as for LB with 1.5 % w/v of agar. Both media types were autoclaved before use.

### LAS and LAGS

*USE: LAS and LAGS are used as counterselection media for allelic exchange*

LB agar with 6 % sucrose (LAS) was prepared by dissolving 4 g of tryptone, 2 g of yeast extract, 6 g of agar powder and 24 g of sucrose in $dH_2O$ to a final volume of 400 ml. LAGS is LAS supplemented with 9 µg/ml gentamicin.

### M9 broth and agar

*USE: Minimal media for selection of desired merodiploid strains*

A 5x M9 media stock was prepared by dissolving 15 g $NA_2HPO_4$, 7.5 g $KH_2PO_4$, 2.5 g $NH_4Cl$, 1.25 g NaCl and 7.5 mg $CaCl_2$ in $dH_2O$ to a final volume

of 500 ml and autoclaved. 100 ml of 5x M9 media stock, 5 ml of 20 % glycerol (autoclaved) and 500 µl of 1 M $MgSO_4.7H_2O$ (filter sterilized) were added to 395 ml of autoclaved $dH_2O$ to make M9 broth. When M9 agar was required 1.5 % w/v agar was added.

**SOC (Super Optimal broth with Catabolite repression)**

*USE: Broth for non-selective outgrowth of bacteria post-transformation*

SOC was prepared by dissolving 5 g of tryptone, 2.5 g of yeast extract and 5 g of NaCl into 200 ml of $dH_2O$. After autoclaving, 50 µl of 2 M $MgCl_2$ (filter sterilized) and 200 µl of 1M glucose (filter sterilised) were added to 1 ml of medium.

## Appendix 2. Reagents

### Phosphate buffered saline (PBS)

8 g NaCl 0.2 g KCl 1.15 g $Na_2HPO_4.7H_2O$ 0.20 g $KH_2PO_4$ pH 7.4 Make up to 500 ml in $dH_2O$ Sterilized by autoclaving

### Tris-EDTA (TE) buffer

10 mM Tris-HCl, pH 8.0 1 mM EDTA, pH 8.0

### Lysis buffer (native)

6.9 g $NaH_2PO_4.H_2O$ 17.54 g NaCl 0.68 g imidazole Adjust pH to 8.0 using NaOH Add water to 1 l

### 50x Tris-acetate-EDTA (TAE) buffer

2 M Tris-HCl 2 M Acetic acid 50 mM EDTA

### 10x Tris-Glycine electrophoresis buffer

30.2 g Tris-HCl pH 8.3 144 g glycine 10.0 g SDS Add $dH_2O$ to 1000ml Dilute to 1x for working stock

### Lysis buffer (denaturing)

13.8 g $NaH_2PO_4.H_2O$ 1.2 g Tris base 480.5 g urea Adjust pH to 8.0 using NaOH Add water to 1 l

# Appendix 3

## Appendix 1: Primers used in this study

| | |
|---|---|
| **fimA_RTPCR_fwd** | ACCGTTCAGTTAGGCCAGGT |
| **fimA_RTPCR_rev** | CCGGTATTGTCGAGGATCTG |
| **fimA2_RTPCR_fwd** | CGTTAATGGCGGTACAGTGC |
| **fimA2_RTPCR_rev** | CTGAGGTACCGGAGAAAGCA |
| **mrkA_RTPCR_fwd** | CGATGCGAACGTTTACCTGT |
| **mrkA_RTPCR_rev** | CAGGTAGCCCTGTTGTTTGC |
| **Fim3A_rtpcr_fwd** | ACCGTCCAGTTGGGCCAGGT |
| **Fim3A_rtpcr_rev** | CCGGTGTTGTCGAGGATCTG |
| **fim3H_rtpcr_fwd** | CGGAAACGATCACTGACTAC |
| **fimH_RTPCR_rev** | GATTAACGATCCTGCGGTGA |
| **fim3K_rt_fwd** | TAAGTCATGCCGAGCTCAAA |
| **fim3K_rt_screen_rev** | CATCGCTTCCCCTCATGC |
| **rpoD_qPCR_fwd** | CACAGCTGAAGCTTCTTGTCC |
| **rpoD_qPCR_rev** | ATCCGGTGCTTCTTCCATC |
| **fimA_qPCR_fwd** | AGTTAGGCCAGGTGCGTTC |
| **fimA_qPCR_rev** | ACGGTGGTATTGCTGCTGTC |
| **fimA2_qPCR_fwd** | CAAAGCGTCTGTTGCTTTCTC |
| **fimA2_qPCR_rev** | GTCGCTGCACTGAAGGATG |
| **mrkA_qPCR_fwd** | TGATGGCACTAAACAGGATGAC |
| **mrkA_qPCR_rev** | CGTTGTCAGTAGACAGCACCAG |
| **Fim3A_qPCR_fwd** | AGTTGGGCCAGGTGCGTTC |
| **Amp_rtpcr_fwd** | TTTGCCTTCCTGTTTTTGCT |
| **Amp_rtpcr_Rev** | ATAATACCGCGCCACATAGC |
| **p7_LacI_fwd** | ACAGGATTTTCGCCTGCTGG |

| | |
|---|---|
| **p7_mcs_rev** | CCATGGTCTGTTTCCTGTG |
| **SC_sacB_mcs_f** | ACAGGAAACAGACCATGGCGGCCGCAACTTTATGCCCATGCAACAG |
| **SC_cat_fimI_r** | TTCCCTGCCTGACTCGGGTCAGCTTGTCTGTAAGCGGATG |
| **pf_fimI_fwd** | GACCCGAGTCAGGCAGGGAAGCTATT |
| **pf_fimI_rev** | AAATTTAGCGTCGTCGGTCCGC |
| **pf_SC_fim2A_mcs_f** | ACAGGAAACAGACCATGGCGGCCGCATGAATGCAAAAATTTTTG |
| **pf_SC_fim2A_fimI_r** | TTCCCTGCCTGACTCGGGTCTTACTCGTATTGCACTTTGAAGG |
| **SC_cat_fim2I_r** | CCCTGTCTGATATTGAAAAGCTTGTCTGTAAGCGGATGC |
| **pf2_fim2I_fwd** | TTTCAATATCAGACAGGGATGCG |
| **pf2_fim2I_rev** | GCCCGATACTTGGCTACAAAATG |
| **pf2_SC_fimA_mcs_f** | AGGAAACAGACCATGGCGGCCGCATGAAAATCAAAACACTGGC |
| **pf2_SC_fimA_fim2I_r** | CCCTGTCTGATATTGAAATTACTCGTACTGCACTTTGAACGTGGCAT |
| **pf_fimG_fwd** | AAATGGACTCATGTGGGCTG |
| **pf_fimG_rev** | GATGCTCTCCTGGCTCAG |
| **SC_sacB_fimH_f** | CTGAGCCAGGAGAGCATCAACTTTATGCCCATGCAACAG |
| **SC_cat_fimH_r** | GGCGAGAGGATATAATCGGTCAAGCTTGTCTGTAAGCGGATGC |
| **Pf_fimK_fwd** | TGACCGATTATATCCTCTCGCC |
| **Pf_fimK_rev** | CGTTTTCTGGCTCAATCCGA |
| **Pf_SC_fim2H_G_fwd** | CTGAGCCAGGAGAGCATCATGAATAAACTTATCCCTCAG |
| **Pf_SC_fim2H_K_rev** | GGCGAGAGGATATAATCGGTCATTACTGATAGATAAATGTTACTCCG |
| **Pf2_fim2G_fwd** | GAAATATCGATTTGCAGGAGCGC |

| | |
|---|---|
| **Pf2_fim2G_rev** | TCACATATTCTCCTTGCTCAAGC |
| **SC_sacB_fim2H_fw** | GCTTGAGCAAGGAGAATATGTGAAACTTTA TGCCCATGCAACAG |
| **SC_sacB_fim2H_rv** | CTTTCACTGACCGTAAATAGCTTGTCTGTAA GCGGATGC |
| **Pf2_fim2K_fwd** | ATTTACGGTCAGTGAAAG |
| **Pf2_fim2K_rev** | CAGATAAGATGGTGATGGTG |
| **Pf2_SC_fimH_G2_f** | GCTTGAGCAAGGAGAATATGTGAATGATGA AAAAAATAATCCCCCTG |
| **Pf2_SC_fimh_K2_r** | CTTTCACTGACCGTAAATTCATTGATAGACA AAGGTGATGCCG |
| **fimHY_2A_sfil_rev** | GCGGGCCCTCCTGGCCCGAATA |
| **fim2HY_A_sfil_rev** | ACGGGCCGTCCTGGCCCAAAA |
| | |
| **Kp_FimA_fwd** | AAAATCAAAACACTGGCAATGATTG |
| **Kp_FimA_rev** | CAGCAGACCAGATTTCATTCC |
| **fim_dwnstrm_flnk_kp** | CAC CCG CGA ATA CGA CGA |
| **fim_upstrm_flank_kp** | ATT TAA CAA TGA CCT GAC GCA |
| **fimG_rev_kleb** | CGT TAA TCA CCG CCT GAA T |
| **fimG_fwd_kleb** | GCG ATC TCT ATA CCT TTA GC |
| **fimC_rev_kleb** | ACG CTG GTT TTG CCC ATC |
| **fimC_fwd_kleb** | ACA GAA GCA GGT CCA GCT |
| **fim_dwn_flnk_ko** | AAA CGG TAC CCA GCG CTT C |
| **fimH3_forward** | TTA TCC CTT TAT TCA CCA CCC |
| **fimH3_reverse** | TAC CCC AGC TGT TGG CTC |
| **fimK_fwd** | GCAGACGATAGGCGAGCA |
| **fimupstrm2_fwd** | Gttttgcagtgaatgctatcc |
| **sp28_fimB_r** | acaggagtggcgcagcaT |
| **Kp_fimA_fwd_inv_R** | CAATCATTGCCAGTGTTTTGATTTT |
| **Sp28_fimC_rev_inv_F** | GATGGGTAAAACCAGCGT |
| **fim3K_fwd_EcoRI** | CGGAATTCAGGAGGACAGGGATGACCGAGT |

| | |
|---|---|
| | ACATCCTTTCT |
| **fim3K_rev_HindIII** | CCCAAGCTTTCATGCCCGGACAAACGC |
| **fim3K_trnscpt_rev** | TCGCCAGCGAATCCACG |
| **sp28_fim_fwd_XhoI** | atat CTCGAG ATGCGGGTATCATCAAGAGG |
| **sp28_fim_rev_Kan1** | GAAGCAGCTCCAGCCTACACACAGGAGTGGC GCAGCAT |
| **sp28_fim_fwd_Kan2** | GGACCATGGCTAATTCCCAtGCAGACGATAG GCGAGCA |
| **sp28_fim_rev_SpeI** | atat TGATCA AATAAGGAATTACTGGAAGCCGG |
| **fim3K_lf-h_SpeI** | atatACTAGT CGACCACCACGGAAACG |
| **fim3K_lf-342_rev2** | GCA GGG AGA AAG GAT GTA CTC GGT CAT CAT TGA TAG AC |
| **Gmf_fim3K_cmp_fwd** | CGACTATATCCTCTCACCCTGCCGAATTGGG GATCTTGAAGTTCCT |
| **fim3K-d_rf_fwd2** | CGT TTG TCC GGG CAT GAG |
| **fim3K_lf_XhoI_rev** | atatCTCGAG ATAAGGAATTACTGGAAGCCGG |
| **mrk_lf_fwd** | ATATTCTGCTGATCCCCGG |
| **mrk_lf_rev_kan1** | GAAGCAGCTCCAGCCTACACTATTTCAATTT CAGTACGTTTGC |
| **mrk_rf_fwd_kan2** | GGACCATGGCTAATTCCCATTACCGAAGAAA TTAACCTGGC |
| **mrk_rf_rev** | GCGATTTAGATCACATTTTTATGG |
| **mrk_lf_fwd2** | AATATTTGCCTGGAAAAACCTCACC |
| **mrk_lf_Kn1_rev2** | GAAGCAGCTCCAGCCTACACACTCCTCTCTG AAAATAGC |
| **mrkA_rf_Kn2_fwd** | GGACCATGGCTAATTCCCAtCGTTGTCAGTA GACAGCACCAGC |
| **mrkA_rf_rev** | CGATGCGAACGTTTACCTGTCTCC |
| **mrk_scrn_del** | CGG ATG CGC AGA AAA TGA CG |

| | |
|---|---|
| **fim3K_sbfI_rev** | tatCCTGCAGGATAAGGAATTACTGGAAG |
| **Vic3oF** | GTTTTCCCAGTCACGACGTTGTA**GGCGAAA TGGCWGAGAACCA** |
| **Vic2oR** | TTGTGAGCGGATAACAATTTC**GAGTCTTCG AAGTTGTAACC** |
| **gapA173oF** | GTTTTCCCAGTCACGACGTTGTA**TGAAATA TGACTCCACTCACGG** |
| **gapA181oR** | TTGTGAGCGGATAACAATTTC**CTTCAGAAG CGGCTTTGATGGCTT** |
| **mdh130oF** | GTTTTCCCAGTCACGACGTTGTA **CCCAACTCGCTTCAGGTTCAG** |
| **mdh867oR** | TTGTGAGCGGATAACAATTTC**CCGTTTTTC CCCAGCAGCAG** |
| **pgi1FoF** | GTTTTCCCAGTCACGACGTTGTA**GAGAAAA ACCTGCCTGTACTGCTGGC** |
| **pgi1RoR** | TTGTGAGCGGATAACAATTTC**CGCGCCACG CTTTATAGCGGTTAAT** |
| **phoE604.1oF** | GTTTTCCCAGTCACGACGTTGTA**ACCTACCG CAACACCGACTTCTTCGG** |
| **phoE604.2oR** | TTGTGAGCGGATAACAATTTC**TGATCAGAA CTGGTAGGTGAT** |
| **infB1FoF** | GTTTTCCCAGTCACGACGTTGTA**CTCGCTGC TGGACTATATTCG** |
| **infB1RoR** | TTGTGAGCGGATAACAATTTC **CGCTTTCAGCTCAAGAACTTC** |
| **tonB1FoF** | GTTTTCCCAGTCACGACGTTGTA**CTTTATA CCTCGGTACATCAGGTT** |
| **tonB2RoR** | TTGTGAGCGGATAACAATTTC**ATTCGCCGG CTGRGCRGAGAG** |
| **mlst_of** | GTTTTCCCAGTCACGACGTTGTA |
| **mlst_or** | TTGTGAGCGGATAACAATTTC |
| | |