Removal of Acetylation by Pneumococcal Esterases Potentiates Neuraminidase Activity for Mucin Utilisation, Colonisation and Virulence

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Statement of originality

The accompanying thesis submitted for the degree of PhD entitled "Removal of acetylation by pneumococcal esterases potentiates neuraminidase activity for mucin utilisation, colonisation and virulence" is based on work conducted by the author in the Department of Infection, Immunity and Inflammation at the University of Leicester mainly during the period between October 2012 and September 2015.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other University.

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Abstract

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The genome of pneumococcal strains contains 4 putative esterase genes (SPD_0534, *estA;* SPD_0932; SPD_1239; and SPD_1506, *axe*). Esterases have been reported to be important for bacterial physiology and virulence in other microorganisms but their role in *S. pneumoniae* is unknown. We hypothesised that esterases potentiate neuraminidase activity by removing acetylation in sialic acid. This hypothesis was tested using isogenic mutants and recombinant esterases in microbiological, biochemical and *In vivo* assays.

The results showed that pneumococcal esterases are specific for short acyl chains, all gene contributed to overall esterase activity but SPD_0534 (EstA) was found to be responsible for main esterase activity. Both Axe and EstA could use acetylated xylan and Bovine Sub-maxillary Mucin (BSM), a highly acetylated substrate, but only EstA was active against tributyrin (triglyceride). Incubation of BSM with either Axe or EstA led to the acetate release in a time and concentration dependent manner, and pre-treatment of BSM with either EstA or Axe increased sialic acid release by subsequent exposure to neuraminidase. qRT-PCR results showed that the expression level of *estA* and *axe* increased when exposed to BSM and in respiratory tissues. Mutation of either *estA* alone or in combination with *nanA* (codes for neuraminidase A), or the replacement of serine 121 to alanine in EstA, reduced the pneumococcal ability to utilise BSM as sole carbon source, sialic acid (Sia) release, colonisation and virulence in a mouse model of pneumococcal infection.

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Abbreviations

µg	Microgram	**CFU**	Colony forming units
µg/ml	Microgram per milliliter	**cfu/ml**	Colony forming units per milliliter
µl	Microliter	**CSP**	Competence stimulating peptide
µm	Micrometer	**cDNA**	Complementary DNA
µmol	Micromole	**CDM**	Chemically defined medium
APS	Ammonium persulphate	**dH2O**	Distilled water
Asp	Aspartate	**DNA**	Deoxyribonucleic acid
BAB	Blood Agar Base	**DMSO**	Dimethyl sulfoxide
BHI	Brain Heart Infusion	**dNTP**	Deoxynucleotide triphosphate
bp	Base pair	**DTT**	Dithiothreitol
BSA	Bovine serum albumin	**EDTA**	Ethylenediaminetetraacetic acid
BgaA	β-galactosidase A	**GalNAc**	*N*-acetylgalactosamine
BSM	Bovine sub-maxillary mucin	**GlcNAc**	*N*-acetylglucosamine
BCIP/NBT	5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium	**g**	Gram
BTB	Bromothymol blue	**His**	Histidine
CBP	Choline-binding protein	**i.n.**	Intranasal

IgG	Immunoglobulin G	**pNP-NANA**	2-*O*-(p-Nitrophenyl)-α-D-*N*-acetylneuraminic acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside	**PMFS**	phenylmethanesulfonyl fluoride
kDa	Kilo dalton	**PVC**	Valent conjugated vaccine
LB	Luria-Bertani medium	**p-NP**	p-Nitrophenyl
LytA	*N*-acetyl-muramoyl-L-alanine amidase	**rpm**	Revolutions per minute
ml	Milliliter	**Sia**	Sialic acid
NanA	Neuraminidase A	**SDS**	Sodium dodecyl sulphate
NADH	Nicotinamide adenine dinucleotide	**SDS-PAGE**	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
ng	Nanogram	**SOAT**	sialate-*O*-acetyltransferase
nm	Nanometre	**Ser**	Serine
Neu5Ac	*N*-Acetylneuraminic acid	**StrH**	β-*N*-acetylglucosaminidase
Neu5Gc	*N*-glycolylneuraminic acid	**TAE**	Tris acetic acid EDTA
Neu5,7Ac$_2$	7-*O*-acetylneuraminic acid	**TBA**	Thiobarbutiric acid
Neu5,9Ac$_2$	9-*O*-acetylneuraminic acid	**TEMED**	Tetramethyethylenediamine
OD	Optical density	**TRs**	Tandem repeats
PBP	Penicillin-binding proteins	**v/v**	Volume per volume
PAF	Platelet-activating factor	**w/v**	Weight per volume
PPV-23	23-valent-pneumococcal-polysaccharide vaccine		

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Chapter One

Introduction:

Overview:

The pneumococcus is one of the most dangerous pathogens causing various diseases such as pneumonia, meningitis, sepsis and bacteraemia. This section will introduce general characteristics and virulence determinants of *Streptococcus pneumoniae*, pathophysiology of pneumococcal diseases, and prevention and therapeutic strategies available for pneumococcal diseases. It will also address the pneumococcal interaction with host glycoproteins and how glycosylation and *O*-acetylation of glycoconjugates might affect pneumococcal survival *In vivo*. It will give an overview of the main characteristics of esterases, and how they may influence the pathogenicity of *S. pneumoniae* by potentiating the activity of other glycosidases such as neuraminidase.

1.1 The pneumococcus:

1.1.1 General characteristics:

Streptococcus pneumoniae (Figure 1.1) was first isolated in 1875 by Edwin Klebs from lung tissue of people suffered from pneumonia. It was described in detail by George M. Sternberg and Louis Pasteur in 1881 (Gihon et al., 1881). In 1883, Anton Weichselbum named it as *Diplococcus pneumoniae* because it typically appears in pairs. This description was accepted until 1974, when it was renamed as *S. pneumoniae* because it grows in chains in liquid medium. *S. pneumoniae* is a Gram-positive, spherical shaped (lancet shape) cell of 0.5-1.5 μm in diameter, encapsulated, non-motile, catalase negative and fermentative microbe. It is an aero-tolerant anaerobic microbe, which can be identified as α-haemolytic under aerobic conditions and β-haemolytic when grown anaerobically on medium containing blood in the presence of 5-10% (v/v) CO_2 (Bergey et al., 1974). It grows at the temperature range between 25-42°C, and is sensitive to ethyl hydrocupreine hydrochloride (optochin), which allows differentiation of the pneumococcus from other streptococci (Stevens and Kaplan, 2000, Moore, 1915).

The pneumococcus has an outer surface of cell wall heavily covered with polysaccharide capsule, which is composed of repeated units of sugars anchored to the outer surface of the bacterial cell wall (Lopez and Garcia, 2004), and is used for serotype classification. Recently, 100 different serotypes have been identified (Bogaert et al., 2004b), 11 of which have been associated with the majority of pneumococcal invasive diseases (Johnson et al., 2010). However, other capsule serotypes are also involved in pneumococcal diseases, as *S. pneumoniae* is able to acquire a new capsule operon and switch its capsule to another type, which allows variation through generations (Dowson et al., 1997). Although *S. pneumoniae* also exists as an unencapsulated form, the capsuleless pneumococcus has not been isolated from patients diagnosed with pneumonia (Catterall, 1999). Below the capsule, a well-designated cell wall exists. It is composed of polysaccharide, teichoic acid, lipoteichoic acid, and some cell wall-associated proteins. These proteins are covalently linked with cell wall polysaccharide, which is known as peptidoglycan (murein) (Bogaert et al., 2004b). More than 500 proteins have been identified as pneumococcal membrane and cell wall associated proteins (Briles et al., 1998).

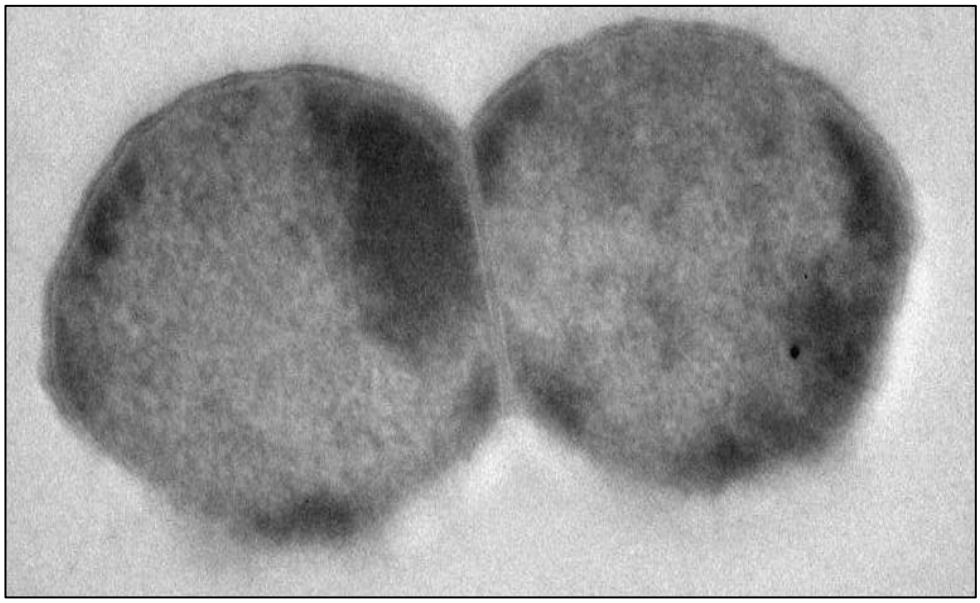

Figure 1.1: Scanning electron micrograph of *S. pneumoniae.*

1.1.2 Pneumococcal epidemiology:

The pneumococcus is one of the most dangerous pathogens in the world (Watson et al., 1993), and is considered to be one of the leading cause of pneumonia, otitis media, meningitis and septicaemia (CDC, 2012). It is the second persistent cause of meningitis, sepsis and otitis media in elderly over the age of 65, immune-suppressed patients and children below the age of 5 (Dagan et al., 1994, Sleeman et al., 2001). This indicates a significant public health problem (Denny and Loda, 1986, Berkley et al., 2005).

The pneumococcal infections can occur in all age groups, however, the most prone are young children and senior individuals, and people who suffer from chronic conditions and immune deficiencies such as acquired immune deficiency syndrome (AIDS) and kidney transplant patients. Amongst these at risk groups, 50-75% of pneumonia cases are due to *S. pneumoniae* (Fedson and Scott, 1999). Although pneumococcal diseases have not been recorded as outbreaks, it occurs in some small communities such as nurseries, childcare centres and pensioner-care houses (CDC, 2013).

Carriage is the first step of invasive pneumococcal diseases. Infants and young children are the main reservoir of pneumococcal carriage. The carriage rate in infants ranges from 27% in developed countries to 85% in developing countries (WHO, 2012). According to the report published by the World Health Organisation (WHO) in 2013, 476,000 out of 8.8 million children under the age of 5 died because of pneumococcal infections (WHO, 2013). The total pneumonia deaths for children under 5 years was reported to be 20% of global mortality, and the number of deaths was over 800,000 for all age groups in 2010 (Lozano et al., 2012). It has been estimated that 14% of child mortality in the world was because of pneumonia, 50% of which was due to pneumococcal infection (Wardlaw et al., 2006). In developing countries, the situation is atrocious. WHO reported that 18% of deaths among children aged less than 5 years are caused by pneumococcal acute respiratory infections (Nga Tong, 2013). It has also been reported that 30-50% of community-acquired pneumonia is attributable to *S. pneumoniae* in Europe and the USA, and 12% of child mortality in Europe and America is attributable to the pneumococcus despite advanced health care provision (Fedson and Scott, 1999). More than 1.2 million cases of pneumonia infections are reported in the United States yearly. Moreover, the pneumococcus is also a serious cause of nosocomial infections. In the UK, it has been reported that 8% of nosocomial pneumonia is attributable to *S. pneumoniae* (Dawn and Michael, 2004).

The progression of pneumococcal pneumonia to meningitis and bacteraemia leads to serious mortality rates. It has been estimated that 60-85% of bacteraemia infections and 47% of meningitis cases was attributable to *S. pneumoniae* in USA (Carbon et al., 2006, Morton and Swartz, 2004). According to the report published by Centre for Disease Control and Prevention in 2012 (CDC, 2012), more than 40,000 people die of pneumococcal bacteraemia, and over 4,400 fatalities were due to pneumococcal meningitis every year in the USA. Another report published by the same organisation in 2005 revealed that *S. pneumoniae* was responsible for nearly 130,000 cases of pneumonia, 6 million cases of otitis media, and 3300 cases of meningitis. 14% of these cases resulted in mortality (CDC, 2005b). These data suggest that pneumococcal infections are serious health problems both in developing and developed countries.

1.1.3 Treatment and prevention of pneumococcal diseases:

***Therapy*:** Penicillin, which is a β-lactam antibiotic (Catterall, 2004), is widely used for treatment of pneumococcal infections. This family includes a wide spectrum of antibiotics such as penicillin G, ampicillin, cephalosporin C and ceftriaxone. The main target of β-lactam antibiotics is penicillin-binding proteins (PBPs) at pneumococcal cell wall peptidoglycan (Charpentier and Tuomanen, 2000). Since its discovery, penicillin reduced the mortality of pneumococcal infections, and was effective against nearly all pneumococcal serotypes (Morton and Swartz, 2004). However, the excessive usage of β-lactam antibiotics led to an increase in prevalence of penicillin resistant strains (Hansman et al., 1971). The resistance of *S. pneumoniae* against penicillin is linked to the modification of penicillin binding proteins (Charpentier and Tuomanen, 2000). The resistance rate varies in different regions. For example, in South Korea, it is up to 70%, whereas no penicillin resistant strain has been reported in the Netherlands (Walsh and Amyes, 2004, Inoue et al., 2004). In the USA, the rate of penicillin resistant pneumococci is approximately 20% (Dawn and Michael, 2004). Most recently, a report published in the USA by the Centre for Disease Control and Prevention revealed that 2.1%, 0.2% and 0,7% of pneumococcal strains isolated from 2,879 individuals are resistant to penicillin, levofloxacin and cefltaxime, respectively (CDC, 2013). A study investigating pneumococcal resistance in eight European countries using 2200 isolates has found that 25-, 2.6-, 5- and 18% were resistant to penicillin, amoxicillin, cefotaxime and cefuroxime, respectively (Reinert et al., 2005). The spread of pneumococcal resistance is dependent upon different factors such as the ability to acquire other microorganisms' DNA that carry antibiotic resistance. This results in new strains that are able to cope with the antibiotic. Therefore, the rising trend of antibiotic resistant strains limits the use of antibiotics.

In addition to β-lactam antibiotics, pneumococcal resistance against other antibiotics has been reported such as macrolides, doxycycline and chloramphenicol (Lynch and Zhanel, 2005). Felmingham et al. (2002) reported that macrolide resistance increased globally due to random usage of macrolide to cure other infectious diseases. For example, pneumococcal resistance rate increased to 80% in South Korea and Japan (Felmingham, 2004). In some European countries, it has been estimated that nearly 27% of all pneumococcal isolates are resistant to macrolides (Reinert et al., 2005). With

regard to tetracycline resistance, the resistance rate has reached 25% in some countries such as Spain, Western Russia and the USA (Jacobs, 2004, Felmingham, 2004). A study published by the Centre for Disease Control and Prevention in 2013 reported that 10.2% of pneumococcal isolates from 2,870 cases were resistant to tetracycline (CDC, 2013) and 28% of pneumococcal strains are resistant to erythromycin

It has been estimated that 20-40% of pneumococcal strains are multi-drug resistant (Van Bambeke et al., 2007). Pneumococcal resistance against trimethoprim/sulfomeoxazole reached up to 50% in some countries such as South Korea and Spain, and 20% in the USA (Felmingham, 2004, Jacobs, 2004). The majority of pneumococcal isolates from Spain and France are found to be resistant to both tetracycline and macrolides (Reinert et al., 2005). Felmingham and Gruneberg (2000) reported that 90% of penicillin resistant pneumococci are also resistant to co-trimoxazole.

Prevention: The pneumococcal antibiotic resistance seems to be a universal concern. Therefore, a new approach is required to eliminate pneumococcal infections. The introduction of pneumococcal immunisation has notably reduced the antibiotic resistance among pneumococcal isolates (CDC, 2005a). Vaccines have been successful in preventing the pneumococcal diseases. Currently, two main types of pneumococcal vaccines are available: the 23-valent-pneumococcal-polysaccharide vaccine (PPV-23) and the protein conjugated pneumococcal vaccine. The 23-valent vaccine, which is known commercially as Pneumovax 23 or Pnu-Immune, immunises against 23 pneumococcal serotypes represented in the vaccine formulation, including 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 20, 22F, 23F and 33F. It has a beneficial impact on reducing invasive pneumococcal infections (Hanna et al., 2006). Although the vaccine is recommended for elderly people and people who suffer from chronic organ failure and diabetes, it does not provide sufficient protection for children under 2 years old. This is because the vaccine stimulates a type-2 immune response that produces antibodies, such as IgG2, from mature B cells. This type of immune response is mainly effective in adults. However, in children under 2 years, this vaccine is inefficient because young children have a weak mature B cell response (Schrag et al., 2001). The vaccine is recommended in some developed countries for adults over the age of 65. Moreover, it has been reported that PPV-23 could not protect

against pneumococcal carriage and common non-invasive diseases such as sinusitis and otitis media (Schrag et al., 2001).

The limited efficacy of PPV-23 led to investigations to develop a vaccine that can be efficient in all age groups, and can induce a high level of memory immune response. As a result, the 7- (PCV-7), 9- (PCV-9), 10- (PCV-10) and 13-valent (PCV-13) conjugated pneumococcal vaccines have been introduced, which consist of purified polysaccharide capsule covalently attached to a nontoxic diphtheria toxoid, which acts as cross-reactive material (Biagini et al., 2003, CDC, 2010), and adjuvant, (Lynch and Zhanel, 2005). They were first licensed for children in the USA in 2000, and in 2006 in the UK. PVC-13 has proved to be an effective vaccine because it can induce the immune system of children from 2 months of age until 13 months (Black et al., 2004, Anttila et al., 1999). The vaccine can induce T-helper cells to produce a T cell dependent response. T-helper cells can convert immature B cells to mature ones (Anttila et al., 1999). Mature B cells produce antibodies enough to provide high level of protection (Anttila et al., 1999).

It was shown that the 13-valent conjugated pneumococcal vaccine reduces the pneumococcal carriage in the nasopharynx (Haddy et al., 2005). Despite its success, protection provided by this vaccine is limited to the small number of pneumococcal serotypes represented in its formulation (1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F). Since its introduction, PCV-13 reduced the mortality among children under 2 years old. A study by Haddy et al. (2005) revealed that the rate of invasive pneumococcal infections was reduced by 67% in children under 2 years old. Other conjugated vaccines also have been effective against pneumococcal infections. PCV-7 (Prenvar), which consists of 7 pneumococcal polysaccharides conjugated to cross reactive material, covered about 80% of invasive pneumococcal strains in France, and was shown to be highly effective (Black et al., 2004). It has been found that PCV-9 is directly linked with the reduction of pneumococcal carriage (Givon-Lavi et al., 2003, Ghaffar et al., 2004). However, other non-vaccine serotypes that have not been included in the vaccine formulation and other resident bacteria will still cause invasive diseases. A study conducted by Kaplan et al. (2004) showed that non-vaccine serotypes, which are not included within the vaccine formulation, become increasingly resistant to penicillin. Kaplan et al. (2004) found that the infections due to non-vaccine serotypes increase rapidly among children under 2 years old. Furthermore, although vaccination

may reduce the rate of pneumococcal infections, the rate of infections by other bacteria can increase. A study by Wiertsema et al. (2011) found that non-typeable *Haemophilus influenzae* has been the dominant cause of otitis media in children vaccinated with PCV-7.

Another issue, which should be taken into consideration, is the high cost of conjugated vaccines. The majority of countries including poor and developing countries cannot afford the cost of conjugated vaccines (Swiatlo and Ware, 2003). As a result, there is an urgent need to develop effective vaccines that can cover all pneumococcal serotypes, effective in all age groups, and are reasonably cheap. An alternative would be immunisation with protein-based vaccine that can be globally found in all pneumococcal serotypes. Bacterial proteins have been shown to elicit a high immune response in all age groups as they induce immune response independently of high memory cell production (Swiatlo and Ware, 2003).

1.1.4 Pneumococcal virulence determinants:

Virulence studies allow understanding of disease mechanism and the identification of microbial targets for effective drug and vaccine design. *S. pneumoniae* has various virulence factors, which play crucial roles in colonisation and invasion. These factors are varied according to their location, function and chemical composition. Moreover, their importance in different tissue sites is also varied.

Pneumococcal capsule, the outer polysaccharide layer is approximately 200-400 nm in thickness, and is the most potent pneumococcal virulence determinant in terms of its contribution to the pathophysiology of pneumococcal diseases (Figure 1.2). There are over 100 different capsular serotypes, each with distinct biochemical compositions. The capsule has anti-phagocytic characteristics, which assists the pathogen to escape or resist phagocytosis of host immune cells by preventing IgG and the complement iC3b deposition (Sorensen et al., 1990). The negative charge of capsular polysaccharide protects the pathogen from removal by respiratory mucus and against host phagocytosis (Kadioglu et al., 2002). The expression of capsule is dependent upon the site of infection. For example, the expression is down regulated in carriage, and up-regulated in blood stream infection in mice. A study reported that there is a clear relationship between colonisation of the pneumococcus and capsule thickness (Martin et al., 2003).

It is generally assumed that encapsulated strains have a potential ability to colonise and invade the epithelial surface of the lung tissue (Crum et al., 2004).

Pneumococcal cell wall is the most potent inducer of host inflammation as it activates the complement system (Catterall, 1999). The stimulation of leukocytes and platelet activating factor (PAF) are attributable to pneumococcal cell wall components. Teichoic acid and lipoteichoic acid enhance the inflammatory effect of pneumococcal cell wall (Mitchell et al., 1997).

The pneumococcal cell surface is also decorated with several protein based virulence determinants (Figure 1.2). Approximately 500 pneumococcal surface proteins have been discovered, some of which are involved in cell wall synthesis (Todar, 2003). Three types of pneumococcal surface proteins have been extensively studied including choline-binding proteins (CBPs), lipoproteins, and LPxTG protein family (Bergmann and Hammerschmidt, 2006).

CBPs are membrane-anchored proteins that have been discovered in the majority of bacterial species (Figure 1.2). These proteins are mainly involved in pneumococcal adhesion with host glycoconjugates (Pagliero et al., 2005). Other proteins of this family bind with secretory immunoglobulin A (sIgA) (Hammerschmidt et al., 1997). CBPs are also involved in human factor H (hFH) recognition (Dave et al., 2001), which regulates the complement system activation and prevents the human tissue from being attacked by complement system (Ferreira et al., 2010). CBPs recruit hFH excessively to evade the complement defence (Ferreira et al., 2010). Sequence analysis of pneumococcal genome revealed that there are 13-16 CBPs sharing the same putative sequence alignment (García et al., 2005). Autolysin LytA amidase (*N*-acetyl-muramoyl-L-alanine amidase), β-*N*-acetylglucosamidase (LytB), β-*N*-acetylmuramidase (LytC), phosphorylcholine esterase (Pce or CbpE), choline binding protein A (CbpA) and pneumococcal surface protein A (PspA) are the well-studied choline binding proteins (CBPs), which are covalently and/or non-covalently anchored to the cell wall peptidoglycan (Garcia et al., 1985, De Las Rivas et al., 2002, Garcia et al., 1999, Vollmer and Tomasz, 2001, Tuomanen, 1999).

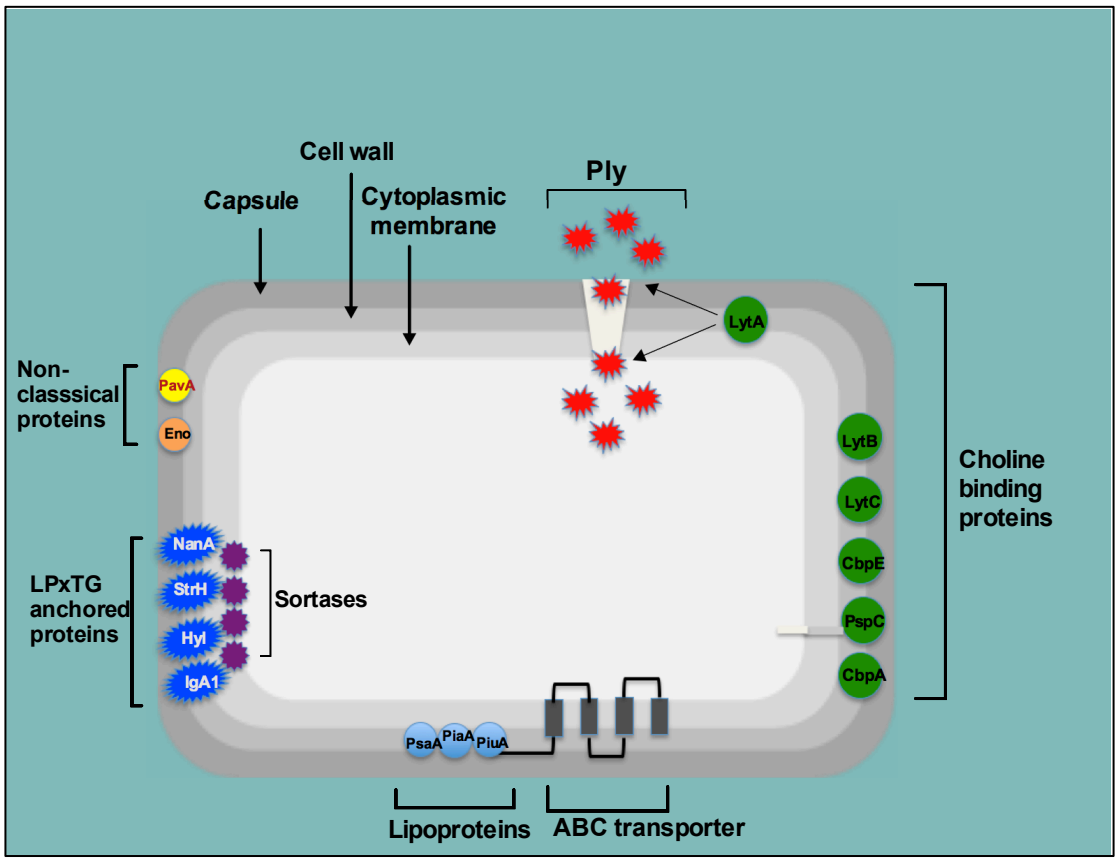

Figure 1.2: Schematic representation of pneumococcal virulence determinants. **Choline binding proteins**: LytA: autolysin A; LytB: endo-β-*N*-acetylglucosaminidase; LytC: 1,4- β-*N*-acetylmuramidase; CbpE: choline binding protein E; PspC: pneumococcal surface protein C; PspA: pneumococcal surface protein A. **LPxTG-anchored proteins:** NanA: neuraminidase A; StrH: β-*N*-acetylglucosaminidase; Hyl: hyaluromate lyase; IgA1: IgA1 protease. **Lipoproteins:** PsaA: pneumococcal surface antigen A; PiuA: pneumococcal iron uptake A; PiaA: pneumococcal iron acquisition. Figure is adapted from Kadioglu et al. (2008).

LytA amidase is an *N*-acetyl-muramoyl-L-alanine amidase, which has a role in hydrolysis of pneumococcal cell wall to release the toxin pneumolysin and inflammatory teichoic acid to the pneumococcal cell surface (Figure 1.2). Binding with choline, which is the main chemical component of cell wall, activates this enzyme, and this reaction increases LytA concentration. The active form of LytA is able to break down pneumococcal peptidoglycan (Romero et al., 2007). LytA is also involved in

pneumococcal cell division (Giudicelli and Tomasz, 1984). The absence of LytA abolished the virulence characteristics of the pneumococcus in experimental mouse infection, showing the importance of LytA for pneumococcal autolysis (Berry and Paton, 2000). Berry et al. (1989) found that mice challenged with a LytA-deficient strain survived longer time than the wild type. It has also been shown that LytA is involved in pneumococcal meningitis. Hirst et al. (2008) investigated the importance of LytA in pneumococcal meningitis using an acute infection model of rats, and found that the pneumococcus lacking either pneumolysin or autolysin caused very moderate or no disease compared to parental strain that was able to cause meningitis 26 h post-infection. It was demonstrated that the antibodies raised against LytA protects mice against otherwise lethal challenge (Canvin et al., 1995).

CbpA, which is also known as pneumococcal surface protein C (PspC), is another example of pneumococcal CBPs (Figure 1.2). It has the adhesive characteristics to facilitate pneumococcal adhesion to host cells. CbpA-deficient pneumococcus could not adhere to cytokine-activated type II host cells (Mitchell, 2000). The positive charge of this protein is advantageous by enhancing the adherence to epithelial surfaces (Swiatlo et al., 2002). It has been shown that CbpA is highly expressed during carriage and invasive infections (Rosenow et al., 1997). CbpA is also able to bind with the polymeric immune receptor (pIgR), which is responsible for transporting the antibodies from host sera to the site of infection, and prevents the antibody efflux (Zhang et al., 2000). Hammerschmidt et al. (1997) showed that CbpA is able to bind with IgA and inhibits the classical complement pathway. Moreover, it can bind to factor H and stops the activation of alternative complement pathway (Dave et al., 2001) or it can inhibit C3 deposition, which is crucial for phagocytosis (Cheng et al., 2000). The deletion of CbpA attenuates pneumococcal colonisation (Rosenow et al., 1997), acute pneumonia (Jounblat et al., 2003), and pneumococcal sepsis (Iannelli et al., 2004) in experimental mouse models.

It has been shown that **PspA** is required for pneumococcal colonisation as well as for pneumonia and bacteraemia. Ogunniyi et al. (2007) found that PspA-deficient pneumococcus could not survive in the blood stream. Another study published by King et al. (2009) revealed the contribution of PspA in pneumococcal secondary infection after viral infection. The authors found that the growth of PspA mutant was

approximately 1800 fold lower than wild type in the mouse nasopharynx. They also found that PspA immunisation significantly reduced pneumococcal secondary infection (King et al., 2009), indicating protective characteristics of the protein. It is widely expressed in most of pneumococcal clinical isolates (Figure 1.2) (Crain et al., 1990). The highly negative charge of PspA prevents complement proteins accumulating on the pneumococcal surface (Jedrzejas et al., 2000), leading to enhanced pneumococcal resistance against phagocytosis (Briles et al., 1997). Immunisation with different domains of PspA produces specific antibodies that provide a full protection against invasive pneumococcal infections, and this makes PspA an attractive target for inclusion into pneumococcal conjugated vaccine (Nabors et al., 2000, Briles et al., 2000b). It also binds covalently with capsular teichoic acid and lipoteichoic acid to disrupt the complement deposition to pneumococcal surface (Kadioglu et al., 2008, Bergmann and Hammerschmidt, 2006). This sophisticated mechanism might have a potential role in immune response (Baril et al., 2006, Hollingshead et al., 2006). Mice immunised with recombinant PspA had less pneumococci in blood and nasopharynx than the non-immunised mice (Wu et al., 1997a). PspA was also reported to inhibit the bactericidal effect of apolactoferrin by binding to host lactoferrin.

S. pneumoniae also expresses more than 42 cell-surface lipoproteins. These proteins may play dual roles; they may modulate the pneumococcal adhesion and facilitate ion and nutrient uptake (Figure 1.2) (Bergmann and Hammerschmidt, 2006). The best example for these proteins is pneumococcal surface antigen A (PsaA). **PsaA** is ATP binding cassette transporter. It is a membrane-associated lipoprotein that is involved in Manganese (Mn^{2+}) and Zinc (Zn^{2+}) uptake (Lawrence et al., 1998, Dintilhac et al., 1997). The consensus sequence of this protein contains a conserved lipid-binding site at N-terminal (Sampson et al., 1994). A study showed that PsaA mutant fails to bind to mammalian cells *in vitro*, and was more susceptible to oxidative stress (Berry and Paton, 1996, Tseng et al., 2002). Intranasal and intraperitoneal challenge with PsaA-deficient pneumococcus revealed that this protein is required for pneumococcal virulence (Berry and Paton, 1996).

In addition, PsaA is also a potent immunogenic agent (Baril et al., 2006). The pneumococcus failed to colonise nasopharyneal cavity of mice immunised with PsaA alone or in combination with PspA (Briles et al., 2000a, Jedrzejas et al., 2000). The

contribution of this protein in pneumococcal virulence is linked to its impact on pneumococcal adhesion (Berry and Paton, 1996, Marra et al., 2002). *In vivo* studies showed that antibody raised against PsaA prevents the pneumococcus to bind to nasopharyngeal cells (Romero-Steiner et al., 2003). However, recent publications showed that it is directly involved in Mn^{2+} and Zn^{2+} uptake (Counago et al., 2014, McDevitt et al., 2011).

Pneumolysin (Ply) is a cholesterol-dependent cytolysin, being the most potent virulence protein of the pneumococcus (Figure 1.2) (Alouf, 2000, Gilbert et al., 2000). This group of proteins function is by creating pores in cholesterol containing cell membranes. Pneumolysin has an intracellular localisation but it is also released outside the pneumococcal cells, either through autolysis mediated by autolysin enzymes such as LytA (Mitchell et al., 1997, Mitchell et al., 1991), or it can be secreted outside cell by changing its structure from soluble to oligomer phase (Lawrence et al., 2015). However, the recent publications proposed a membrane localisation for Ply (Price and Camilli, 2009).

Early studies demonstrating the involvement of Ply in pneumococcal invasiveness were done in Leicester, UK. Leicester scientists found that mice immunised with inactivated pneumolysin toxoid survived longer when they were subsequently infected with the lethal dose of the pneumococcus (Alexander et al., 1994). Ply was found to inhibit the ciliary beating of epithelial cells. This action allows the pneumococcus to colonise efficiently (Feldman et al., 2002). Ply can also modulate the immune system cell function during the invasion, for example, by inducing the release of chemokines such as tumor necrosis factor alpha (TNF-α) and interleukin-1β, and by adversely affecting the phagocytic killing (Hirst et al., 2002, Houldsworth et al., 1994). Ply also stimulates both classical and alternative complement pathway (Mitchell et al., 1991, Paton et al., 1984). Elimination of haemolytic activity of Ply does not abolish inflammation caused by pneumococcal infections (Alexander et al., 1998), suggesting that there are other roles of Ply. Indeed, it was found that Ply could stimulate interferon γ synthesis (Baba et al., 2002), could inhibit the production of hydrogen peroxide from macrophages (Segal, 2005) and it is involved in leukocyte migration and chemotaxis (Paton and Ferrante, 1983).

Strains that are not able to produce pneumolysin are not as virulent as cytolytic

pneumolysin producing strains. In an acute infection model of pneumonia, it was shown that Ply is needed to cause pneumonia (Kadioglu et al., 2002). Orihuela et al. (2004) demonstrated the involvement of Ply in pneumococcal bacteraemia. It has been demonstrated that Ply is required for pneumococcal spread from the respiratory tract to bloodstream (Berry et al., 1999, Orihuela et al., 2004). Ply is also involved in pneumococcal meningitis. Braun et al. (2002) demonstrated that a Ply in combination with H_2O_2 could damage the central nervous system. It was found that Ply knock out strain could not cause meningitis in mice.

LPxTG proteins are covalently linked with pneumococcal cell wall via a C-terminal LPxTG motif. These include neuraminidase (Nan), IgA1 protease, hyaluronate lyase (Hyl), β-*N*-acetylglucosaminidase (StrH), and high-temperature requirement A (HtrA) protein (Figure 1.2). This linkage occurs after the cleavage of LPxTG motif from cell wall peptidoglycan by pneumococcal sortase (Paterson and Mitchell, 2004, Bergmann and Hammerschmidt, 2006). Sequence analysis of pneumococcal genome revealed that there are at least 17 putative LPxTG proteins (Tettelin et al., 2001).

IgA1 protease belongs to Zn^{2+} metalloproteinase family that is produced along with other metalloproteinases such as ZmpB, ZmpC and ZmpD (Camilli et al., 2006). Most of virulent pneumococcal strains produce these enzymes suggesting the involvement in virulence (Oggioni et al., 2003). IgA1 protease is able to break human IgA down at the hinge site into two fragments, Fab and Fc (Paton et al., 1993, Romanello et al., 2006, Kilian et al., 1996). IgA1 is produced in human airway and it represents about 90% of total IgA in the body (Wani et al., 1996, Poulsen et al., 1998). IgA1 is able to neutralise pneumococcal toxins and prevents bacterial adhesion. The cleavage of IgA is advantageous for pneumococcal adhesion as Fab fragment enhances the binding of phosphorylcholine protein (ChoP) to platelet activating factor receptor (rPAF) (Weiser et al., 2003, Tyler and Cole, 1998, Ring et al., 1998). Polissi et al. (1998) reported the importance of IgA1 protease in pneumococcal pneumonia and bacteraemia. Inactivation of human IgA1 by virulent pneumococcal strains is clearly linked with the function of IgA1 protease, as IgA1 protease deficient pneumococcus could not break human IgA1 (Janoff et al., 2014).

Another zinc metalloproteinase, **ZmpC**, is important for pneumococcal survival in lungs. Absence of ZmpC was attributed to mortality delay of 75% of experimentally

infected mice (Oggioni et al., 2003). ZmpC is also able to break down human matrix metalloproteinase 9 (MMP-9), which is responsible for repairing and remodelling the damaged human extracellular matrix components such as collagen and glycoproteins. Gene regulation analysis of human matrix metalloprotein expression revealed that MMP-9 is induced by the heat-killed pneumococcus (Michel et al., 2001). Deletion of *zmpB* and *zmpC* attenuate pneumococcal virulence in murine model of infection (Blue et al., 2003, Chiavolini et al., 2003).

Hylaronate lyase (Hyl) is another example of a LPxTG protein. It damages the extracellular matrix layer of human cells to release nutrient for the pathogen (Kadioglu et al., 2008, Berry et al., 1994). This protein is able to cleavage the extracellular matrix composes hyaluronan of host cell. Hyl very likely breaks down the linkage between N-acetyl-β-D-glucosamine and D-glucuronic acid in hyaluronan. It is expressed in most of pneumococcal serotypes (Humphrey, 1948, Jedrzejas, 2001). The protein structure analysis of this enzyme revealed that it contains LPxTG motif at C-terminal anchored to cell wall peptidoglycan (Jedrzejas, 2004). Acute infection model of mice revealed that pneumococcal meningitis is enhanced when Hyl is administered intranasally, suggesting the involvement of Hyl in pneumococcal meningitis (Zwijnenburg et al., 2001). In addition, it has been shown that Hyl enhances the damage of ciliated epithelial cells by Ply (Feldman et al., 2007). This strongly supports the idea that Hyl disrupts the extracellular matrix of epithelial cells making it accessible for Ply to penetrate. Although immunisation with Hyl could not protect mice against lethal pneumococcal challenge, Hyl could still be considered as vaccine candidate as it has shown a synergistic role with other virulence factors.

1.1.4.1 Pneumococcal nutrient metabolism as a virulence attribute:

In order to understand how *S. pneumoniae* colonises asymptomatically and causes invasive diseases, it is vital to understand how the pathogen utilises host sugars as carbon source. There is a well-established link between pneumococcal virulence and sugar acquisition (Yesilkaya et al., 2009). It is widely known that the pneumococcus is only isolated from live surfaces and no literature has reported an environmental reservoir. In addition, one third of pneumococcal genome is involved in sugar utilisation pathways, including carbohydrate uptake systems and glycosidases (Tettelin et al., 2001, Marion et al., 2011, Manco et al., 2006, Terra et al., 2010, Marion et al., 2012).

The pneumococcus is able to utilise a wide range of sugars *in vitro*, including 32 carbohydrates such as glucosides, galactosides and polysaccharides (Bidossi et al., 2012, Terra et al., 2015). Although the free carbohydrates are scarce in the respiratory tract (Philips et al., 2003), the pneumococcus relies on host glycoconjugates for nutritional requirements (King, 2010). For instance, the level of free glucose in the respiratory tract is below the pneumococcal requirements (Philips et al., 2003). Therefore, the complex host glycoproteins appear to be the sole source of carbon for the pneumococcus in the respiratory tract.

Pneumococcal glycoconjugate utilisation has a key role for survival and virulence. The mucin is a very rich source of galactose and *N*-acetylgalactosamines (Terra et al., 2010). *S. pneumoniae* is a strictly fermentative pathogen. It completely relies on glycolytic metabolism for energy requirements (Hoskins et al., 2001). The respiratory metabolic system is completely absent in pneumococcal genome. Therefore it is not able to obtain energy by respiration (Tettelin et al., 2001).

Other proteins are also linked with host sugar metabolism. These include pyruvate formate lyase (PFL), lactate dehydrogenase (LDH) and pyruvate oxidase (SpxB) (Yesilkaya et al., 2009, Gaspar et al., 2014, Spellerberg et al., 1996). The involvement of PFL in pneumococcal galactose metabolism and survival was shown, as it is responsible for ATP generation for energy requirements (Yesilkaya et al., 2009). A knockout strain, *pflB* that encodes for PFL, has shown a reduction in ATP synthesis. The elimination of PFL synthesis leads to decrease in the level of acetyl-CoA, which is involved in fatty acid and choline synthesis of *S. pneumoniae* (Yesilkaya et al., 2009). Intravenous inoculation of a LDH isogenic mutant failed to cause bacteraemia. It also could not cause pneumonia. The importance of SpxB is linked with pneumococcal ability to adapt to various environments *In vivo*. It converts pyruvate to acetyl phosphate and CO_2. *S. pneumoniae* with a *spxB* deletion failed to compete with other respiratory colonisers. In addition, the deletion of *spxB* led to decrease in adhesion proteins and capsule production (Spellerberg et al., 1996).

There are at least 10 extracellular pneumococcal glycosidases. These glycosidases have specificity for *N*- and *O*-linked glycoproteins (King, 2010). For example, β-galactosidase C (BgaC) and *O*-glycosidase (Eng) are able to cleave *O*-linked glycans (Marion et al., 2009, Terra et al., 2010, Jeong et al., 2009). On the other hand, *N*-linked

glycans can be broken down by pneumococcal neuraminidase A (NanA), neuraminidase B (NanB), neuraminidase C (NanC), *N*-acetylglucosaminidase (StrH) and β-galactosidase A (BgaA) (King et al., 2006, Burnaugh et al., 2008). This break down is sequentially processed starting from terminal sialic acid cleavage by NanA to galactose break down by BgaA. Modifications in glycoproteins might hinder the sequential cleavage of sugars.

Sugar transport systems import sugars for catabolism once they are released by the glycosidases. Up to date, three sugar transport systems have been established: the secondary carriers, the ATP-binding cassette transporters (ABC transporters), and phosphoenolpyruvate-dependent carbohydrate phosphotransferase systems (PTS systems). The secondary carriers translocate carbohydrates to electrochemical gradients. ATP transporters exhaust ATP to uptake carbohydrates. On the other hand, PTS system uptakes carbohydrates by phosphorylation process (Davidson et al., 2008, Konings et al., 1994, Postma and Lengeler, 1985).

The importance of pneumococcal exoglycosidases including NanA and how mucin *O*-acetylation might affect the sequential cleavage of sugars will be explained in the next section.

1.1.5 Pathophysiology of pneumococcal diseases:

A comprehensive understanding of the pneumococcal determinants of virulence and pathophysiology of disease are essential to combat the pneumococcal diseases and to find the best therapeutic and protective solutions for the problem since there is a rising trend of antibiotic resistant clones and the existing pneumococcal vaccines do not provide universal protection. Pathophysiology of disease occurs due to interaction of host and the microbe, and requires understanding of the factors affecting colonisation, adhesion and invasion, and host response to the pneumococcus. As the host response to the pneumococcus is beyond the scope of this study, it will not be covered in detail.

Factors affecting colonisation: *S. pneumoniae* colonises the nasopharynx of healthy individuals asymptomatically without causing disease, also known as carriage. Colonisation is the first step of invasive disease (Jacobs, 2004, Kadioglu et al., 2008). Therefore it is important to understand the factors affecting the carriage. During carriage, pneumococci can be spread between individuals by direct transmission of the

microbe by respiratory droplets. The carriage might be maintained for a long time without causing disease before clearing off or maintained by separate pneumococcal serotypes (Kadioglu et al., 2002). It is possible that more than one serotype resides in nasopharynx at the same time. This phenomenon is common in children under the age of 5. Conversely, the pneumococcus can also migrate and spread through the airways to lower respiratory tract to cause pneumonia. It can pass through the Eustachian tube to cause otitis media. The microbe may also penetrate into the blood stream and infect meninges of central nervous system to cause bacteraemia and meningitis, respectively (Figure 1.3) (Kadioglu et al., 2008).

The transition of pneumococcal carriage to invasive disease is dependent upon different factors such as the age, immunisation status and the population exposed to pneumococci. The most frequent age groups prone to pneumococcal infections are children under the age of 5 years old and elderly people. The age can also be linked to the immune system of individuals (WHO, 2013). The translocation from the nasopharynx to cause invasive disease is influenced by the immune status of the host (AlonsoDeVelasco et al., 1995). The immunisation strategy might also affect the transition process as some vaccines prevent certain serotypes allowing other colonised serotypes to develop to invasive phase (Kaplan et al., 2004). However, the mechanism is still unclear whether carriage serotypes can cause invasive disease. Invasive serotypes are not associated with colonisation as these serotypes do not need a long time to colonise and they cause invasive disease (Sleeman et al., 2006). On the other hand, non-invasive serotypes such as serotype 6, 9, 14 and 23 reside in the nasopharynx for a long time and their ability to cause disease is dependent upon the host immune system (Pineda et al., 2002).

The pneumococcal colonisation excessively induces the host innate immune system and its components such as secretory IgA (sIgA) and lactoferrin (Shaper et al., 2004, Janoff et al., 1999). These components delay the adherence of pneumococci to nasopharyngeal tissue and allow the phagocytosis to occur. Pneumococcal capsule prevents the pathogen from opsonisation by sIgA, and pneumococcal IgA1 protease can damage IgA (Fasching et al., 2007). The Fab site that is cleaved from sIgA can be bound to the pneumococcal choline binding proteins and enhances the pneumococcal adhesion to host epithelia (Weiser et al., 2003). The complement cascade can also be activated in

early stage of pneumococcal colonisation (Paterson and Mitchell, 2006). This activation leads to the cleavage of complement pathway components that directly cause bacterial lysis.

The rapid immune response against the carriage limits the existence of colonisation (Soininen et al., 2001). This is supported by the fact that in healthy individuals, the pneumococcus tends to colonise asymptomatically more than causing disease. This might be attributable to host immune system that can generate antibodies that are specific to pneumococcal components, which prevent the invasive disease (Musher et al., 1997). Pneumococcal colonisation is commonly found in early childhood. The rate of colonisation drops from 50% to 20% after the age of 5 years old (Abdullahi et al., 2008). The dramatic decrease in pneumococcal carriage is attributable to the development of immune system of children after the age of 5, as after this age children can produce antibodies specific to pneumococcal capsular polysaccharides (Douglas et al., 1983). It has also been found that the production of anti-capsular IgG is age dependent. IgG2 is produced less in children under the age of 5, and this increases gradually (Soininen et al., 2001). Certain pneumococcal strains can colonise the nasopharynx such as serotype 6, 19, 14 and 23 (Kadioglu et al., 2002, Soininen et al., 2001). However, it has been reported that some capsular serotypes that cause invasive disease such as serotype 1 and 2 do not tend to colonise the nasopharynx of healthy people (Brueggemann et al., 2004). A study by Gray et al (1980) found that certain pneumococcal serotypes that commonly colonise individuals protects them from highly invasive serotypes. This finding is in agreement with the study, which showed that children immunised with PCV-7 were protected against pneumococcal diseases caused by other serotypes not represented in the vaccine (Kaplan et al., 2004).

Certain factors might affect pneumococcal colonisation such as interspecies competition, viral infections, and other respiratory microbiota such as *H. influenzae* and *Neisseria meningitidis*. Certain serotypes are associated with carriage, and they prevent other serotypes colonising the nasopharynx by producing serotype specific peptides. These small sequences of amino acids, which are known as bacteriocins (pneumocins), are able to kill other serotypes (Dawid et al., 2007), and the pneumococcus can acquire DNA from these killed bacteria as it is naturally competent. This characteristic allows gain of competitive factors (Lux et al., 2007).

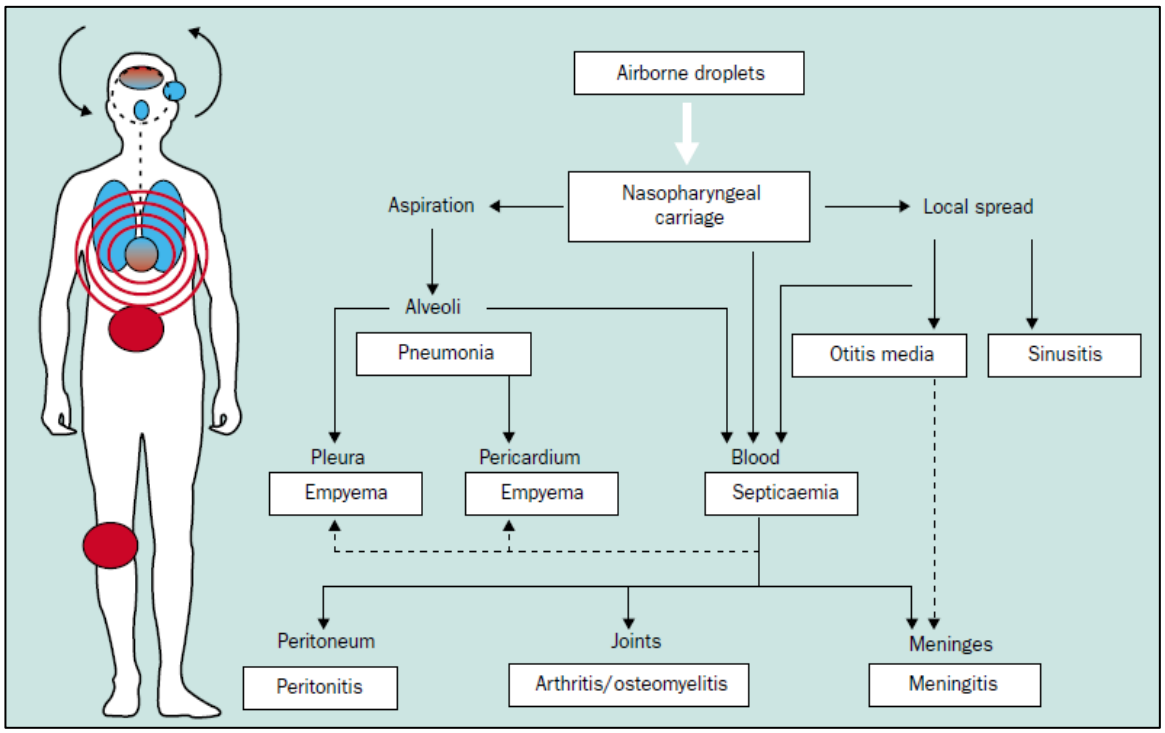

Figure 1.2: Pathogenesis of the pneumococcus. The diagram shows the systematic dissemination of pneumococci through the host tissues. Infections start with nasopharyngeal colonization. The microbe then spread to cause noninvasive disease such as otitis media and sinusitis, and invasive disease such as meningitis, pneumonia and septicemia or bacteremia. The arrows around the head represent the circular dissemination from nasopharynx to meninges through middle ear (Bogaert et al., 2004a).

Furthermore, the production of toxic reactive oxygen species of hydrogen peroxide by *S. pneumoniae* may provide competitive advantage over other respiratory bacteria in nasopharynx. The pneumococcus can produce up to 1 mM hydrogen peroxide as a by-product of its fermentative metabolism (Pericone et al., 2003, Yesilkaya et al., 2000). It was shown that the hydrogen peroxide produced by the pneumococcus could kill other species on the nasopharyngeal surface such as *H. influenzae* and *N. meningitidis* (Pericone et al., 2000).

Viral diseases are also known to be a predisposing factor for pneumococcal infections. They occur, for example, after influenza infections (AlonsoDeVelasco et al., 1995). Viral infection can facilitate pneumococcal transition to the invasive state by stimulating host receptor such as Toll-like receptors (TLRs) and cytosolic NOD-like receptors (NLRs), allowing pneumococcal cells to adhere to host epithelia and cause infection (Hakansson et al., 1994). The viral neuraminidase may enhance pneumococcal adhesion to lung epithelial cells by cleaving sialic acid (Sia) from glycosphingolipids on the surface of these cells. As a result, the cell receptors may be unmasked for pneumococcal adhesion (Krivan et al., 1988). Watson et al. (2006) established a clear link between influenza and pneumococcal diseases such as pneumonia. It has also been shown that there is a temporal relationship between seasonal pneumococcal and influenza infections related to respiratory syncytial viral circulation (Talbot et al., 2005). It has also been suggested that the high mortality of the Spanish viral flu epidemic during the first world war was due to the secondary pneumococcal infection post viral infection (Brundage and Shanks, 2008).

Pneumococcal adhesion: For successful pathogenesis, *S. pneumoniae* must have efficient mechanisms for adhesion to host cells. Once the pneumococcus reaches the mucosal surface, it encounters the excessive secretion of the mucosal barrier. The mucus is known to sweep all microbial cells from host airway by cilia movement (Bogaert et al., 2004c). As a result of this interaction, the pneumococcus expresses a heavily sugar coated layer, the capsule. The capsule keeps pneumococcal cells away from mucus, as both the capsule and mucus are negatively charged polysaccharides (Nelson et al., 2007). However, it has been shown that the expression of capsule is reduced after the adhesion to epithelial cells (Hammerschmidt et al., 2005). This action might be advantageous to the pathogen as it enhances the adhesion and increases pneumococcal pathogenesis.

It has been found that one third of pneumococcal genes are linked to the pathogen's attachment (Orihuela et al., 2004). During colonisation, some of pneumococcal proteins are proposed to be involved in interaction with host cells. Amongst these enzymes, phosphoryl choline protein (ChoP), which is a cell wall anchored protein, is found to be one of pneumococcal proteins that interrupt host cell signalling by interacting with platelet-activating factor (PAF) of host cells. This mechanism provides more time for

pathogen to adhere to epithelial cells (Cundell et al., 1995a). Choline binding protein A (CbpA) is also involved in pneumococcal adhesion. This protein binds to the host cell secretions (Rosenow et al., 1997).

Pneumococcal phase variation has a key role in adhesion. Pneumococci can adjust their capsule thickness from opaque (thick) to transparent (thin) (Cundell et al., 1995b, Weiser et al., 1994). The existence of an opaque phase or transparent phase is infection site dependent. For example, the pneumococcus colonises the nasopharynx in the opaque phase as it hinders the mucociliary action and allows the microbe to escape from opsonophagocytosis. However, the capsule thickness is disadvantageous for pneumococcal invasiveness, therefore, the transparent phase is ideal for adhesion to host epithelia (Weiser et al., 1994).

S. pneumoniae adheres to the epithelial surface of the nasopharynx by attaching to host glycoconjugates. This attachment occurs by producing exoglycosidase enzymes that are important for invasion and nutrient release such as neuraminidase A (NanA), β-galactosidase (BgaA) and *N*-acetylglucosaminidase (StrH) (King et al., 2006). These enzymes are involved in the sequential cleavage of sugars from host glycoconjugates providing the carbon source for pneumococcal growth. Exoglycosidases are also involved in deglycosylation of host immune components such as IgA and lactoferrin. Matthias et al. (2008) found that exoglycosidases enhance pneumococcal invasion by inhibiting the opsonophagocytosis. Recently, the involvement of BgaA in pneumococcal adherence has been reported (Limoli et al., 2011). Recombinant BgaA binds to epithelial cell receptors acting as an adhesin (Limoli et al., 2011). Because the hypothesis of this thesis centres on factors effecting neuraminidase activity, a detailed discussion on neuraminidases will be provided in section 1.2.

Pneumococcal invasion: After adherence, the second stage of pneumococcal infection is the penetration through host epithelial cells. The penetration of pneumococcal cells through epithelia stimulates the production of cytokines such as tumour necrosis factor (TNF) and interleukin 1 (IL-1), which stimulates the production of platelet activating factor receptor (rPAF). The latter component binds to the pneumococcal phosphorylcholine (ChoP) facilitating the migration into the epithelia (Cundell et al., 1995a). The other choline binding protein involved in pneumococcal invasion is pneumococcal surface protein C (PspC). This protein can bind to polymeric

immunoglobulin receptor (pIgR) enhancing the pneumococcal adhesion (Elm et al., 2004). It has been found that pIgR is up-regulated in response to pneumococcal invasion (Zhang et al., 2000).

The pneumococcus is prone to removal by phagocytosis (Janoff et al., 1999). The most important pneumococcal factor at this stage is the capsule. The production of the capsule is required for invasion as it prevents the opsonophagocytosis (Hyams et al., 2010). On the other hand, the phase variation of the pneumococcus from opaque to transparent phase allows pneumococci to adhere to epithelial cells (Weiser et al., 1994). In addition to the capsule, the pneumococcus needs other virulence factors that are involved in the invasion, such as pneumolysin (Ply), which is required for the penetration by inhibiting the ciliary beating of epithelial cells. It is involved in epithelial cell damage (Feldman et al., 2002). When the microbe reaches the lower respiratory tract, the pneumococcus adheres to bronchoepithelial cells (Weiser et al., 1994).

After passing the host epithelia, the pneumococcus has to overcome another host barrier, the extra-cellular matrix (ECM). The main virulence factor involved in the cleavage of hyaluronan component of the ECM is hyaluronate lyase. The digestion of the hyaluronan layer facilitates the pneumococcal invasion (Jedrzejas et al., 2002). The administration of hyaluronate lyase intranasally promotes pneumococcal invasiveness *In vivo* (Zwijnenburg et al., 2001). In addition, pneumococcal enzymes such as enolase (Eno) convert plasminogen to plasmin that can lyse other ECM components such as fibrin and laminin (Bergmann et al., 2001). The Fibronectin component can be degraded by pneumococcal adhesion and virulence factor A (PavA) and B (PavB) (Holmes et al., 2001, Jensch et al., 2010). The ability of pneumococcal cells to translocate from airway epithelia to bloodstream is dependent upon PavA. It was shown that PavA knockout strain could not survive in mouse nasopharynx, and could not cause pneumonia and septicaemia (Kadioglu et al., 2010).

Once the pneumococcus invades the host epithelial layer, it penetrates into endothelia. Pneumococcal cells are able to bind to endothelial cells such as alveolar type 2 cells. This type of cell can stimulate the production of platelet activating factor receptor (rPAF), which was shown to be a target receptor for pneumococcal binding proteins (Cundell and Tuomanen, 1994). The damage to the lung endothelia occurs due to the host inflammatory response.

The pneumococcal attachment to alveolar cells seems to be advantageous, as these cells provide passage of pneumococcal cells through endothelia into the blood stream. This mechanism is called endocytosis (Talbot et al., 1996). The bloodstream provides a good environment for *S. pneumoniae* to replicate and cause a high level of inflammatory response. However, the host classical complement pathway prevents pneumococcal dissemination in bloodstream (Jarva et al., 2003). In the bloodstream, the transparent phase of the pneumococcus is favourable as the absence of capsule prevents opsonisation and phagocytosis (Kadioglu et al., 2008). Complement factor 1q (C1q) and C3b deposition are inhibited by pneumococcal surface protein A (Jarva et al., 2003), and pneumococcal surface protein C prevents opsonophagocytosis by blocking C3 convertase pathway (Li et al., 2007).

During pneumococcal bacteraemia, it was shown that the toxin pneumolysin is important for pneumococcal survival in bloodstream. The toxin recognises IgG and activates classical complement pathway, optimising pneumococcal invasion (Alcantara et al., 2001). Pneumococcal lacking pneumolysin failed to survive in murine bacteraemia model (Orihuela et al., 2004). Another immunogenic pathway occurring during pneumococcal bacteraemia is due to the production of C-reactive protein (CRP). CRP recognises phosphorylcholine of pneumococcal cell wall and interacts with CIq components, enhancing classical pathway of complement (Claus et al., 1977). This protein is found in humans, and was shown to inhibit pneumococcal binding with platelet activating factor receptor (rPAF) *in vitro* (Gould and Weiser, 2002).

The other barrier for the pneumococcus after the bloodstream is the host meninges leading to cause meningitis. At this stage of infection the host immune system cannot clear the pathogen and therapeutic intervention is the only viable solution for pneumococcal sepsis and meningitis (Ring et al., 1998, Weber and Tuomanen, 2007). Pneumococcal meningitis starts when pneumococci reach the endothelia of micro-vessels in host brain. The pneumococcus is capable of binding to rPAF of brain endothelia, which allows the pneumococcus to translocate from endothelia to meningeal cells (Ring et al., 1998). Once again, the transparent phase of the pneumococcus is favourable in meningitis because thin capsule exposes phosphorylcholine more efficiently, and this allows better adhesion to host rPAF (Weber and Tuomanen, 2007). Pneumococcal components that are released from lysed pneumococci due to treatment

with antibiotics increases host inflammatory response, as they are good immunogenic factors (Nau and Eiffert, 2002).

Pneumococcal pneumolysin is also involved in meningitis. In a mouse model of meningeal infection, pneumococci devoid of pneumolysin resulted in less severe signs of meningitis and delay in causing infection although no significant difference was seen in comparison to parental strain in terms of inflammatory response (Wellmer et al., 2002).

1.2 Pneumococcal interaction with host glycoproteins:

Overview:

The pneumococcus interacts with host glycoproteins in order to colonise in the respiratory tract. The mucosal barrier is covered with mucus and is responsible for defending the host against physical confrontations and pathogenic impact of microorganisms. The mucus has viscous characteristics and it can entrap pathogens and dust particles, which are then removed by ciliary effect of epithelia. The major macromolecular component of mucus is mucin, which is a high molecular weight glycoprotein. The thickness of mucin can be the best basement for pneumococcal cells to initiate attachment and start digging through the barrier by the activities of pneumococcal exoglycosidases such as NanA, β-*N*-acetylglucosaminidase (StrH), and β-galactosidase A (BgaA). These enzymes are able to cleave sugar components of mucin including sialic acid, galactose and *N*-acetylglucosamine. This section will cover the aspects of pneumococcal interaction with host glycoproteins.

1.2.1 General characteristics and classification of glycoproteins:

Mucus is the first physical barrier that coats all non-keratinized epithelial surfaces of the mammalian host (Varki and Sharon, 2009). The majority of pathogens have to pass through this barrier to cause infection. The mucus layer is considered to play a key role in the host innate immune defense. It can delay the movement and penetration of pathogens (Thornton et al., 2008). The chemical composition of mucus also allows it to combat foreign particles and microbes as it contains secretory IgA (sIgA), lysozymes, lactoferrins, cathelicidins, defensins and pentraxins (Rogan et al., 2006), which are able to kill or suppress the growth of invading bacteria. It also contains other macromolecules such as hormones, cell adhesion and transport proteins, and cytoplasmic and nucleoplasmic proteins. The existence of different biological molecules in the mucosal layer reflects the functional effect of mucus in enzymatic catalysis, immunological protection, ion transport, structural support and cell adhesion (Allen and Kisailus, 1992).

Mucus is composed of mucin and other inorganic substances such as salts, glycolipids and free sugars. The mucin is secreted in different parts of body such as the eyes, sinus, intestine and respiratory tract. The mucin secretion is controlled by the cells that secrete mucin as defensive function (Schuhl, 1995, Thornton et al., 2008). The lungs secrete mucin approximately 5-50 μm in thickness clearing the airways regularly (Schuhl, 1995).

Mucin is a family of polymerizing molecules that is secreted directly from special cells called goblet cells and/or sub-mucosal cells (Davies et al., 1996). These cells normally secrete mucin in dehydrated phase and this can be released to the surface of epithelia protecting the airways (Thornton et al., 2008). The molecular weight of this macromolecule is in range of 2-50 mega Dalton (Davies et al., 1996, Thornton et al., 1990). Mucin is mainly composed of *O*- and *N*-linked glycoproteins that contain a large domain of polypeptides that is rich in threonine/serine and asparagine residues. This domain is covalently attached with a carbohydrate side chain containing *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose, mannose, fucose, and sialic acid. The *O*-glycan linkage can be sialylated or sulfated, increasing the complexity of mucin.

The integration between polypeptide domain and sugars side chain provides the viscosity of mucin. Moreover, the disulphide linkage within cysteine residues at N- and

C-termini are important for the thickness of mucin (Thornton et al., 2008). These structural and biological characteristics of mucin provide protection from innate protease activity and are responsible for mucin's defense properties (Thornton et al., 2008).

There are more than 17 mucin genes (17 MUCs) responsible for the production of two types of mucins in humans (Thornton et al., 2008). These can be divided into two types of mucin according to its structure: monomeric mucin, which is located on the surface of epithelia and oligomer mucin, which is secreted and freely released (Thornton et al., 2008). These mucin types can also be divided into two further groups depending upon the type of mucin secretion: cell-tethered mucin genes *MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17,* and *MUC20*; and secreted mucin genes *MUC2, MUC5AC, MUC5B, MUC6, MUC19, MUC7* and *MUC8* (Shankar et al., 1997, Rose and Voynow, 2006, Buisine et al., 1999). Apart from *MUC6*, all secretory mucin genes are expressed in epithelial surface of airways (Buisine et al., 1999, Vinall et al., 2000).

Mucin is the major component of mucus. Therefore, it is vital to understand how bacteria interact with mucin. This interaction must be important for microbial colonisation and invasion. Therefore this relationship will be covered in detail.

1.2.1.1 Mucin-pathogen interactions:

Respiratory tract commensals and pathogens manage to cause infection after combating the host innate defenses and stimulating the inflammatory response of the mucosa. It has been reported that *Pseudomonas aeroginosa*, *H. influenzae*, *Staphylococcus aureus* and *S. pneumoniae* adhere to the mucosal surface of the respiratory tract (Vishwanath and Ramphal, 1984, Davies et al., 1995, Shuter et al., 1996). Bacterial attachment to mucin is linked with the structural properies of mucin itself. This structure enhances bacterial attachment and entrapment to the mucosal surface allowing the removal by cilia beating (Thornton and Sheehan, 2004). However, some commensal bacteria manage to interact with host glycan without causing any disturbance or biological effect (Varki and Lowe, 2009).

The microbe uses a range of strategies to interact with mucin in order to colonise and invade by utilising mucin for nutritional sources (King, 2010, Yesilkaya et al., 2008).

Salivary glands produce high level of mucin that can be a good target for oral commensals and pathogens. Oral bacteria synthesise sialidase for revealing the underlying sugars that can be used as carbon source (Byers et al., 1999). All viridans streptococci produce sialidases, for example, *Streptococcus oralis*, *Streptococcus intermedius* and *Streptococcus mitis* (Byers et al., 1996). Most of viridans streptococci are also able to utilise sialic acid for growth (Takao et al., 2010). In some cases, mucin can be cleaved by sialidase-producing bacteria providing utilisable sugars to streptococci that do not produce sialidase (Takao et al., 2010). Studies by van der Hoeven et al. (1990) and Van der Hoeven and Camp (1991) showed that *S. mutans* grows poorly on media containing mucin, and they need accessible sugar to start growth. Other oral pathogens also encode sialidases to support attachment and biofilm formation. For example, *Porphyromonas gingivalis* expresses sialidase that is involved in capsule formation, complement resistance and infection (Li et al., 2012). The biofilm formation and adhesion of *Tanneralla forsythia* is linked with sialidase activity of this bacterium (Roy et al., 2011, Honma et al., 2011).

Other respiratory pathogens that cause infection in the host airway are known to utilise host mucin as a nutrient and to enhance attachment. Sialidases and tripartite ATP-independent periplasmic transporters (TRAP transporters) encoded by *H. influenzae*, *P. aeruginosa* and *Corynebacterium diphtheriae* were clearly linked with the ability of these pathogens to utilise mucin and cause infection. *H. influenzae* can release sialic acid from host mucin and transport it to cells (Lichtensteiger and Vimr, 2003, Severi et al., 2005). It has been shown that *H. influenzae* scavenges sialic acid for cell wall decoration, as this pathogen needs sialic acid for cell wall biosynthesis. This modification allows the pathogen to escape from host immune defence (Bouchet et al., 2003). *P. aeruginosa* does not encode sialidases. However, it has been reported that it expresses some sialidases-like enzymes. These enzymes are reported to be potent factors for *P. aeruginosa* attachment to epithelia (Davies et al., 1999). *C. diphtheriae*, a Gram positive pathogen that colonise the mucosa of the upper respiratory tract and also cause diphtheria, encodes extracellular sialidase that cleave sialic acid from mucosa (Kim et al., 2010). Although *C. diphtheriae* does not have Sia transporter, it releases Sia to reveal underlying sugars within the host glycoprotein. Nasopharyngeal co-colonisation with other bacterial species affects the interaction of these pathogens with mucin.

Gastrointestinal mucin is also a good target for commensal and pathogenic bacteria. Most of these species express sialidases that cleave host sialic acid for either nutritional purpose or unmasking the mucin for sequential cleavage of mucin sugar content. The best example of these species is *Escherichia coli*, *Vibrio cholera*e, *Helicobacter pylori*, *Bacteriodes* spp., *Clostridium* spp. and *Bifidobacterium longum* (Aspholm et al., 2006, Byres et al., 2008, Moncla et al., 1990, Moustafa et al., 2004, Fraser, 1978). It has been shown that the gastric mucin synthesis is adversely affected by lipopolysaccharides of *H. pylori* (Slomiany and Slomiany, 2006). *H. pylori* encodes different enzymes that hinder the activity of mucin. For example, it can bind to mucin by different adhesins, and it also produces glycosidases to degrade mucin and provide nutrition sources. It has been shown that *Cronobacter sakazakii*, which is the most severe pathogen causing fatal infections in neonates and infants, is able to use sialic acid as carbon source (Joseph et al., 2013).

Interestingly, bacterial species that do not encode sialidases can scavenge sialic acid that is released by other sialidase-producing bacterial species. For example, *Bacteriodes thetaiotaomicron* produces a number of glycosidases including sialidase, but it cannot scavenge the released sialic acid (Marcobal et al., 2011). However, *E.coli* that does not produce sialidase but a sialic acid lyase that can utilise the released sialic acid (Vimr et al., 2004, Severi et al., 2007). The sialidase in *B. thetaiotaomicron* appears to release sialic acid from host mucin to make other sequential sugar components accessible. The gastrointestinal pathogens produce sialidase to cleave Sia from mucosa to reveal the specific receptors for adhesins. For example, *V. cholerae* cleaves sialic acid from mucosa to access to the underlying receptor for cholera toxin GM1 (Moustafa et al., 2004). Similarly, *Clostridium perfringens*, which is the most dangerous pathogen that cause enterotoxemia, encodes sialidase for two reasons: firstly to unmask mucosa for other virulence factors such as epsilon toxin and alpha toxin, and secondly the released sialic acid can be utilised by the pathogen for nutrient sources (Li et al., 2011).

1.2.1.2 *Streptococcus pneumoniae*-mucin interaction:

Although mucin plays a key protection role against bacteria, it is also a potent target for several respiratory pathogens. Airway mucin can be beneficial for commensals and pathogens as they produce sialidases that facilitate colonisation and invasion of respiratory tract. One example of a respiratory pathogen that successfully interacts with

mucin is *S. pneumoniae*. The pneumococcus escapes the mucosal clearing, since the negative charge of capsule repulses the pathogen away from sialic acid (Nelson et al., 2007). The second way of pneumococcal mechanism to combat removal by mucin is the production of exoglycosidases including NanA, BgaA and StrH (King, 2010). In addition, the activity of the pore forming toxin, pneumolysin, decreases the ciliary beating of epithelia (Gilbert et al., 2000).

NanA activity is present in all pneumococcal serotypes, and is linked with pneumococcal carriage and invasiveness (Kelly and Greiff, 1970). It is involved in pneumococcal fitness by providing accessible carbon source. NanA is covalently linked to cell wall peptidoglycan by C-terminal LPxTG motif. The biological substrate of NanA is sialic acid containing glycoconjugates such as mucin. It cleaves terminal sialic acid from glycoproteins, and unmasks the substrate for other pneumococcal exoglycosidases such as β-galactosidase (BgaA), *N*-acetylglucosaminidase (StrH), and glycanase (Manco et al., 2006, King et al., 2006). Pneumococcal neuraminidases can cleave the α2-3, α2-6 and α2-8 glycosidic linkages between galactose and terminal sialic acid, releasing sialic acid, and thus, exposing the next sugar component (King et al., 2006, Schauer, 1982). In addition, the pneumococcus can utilise the released sugars as carbon source (Marion et al., 2011). Moreover, the importance of pneumococcal sialidases in colonisation, biofilm formation and adhesion has been shown (King, 2010, Manco et al., 2006, Parker et al., 2009, Trappetti et al., 2009). It has been reported that glycoprotein induces sialidases from *S. pneumoniae* (Gualdi et al., 2012). Although NanA is the major neuraminidase in *S. pneumoniae*, also NanB is encoded for total neuraminidase activity (Manco et al., 2006). A study found that there is a compensatory effect between both NanA and NanB (Burnaugh et al., 2008). However, the specific activity of NanA is 100 times higher than NanB (Berry et al., 1996).

In addition to its role in nutrition, neuraminidase activity is also required for pneumococcal attachment and the microbe's interaction with the host immune system. The activity of NanA and NanB is reported to be required for the initial attachment of pneumococcal cells to epithelial surface by modifying the glycoconjugates barrier (Andersson et al., 1983, Camara et al., 1994). Manco et al. (2006) reported the requirement of NanA and NanB for pneumococcal invasion of the respiratory tract. However, NanA activity was not found to be essential for pneumococcal sepsis (Berry and Paton, 2000, Paton et al., 1997), as a NanA-deficient mutant was able to cause

infection when given intraperitoneally. With regard to pneumococcal meningitis, it has been indicated that the active domain of NanA, but not lectin like domain, is partially involved in meningitis causing in mouse model (Uchiyama et al., 2009).

Moreover, NanA is able to deglycosylate the human IgA, lactoferrin and alternative pathway components (Dalia et al., 2010, Matthias et al., 2008). It has been demonstrated that pneumococcal exoglycosidases including NanA are able to deglycosylate host defence molecules, which are glycoproteins, by sequential cleavage of sugars (King et al., 2006). The ability of pneumococcal exoglycosidases to desialylate human immune components has been studied by King et al. (2004), such as secretory components, lactoferrins and IgA1. The structural characteristics of these immune components share the common glycosylation (Hughes et al., 1999, van Berkel et al., 1996). It has been reported that pneumococcal NanA inhibits the activity of sialic acid-binding immunoglobulin-like lectins (Siglecs). Siglecs are immune-receptors that are *cis*-masked with sialic acid, hindering the receptor from pathogens binding (Varki and Angata, 2006). Sia can be cleaved from Siglecs by the activity of NanA (Razi and Varki, 1999).

The activities of sialidases are more far reaching than previously thought. It has been reported that sialic acid occurrence in airway mucosa acts as a molecular signal for *S. pneumoniae* to colonise and translocate to lower respiratory tract causing pneumonia (Parker et al., 2009). The pneumococcus also degrades respiratory mucin and potent immune molecules, which are glycosylated components (Chuang and Morrison, 1997, Rose and Voynow, 2006).

The pneumococcal interaction with host mucin is enhanced by prior infection with influenza viruses. This interaction has been extensively studied. Viruses damage the architecture of host glycoprotein and alter the receptors allowing pneumococci to adhere efficiently (McCullers, 2004). It has been reported that pneumococcal adhesion is reduced in mice challenged with inactive influenza virus neuraminidase, as viral neuraminidase potentiates pneumococcal attachment (Peltola and McCullers, 2004). A very recent study revealed that influenza infection promotes pneumococcal growth and enhances colonisation (Siegel et al., 2014). They also found that influenza infection provides carbon source required for pneumococcal replication. Certain viral infections are reported to enhance pneumococcal colonisation by up-regulating rPAF, which is

used by *S. pneumoniae* for adherence (van der Sluijs et al., 2006). However, a study by McCullers et al. (2008) refutes the involvement of rPAF in pneumococcal post viral infections. Viral attachment to tracheal cells *in vitro* showed a severe disruption in tracheal epithelia but it does not enhance pneumococcal attachment. However, this damage might be beneficial for the pneumococcus as it decreases the viscosity of mucus (Pittet et al., 2010).

The sequential cleavage of sugars from host glycoproteins has a key role in pneumococcal attachment. The first sugar could be cleaved from sialo-glycoconjugates is Sia, which can be released by pneumococcal neuraminidases (Corfield, 1992). This allows other exoglycosidases to cleave other sugars that are important for pneumococcal nutrition. Two features affect the cleavage of sequential sugars from host glycoproteins: First, the nature of host glycoprotein and glycosidic linkages between sequential sugars such as α2-3, α2-6 and α2-8 linkages (King, 2010). The second potent effect is the occurrence of side chain hydroxyl groups at different sites such as 4-*O*-acetyl, 7-*O*-acetyl, 8-*O*-acetyl and 9-*O*-acetyl groups. It is well documented that bacterial and viral sialidases activity is reduced by high occurrence of *O*-acetylation of sialic acid (Schauer, 1982, Varki and Schauer, 2009). These two modifications might affect pneumococcal attachment and invasiveness to host glycoproteins. Therefore, the functional structure of glycoproteins and *O*-acetylation of sialic acid will be discussed in detail in the next section.

1.2.2 Glycosylation of glycoconjugates:

The mucin backbone is heavily glycosylated by different sugar residues. These sugars are linked to threonine/serine or asparagine residues in protein backbone with *O*-glycosidic and *N*-glycosidic linkages (Figure 1.4). The frequency of serine/threonine residues makes the polypeptide backbone highly hydroxylated and typically appears as tandem repeats (TRs). The TRs determine the range of carbohydrates attached to apomucin. TRs vary from 5 amino acids repeat to 395 amino acids repeat, that are heavily attached to sugar macromolecules (Rose and Voynow, 2006). TRs are linked with each other by non-repeat arbitrary amino acids (Crepin et al., 1990, Toribara et al., 1993). It has been reported that there are nearly 30 different monosaccharide chains covalently attached with 100 amino acid residues (Lamblin et al., 1991). A study has

established that more than 150 different sugar chains can be attached to apomucin secreted in respiratory tract of a single individual (Breg et al., 1988, Klein et al., 1988).

The glycosylation process occurs at the end step of mucin biosynthesis in *N*- and *O*-linked forms (Rose and Voynow, 2006). *O*-linked glycosylation starts with *N*-acetylgalactosamine (GalNAc) by linkage with oxygen of threonine/serine residues (Ser/Thr) at polypeptide backbone (Rose and Voynow, 2006). GalNAc also links with galactose (Gal) with β1-3 *N*-glycosidic linkage; galactose (Gal) is linked with terminal sugar sialic acid (Neu5Ac), in α2-3 and α2-6 *N*-glycosidic linkages. *N*-linked glycosylation occurs by the linkage of *N*-acetylglucosamine (GlcNAc) to nitrogen of asparagine residue within the polypeptide backbone. GlcNAc is sequentially linked with mannose (Man), which is linked with GlcNAc from other side chain by β1-4 linkage. GlcNAc is linked to Gal by β1-4 *N*-glycosidic linkage, and the latter sugar is linked with terminal sialic acid (Sia) by α2-3 and α2-6 glycosidic linkages (Figure 1.4) (Marion et al., 2009, King et al., 2009, Varki and Sharon, 2009). The existence of either sialic acid or fucose peripheral units provides hydrophobicity and electronegative charge on the surface of mucin (Rose, 1992, Rose and Voynow, 2006).

1.2.3 Post-translational modifications of glycoproteins:

The glycosylation of proteins involves several post-translational modifications involving sulfation, phosphorylation, methylation, pyruvation, acetylation and acylation. The result of all of these modifications can be varied depending on species and tissues within the same species (Rini et al., 2009, Freeze and Elbein, 2009). For example, the acetylation level in respiratory tract and gastrointestinal mucin is relatively higher than other tissues. Similarly, *O*-acetylation level in human tissues is completely different than other species (Corfield et al., 1992, Thornton et al., 2008). The terminal sialic acid can be acetylated. It is widely known that *O*-acetyl groups influence sialic acid metabolism by preventing Sia cleavage from glycoconjugates, by increasing sialic acid synthesis, and by maintaining sialic acid function. *O*-acetylation of sialic acid may also change its structural function.

Bacterial sialidases recognise and interact with host sialic acid, and this interaction might be affected by *O*-acetyl modifications (Varki, 1992). As the aim of this study is on the protective role of sialic acid *O*-acetylation against pneumococcal neuraminidase activity, sialic acid composition and its *O*-acetylation will be discussed in detail.

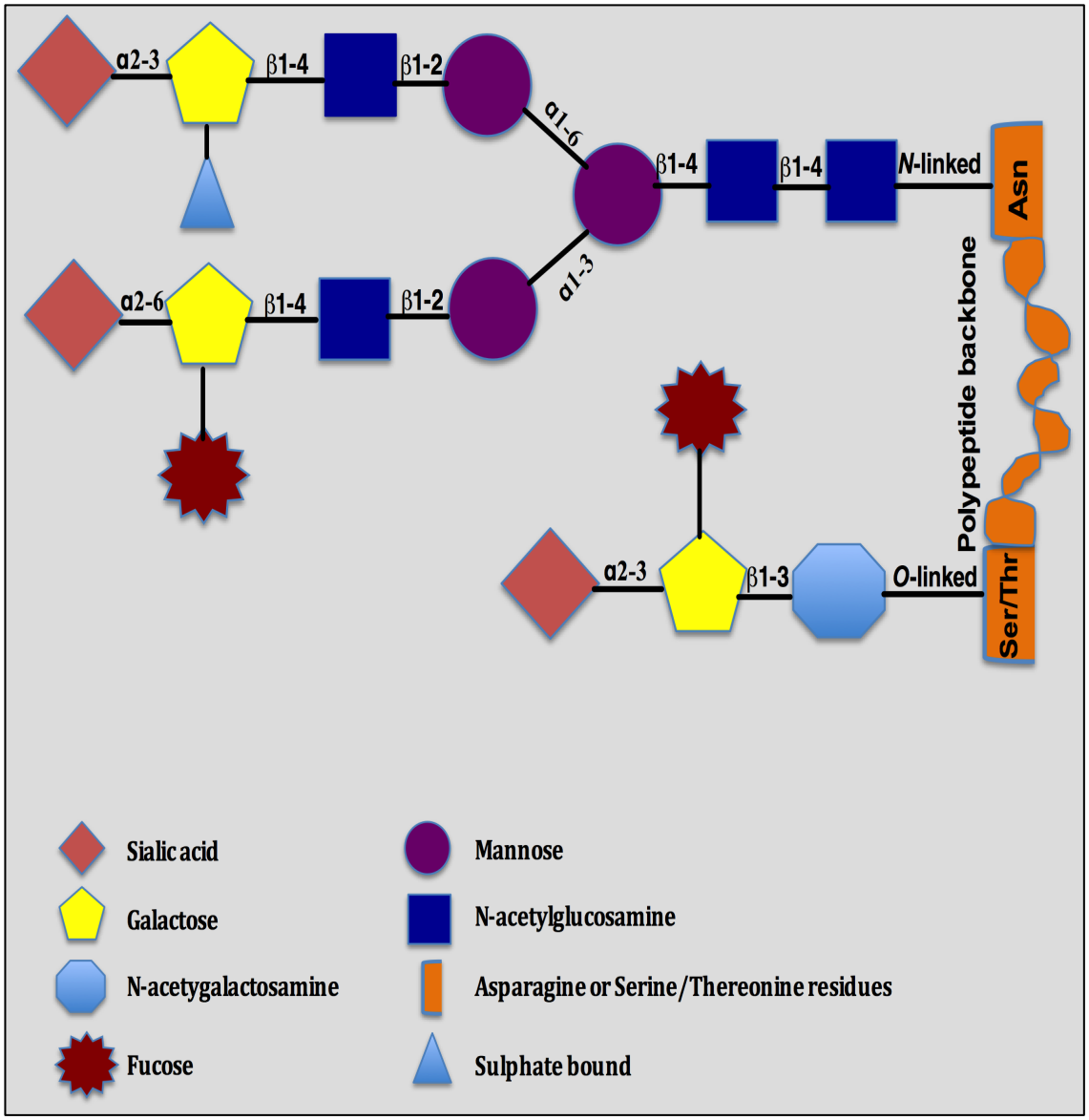

Figure 1.4: Schematic representation of glycoprotein glycosylation. *N*-glycosidic linkage links sugar residue with nitrogen side chain of aspargine residues (Asn). *O*-glycosidic linkage links sugar residues with oxygen side chain of serine/threonine residues. The linkages between sugar residues are indicated over the lines. Figure is adapted from King (2010) and Rose and Voynow (2006).

1.2.4 Sialic acid composition:

Sialic acid is the terminal sugar of *O*- and *N*-linked glycans, and is found in mammals, bacteria (Angata and Varki, 2002), protozoa and viruses (Varki, 1992, Schauer and Kamerling, 1997). Sialic acids are electronegatively charged monosaccharides with a 9-carbon backbone (Figure 1.5). The electronegative charge of sialic acid occurs due to the addition of carboxylate group at first carbon, which is ionised at physiological pH. Most of these modifications occur in the Golgi apparatus of eukaryotic cells (Varki and Schauer, 2009).

Sialic acids are derived from 5-amino-3,5-dideoxy-D-glycero-D-galacto-non-2-ulosonic acid (neuraminic acid; Neu). Generally, sialic acid is linked with other glycan substituents in different α-glycosidic linkages. For example, it links with galactose (Gal) by α2-3 and α2-6 linkage, with *N*-acetylgalactosamine (GalNAc) by α2-3 and α2-6 linkages, with N-acetylglucosamine (GlcNAc) by α2-6 linkage, and with each other by α2-8 linkage (Schauer and Kamerling, 1997).

The most frequent sialic acids in living cells are *N*-Acetylneuraminic acid (5-acetamido-3,5-dideoxy-D-glycero-D-galacto-non-2-ulopyranosonic acid; Neu5Ac) and *N*-glycolylneuraminic acid (5-hydroxyacetamido-3,5-dideoxy-D-glycero-D-galacto-non-2-ulopyranosonic acid; Neu5Gc), which are derived from Neu5Ac by hydroxylation of *N*-acetyl group (Angata and Varki, 2002, Schauer and Kamerling, 1997). These two types of sialic acids are considered as the basis of more than 50 different types of sialic acids (Varki and Schauer, 2009). One sialic acid molecule may carry up to four acetyl residues at 4-, 7-, 8- and 9- carbon sides as well as *O*-methyl, *O*-lactyl, *O*-sulphate and phosphate group, which are combined with an *N*-acetyl or *N*-glycolyl groups (Figure 1.5).

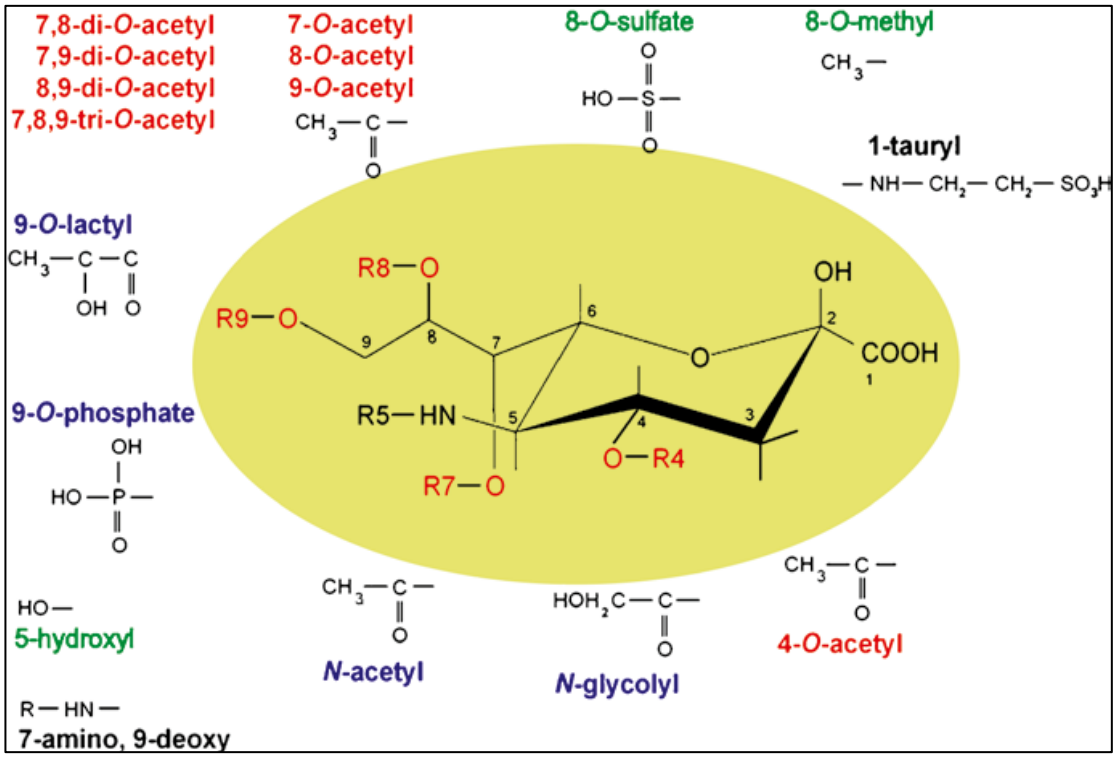

Figure 1.5: Natural composition of sialic acid presenting different types of sialic acids and their combinations and substituents in tissues and cells. The following substitutions can occur: R1: gives the negative charge for Sia; R2: binds to Gal, GalNAc, GlcNAc, Sia, or Neu5Gc by α- linkages; R4: 4-*O*-acetyl substituent or links to Fucose and galactose; R5: N-acetyl, *N*-glycolyl, hydroxyl, *N*-acerimidoyl, *N*-glycolyl-*O*-methyl and *N*-glycolyl-*O*-2-Neu5Gc; R7: 7-*O*-acetyl and *N*-acetyl; R8: 8-*O*-acetyl, methyl, sulphate, Sia and Glc; R9: 9-*O*-acetyl, lactyl, phosphate, sulphate, Sia and OH substituted by H. the Figure is adapted from Schauer et al. (2011).

1.2.5 *O*-acetylation of sialic acid:

O-acetylation of sialic acid is the most frequent modification that occurs (Varki, 1992). This modification is commonly generated at the exocyclic chain of sialic acid. The occurrence of acetylation is typically *O*-acetyl groups at positions 4-, 7-, 8- and 9- (Figure 1.6) (Diaz et al., 1989, Schauer, 1982). The occurrence of *O*-acetylation seems to be a regulated process and can be molecule- and tissue-specific, and is dependent on

development stage (Muchmore et al., 1987). Acetylation is influenced by immune response, cell growth and differentiation, cell adhesions and cell malignancy (Schauer, 2004, Angata and Varki, 2002, Klein and Roussel, 1998).

O-acetylation occurs within the Golgi by enzymes called sialate-*O*-acetyltransferases (SOATs). There are three SOATs enzymes: sialate-4-*O*-acetyltransferase, sialate-7-*O*-acetyltransferase and sialate-9-*O*-acetyltransferase (Schauer et al., 2011). These enzymes are fully active at pH 6.5. They transfer activated *O*-acetyl groups from acetyl coenzyme A (AcCoA) to Golgi membrane. The *O*-acetylation starts when SOAT esterifies C7 and C9 of CMP-Neu5Ac to produce 7- and 9-*O*-acetylneuraminic acid (Neu5,7Ac_2 and Neu5,9Ac_2). The migration of 7- and 8-*O*-acetyl groups to 9-*O*-acetyl group can also occur yielding Neu5,9Ac_2 (Kamerling et al., 1987). The occurrence of 8-*O*-acetylation is less frequent than 7- and 9-*O*-acetylation. However, it has been reported that Bovine sub-maxillary mucin (BSM) contains all types of *O*-acetylation including 8-*O*-acetylation (Schauer et al., 2011). 4-*O*-acetylation seems to be limited to some organisms such as in the guinea pig liver and submandibular mucin of the horse (Iwersen et al., 2003, Schauer et al., 2011). This type of *O*-acetylation does not occur in humans.

O-acetylation occurs very likely due to a host's response to environmental and bacterial stimuli. *O*-acetylation of newborn rat colon mucin is absent prenatally, and it occurs after birth indicating that *O*-acetylation of sialic acid might occur in response to bacterial and viral products and environmental stimuli. A study by Muchmore et al. (1987) found that aborted human fetuses that were shown to have intrauterine infection had low level of *O*-acetylation. It has been also proposed that bacterial neuraminidase present in the newborn colon induces the occurrence of sialic acid *O*-acetylation (Muchmore et al., 1987).

The *O*-acetylation of sialic acid can be removed by two types of enzymes. These enzymes are sialate-pyruvate lyase and esterases (Schauer et al., 2011). Sialate-pyruvate lyase participates in the regulation of *O*-acetylation in most animal species. It works along with *O*-acetyletransferase enzyme to regulate the level of *O*-acetylation in glycoproteins and other tissues (Schauer et al., 2011). This regulation is dependent upon different factors such as age and health status (Schauer et al., 2011, Schauer et al., 1988). Viral esterases can recognise terminal sialic acid and remove *O*-acetylation from

glycoproteins (Herrler et al., 1987). The esterase activity seems to be specific upon the *O*-acetylation type. For example, viral esterases could hydrolyse 7- and 9-*O*-acetyl groups of host cell membrane Sia (Smits et al., 2005, Herrler et al., 1985, Harms et al., 1996a). Esterases are also involved in *O*-acetylation regulation and they determine the level of *O*-acetylation in glycoconjugates. The ratio of *O*-acetylation is dependent upon the relative activity of *O*-acetyltransferases and esterases (Shen et al., 2002). Esterases are frequently distributed amongst animals as well as bacteria and viruses (Diaz et al., 1989, Schauer and Shukla, 2008, Schauer et al., 1988).

O-acetylation of sialic acid within mucin makes it a poor substrate for bacterial sialidases. Supporting this idea is *C. sakazakii*, which causes fatal infections in neonates, which can utilise sialic acid from intestinal mucosa (Joseph et al., 2013). Bovine sub-maxillary mucin was shown to be 60% resistant against group A streptococcal neuraminidase (GASN), whereas the enzyme displayed a total resistance against porcine sub-maxillary mucin demonstrating that the acetylation level of sialic acid limits the activity of neuraminidases (Davis et al., 1979). Another study on 23 isolates of human faecal bacteria found that highly acetylated sialic acid was cleaved from mucin slower than non-*O*-acetylated sialic acid (Corfield et al., 1992).

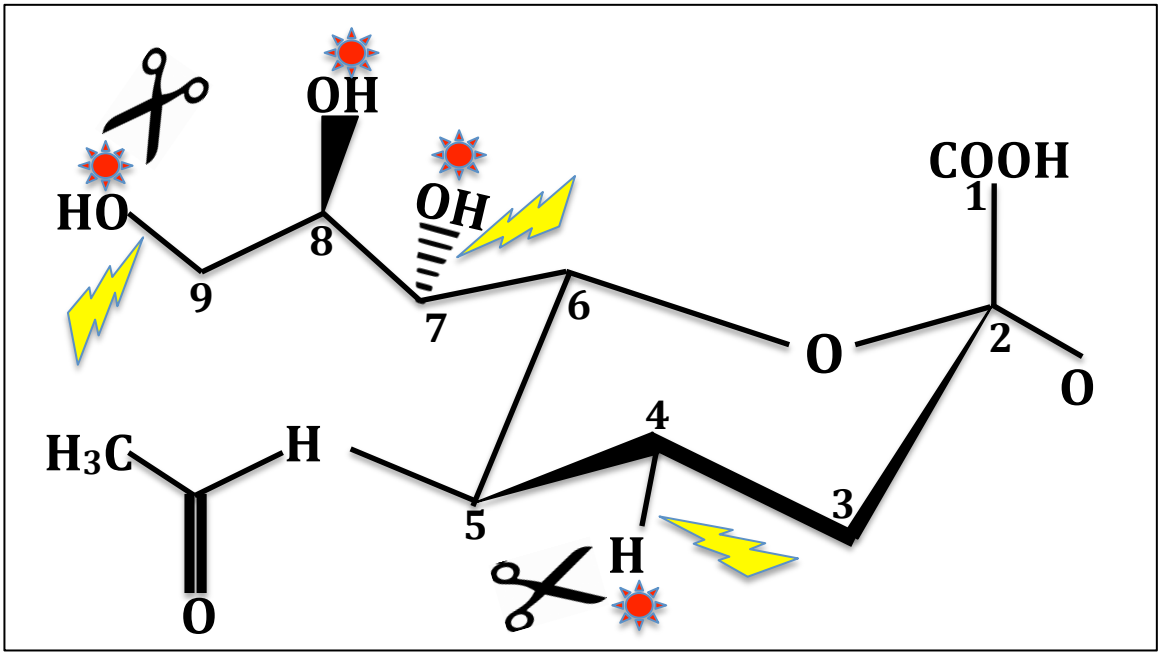

Figure 1.6: Natural occurrence of sialic acid *O*-acetylation. The *O*-acetylation can be in different glycerol side chain including 4-, 7-, 8- and 9-*O*-acetyl groups. The acetylation occurs in mono-*O*-acetylated or di-*O*-acetylated. *O*-acetyl group can migrate from 7- and 8-*O*-acetyl groups glycerol side chain to 9-*O*-acetyl group. The red stars represent *O*-acetylation of sialic acid. These O-acetyl side chains are the expected targets for esterases. The symbols of yellow flashed arrows show the specificity of sialate-4-*O*, sialate-7-*O* and sialate-9-*O*-acetyltransferases that are involved in *O*-acetyl metabolism. The scissors symbolized the hydrolysis of 4- and 9-*O*-acetyl groups by sialate-4-*O* and sialate-9-*O*-acetylesterases. The Figure is adapted from Schauer et al. (2011).

1.2.6 The function of sialic acid *O*-acetylation:

It is widely known that *O*-acetyl groups influence sialic acid metabolism by preventing sialic acid cleavage from glycoconjugates, by increasing sialic acid synthesis, and by maintaining sialic acid function. The terminal sialic acid and its modifications can be a target for bacterial proteins and toxins. Bacterial intracellular and extracellular sialidases degrade host mucin. Although the removal of sugars from host glycoproteins is dependent upon initial action of sialidases, the *O*-acetyl modifications make this cleavage less efficient (Schauer et al., 1995).

The main role of sialic acid *O*-acetylation is protective. *O*-acetylation can hinder the activity of human sialidases and acylneuraminate lyase against glycoconjugates. These enzymes degrade sialic acid from glycoprotein molecules (Schauer et al., 2001, Corfield, 1992). *O*-acetylation of sialic acid provides a hydrophobic moiety that affects the size, the charge and conformation of glycoproteins. These parameters determine the biological function of glycoconjugates (Siebert et al., 1996). It has been established that *O*-acetylation could affect the activation of complement pathways. For example, Shi et al. (1996) found that *O*-acetylation affects the complement system. It can bind to type-I lectins and influences tissue homing. The tryptic cleavage of complement factor H is abrogated by the recognition of *O*-acetylated sialic acid (Koistinen, 1992). Furthermore, it has been found that *O*-acetylation might affect the antigenicity of molecules (Larkin et al., 1991, Schauer, 1988, Schauer, 1982). The binding of monoclonal antibody GD3 to T-lymphocytes is stimulated by *O*-acetylation of C7 glycerol side chain (Kniep et al., 1995). These data indicate the biological significance of *O*-acetylation (Varki et al., 1991).

Although bacteria can use the released sialic acid as carbon source, the essence of this cleavage in other bacteria is to access to the underlying sugars such as galactose and *N*-acetylgalactosamine (Byers et al., 1996, Byers et al., 1999, Corfield, 1992). *O*-acetylation in the intestinal tract mucin is relatively high (Robbe et al., 2003). This acetylation tends to be protective from enteric bacterial species (Corfield et al., 1992). However, some pathogens adapt their virulence factors to cope with this protection. Indeed the most abundant factor is bacterial esterases that enhance the activity of sialidases by unmasking terminal sialic acid.

Bacterial sialic acid *O*-acetylation is clearly linked with pathogenicity. It can occur in capsule and lipopolysaccharides of *E. coli* (Dmitriev et al., 1977b), *Shigella dysenteriae* (Dmitriev et al., 1977a) and *K. pneumoniae* (Lindberg and Lonngren, 1972). The capsular polysaccharide of *Salmonella typhi*, which is an important virulence factor, is highly *O*-acetylated indicating that it enhances the activity of the polysaccharide (Bystricky and Szu, 1994). Nearly 85% of sialic acid from *N. menigitidis* is acetylated at positions C7 and C8 glycerol side chain (Arakere and Frasch, 1991). *O*-acetylation is found to be an immune response signal. Immunisation with *O*-acetyl residues from *E. coli* K1 capsular polysaccharide stimulates the immunity of mice more efficient than

immunising with de-*O*-acetylated polysaccharides (Orskov et al., 1979). It has also been reported that *O*-acetylation of sialic acid might prevent cell apoptosis caused by cancer and neurodegenerative disorders (Malisan and Testi, 2002).

The diversity of sialic acid and its modifications have a key role in pathogen-host interaction. For example, α2-6 linkage of sialic acid is recognizable by influenza A virus and it is directly involved in upper respiratory tract viral infection (Gagneux et al., 2003). It has also been reported that sialic acid is recognized by *V. cholerae*, *H. influenzae*, *H. pylori*, *S. aureus*, *Clostridium* spp., *P. aeruginosa* and *S. pneumoniae* (Aspholm et al., 2006, Moustafa et al., 2004, Fraser, 1978, King, 2010). This recognition is dependent upon the benefits of sialic acid that can be advantageous to the pathogen. For example, *H. influenzae* takes up sialic acid for its cell wall decoration as a way of avoiding the host immune system. Other pathogens use sialic acid as carbon source (Marion et al., 2011).

Sialidase activity is strongly influenced by the *O*-acetylation. It has been reported that the activity of sialidases is increased by 50-80% when all *O*-acetyl group are removed from sialic acid (Schauer and Kamerling, 1997, Schauer, 2004). Approximately, 5-10% of human nasal mucin is 9-*O*-acetylated (Reuter et al., 1988). The removal of *O*-acetylation by esterases accelerates the cleavage of sialic acid by NanA. Hence, pneumococcal esterase might influence the cleavage and utilisation of sequential sugar within host mucin (Herrler et al., 1985, Corfield, 1992). This project will seek evidence to support the hypothesis that esterase potentiates pneumococcal neuraminidase by unmasking the terminal sialic acid of host glycoprotein, and the potentiation of neuraminidase activity leads to increase utilisation of host glycoproteins and contributes to pneumococcal virulence. Therefore, the next section will introduce the structure and function of bacterial esterases.

1.3 Esterases

Overview:

Although past research revealed many unknown aspects of pneumococcal biology, further work is required for a comprehensive understanding of the microbe's virulence. One of the fields that have not been studied in sufficient detail is the role of esterases in pneumococcal biology. It is widely known that esterases are widespread in bacteria, and their contribution to virulence of many medically important bacteria have been demonstrated. Their wide ranging substrate specificity suggests that they are involved in different microbial pathways such as nutrient uptake and catabolism (Bornscheuer, 2002). This section will cover the importance of esterases for pathogenic bacteria.

1.3.1. Esterases' structure and classification:

Lipases (EC 3.1.1.3) and esterases (EC 3.1.1.1) are hydrolytic enzymes. These enzymes are able to cleave ester bounds into acid and alcohol. The main difference between esterases and lipases is substrate preference. Esterases (EC 3.1.1.1) can hydrolyse water-soluble esters and short chains of aliphatic esters, whereas lipases (EC 3.1.1.3) have an activity against water insoluble and long chain triglycerides (Arpigny and Jaeger, 1999, Faiz et al., 2007, Eggert et al., 2002). Both of these enzymes are defined as α/β-hydrolases (Figure 1.7) (Patel, 2007, Bornscheuer, 2002, Ollis et al., 1992). Esterases obey classical Michaelis-Menten kinetics, whereas lipases can react with a minimum concentration of substrate before giving a high activity (Bornscheuer, 2002).

Esterases can also be differentiated from lipase by the interfacial activation reaction, which is a typical reaction in lipases. Structural characterization of lipases showed that interfacial activation is caused by a flexible hydrophobic α-helix domain, which plays as a role as a lid to cover the active site of lipase when it reacts with high concentrations of substrate. This phenomenon does not occur in the presence of a minimum substrate concentration because the lid moves aside, exposing the active site to substrate (Ejima et al., 2004). Esterases are also able to catalyse, esterify, and aminolyse substrates and they do not require cofactors (Mnisi et al., 2005, Godinho et al., 2011).

The process of esterase's function in ester hydrolysis composes of four main steps: the first step starts with the active site serine binding to substrate, and a tetrahedral intermediate is formed, which is stabilized by catalytic His and Asp residues. The alcohol is released in the second step, and a complex of acyl-enzyme is generated. The third step starts by esterification of substrate by forming a tetrahedral intermediate again. The last step yields either acid or ester, and the free enzyme (Bornscheuer et al., 1999, Patel, 2007, Schmidt et al., 2004). This reflects the typical model of liberating acetyl groups from partially acetylated substrates (Christakopoulos et al., 1999).

Crystal structure analysis of esterases revealed that esterases have a typical α/β-hydrolase fold (Bornscheuer, 2002). These folds consist of 6 α-helixes and 8 β-strands (Figure 1.7). The majority of esterases have a typical catalytic triad of serine-histidine-aspartate (Ser-His-Asp). Serine is usually in the active site of esterases, and it is conserved in characteristic consensus sequence of Gly-X-Ser-X-Gly (where X represents an arbitrary amino acid residue) around the active site serine (Bornscheuer,

2002). Some esterases have a conserved consensus sequence of Gly-X-Ser-Leu motif, which is localised close to N-terminal (Arpigny and Jaeger, 1999). Recently, a consensus sequence of Gly-X-X-Leu motif have been identified in some esterases such as class C β-lactamases (Wei et al., 1995).

The BRENDA database has revealed that there are 745 types of triacylglycerol lipases and 696 types of carboxylesterases (Pharkya et al., 2003, Schomburg et al., 2004). Arpigny and Jaeger (1999) have classified and characterized all bacterial lipolytic enzymes to 8 different families (block L in ESTHER database http://bioweb.ensam.inra.fr/ESTHER) according to the conserved sequence motifs and biological properties of these lipolytic enzymes. The authors also relied on 3D structures of these enzymes where available. A total of 53 sequences of lipolytic enzymes were compared and divided into 8 different families using sequence similarity searches, sequence comparison and protein alignment software. Family I consists of true lipases and it was divided into a further 6 subfamilies. This family consists of large number of lipases with molecular weight ranging between 30-75 kDa. Subfamily I.1 (30kDa) and I.2 (33kDa) contain two aspartic acid residues that bind to Ca^{2+}, and two cysteine residues that form disulphate linkages within the enzyme (Arpigny and Jaeger, 1999). By contrast, I.3 (50-65kDa) does not have cysteine residues and *N*-terminal signal peptide. The molecular weight of enzymes in subfamilies I.4 and I.5 is 20 and 45kDa, respectively. Both subfamilies have a conserved consensus sequence of Ala-X-Ser-X-Gly as Glycine is replaced by Alanine. The last subfamily, I.6 enzymes are known to be secreted enzymes via protease cleavage at the *N*-terminal.

Family II and III of lipolytic enzymes have a conserved sequence Gly-Asp-Ser-(Leu) motif with 20% amino acid sequence identity with the human platelet-activating factor acetylhydrolase (PAF-AH). Family IV are hormone-sensitive lipases with a significant identity with mammalian hormone-sensitive lipases (HSL). Family V enzymes have the same conserved catalytic triad of Ser-His-Asp as epixode hydrolase, dehalogenases and haloperoxidases. Family VI also have the same conserved triad but they resemble eukaryotic lysophospholipases. Family VII and VIII are similar to eukaryotic acetylcholine esterases and class C β-lactamases, respectively.

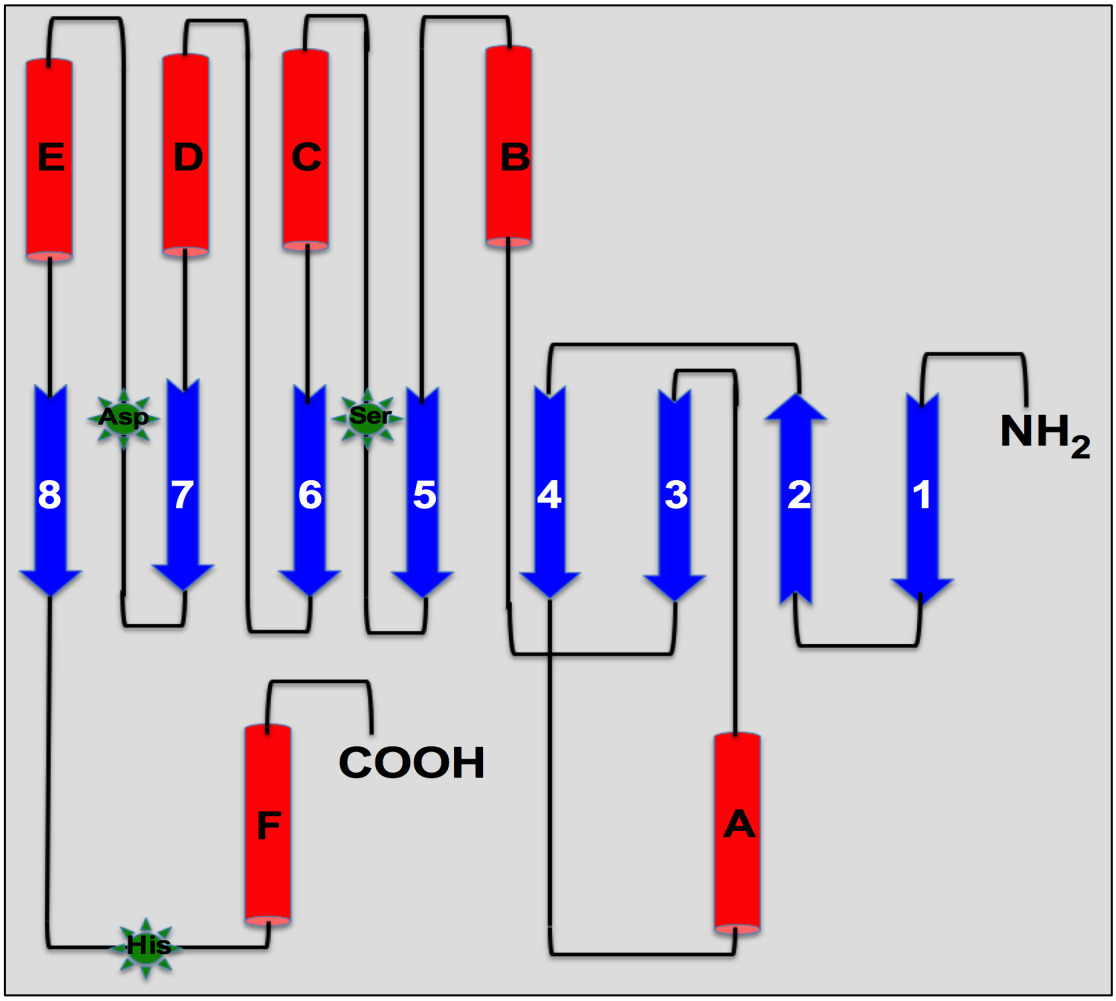

Figure 1.7: Schematic representation of the α/β-hydrolase fold of esterase. α-Sheets (1-8) are shown as blue arrows, β-helices (A-F) as red columns. The relative positions of the amino acids of the catalytic triad are indicated as green stars. The Figure was adapted from Bornscheuer (2002).

1.3.2 Functions of esterases:

Esterases have diverse functions in animal cells and microorganisms. They appear to be the most efficient enzymes that cleave *O*-acetyl groups from *O*-acetylated sialic acid (Schauer et al., 2011). *O*-acetyl groups on Sia prevent cleavage of the mucinous barrier on epithelial surface by pathogens, and serves against colonisation and eventual devastation of epithelia. Srinivasan and Schauer (2009) suggested that de-*O*-acetylation and hydrolysis of sialic acid molecules by esterase could facilitate the cleavage of sialic

acid and then degradation of *N*-acetyl or *N*-glycolyl groups from bovine submandibular gland mucin. Evidence regarding esterase activity on *O*-acetyl groups is studied in detail in viruses. For example, Herrler et al., (1985) showed that neuraminate-*O*-acetylesterase, which is also known as influenza C virus receptor destroying enzyme, releases *O*-acetyl groups from *N*-acetyl-9-*O*-acetylneuraminic acid. Smits et al., (2005) demonstrated that different types of enteric and respiratory viruses utilise sialic acid as a receptor and initial attachment is mediated by esterase. Some viruses use acetyl esterase as an initial attachment to epithelial cells by binding with 9-*O*-acetylated sialic acid (Schultze and Herrler, 1992).

The function of bacterial lipolytic enzymes in pathogenesis and virulence is not well annotated. The limited available evidence suggests their importance in microbial virulence. For example, esterases and lipases were shown to be important for the virulence of *S. pyogenes*, *S. aureus* (Jaeger et al., 1994), *Burkholderia cepacia* (Mullen et al., 2007), and *Mycobacterium tuberculosis* (Lun and Bishai, 2007). It has been reported that esterases are involved in bacterial sporulation (Higerd, 1977). The contribution of lipases/esterases to microbial virulence was attributed to their ability to modify host cell lipids (Mumy et al., 2008). For example, Guo et al. (2010) found that Rv0045c protein, which is a novel mycobacterial esterase, is involved in ester/lipid metabolism. Carboxyl esterase of *M. tuberculosis* coded by Rv3487c was linked to mycobacterial ability to cause lung infection in mice (Zhang et al., 2005, Camacho et al., 1999). It has been reported that lipases from *S.aureus* play a role in the pathogenesis and localisation of the microbe through the superficial tissues into deep tissues to cause subcutaneous infections (Rollof et al., 1987).

It has been found that *P. aeruginosa* has an outer membrane esterase (EstA) (Wilhelm et al., 1999). EstA was found to be important for *P. aeruginosa* lipid production, cell attachment and biofilm formation (Wilhelm et al., 2007). A novel carboxylesterase (EstV) was identified in *H. pylori*, an agent associated with gastric ulcers (Ruiz et al., 2007). It has also been reported that esterases are able to convert some inactive bacterial toxins into active toxins. For example, esterase (CyaC) from *Bordetella pertussis* converts inactive adenylate cyclase-hemolysin (CyaA), which is a key virulence factor in this pathogen, into active toxin (Westrop et al., 1996).

The involvement of esterases in antibiotic resistance has also been reported. Esterases are able to modify the active site of antibiotics by cleaving the macrolactone ring within the chemical structure of macrolides (Wright, 2005). Many bacterial species including *E. coli* (Barthelemy et al., 1984), *Providencia stuartii* (Plante et al., 2003), *S. aureus* (Wondrack et al., 1996), *Pseudomonas* spp. (Kim et al., 2002) and *K. pneumoniae* (Yong et al., 2009) are known to use macrolides esterases for antibiotic resistance. Two erythromycin esterases, EreA and EreB, have been structurally characterised in *E. coli* (Barthelemy et al., 1984).

Esterases were also reported to be beneficial immunogens. Some studies showed that passive immunisation of mice by monoclonal antibody, which was raised using viral esterase, protects animals from otherwise lethal viral infection by the inhibition of viral spread through the nervous system of mice (Yokomori et al., 1992). Liu et al., (2007) found that immunisation with *S. pyogenes* esterase, protected mice against skin infections. Nevertheless, the information relating to their mechanism of action is limited. Group A streptococcal secreted esterase was shown to hydrolyse platelet activating factor and to prevent the recruitment of neutrophils (Liu et al., 2012). In addition, it has been established that lipase C (LipC) in *M. tuberculosis*, a novel cell wall esterase, stimulates the production of pro-inflammatory cytokines and chemokines in macrophages (Shen et al., 2012).

Esterase are also involved in microbial nitrosative stress resistance. Potter et al. (2009) found that *estD*, which codes for esterase D (EstD), is important for nitrosative stress resistance of *Neisseria gonorrhoeae*. The authors also found that an *estD* mutant was unable to attach to human cervical epithelial cells. S-formylglutathione hydrolase produced from *Paracoccus denitrificans* is homologous to human esterase D (hEstD). This enzyme protects the microbe from formaldehyde toxicity (Harms et al., 1996b). Similarly, protein encoded by *adhC-estD* operon in *H. influenzae* is involved in nitrosative stress resistance (Kidd et al., 2007). It was found that the deletion of *adhC*, which encodes for alcohol dehydrogenase C (AdhC), does not attenuate the nitrosative stress resistance. However, the deletion of *adhC-estD* operon abolished the nitrosative stress resistance of *H. influenzae*, indicating that EstD is involved in nitrosative stress detoxification. Gonzalez et al. (2006) found that *frmB* and *yeiG*, which code potent enzymes that are involved in S-formylglutathione detoxification in *E. coli*, are carboxyl esterases, and contain typical serine catalytic active site.

Esterases manifest specificity depending on the position of acetylation in sialic acid, as acetylation can occur at one of the nine carbons, commonly occurring in C4, C7 and C9. Regl et al., (1999) identified a viral sialate-4-*O*-acetyl esterase that removes 4-*O*-acetyl group from *N*-acetyl-*O*-neuraminic acid. It has been reported that influenza A and B viruses target 9-*O*-acetylated sialic acid, whereas influenza C virus targets 4-*O*-acetylated sialic acid (Schauer et al., 2011). The position of *O*-acetylation determines viral attachment to host glycoprotein. It has been found that the individuals are less resistant against type A and B influenza viruses than type C because it was found that 20% of nasal mucin composes of 9-*O*-acetylated sialic acid.

One of the presumed roles of esterases is the removal of *O*-acetylation from sialic acids. *O*-acetylated sialic acid are commonly found in the terminal ends of glycoconjugates, such as mucin (Smits et al., 2005, Srinivasan and Schauer, 2009). The initial removal of sialic acid from host glycoconjugates by neuraminidases is extremely important for the complete breakdown of the complex sugar-rich molecules. Once the sialic acid is removed, then host glycoconjugates can be sequentially cleaved by other microbial glycosidases, such as galactosidases, and the released sugars can be utilised as nutrient by invading microbes (Marion et al., 2011, Yesilkaya et al., 2008, Severi et al., 2007).

1.3.3 Pneumococcal esterases:

The sequenced *S. pneumoniae* strains D39 (serotype 2) and TIGR4 (serotype 4) have 4 putative esterases: acyl-ACP thioesterase (*SPD1239/SP1408*), tributyrin esterase (*SPD0534/SP0614*), phosphoesterase (*SPD0932/SP1052*), and acetyl-xylan esterase (*SPD1695/SP1506*) (the gene codes have 'SPD' tag for D39 and 'SP' for TIGR4 strains). The sequence analysis database has shown that all of these genes more likely encode putative esterases. The amino acid sequence analysis has shown that 4 of them are composed of typical characterisatics of esterases. However, no published data has shown their function and involvement in pneumococcal physiology or virulence.

Among pneumococcal putative esterases, tributyrin esterase (SPD_0534, EstA) has been characterised structurally (Kim et al., 2008a), but no detail is available for its functional role. In a signature tagged mutagenesis study, this enzyme was reported to be required for lung infection in an experimental animal model (Hava and Camilli, 2002). Kang et al., (2009) showed the induction of nitric oxide and pro-inflammatory cytokine production in macrophages by EstA. Another study by Gebru et al. (2011) found that

EstA activates Janus kinase 2 (JAK2) as an inflammatory response in macrophages *in vitro*. However, the detailed function of EstA in pneumococcal virulence is not known, and the other pneumococcal esterases have not been studied at all. Hence, I hypothesise that the esterases are important for pneumococcal growth and virulence.

1.4 Aims and objectives:

The aim of this project is to identify the involvement of esterases in pneumococcal physiology and virulence. The sequenced *S. pneumoniae* strain D39 (serotype 2) has 4 putative esterases annotated as: acyl-ACP thioesterase (SPD_1239), tributyrin esterase (*estA*, SPD_0534), phosphoesterase (SPD_0932), and acetyl-xylan esterase (*axe*, SPD_150*6*). The information on the functional role of these esterases is sparse. I hypothesized that esterases are important for pneumococcal growth and virulence. Esterases are known as de-*O*-acetylating agents of natural and organic acetylated substrates. My hypothesis is to find out whether pneumococcal esterases are involved in sialic acid cleavage and how the removal of *O*-acetyl groups from sialic acid potentiates pneumococcal utilisation of host glycoproteins. I also aimed to characterise the pneumococcal esterases using synthetic and natural substrates. Subcellular localisation of esterases was also investigated. A mouse model of pneumococcal infection was also used to determine the esterases' involvement in pneumococcal colonisation and virulence.

Chapter Two

Materials and Methods:

2.1 Biological and chemical materials:

Unless otherwise stated, all chemicals used in this study were purchased from Sigma Aldrich (Germany), New England Biolabs (UK), Fisher (UK) or Oxoid (Thermo Scientific, UK).

2.2 Bacterial strains and plasmids:

Bacterial strains and plasmids used in this study are listed in Table 2.1 and Table 2.2, respectively.

Table 2.1: Bacterial strains used in this study.

Strains	Genotype	Source
E. coli BL21 DE3	*F-ompT hsdsB (rb-mb)gal dcm (DE3)*	Agilent Technology Ltd., UK
E. coli DH5 α	*F^-, φ 80dlacZ ΔM15, Δ(lacZYA -argF)U169, deoR , recA1 , endA1 , hsdR17 (rK^-, mK^+), phoA, supE44 , λ^-, thi -1, gyrA96 , relA1*	In-Fusion® HD Cloning Kit, Clontech, USA
S. pneumoniae D39	Virulent type 2 strain	The National Collection of Type Cultures, London, UK Strain NTCC 7466

Strains	Genotype	Source
S. pneumoniae TIGR4	Virulent type 4 strain	The National Collection of Type Cultures, London, UK Strain NTCC BAA-334
ΔSPD0534 (*ΔestA*) in D39 *ΔSP0416* in TIGR4	Tributyrin esterase, putative	This study
ΔSPD1506 (*Δaxe*) in D39 *ΔSP1695* in TIGR4	Acetyl xylan esterase, putative	This study
ΔSPD1239 in D39 *ΔSP1408* in TIGR4	Acyl-ACP-thioesterase, putative	This study
ΔSPD0932 in D39 *ΔSP1052* in TIGR4	Phosphoesterase, putative	This study
ΔestAnanA in D39	Tributyrin esterase-neuraminidase A double mutant	This study
ΔaxenanA in D39	Acetyl xylan esterase-neuraminidase A double mutant	This study
ΔestAaxe in D39	Tributyrin esterase-acetyl xylan esterase double mutant	This study
ΔnanA in D39	Neuraminidase A	Dr Hasan Yesilkaya
estAComp in D39	Tributyrin esterase complemented	This study

Strains	Genotype	Source
axeComp in D39	Acetyl xylan esterase complemented	This study
axeComp^{S181A}	Acetyl xylan esterase modified complemented	This study
estAComp^{S121A}	Tributyrin esterase modified complemented	This study

Table 2.2: Plasmids used in this study.

Plasmids/phenotype	Aim of use	Source
pR412/spectinomycin resistance	Mariner mutagenesis	Dr Marc Prudhomme CNRS-Universite Paul Sabatier Toulouse France (Martin et al., 2000)
pCEP/ Kanamycin resistance	SOEing mutagenesis and complementation	Dr Marc Prudhomme CNRS-Universite Paul Sabatier Toulouse France (Guiral et al., 2006)
pLEICS-01/ Ampicillin resistance	Proteins expression	PROTEX, University of Leicester

2.2 Growth conditions and media:

All media were prepared in distilled water, and sterilised by autoclaving at 121°C, 15 psi pressure for 15 min unless otherwise stated.

E. coli was grown on Luria broth (LB) or Luria agar (LA) (LB: 10g/l of NaCl, 5 g/l of yeast extract and 10 g/l trypticase peptone; LA: LB with the addition of 1.5% w/v bacteriological agar) (Oxoid, UK) at 37°C. The cultures were grown by shaking at 220 rpm. When required, ampicillin and kanamycin were added to *E. coli* cultures at 100 µg/mL and 150 µg/mL (Sigma, UK), respectively.

S. pneumoniae strains were grown at 37°C in micro-aerobic conditions either in brain heart infusion broth (BHI) (Oxoid, UK) or on blood agar base (Oxoid, UK) supplemented with 5% (v/v) defibrinated horse blood in a candle jar to promote the anaerobic conditions. Blood agar was prepared as described in manufacturer's instructions and sterilised. Horse blood, 5% (v/v), was added to the medium after cooling it down to 45°C. When needed, spectinomycin and kanamycin (Sigma, UK) were added to pneumococcal culture at 100 µg/mL and 250 µg/mL, respectively.

Chemically defined medium (CDM) was also used to grow *S. pneumoniae* (Kloosterman et al., 2006, van de Rijn and Kessler, 1980). The composition of CDM consists of basal solution, amino acids, vitamins, micronutrients, nitrogenous bases, sodium pyruvate and choline-HCl. Basal solution (Table 2.3) was prepared in 870 ml distilled water, and pH was adjusted at 6.5. The solution was then autoclaved at 121°C for 15 min. Micronutrients (Table 2.4) were prepared by dissolving in water and were kept at 4°C. All amino acids were dissolved in distilled water, and filter sterilised (Table 2.5). Nitrogenous bases (Table 2.6) were dissolved in 0.1 M NaOH and were kept at 4°C. Vitamins solution (Table 2.7) was prepared by dissolving riboflavin in 75% of total volume of the solution, and heated up to 70°C. D-biotin and folic acid were dissolved in 2 M NaOH in small aliquots. Other vitamins were added accordingly after riboflavin solution is cooled to 30°C. The solution was then kept at 4°C until needed. Pyruvate and choline-HCl solution was prepared as described in Table 2.8. The composition of CDM prepared is given in Table 2.9. Either glucose (55mM) (Kloosterman et al., 2006) or bovine sub-maxillary mucin (BSM) (Terra et al., 2010) were added as carbon source for pneumococcal growth.

S. pneumoniae strains were grown in a chemically defined medium (CDM) supplemented with different concentrations of BSM for 24 hours. The growth of strains was measured by determining colony-forming unit (CFU/ml) on blood agar after 0,1,3,6,12 and 24 hours incubation.

Table 2.3: Composition of basal solution used in CDM preparation.

Chemicals	Stock as g in 870 ml water
Na_2-β-glycerophosphate	21.0
KH_2PO_4	1.0
$(NH_4)_3$ citrate	0.6
Na-Acetate	1.0
Cysteine-HCl	0.4

Table 2.4: Micronutrients used for CDM preparation.

Micronutrients	Stock (g/l)	Concentration (mg/l)
$MgCl_2$	20	200
$CaCl_2$	3.8	38
$ZnSO_4$	0.5	5

Table 2.5: Amino acids added to CDM.

Amino acids	Stock as g/l	Final concentration as mM
Alanine	3	2.69
Arginine	1.55	0.71
Asparagine	4.4	2.34
Aspartate	5.25	3.00
Glutamate	6.25	2.46
Glutamine	4.9	2.68
Glycine	2.2	2.34
Histidine	1.9	0.72
Isoleucine	2.65	1.62
Leucine	5.7	3.48
Lysine	5.5	2.41
Methionine	1.55	0.83
Proline	8.45	5.87
Serine	4.25	3.23
Threonine	2.8	1.88
Tryptophan	0.65	0.25
Valine	4.05	2.77
Phenylalanine	3.45	1.67

Table 2.6: Nitrogenous bases used for CDM preparation.

Nitrogenous bases	Stock (g/l) of 0.1 NaOH	Concentration (mg/l)
Adenine	1	10
Uracil	1	10
Xanthine	1	10
Guanine	1	10

Table 2.7: Composition of vitamins added to CDM.

Vitamins	Stock (mg/l)	Concentration (mg/l)
Na-p-Aminobenzoate	500	5
D-biotin	250	2.5
Folic acid	100	1
Nicotinic acid	100	1
Ca(D+)Pantothenate	100	1
Pyridoxamine-HCl	250	2.5
Pyridoxine-HCl	200	2
Riboflavin	100	1
Thiamine-HCl	100	1
DL-6,8-Thioctic acid	150	1.5
Vitamin B_{12}	100	1

Table 2.8: Other chemicals added to CDM**.**

Chemicals	Stock as g/l	Concentration as mM
Pyruvate	10	1.1
Choline-HCl	2.5	0.072
Glucose	500	55.5
BSM	5	0.5%

Table 2.9: CDM composition. After preparing all solutions described in Tables 2.3, 2.4, 2.5, 2.6, 2.7 and 2.8, the following volumes were mixed to obtain final CDM preparation.

Chemicals	Volume (ml)
Basal solution	870
Aminoacids solution	80
Micronutrients solution	10
Nitrogenous bases solution	10
Vitamins solution	10
Pyruvate	1
Choline-HCl	4
Glucose	20

2.4 Colony Forming Unit assay (Miles and Misra assay):

Bacterial growth was measured spectrophotometrically at 500 nm by recording the increase in absorbance, or by quantification of CFU/ml using the Miles and Misra technique (Miles et al., 1938). Briefly, 20 µl of bacterial culture was mixed with 180 µl of PBS, pH 7.0, in a 96 well microtitre plate. This was followed by a 10-fold serial dilution of the mixture, and plating of 50 µl of each dilution on blood agar plates. The plates were allowed to dry at room temperature, and were incubated overnight at 37°C in a candle jar. The colonies were then counted from the sections that had between 30 and 300 colonies. The colony-forming unit per ml (CFU/ml) was calculated using the following formula:

CFU/ml=number of colonies X dilution factor X the volume plated (50 µl) /1000

2.5 Pneumococcal chromosomal DNA preparation:

Pneumococcal DNA was extracted according to the method of Saito and Miura (1963). After overnight growth, the pneumococcal culture was centrifuged at 3500 rpm for 10 min using Avanti J-E centrifuge (Beckman Coulter, USA). The pellet was re-suspended in 400 µl TE buffer containing 25% (w/v) sucrose, 60 µl of 500 mM EDTA, 40 µl of 10% (w/v) SDS and 2 µl of proteinase K (from 12.5 mg/ml stock). The mixture was incubated at 37˚C for 1-2 hours until the lysate was clear, and was centrifuged at 13,000 rpm (Hettich MIKRO 22R, Germany) for 5 min. The supernatant was transferred to a new tube, and an equal volume of liquidified phenol washed in Tris buffer pH 7.6 was added to the mixture and gently mixed until an emulsion formed. Then the emulsion was centrifuged at 13,000 rpm for 10 min. The upper aqueous phase was transferred to a fresh tube without disturbing the white protein layer. An equal volume of chloroform: isoamylalcohol (24:1 ml) was added and centrifuged at 13,000 rpm for 10 min. A 500 µl of upper phase was mixed with 2.5 ml of 100% ethanol and 20 µl of 3 M sodium acetate, pH 5.2, was added. The sample was then centrifuged at 13,000 rpm for 5 min and supernatant was discarded. Then, 500 µl of 70% (v/v) ethanol was added to wash the pellet, and the sample was centrifuged at 13,000 rpm for 5 min. The ethanol was discarded and pellet was allowed to dry prior to re-suspending it in 250 µl of TE buffer pH 7.0. The extracted DNA was kept at 4 ˚C until use.

2.6 Purification and extraction of the PCR products:

All PCR products were purified and extracted using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). This system is based on the ability of DNA to bind to silica membranes in the presence of chaotropic salts. The protocol was done according to the manufacturer's instruction. By using this kit, all DNA fragments could be extracted and purified including PCR products and DNA recovered from agarose gels. Briefly, the protocol started with weighing a 1.5 ml micro-centrifuge tube for each DNA fragment to be isolated. The DNA fragments were excised in a minimal volume of agarose using a clean scalpel or razor blade and were then transferred to the weighed micro-centrifuge tube. 10 µl of membrane Binding Solution (guanidine isothiocyanate 4.5 M) was added for every 10 mg of agarose gel slice, and the mixture was vortexed and incubated at 50-65°C for 10 min or until the gel slice was completely dissolved. The dissolved gel was transferred into a SV minicolumn assembly. The column was incubated for 1 min and then centrifuged at 14,000 rpm for 1 min using bench top micro-centrifuge and the supernatant was discarded. The recovered DNA was washed by adding 700 µl of Membrane Wash Solution (10 mM potassium acetate pH 5.0, 80% ethanol and 16.7 µM EDTA pH 8.0) to the SV Minicolumn. The column was centrifuged for 1 minute at 14,000 rpm using bench top micro-centrifuge. The supernatant was discarded and the washing step was repeated with 500 µl of Membrane Wash Solution. The SV Minicolumn was transferred to a fresh 1.5 ml micro-centrifuge tube, and the bound DNA was eluted by 50 µl of nuclease-free water by centrifugation for 1 min at 14,000 rpm after incubating the column for 1 min at room temperature. The SV Minicolumn was discarded and the micro-centrifuge tube containing the eluted DNA was stored at –20°C until needed.

The confirmation of successful recovery of the purified and extracted plasmid was done using agarose gel electrophoresis, and visualizing under a UV transilluminator system (UVP, USA) equipped with an Olympus SP-500UZ digital camera. The yield of DNA was determined spectrophotometrically at 260 nm by using NanoDrop 1000 (Thermo Scientific, USA).

2.7 Small-scale plasmid extraction (Mini Prep kit):

The plasmid DNA was extracted according to manufacturer's instruction (QIAprep®, Qiagen). Briefly, one single colony from fresh *E. coli* culture was picked and inoculated

in a 10 ml LB supplemented with ampicillin (100 µg/ml). The culture was incubated at 37°C overnight with shaking at 220 rpm. Next day, the culture was harvested by centrifugation at 3500 rpm for 10 min at room temperature. The pellet was then re-suspended in 250 µl Buffer P1, and transferred to a 1.5 ml a micro-centrifuge tube; 250 µl of Buffer P2 was then added and the preparation was mixed by inverting the tube gently, 4 to 6 times (avoiding vortex as it might shear the genomic DNA), to homogenise the suspension.

A 350 µl of Buffer N3 was then added, and the tube was immediately mixed by inverting it 4 to 6 times until a cloudy suspension had been formed. The suspension was then centrifuged for 10 min at 13,000 rpm in a bench top micro-centrifuge (Micro centaur), and the supernatant was transferred to the QIAprep column without disturbing the pelleted protein layer. The column was then centrifuged for 1 min at 13,000 rpm and the flow-through was discarded. To remove any trace of nucleases, 0.5 ml of Buffer PB was added to the column, which was then centrifuged at 13000 rpm, and the flow-through was discarded. The column was then washed by adding 750 µl of Buffer PE and centrifuging at 13,000 rpm for 1 min. The column was once again centrifuged for 1 min to eliminate the residual buffers and ethanol that could interfere with the subsequent enzymatic reactions. The column was finally placed into a fresh micro-centrifuge tube and the recovered plasmid DNA was eluted by adding 50 µl of Buffer EB. The recovered plasmid DNA was stored at -20°C for further use.

2.8 Large-scale plasmid extraction (Maxi Prep kit):

Maxi Prep plasmid purification was done using Maxi Prep kit (High Speed Plasmid Maxi Kit 10, Qiagen, UK). Briefly, one single colony from fresh *E. coli* culture was picked, and was inoculated in a 150 ml LB supplemented with appropriate antibiotic. The culture was then harvested by centrifugation at 6500 rpm for 15 min using an Avanti J-E centrifuge (Beckman Coulter, USA). The supernatant was removed, and the pellet was re-suspended with 10 ml P1 buffer. The suspension was mixed thoroughly by adding 10 ml P2 buffer and vigorously inverting for 4 to 6 times. The mixture was incubated at room temperature for 5 min. After the incubation, 10 ml of chilled P3 buffer was added to the lysate, and was mixed immediately and thoroughly by inverting for 4 to 6 times. The mixture was immediately poured into the barrel of the QIAfilter Cartridge. The QIAfilter Cartridge was then incubated at room temperature for 10 min.

The cell lysate was passed through QIAfilter Cartridge into the equilibrated HiSpeed Maxi Tip by pressing the plunger gently. The cleared lysate was left to enter the resin by gravity flow. After that, the bound DNA was washed with 60 ml of QC buffer. The bound DNA was then eluted from the HiSpeed Maxi Tip by adding 15 ml of QF buffer. The eluted DNA was precipitated by adding 10.5 ml of isopropanol, and the solution was incubated at room temperature for 5 min. The precipitated DNA was then transferred into a 30 ml syringe and was filtered by inserting the plunger and pressing it constantly and gently. This allows DNA binding into the QIAprecipitator Maxi Module and removes isopropanol residues. The QIAprecipitator was then removed from the syringe and the plunger was removed. The QIAprecipitator was then re-attached to the syringe and 2 ml of 70% (v/v) ethanol was added into the syringe. The recovered DNA was then washed by inserting the plunger into the syringe and passing the ethanol through the QIAprecipitator. The latter step was repeated two times without adding 70% (v/v) ethanol to dry the membrane. The outlet nozzle of QIAprecipitator was then dried with absorbent paper to prevent ethanol carryover. To elute DNA, the plunger of a 5 ml syringe was removed and the QIAprecipitator was attached onto the outlet of the 5 ml syringe. 1 ml TE buffer was added into the syringe and the plunger was inserted. The eluted DNA was collected into clean micro-centrifuge tube by pressing the plunger constantly and gently.

2.9 Mutagenesis:

All mutants were constructed in this study using one of two methods: mariner mutagenesis (Martin et al., 2000, Yesilkaya et al., 2009) and gene splicing by overlap extension mutagenesis (Horton et al., 1990). Both strategies are explained in detail below:

2.9.1 Mariner mutagenesis:

Mariner mutagenesis was used for mutant construction (Lampe et al., 1996). For this, target genes were amplified using primers listed in Table 2.10. *Himar I* transposase enzyme was used to insert the antibiotic cassette into the target genes (Lampe et al., 1996). The components of transposition reaction are listed in Table 2.11. The reaction mix was incubated at 30°C for 6 h. The content of the mixture was recovered using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) as described in section 2.4. Next, gap repairing was done by setting up the reaction listed in the Table 2.12. The

reaction was incubated at 16°C for 30 min, and then was stopped at 75°C for 10 min. The ligation reaction was set up as described in Table 2.13. The reaction was incubated overnight at 16°C and the reaction was terminated by heating at 75°C for 10 min. The transposon reaction was then purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) as described in section 2.6. The purified amplicons were transformed into *S. pneumoniae* wild type as described in the section 2.9.3.

Table 2.10: Oligonucleotides used in mariner mutagenesis.

Gene/Symbol		**Primer Sequence (5′–3′)**
SPD1506	Forward	TACAGACAAGCCTGACGATAGC
	Reverse	TGCATTGATTACATAGATATGC
SPD1239	Forward	CGTCACCAAGAGTGTTGGAA
	Reverse	CCGGTTTTGAAAGTCGTCAT
SPD0534	Forward	GTATCAGGGCACGGAAATGT
	Reverse	CCACTCTCTTACTGCGAGCAT
SPD0932	Forward	GATAGCGTTGAATACACCAAAGATT
	Reverse	GAGTTCCTCATCCGCTATATATGG
MP127 *	Reverse	CCGGGGACTTATCAGCCAACC
MP128 *	Forward	TACTAGCGACGCCATCTATGTG

*: Spectinomycin mini cassette specific primers (Martin et al., 2000).

Table 2.11: Transposition reaction used in mariner mutagenesis.

Component	Volume as µl
Himar I transposase	0.5
10 X transposase buffer	2.0
Glycerol	2.0
Acetylated BSA 10 mg/ml	0.5
Target gene (250 ng)	5.0
pR412 donor plasmid (spectinomycin resistant) (250 ng)	5.0
Nano pure water	6.0
Total volume	20.0

Table 2.12: Gap repair reaction for mariner mutagenesis.

Component	Volume as µl
NEB buffer 2	3.5
BSA (NEB)	0.15
dNTPs (2mM)	1.5
T4 DNA polymerase (3U/ µl)	1.5
Transposition reaction DNA	30
Total reaction	36.15

Table 2.13: Ligation reaction for mariner mutagenesis.

Component	Volume (µl)
E. coli DNA ligase	2
10 X ligase buffer	4
Contents of Table 2.12	36.15

2.9.2 Gene splicing by overlap extension mutagenesis (SOEing PCR):

Mutagenesis by SOEing PCR was achieved according to the protocol described by Horton (1995). This method was used for two purposes: for insertion-deletion mutation, and for replacement of selected amino acid residues. The Figures 2.1 and 2.2 illustrate the strategy for SOEing PCR. For insertion deletion mutation, the procedure consisted of two steps of PCR. In the first step, 800 bp of upstream and downstream of target gene was amplified using SPDXUP/F and SPDXUP/R, and SPDXD/F and SPDXD/R primer sets, respectively (where X indicates the gene designations) (Table 2.14). These primers were designed to contain a homologous region for kanamycin resistance gene (Kan^R). The Kan^R gene was amplified from the plasmid pCEP (Table 2.2) using the primers Kan^R/F and Kan^R/R that are listed in Table 2.14. The second step of PCR consisted of fusing the upstream and downstream target gene fragments with Kan^R cassette using SPDXUP/F and SPDXD/R primers (Table 2.14).

The second aim of SOEing PCR was with replace serine (S^{121} and S^{181} for EstA and Axe, respectively) to alanine to investigate serine's role in the catalytic activity of esterases. SOEing PCR mutation was done according to the method described by Horton (1995) with some modifications. The strategy of mutation is presented in the Figure 2.2. Briefly, in the first PCR, the left flank and right flank of target genes were amplified using modified primers to amplify the gene of interest into two flanks with the intended mutation (Table 2.14). The flanks carry a homologous region for each

other to mediate fusion (Figure 2.1). The second round of PCR was achieved to fuse the left and right flanks using $SPDX^{S-A}$/F1 and $SPDX^{S-A}$/R2 primers where X indicates the gene designation and S-A indicates serine active site that was replaced to alanine (Table 2.14).

2.9.2.1 PCR mix composition:

The PCR mix consisted of 2X 25 µl of PrimeSTAR HS premix (25 µl PrimeSTAR HS DNA Polymerase (1.25 U), 2X dNTP mixture 0.4 mM each, 2X PrimeSTAR buffer containing 2 mM Mg^{2+}) (Takara, Japan), 2 µl of 15 ng/µl of either pneumococcal DNA or the plasmid pCEP, 2 µl of gene specific forward and reverse primer mix (1 pmol each/reaction) and 21 µl of molecular grade water. For joining flanks, 2 µl of product from first round PCR (20 ng/ µl) were mixed and used as a template in a 30 cycles of amplification reaction (Table 2.15). The amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) as described in section 2.4.

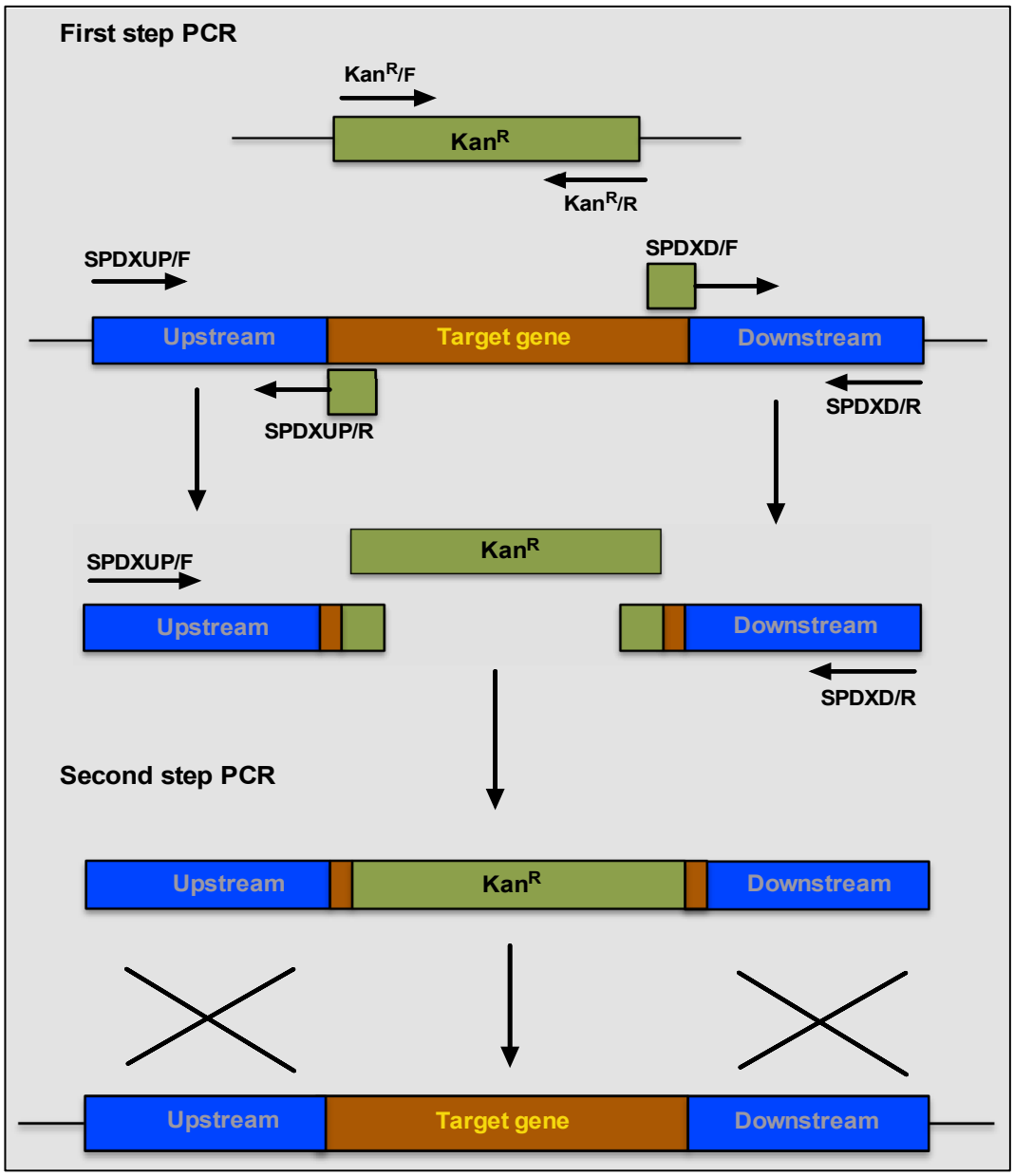

Figure 2.1: Illustration of SOEing PCR steps for allelic insertion deletion mutagenesis and gene knockout by homologous recombination. X in primers designation refers to the gene number. The bold crosses represent the homologous recombination during transformation.

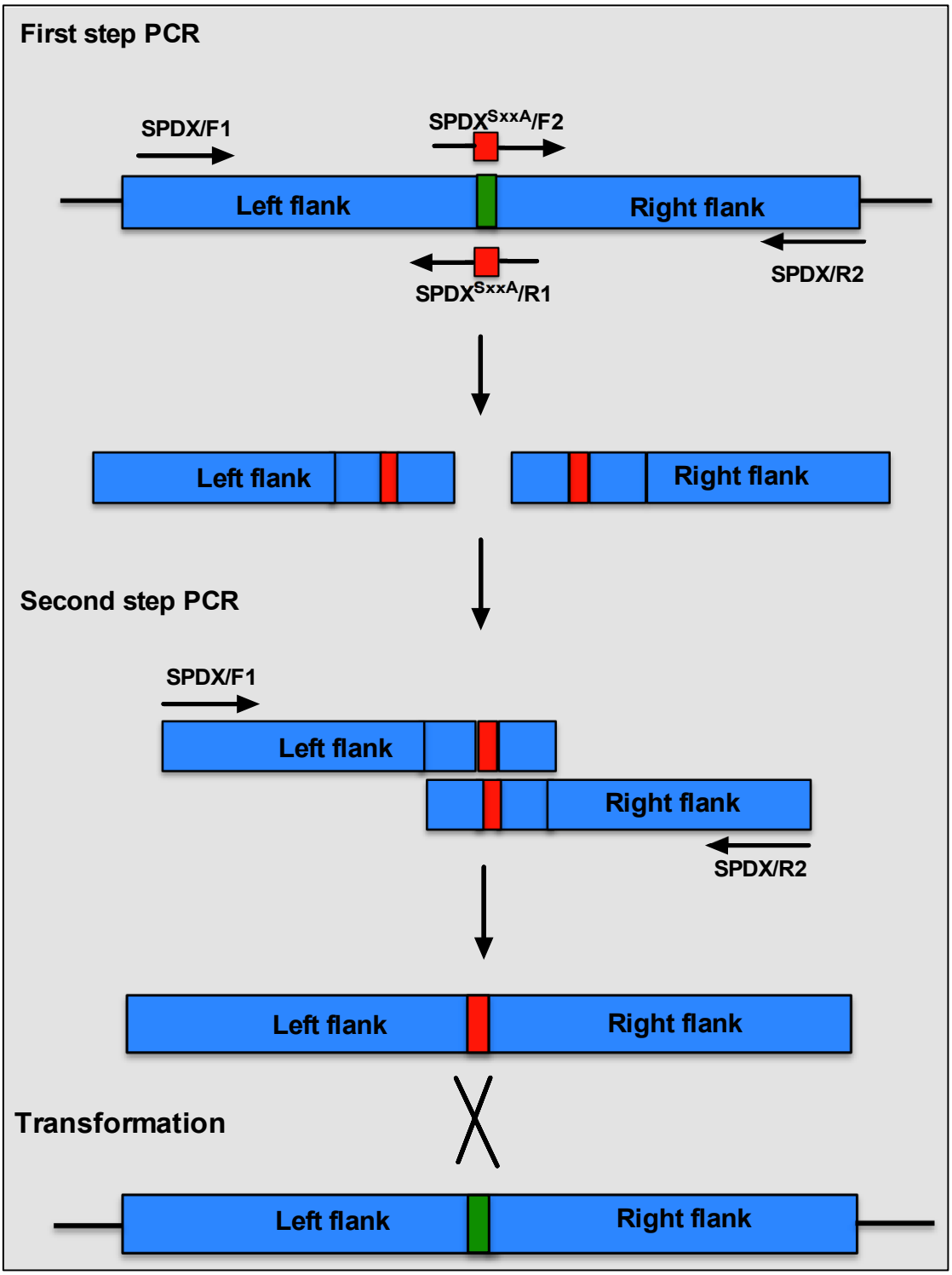

Figure 2.2: Schematic representation of amino acid replacement using Splicing Overlap Extension PCR (SOEing PCR). SPDX/F1 and SPDX/R2 refer to gene specific primers. SPDX^{SxxA}/F2 and SPDX^{SxxA}/R2 refer to mutagenic site primers that carry replaced nucleotide (red rectangle) with wild type nucleotide (green rectangle) where xx refers to the serine active site being replaced with alanine.

Table 2.14: Oligonucleotides used for SOEing PCR.

Gene/Symbol	Primer Sequence (5′–3′)
SPD1506UP/F	ACCAGATCATGCAGGATTTCAAGGAGAGAAAGACGGATA
SPD1506UP/R	**ACGGATCCCCAGCATGCGCGC*ATTTTTCACTTCCCCATCC
SPD1506D/F	**TTACTGGATGAATTGTTTTAG*ATGCCTGAGTATGCTCACG
SPD1506D/R	AAAGATAGATTCTATCACTTTTTATAAGTGTTAGTAAAT
SPD0534^{S121A}/F	**CGCTGGTCTT***G***CTATGGGAGGCT
SPD0534^{S121A}/R	**AGCCTCCCATAG***C***AAGACCAGCG
SPD1506^{S181A}/F	**CTATGGTGCC***G***CACAAGGAGGGG
SPD1506^{S181A}/R	**CCCCTCCTTGTG***C***GGCACCATAG
Kan^{R}/F	GCGCGCATGCTGGGGATCCGT
Kan^{R}/R	CTAAAACAATTCATCCAGTAA
SPD0523/F	TCGTTGAAATAAACAGAGAG
SPD0523/R	CTAAGTCAGTCTCTCTTCTAAT
SPD1506/F	GCTTTCTCTAAGGAAAACTT
SPD1506/R	TTATTTTAGATATTTAAAAG

*: The nucleotides italicized are complementary to 5′ and 3′ of kanamycin resistance gene.

**: The nucleotides italicized represent the replacement of thiamine to guanine to change the amino acid serine to alanine.

Table 2.15: PCR conditions used for SOEing PCR mutagenesis.

Step	Description	Cycles	Temperature	Minutes:Seconds
1	Initial denaturation	1	98°C	00:10
2	Amplification	30	98°C	00:10
			55°C	00:05
			72°C	01:00
3	Final hold	-	4°C	-

2.9.3 Transformation of *S. pneumoniae*:

Transformation of DNA to the pneumococcal strains was done according to the method described by Bricker and Camilli (1999). A hundred microliter of an overnight culture of pneumococci was diluted at a ratio of 1:100 (v/v) in fresh BHI. The culture was incubated at 37˚C in a candle jar until $OD_{(600)}$ had reached between 0.03-0.06. At this stage, 860 µl of pneumococcal culture was added into a 1.5 ml sterile tube and mixed with 100 µl of 100 mM NaOH, 10 µl of 20% bovine serum albumin (BSA), 10 µl of 100 mM $CaCl_2$, 2 µl of 50 ng/ml of competence stimulating peptide (CSP), and 5-10 µl of DNA containing the mutated genomic region. The reaction was incubated at 37˚C for 3 hours. Every hour over the 3 hours incubation periods, 330 µl of bacterial culture was plated out on blood agar supplemented with appropriate antibiotics. The plates were incubated overnight in a candle jar at 37˚C.

2.9.4 Confirmation of pneumococcal transformation:

Successful transformation was confirmed by colony PCR according to the method described by Trower (1992). In this technique, a single colony of grown transformant was used as a template to amplify the mutated region. Transformants isolated from

selective medium were grown overnight on 10 ml of BHI supplemented with appropriate antibiotic. Next day, 1 ml of the culture was centrifuged and the pellet was resuspended in 500 µl PBS. The suspension was heated on a hot plate at 95˚C for 5 min. The heated mixture was then cooled on ice, and spun down at 13000 rpm for 1 min using a bench-top microcentrifuge. The supernatant was transferred into fresh eppendorf tube, and was used as template for PCR. Transposon-specific primers, MP127 and MP128 (Table 2.10), together with an appropriate target gene primer, were used to amplify the mutated region. The entire mutated locus was also amplified using primers located upstream and downstream of target gene. In addition, the presence of antibiotic resistance cassette was interrogated in the transformants; 20 µl reaction mixture contained 10 µl of HotStarTaq *Plus* DNA polymerase (Qiagen, UK), 2 µl of gene specific primers (1 pmol each), 2 µl of cell lysate, and 6 µl of molecular grade water. The thermo cycler was set to run 35 cycles of amplification using the conditions described in Table 2.16. The PCR products were examined on agarose gel (1% w/v) to confirm the amplification. After that, when needed, the amplicons were extracted and purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) as described in section 2.4.

Table 2.16: PCR conditions used for colony PCR to confirm transformation.

Step	Description	Cycles	Temperature	Minutes : Seconds
1	Activation (Initial denaturation)	1	95°C	05:00
2	Amplification	35	94°C	00:45
			55°C	00:45
			72°C	03:00
3	Final extension	1	72°C	10:00
4	Final hold		4°C	-

2.9.5 Confirmation of mariner mutagenesis by DNA sequencing:

The template for sequencing was prepared by single primer PCR as described by Karlyshev et al., (2000). This PCR consisted of three rounds of amplification. The first round was a linear PCR using a single strand of chromosomal DNA of mutants and MP128 primer (Table 2.10) and consisted of 30 cycles of 90˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 2 min. The second round amplified the region flanking the insertion site using the products of the first round as template and cycling parameters were: 10 cycles of 90˚C for 30 sec, 30˚C for 30 sec, and 72˚C for 2 min. The last round amplified the second round products and the conditions for amplification were: 30 cycles of 90˚C for 30 sec, 55˚C for 30 sec, and 72˚C for 2 min. The products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and were sequenced by the Protein & Nucleic Acid Chemistry Laboratory (PNACL) at the University of Leicester using MP127 primer (Table 2.10).

2.10 Complementation of isogenic mutants:

In order to rule out that the observed effects were not due to polar effect but due to the mutation of esterase genes, genetic complementation of the mutants was achieved using the method described by Guiral et al. (2006). This technique uses a plasmid, pCEP, which has ability to replicate in *E. coli*, but not in *S. pneumoniae*. It contains a kanamycin resistance gene. It also has more than 2 kb of pneumococcal DNA homologous to region surrounding *amiA*, which is considered to be transcriptionally silent site in the pneumococcus. Therefore, pCEP allows insertion of the gene of interest in a chromosomally silent site, downstream of *amiA* operon.

The esterase isogenic mutants were also complemented with modified $estA^{S121A}$ and axe^{S181A}. The replacement of putative serine active site to alanine was achieved by SOEing mutagenesis as described in section 2.3.2. SOEing products carrying modified putative active site were amplified using pCEP dependent primers (Table 2.17) that incorporate restriction sites homologous to 5′ and 3′ of pCEP.

2.10.1 Cloning of wild type and modified esterase genes into pCEP:

estA (SPD0534), $estA^{S121A}$, *axe* (*SPD1506*) and axe^{S181A} genes were amplified either from D39 or from a recombinant plasmid carrying serine active site replacement to alanine, using the primers that incorporate desired restriction sites (Table 2.17). The

PCR master mix reaction was prepared as described in section 2.9.2. The PCR condition is described in the Table 2.15.

Table 2.17: Oligonucleotides used for genetic complementation.

Gene/Symbol		Primer Sequence (5′–3′)
SPD0534Comp/F	Forward	*CATG*CCATGG*TCGTTGAAATAAACAGAGAG
SPD0534Comp/R	Reverse	*ACG*GGATCC*CTAAGTCAGTCTCTCTTCTAAT
SPD1506Comp/F	Forward	*CATG*GTATAC*GCTTTCTCTAAGGAAAACTT
SPD1506Comp/R	Reverse	*ACG*GGATCC*TTATTTTAGATATTTAAAAG
malF2	Forward	**GTCAACTGTAGTGGGTTGAAGTCAGC
pCEPR2	Reverse	**CCTTTTCCCGTTCCACATCATAGGTG
SPD0534^{S121A}/F	Forward	***CGCTGGTCTT***G***CTATGGGAGGCT
SPD0534^{S121A}/R	Reverse	***AGCCTCCCATAG***C***AAGACCAGCG
SPD1506^{S181A}/F	Forward	***CTATGGTGCC***G***CACAAGGAGGGG
SPD1506^{S181A}/R	Reverse	***CCCCTCCTTGTG***C***GGCACCATAG

*: The nucleotide italicized *CCATGG* are complementary to *NcoI,* *GGATCC* is complementary to *BamHI*, *GTATAC* is complementary to *Bstz17I* restriction sites. **: The recognition sites for these primers are located immediately upstream and downstream of the cloning site, respectively. ***: The nucleotides italicized in bold represent the replacement of thiamine to guanine to change the amino acid serine to alanine.

The inserts and pCEP were digested using desired restriction enzymes (New England Biolabs Inc., UK) to prepare it for ligation. The restriction enzymes *Nco*I/*BamH*I and *Nco*I/*Bstz*17I were used to digest *estA* and *axe*, respectively. The same restriction enzymes were used to digest pCEP to create compatible ends. The digestion reaction was set up as described in Table 2.18. The reaction was incubated for 2 h at 37°C in water bath.

Table 2.18: Restriction digests of pCEP or amplified DNA fragments.

Reaction composition	Reaction	Control
pCEP or linear DNA	1 µg	1 µg
NcoI, Bstz17I or BamHI	1 µl (10U)	---
Buffer	5 µl	6 µl
DNase free water	Top up to 20 µL	Top up to 20 µl

The ligation was set up as described in Table 2.19. The ligation reaction was mixed gently by pipetting up and down. The reaction was then incubated overnight at 16°C. The T4 ligase activity was then heat inactivated at 75°C for 10 min, and ligates stored at -20°C until needed.

Table 2.19: Components of the ligation reaction for genetic complementation of the mutants.

Components	Volumes and concentrations
pCEP	5 µl at concentration 30ng/µl
Insert	5 µl at concentration 80ng/µl
Ligation buffer	2 µl
T4 ligase	1 µl (400 U)
DNase free water	6 µl

An aliquot of ligation mixture was transformed into *E.coli* BL21 DE3 as described by the manufacturer (Agilent Technology Ltd., UK). Briefly, an aliquot of competent *E. coli* BL21 DE3 cells was thawed on ice, and mixed with 1 µl of 50-60 ng/µl recombinant plasmid pCEP. 2 µl of β-Mercaptoethanol (β-ME) was then added to the mixture. The tube was incubated on ice for 30 min with a gentle mix for every 2 min, followed by a heat shock at 42°C for 45 sec. The tube was immediately put on ice for 2 min., and 500 µl of preheated NZY+ broth medium (4 g NZ amine (enzymatic casein hydrolysate), 2 g of yeast extract, 2 g of NaCl and dH_2O to a final volume of 400 ml) at 42°C was then added. The transformed bacteria were incubated at 37°C for 1 hour on a shaking platform (220 rpm) to promote the recovery of bacteria. Finally, the transformants were selected on LA supplemented with 150 µg/ml of kanamycin. The presence of insert in selected transformants was confirmed by colony PCR using malF2 and pCEPR2 primers (Table 2.17) whose recognition sites are located immediately upstream and downstream of the cloning site, respectively. One of the transformants containing the insert was used for recombinant plasmid extraction using Mini-Prep kit (QIAprep Spin Miniprep Kit, Qiagen, UK) as described in section 2.7. The resultant plasmids were sent for sequencing to eliminate the possibility of any mutations using

malF2 and pCEPR2 primers (Table 2.17).

The purified plasmids were then transformed into *ΔestA* and *Δaxe* isogenic mutants as described in section 2.9.3 (Bricker and Camilli, 1999). Transformation was confirmed by colony PCR according to the method described by Trower (1992) as described in section 2.9.4. The primers malF2 and pCEPR2 (Table 2.17) confirmed incorporation of desired inserts into the pneumococcal genome.

2.11 *In vitro* gene expression studies:

This assay was done to check the expression level of genes of interest. This assay relies on the quantification of mRNA. For *in vitro* gene expression bacterial strains were grown on minimal medium supplemented with the selected nutrient. The gene expression analysis was done using mid exponential phase cultures.

2.11.1 RNA extraction:

The Trizol method was used to extract RNA (Stewart et al., 2002). Briefly, pneumococci were grown in 10 ml BHI or CDM supplemented with BSM until mid-exponential phase. Pneumococci were then pelleted by centrifugation at 3000 rpm for 10 min, and the pellet was resuspended in 500 µl of Trizol reagent; 100 µl of chloroform was then added and mixed by vortexing for 15 seconds. Samples were transferred into Hybaid Lysing Blue tubes and sonicated at 6.5 power for 45 seconds twice with 15 seconds cooling intervals using a PowerLyzer ^{TM} 24 homogeniser (MO BIO, USA). The cell lysates were centrifuged at 13,000 rpm for 15 minutes at 4°C. The aqueous phase was transferred into a fresh tube, 250 µl of isopropanol was added to precipitate nucleic acid by vortexing for 15 seconds, and the tube was incubated for 5 minutes at room temperature. The tubes were then centrifuged at 13000 rpm for 10 minutes at 4°C. The isopropanol was discarded, 500 µl of 75% ethanol was added, and the pellet was recovered by centrifuging at 13,000 rpm for 10 minutes at 4°C. Ethanol was removed and the pellet was dried for 15 minutes at room temperature. The dried pellet was then re-suspended with 87.5 µl of DNase-RNase free water. The recovered RNA was kept at -80 until use.

2.11.2 DNase treatment:

The extracted RNA was treated with TURBO^{TM} DNase I (Ambion, UK) to remove the

residual chromosomal DNA. 1 µl (2U) of TURBO DNase I was added to 200 µg of RNA and incubated for 20 min in a water bath at 37°C. DNase I was inactivated by adding 5 µl of DNase inactivation solution according to the procedure described by the manufacturer (Ambion, UK). The mixture was then incubated at room temperature with gentle mixing. The mixture was then centrifuged at 13,000 rpm for 1.5 min and the supernatant containing treated RNA was transferred into a fresh tube. The purified RNA was then stored at -80°C until use. The quantity and integrity of RNA was checked spectrophotometrically using a NanoDrop 1000 (Thermo Scientific, UK) at 230 nm, and by 1% (w/v) agarose gel electrophoresis.

2.11.3 Synthesis of complementary DNA (cDNA):

SuperScript III reverse transcriptase (Invitrogen, UK) was used to synthesize first strand complementary DNA (cDNA) according to the procedure described by the manufacturer (Invitrogen, UK). Approximately 5 µg of total DNase treated RNA was mixed with 1 µl of 50 µM random primers and 1µl of 10 mM dNTP mix in a total volume of 20 µl by topping up with DNase-RNase free water. The mixture was heated at 65°C for 5 min, and the reaction was incubated on ice for 1 min. After that, 4 µl of 5X First-Strand Buffer, 2 µl of 0.1 M dithiothreitol (DTT) and 1 µl of SuperScript III were added to the reaction. The tubes were incubated at 25°C for 5 minutes followed by incubation at 42°C for 50 minutes. Heating at 70°C for 15 min then stopped the reaction.

2.11.4 Quantitative reverse transcriptase Polymerase Chain Reaction (qRT-PCR):

qRT-PCR was performed using Corbett RG-6000 PCR system. 2 µl (15 ng) cDNA was mixed with 2 µl of gene specific primers (Table 2.20) and 10 µl 2X SensiMix SYBR Master mix (Bioline, UK). The reaction was topped up to 20 µl by adding 6 µl of molecular grade water. Following conditions were used for amplification: 1 cycle initial denaturation at 95°C for 10 min; 40 cycles of amplification at 95°C for 20 sec, 58°C for 35 sec and 72°C for 20 sec; 1 cycle of DNA melting at 95°C for 1 min. The expression levels of the genes were normalized to the expression of *gyrB,* the housekeeping gene. The comparative threshold cycle C_T method was used to analyse the results (Livak and Schmittgen, 2001).

Table 2.20: Gene specific primers for real-time PCR.

Primers	Sequence (5′–3′)
SPD1239-RT/F	GAAACGGAAGCCTTGAG CTA
SPD1239-RT/R	CGGTCCATGAGAACAAA GGT
SPD0534-RT/F	GGCTTAAGCGGACCAAT GTA
SPD0534-RT/R	CAGAACCTGTGGCAATTC CT
SPD0932-RT/F	AGTGCAGCAGATAAATTG CAT
SPD0932-RT/R	AGTGCAGCAGATAAATTG CAT
SPD1506-RT/F	GGGATGGGGAAGTGAAA AAT
SPD1506-RT/R	ATGCGTGCATAGACCTTT CC
NanA-RT/F	GTGAAAATGGGATGGTCCAC
NanA-RT/R	CACTTTCGCTTCGGTAGG AG
gyrB-F	TGATGACCGATGCCGATG
gyrB-R	TTGGGCAATATAAACATAACCAGC

2.12 Cloning and expression of recombinant enzymes:

EstA, EstA^{S121A}, Axe, Axe^{S181A} and NanA enzymes were cloned and expressed in the plasmid pLEICES-01. The plasmid pLEICES-01 carries 6XHis Tag, which allows purification of proteins using metal affinity column. The cloning step was done in collaboration with Dr Yang (PROTEX, Department of Molecular and Cell Biology, University of Leicester) as described below. The overview of the procedure is given in Figure 2.3.

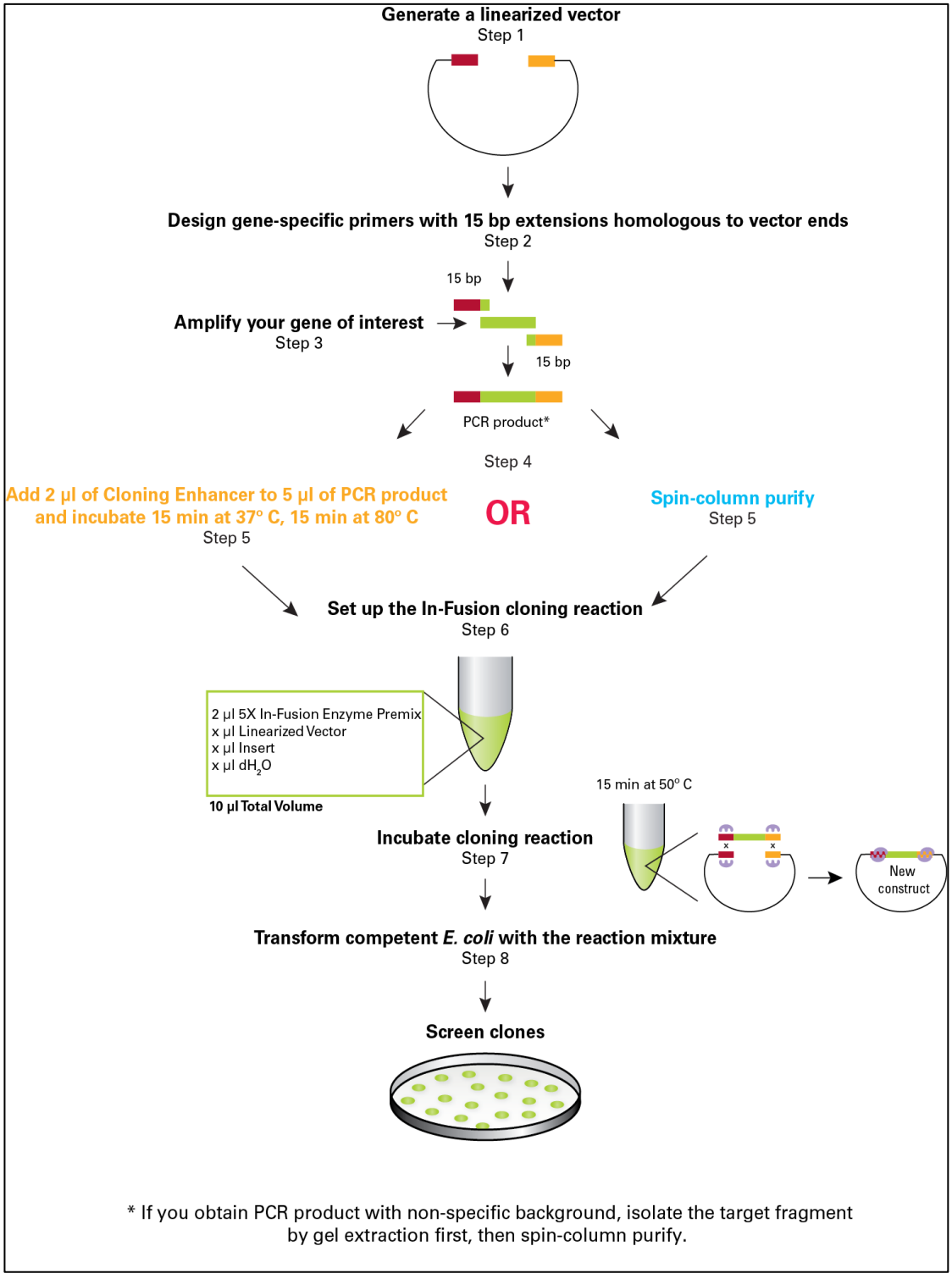

Figure 2.3: In-Fusion HD Protocol Overview. The figure was adapted from Clontech Laboratories.

2.12.1 Amplification of genes for cloning:

pLEICS-01 was used to express proteins. This vector incorporates 6XHIS tag at the *N*-terminal region of protein to be expressed. Gene cloning primers were designed to incorporate 15 nucleotide complementary regions of either side of multiple cloning site of pLEICS-01 (Table 2.21). *estA*, $estA^{S121A}$, *axe*, axe^{S181A} and *nanA* were amplified using the proofreading DNA polymerase PrimeSTAR HS (Takara, Japan). The PCR conditions are shown in Table 2.15. The Thermo cycler (Biometra, Germany) was set to run a 30 cycles of amplification. The PCR master mix reaction consisted of 2X 25 µl of PrimeSTAR HS premix (1.25 U/25 µl PrimeSTAR HS DNA Polymerase, 2X dNTP mixture 0.4 mM each, 2X PrimeSTAR buffer containing 2 mM Mg_2^+) (Takara, Japan), 2µl of 15 ng/µl of either D39 pneumococcal DNA or SOEing product constructed to replace serine of the active site with alanine, 2 µl of gene specific forward and reverse primer mix (1 pmol each/reaction) (Table 2.21) and 21 µl of molecular grade water. The successful amplification was confirmed by the agarose gel electrophoresis. The amplicons were then purified and extracted to remove salts and primer-dimers using Wizard® SV Gel and PCR Clean-Up System (Promega, USA) as illustrated in section 2.6.

Table 2.21: Primers used for cloning.

Gene/Symbol	Primer Sequence (5′–3′)
Axe/F	**TACTTCCAATCCATG*AAAAATCCAGCTTTGCTA
Axe/R	**TATCCACCTTTACTGTCA*TTTTAGATATTTAAAAGGAAT
EstA/F	**TACTTCCAATCCATG*GCAGTGATGAAAATCGAGTA
EstA/R	**TATCCACCTTTACTGTCA*AGTCAGTCTCTCTTCTAATT
NanA/F	**TACTTCCAATCCATG*ATGATTGTAGGAGCAGTGG
NanA/R	**TATCCACCTTTACTGTCA*TTTATTGTTCTCTCTTTTTC

*: The nucleotides italicized are complementary to the N- and C-terminals of cloning site in pLEICS-01 plasmid.

2.12.2 Gene cloning:

The cloning step was done by Dr Yang (PROTEX, Department of Molecular and Cell Biology, University of Leicester). The cloning procedure followed the method described in In-Fusion® HD Cloning Kit (Clontech, USA) instructions. Briefly, amplicons of the genes of interest were mixed with linearized pLEICS-01 in the presence of 2 µl of 5X In-Fusion enzyme premix and 1 µl of cloning enhancer. The reaction was incubated first at 37°C for 15 min, and then at 80°C for 15 min. Finally, the reaction was placed on ice. The recombinant plasmid was then transformed into *E. coli* DH5α as described in section 2.7.3. The transformants were selected on LA plates supplemented with 100 µg/ml of ampicillin (Fig. 2.3).

The cloned pLEICS-01 was then extracted and purified from transformants using QIAprep spin Miniprep (Qiagen, UK) as described in section 2.5. The recombinant plasmids were sent for sequencing to eliminate the possibility of any mutations, using pLEICS-01 plasmid dependent primers (T7 promoter and terminator primers). The gene

sequencing was done at Protein & Nucleic Acid Chemistry Laboratory (PNACL)/ University of Leicester.

Subsequently, the recombinant plasmid was transformed into *E. coli* BL21 (DE3) for expression as instructed by the manufacturer (Agilent Technology Ltd., UK) as described in section 2.10.1. The transformants were selected on LA plates supplemented with 100 µg/ml of ampicillin. The successful cloning was confirmed by PCR primers listed in Table 2.21, and the conditions used described in Table 2.16. Glycerol stock was prepared from one of the transformants, and was kept in -80°C until use.

2.12.3 Expression of recombinant proteins:

To express the recombinant proteins, the *E. coli* strains carrying the desired recombinant plasmid were grown in LB medium supplemented with 100µg/ml ampicillin at 37°C with constant shaking (220 rpm). When the OD_{600} had reached between 0.5-0.8, the temperature was lowered to 25°C, and the expression of the cloned genes were induced by adding 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 15 hours, the bacteria were harvested by centrifugation at 10,000 rpm for 30 min at 4°C using a precooled Avanti J-E centrifuge (Beckman Coulter, USA). The supernatant was discarded, and the pellet was kept at -80°C for 1 hour, and then re-suspended with binding buffer containing 50 mM Tris-HCl and 300 mM NaCl, pH 7.45. After that, 3 µl of ProteoGuard™ EDTA-Free protease inhibitor cocktail (Takara Bio Europe, France) and 2 µl of Ambion® TURBO™ DNase (Invitrogen, UK) were added to the cell suspension. The suspension was sonicated at 50% of the maximum power of sonicator (Sanyo Soni Prep, UK) for 10 to 15 cycles for 20 sec each, with a 45 sec cooling intervals in order to prevent protein denaturation due to overheating. The lysate was then centrifuged at 25,000 rpm for 30 min at 4°C using a precooled Avanti J-E centrifuge (Beckman Coulter, USA) to remove the cell debris. The supernatant was passed through 0.45µm micro-filter (Fisher Scientific, UK) to remove minor cell debris.

2.12.4 Purification of recombinant proteins:

TALON Metal Affinity Resin (Clontech, USA) was used to purify the recombinant protein. The resin (2 ml) was added into the column, and calibrated with 30 ml of binding buffer. The buffer was allowed to run through by gravity flow. The clear cell lysate was then applied and passed through the column 3 times. The column was then

washed with 50-70 ml binding buffer to wash away all non-desired proteins that were not tagged, i.e *E. coli* proteins. Different concentrations of imidazole (20 mM Tris, 150 mM NaCl, and 20, 40, 100, 300, or 500 mM Imidazole, pH 7.45) were prepared in binding buffer to form elution buffer, and were used to elute the desired protein from the resin. The eluted protein was kept on ice until needed.

2.12.5 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS PAGE):

The analysis of the recombinant proteins was done by 10% (w/v) Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE), and subsequent staining with Coomassie blue. The gel was made as described in Table 2.22 and 2.23.

Recombinant protein samples were prepared for electrophoresis by adding 10X SDS loading buffer (6.25 ml of 0.5 M Tris-HCl pH 6.8, 1g of SDS, 10 mM DTT, 50 mg bromophenol blue, 10 ml glycerol and 6.25 ml dH_2O). The samples were then heated at 95°C for 1-2 min on hot plate, loaded on SDS gel, and were run in 1X SDS running buffer. The Precision Plus protein marker (Bio-Rad, UK) was run along with samples to estimate the molecular weight of the eluted recombinant protein. The electrophoresis was done at 150 mV.

Table 2.22: Composition of 10% (w/v) SDS PAGE gel.

Component	Quantity
Distilled water	3.4 mL
30% (w/v) Acrylamide bisacrylamide	7.5 mL
1.5 mM Tris HCl, pH 8.8	3.8 mL
10% (w/v) SDS	150 µL
10% (w/v) Ammonium persulphate (APS)	150 µL
Tetramethylethylenediamine (TEMED)	6 µL

Table 2.23: Composition of 5% (w/v) SDS PAGE gel.

Component	Quantity
Distilled water	3.4 mL
30% (w/v) Acrylamide bisacrylamide	0.83 mL
1 mM Tris HCl, pH 8.8	0.63 mL
10% (w/v) SDS	50 µL
10% (w/v) Ammonium persulphate (APS)	50 µL
Tetramethylethylenediamine (TEMED)	5 µL

To visualise the recombinant protein samples, the SDS gel was stained with staining solution (0.4 g of 0.1% (w/v) Commasie brilliant blue, 40% (v/v) methanol, 10% (v/v) acetic acid and dH_2O topped up to a final volume of 400 ml) for 45 min. The gel was then treated with de-staining solution (25% v/v isopropanol, 10% v/v acetic acid and dH_2O topped up to 400 ml), and the solution was replaced 3 times after 20 min incubation. Finally, the gel was washed with dH_2O, and then scanned.

After confirming the identity of the recombinant proteins, the eluted proteins were dialysed with binding buffer and concentrated to get high concentration of recombinant protein. The protein concentration was measured according to the method described by Bradford (1976) as described in section 2.13.2.

2.12.6 Western Blot assay:

This assay was done according to the method described by Towbin et al. (1979). Briefly, the recombinant proteins were separated by SDS PAGE gel. Nitrocellulose membrane paper, filter papers and sponges were equilibrated with transfer buffer (48 mM Tris Base, 39 mM Glycine, 20% (v/v) methanol, 0.037% (w/v) SDS) for 30 min prior to blotting. The reaction was assembled in order to allow the protein to migrate from SDS PAGE gel to nitrocellulose membrane (migration from negative phase to positive phase) (Figure 2.4). Electro-blotting was done for 1 h at 230 mAmp. The membrane was blocked with blocking buffer (phosphate buffer saline (PBS) pH 7.5 and skimmed milk 5% (w/v)) overnight. The nitrocellulose membrane was then washed thoroughly with washing buffer (PBS pH 7.5 and 0.05% (v/v) Tween 20), and incubated with Anti-His Tag primary antibody (Sigma Aldrich Ltd, UK) for 2 h at room temperature. To remove the excess antibody, the membrane was washed with washing buffer three times for 5 min. The membrane was then incubated with anti mouse IgG Fc specific alkaline phosphatase secondary antibody (Sigma Aldrich Ltd, UK) for 1-2 h. After that, the membrane was rinsed with washing buffer 3 times. To develop the antigen-antibody reaction, the membrane was treated with 5 ml 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium (BCIP/NBT) (Sigma Aldrich Ltd, UK) for 10-15 min. Finally, after observing clear bands, the reaction was stopped by washing the membrane with distilled water. The membrane was then scanned to keep a digital image.

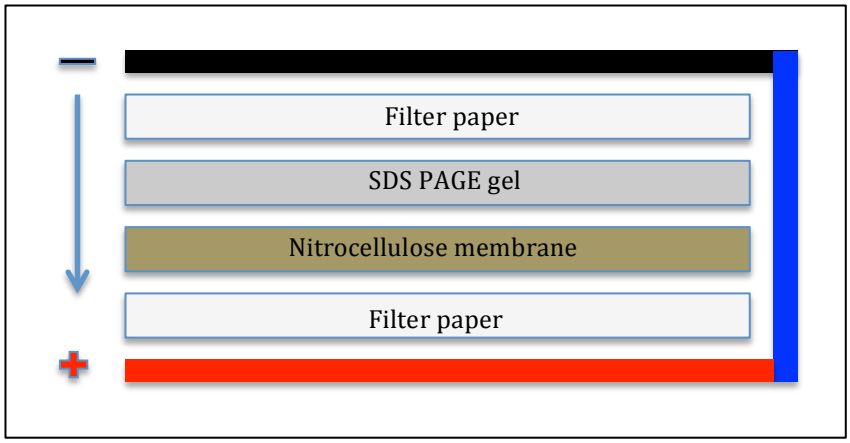

Figure 2.4: Schematic representation of Western Blot assembly (Sandwich assembly). The assembly allows protein to migrate from negative pole (SDS PAGE gel) towards the positive pole to blot on the nitrocellulose membrane. The red, blue and black lines represent the cassette used to assemble the western blot components.

2.12.7 MALDI-TOF analysis for protein identification:

The purified recombinant proteins were analysed by matrix-assisted laser desorption ionization – time of flight (MALDI-TOF) mass spectrometry in collaboration with PNACL, University of Leicester to confirm the identity of recombinant proteins. This analysis depends on the trypsin digestion that cleaves carboxyl side of the amino acids lysine and arginine. This digestion results in peptides that can be analysed to find out the identity of these proteins.

2.13 Enzyme activity assay:

Esterase activity was assayed using chromogenic substrates. In addition, esterase activity was assayed using organic substrates such as BSM, tributyrin, and acetylated xylan.

2.13.1 Cell free lysate preparation:

Overnight cultures of *S. pneumoniae*, grown either in 10 ml BHI or CDM, were centrifuged at 4°C, 5000 rpm for 10 min (Allegra® X-22R Series, Deckman coulter, USA). The pellet was re-suspended in 1 ml chilled 50 mM Tris-HCl buffer, pH 7.5, and

was sonicated for 5 cycles of 20 sec, each with a 1 min cooling time in between cycles at 50% of the maximum power of sonicator (Sanyo Soni Prep 150, Japan) (Yesilkaya et al., 2000). The lysate was centrifuged at 13000 rpm at 4°C (Hettich MIKRO 22R, Germany), and the clear lysate was stored at -80°C until required.

2.13.2 Protein concentration measurement:

The protein concentration was measured according to the method described by Bradford (1976). 1000 µg/mL of bovine serum albumin (BSA) was serially diluted to prepare the standards. The protein concentrations in the samples were quantified using the standard curve prepared with the known concentrations of BSA.

2.13.3 Enzyme activity assays by using chromogenic substrates:

Esterase activity was assayed using five different p-Nitrophenyl esters: p-Nitrophenyl acetate (p-NPA), p-Nitrophenyl butyrate (p-NPB), p-Nitrophenyl hexanoate (p-NPH), p-Nitrophenyl octanoate (p-NPO) and p-Nitrophenyl decanoate (p-NPD), which are composed of 2, 4, 6, 8 and 10 carbon chain esters, respectively, as described by Chungool et al. (2008). Briefly, 0.1 ml of 0.04 M substrate, dissolved in 50% methanol, was mixed with 0.1 ml of 0.5 M sodium phosphate buffer, pH 7.5, and 0.5 ml of different concentrations of cell free lysate or purified recombinant protein. The reaction volume was brought to 1 ml with distilled water and incubated at 50°C for 10 min. Placing the tube on ice for 5 min stopped the reaction, and the absorbance of released p-nitrophenol was measured photometrically at 405 nm. One unit of enzyme activity was defined as the amount of enzyme that produced 1 µmol p-nitrophenol per min per milligram of protein under standard assay conditions.

Neuraminidase activity was assayed using 2-*O*-(p-Nitrophenyl)-α-D-*N*-acetylneuraminic acid (pNP-NANA; Sigma, UK) as substrate according to the method described by Manco et al. (2006). 25 µl of 150 µg/ml recombinant NanA was added in triplicate to each well of a 96-well plate, and mixed with 25 µl of 0.3 mM pNP-NANA diluted in sodium phosphate buffer at pH 6.6. The reaction was then incubated for 2 h at 37°C. The reaction was stopped by adding 100 µl ice-cold 0.5 M Na_2CO_3 into each well. The activity of NanA was measured by reading the absorbance of released p-nitrophenol at 405 nm in a micro-plate reader (Bio-Rad, United Kingdom). 25 µl of 40 mM Tris was added to blank reaction well and served as control. One unit of enzyme activity was

defined as the amount of enzyme that produced 1 µmol p-nitrophenol per min per milligram of protein under standard assay conditions.

2.13.4 Enzyme activity assays using natural substrates:

The activity of esterases on tributyrin and acetylated xylan was determined as essentially described previously (Smeltzer et al., 1992, Martinez-Martinez et al., 2007). To qualitatively determine tributyrin esterase activity, 0.5% (v/v) of tributyrin was suspended in 50 mM Tris (pH 8.8) and 25 mM $CaCl_2$ and then embedded into 2% (w/v) standard agarose. Different concentrations of esterases were spotted onto agar. Commercial lipase from *Staphylococcus aureus* was used as a positive assay control (Sigma Aldrich, UK). The zone of clearance indicated the presence of tributyrin esterase activity. For quantitative assay, 250 U of recombinant esterase was mixed with 0.5% (v/v) of tributyrin suspension embedded in 0.8% (w/v) low melting point agarose. The decrease in absorbance at 405 mM was recorded over time. The activity of tributyrin esterase in the sample was quantified using a standard curve generated with the known concentrations of commercial lipase, as the lipase from *S. aureus* can use tributyrin as substrate.

Acetyl xylan activity in the recombinant esterases was assayed by using birchwood xylan as substrate according to the previously described method (Johnson et al., 1988). To prepare substrate, 10 g of xylan was dissolved in 250 ml dimethyl sulfoxide (DMSO) with gentle stirring, solubilised by heating to 55 °C for 20 min in the presence of K_3BO_3. 200 ml of preheated acetate anhydride was then added, and the substrate mixture was dialysed against running water at 4°C for 5 days. The dialysed reaction was then lyophilized and kept at -20°C until needed. To check the activity of esterase on acetylated xylan, different concentrations of recombinant esterase was mixed with 150 µl of substrate solution, which was prepared by mixing 5% (w/v) of acetylated xylan with 0.01% (w/v) bromothymol blue (BTB), as indicator, and 5 mM of sodium phosphate buffer (pH 7.3), into 96-well plate. The reaction mixture was incubated for 30 min with stirring and the centrifuged at 4°C for 20 min. After incubation, the absorbance of supernate at OD_{616} was recorded. The decrease in absorbance indicated enzyme activity, which was calculated by generating a standard curve using acetic acid. One unit of activity was defined as the formation of 1 µmol of acetic acid per minute under the standard reaction conditions.

The assay was done to measure *O*-acetylation level of *O*-acetyl groups in BSM. The released acetate from BSM was measured according to the method described by Herrler et al. (1985). To determine esterase activity on BSM, 5 mg of BSM was incubated with different concentrations of recombinant esterases in a total reaction volume of 200 µl in PBS, pH 7.5. After different durations of incubation at 37°C, the reaction mixture was centrifuged at 5000 rpm and the released acetate was assayed enzymatically using a commercial acetate test kit (Megazyme, Ltd.). A sample of BSM incubated with esterase at 4°C served as a control. The value of the control sample was subtracted from the samples incubated at 37°C.

2.13.5 Kinetic characterisation of the recombinant esterases:

Prior to measuring the enzyme kinetics of the recombinant protein, pH optimisation was done to find out the best buffer for esterase activity. Two buffers were used: sodium citrate buffer with a pH range of 3.0 to 6.0, and sodium phosphate buffer with a pH range of 6.0 to 8.5 with 0.5 increments. Esterase activity was measured as described in section 2.13.3 (Chungool et al., 2008).

In addition, before the assay of *Km* and *Vmax*, the optimum concentration of enzyme was identified, using different concentrations of enzyme while keeping substrate concentration fixed. All tests were done in triplicate.

For substrate variation, p-NPA concentration was varied from 0 to 25 mM with 2.5 mM increments. The amount of the recombinant protein used per reaction was 125 ng/ml as a constant concentration for enzyme kinetics. The reaction mixture contained 20 µl of different concentrations of substrate, 20 µl of 0.5 M sodium phosphate buffer pH 7.5, 100 µl of 125 ng/ml of the recombinant enzyme, and the reaction was brought to 200 µl by adding distilled H_2O. The reaction mixture was incubated for 2 hours in a microplate reader (Varioskan Flash, Thermo Fisher) at 50°C, and the absorbance was recorded at 405 nm every minute during the incubation. The test was performed in triplicate. The kinetic curve was generated by plotting velocity against substrate concentration and non-linear regression analysis was used to determine the kinetic parameters *Vmax* and *Km* using the Michaelis Menten model**.** The Prism Graph Pad 6.0 software (GraphPad 6.0 Mac Software, Inc.) was used for analysis.

2.13.7 Investigation of esterases' potency on neuraminidase activity:

This assay was done to find out the synergism between esterase and NanA activity for the cleavage of Sia from BSM according to the method described by Corfield et al. (1986). 5 mg of BSM (Sigma Aldrich, UK) dissolved in 200 µ1 of PBS at pH 7.5 was incubated with 100 µ1 of 250 µg/ml of recombinant esterase. After incubation for predetermined time intervals at 37°C, 100 µ1 of recombinant NanA at a concentration of 200 µg/ml was added to the reaction mixture, and incubated further for different time points. Placing the tubes into ice stopped the reaction. The reaction tubes were then centrifuged at 13000 rpm for 5 min. the released sialic acid was measured according to the method described in section 2.14.

2.14 Free sialic acid measurement:

Free sialic acid (Sia) was measured according to the method described by Warren (1959) and Sobenin et al. (1998). Briefly, for bound Sia measurement, 100 µl of 96% of H_2SO_4 was added to 100 µl BSM sample and the mixture was incubated at 80°C for 1 h. 20 µl of 0.2 M periodate reagent (0.2 M sodium periodate in 0.1 M H_3PO_4) was then added. The reagent was also added into the correction samples with no sodium periodate and served as control. The reaction tubes were vortexed, and incubated for 20 min at room temperature. 100 µl of 10% *m*-arsenite reagent (10% (w/v) sodium arsenite in 0.1 N H_2SO_4 with 0.5 M Na_2SO_4) was added to the reaction mixture and vortexed until a yellow-brownish colour formed and then disappeared; 250 µl of 0.6% of thiobarbutiric acid reagent (0.6% (w/v) of thiobarbutiric acid (TBA) in 0.5 M of Na_2SO_4) was then added and thoroughly vortexed. The reaction tubes were boiled for 15 min, and were cooled at 20°C. Then, 1 ml of cyclohexanone was added to the reactions and vortexed twice for 10 seconds. Subsequently, the reaction mixtures were centrifuged at 1200 rpm for 7 min using a microcentrifuge. The density of the organic phase was determined at 549 nm against the correction samples using quartz cuvettes.

2.15 Subcellular localisation of esterases:

This assay was done to assess the cellular localisation of esterases in *S. pneumoniae*. Three fractions were prepared from the whole cell lysate: cell wall, membrane and cytoplasmic fractions according to the method described by Cole et al. (2008) with some modifications. The procedure started by streaking D39 wild type on a fresh blood

agar plate which was incubated overnight at 37°C with 5% CO_2. A single and well-isolated colony was inoculated into 10 ml BHI broth and incubated overnight at 37°C without shaking. Next day, 200 µl of the overnight culture was sub-cultured into fresh 10 ml BHI broth and was grown until late exponential phase. The culture was harvested by centrifugation at 5000 rpm for 20 min at 4°C. The pellet was kept on ice for 5-10 min, and resuspended with 5 ml chilled TE buffer (1M Tris-HCl and 0.5M EDTA) containing 1mM phenylmethanesulfonyl fluoride (PMSF) by pipetting up and down. The suspension was then centrifuged at 5000 rpm for 10 min at 4°C. The latter step was repeated 3 times. The pellet was resuspended in 1ml of TE buffer containing 20% (w/v) sucrose, 100 mg/ml lysozyme and 5000 U/ml mutanolycin. The reaction mixture was incubated at 37°C for 2 h with shaking at 200 rpm, and was centrifuged at 50,000 rpm for 5 min at 4°C using a bench-top ultracentrifuge (Beckman Coulter TL-100). The supernatant was collected in small aliquots and served as cell wall fraction. The pellet was then resuspended in 1 ml TE, and sonicated (6-8 amplitude) in cycles of 15 sec followed by 45 sec cooling intervals. The lysate was then centrifuged at 10,000 rpm for 5 min to remove cell debris. The supernatant was transferred into fresh tubes and centrifuged at 50,000 rpm for 5 min using bench-top ultracentrifuge (Beckman Coulter TL-100). The supernatant was transferred into fresh tubes and served as cytoplasmic fraction. The pellet was resuspended in 1ml TE buffer and served as cytoplasmic membrane fraction. All fractions were kept in -20°C until use.

The fractions were run on SDS PAGE gel and blotted on nitrocellulose membrane using electro-blot (Bio-Rad) as described in sections 2.12.5 and 2.12.6. The nitrocellulose membrane was incubated with polyclonal antibody, which was raised against recombinant proteins in mice as described in section 2.16.5. The membrane was washed with washing buffer (0.5% (v/v) Tween20 in PBS), and then was incubated with secondary anti-FC antibody for 1 hour at room temperature. The membrane was then developed by NCIP/NBT developing solution for 10 min until the clear bands were visible. Finally, the membrane was washed with distilled water to stop the reaction, and was scanned.

2.16 Virulence studies:

To study esterases' role *In vivo,* two mouse models of pneumococcal infection were used: acute infection model or also known as pneumonia model which involves

virulence testing by determining survival time after intranasal infection, and nasopharyngeal colonisation model. Infection of mice was kindly done by Dr Hasan Yesilkaya.

2.16.1 Pneumococcal stock preparation:

Single colonies of pneumococcal strains were streaked on blood agar plates and incubated overnight at 37°C in the presence of 5% CO_2. Optochin sensitivity was tested. The following day, 10 ml of BHI was inoculated with a sweep of colonies from overnight cultures of pneumococci. The inoculated cultures were then incubated at 37°C until OD_{600} had reached 1.4-1.6. The cultures were then centrifuged at 3,500 rpm for 10 min using Allegra X-22 centrifuge (Beckman Coulter), and the supernatant was then discarded. The pellet was re-suspended with 1 ml of fresh BHI containing 20% (v/v) of filter sterilised foetal calf serum; 700 µl of re-suspended culture was then added into 10 ml of fresh BHI-serum medium, and the culture was incubated until the OD_{500} had reached 1.6. The cultures were then distributed into 500 µl aliquots, and were kept at -80°C until use. To confirm pneumococcal viability, one aliquot of each pneumococcal strain was thawed and CFU/ml was checked as described in section 2.4.

2.16.2 Animal passage of pneumococci:

All strains were subjected to animal passage in order to standardise the virulence and minimize any possibility of undesired pneumococcal mutations (Canvin et al., 1995). Briefly, 10 ml of BHI overnight culture inoculated with pneumococcal strains were centrifuged at 3,500 rpm for 10 min. The pellet was then re-suspended with 5 ml PBS and 500 µl of which was injected intraperitoneally into mice using 1 ml insulin syringe. After 24 h, infected mice were anesthetized with 2.5% isoflurane (v/v) (Isocare, UK) over oxygen (1.5 to 2 litres/min), and culled by neck dislocation. Approximately, 50 µl of blood was collected by cardiac puncture using 2 ml syringe (Terumo) and BD Microlance 21 G X 5/8" needle (Bechton Dickinson) attached. The collected blood was then added into 10 ml of fresh BHI and incubated overnight at 37°C. Following day, the culture was collected without disturbing the pelleted blood and transferred into fresh universal tubes. The cultures were then centrifuged at 3,500 rpm for 10 min, and re-suspended with 1 ml BHI containing 20% (v/v) of foetal calf serum. 700 µl of re-suspended culture was then added into 10 ml of fresh BHI-serum medium, and OD_{500}

was adjusted at 0.7 as starting culture. The incubation was then proceeded until the OD_{500} had reached 1.6. The cultures were then distributed into 500 µl aliquots, and were kept at -80°C until use. To confirm pneumococcal viability, one aliquot of each pneumococcal strain was thawed and CFU/ml was checked as described in section 2.4.

2.16.3 Mouse model of acute infection:

In this study, 8 to 9 week old female outbred MF1 mice (Charles & Rivers, UK) were used. The mice were left for 1 week after arrival to habituate. *In vivo* studies were performed under appropriate project (document no: 60/4327) and personal (document no: 80/10279) licenses according to the UK Home Office guidelines and local ethical approval. Where indicated, the procedures were carried out under anesthetic with isoflurone. Mice were housed in individually ventilated cages in a controlled environment, and were frequently monitored after infection to minimize suffering.

Mice were anesthetized with 2.5% isoflurane (v/v) (Isocare, UK) over oxygen (3 litre/min), and infected with $2x10^6$ CFU/mouse in 50 µl PBS, pH 7.0, through the intranasal route. The mice were kept vertically for 10 seconds to allow the dose to be inhaled. Following infection, the inoculum dose was confirmed by viable count by plating on blood agar plates as described in section 2.4. All mice were scored for the signs of disease (starry coat, hunched and lethargic) for 7 days (Morton et al., 1990).

At 24 and 36 hours post-infection, 20 µl of blood was collected from all infected mice by tail bleeding. The procedure started with placing mice into the static 37°C incubator to increase the visibility of veins. Tail bleeding processed by placing each mouse into an apparatus that keeps mice under control while taking the blood. The blood was taken and collected using 1 ml insulin syringe. Bacterial titre (CFU/ml) in the blood was determined by plating serial dilutions of blood using Miles and Misra method (Miles et al., 1938) as described in section 2.4. Mice were culled immediately after developing sign of lethargy, and the time to this point was considered as the 'survival time'.

2.16.4 Mouse model of nasopharyngeal colonisation:

Female outbred MF1 mice (Charles River, UK) were used in this study. All the infection process was same as above except that the inoculum dose was reduced to $5x10^5$ CFU/mouse and this dose was administered in 20 µl PBS rather than 50 µl, which

ensures that the pneumococci do not disseminate into the lower respiratory tract. At 0, 3 and 7 days post-infection, 5 mice were culled, the nasopharynx was dissected, and tissue samples were collected into 5 ml PBS. The tissues were then homogenized using ultra-turrax homogeniser (IKA-Werk, Germany). Bacterial count in nasopharyngeal tissue was determined by plating serial dilutions of homogenized tissues on blood agar plates, and CFU count was determined as described in section 2.4.

For sialic acid level of nasopharyngeal lavage of mice colonised with pneumococci, the mice were culled 7 days post-infection and nasopharyngeal lavage was performed by washing the nasopharyngeal cavity of mice with 500 µl of PBS. The samples were prepared for bound sialic acid level assay as described in section 2.14.

2.16.5 Preparation of anti-sera against recombinant esterases:

Ten weeks old female MFI outbred mice (Charles River, UK) were injected intraperitoneally with a 25 mg of recombinant protein and 33 µl of Imject Alum adjuvant (Perbio Science, Cramlington, UK) and 67 µl of PBS. The control group was received only adjuvant and PBS. Injections were repeated three times at fortnightly intervals. Two weeks after the last injection, mice were anesthetized with 2.5% (v/v) isoflurane (Isocare, UK) over oxygen (1.5 to 2 liters/min) and blood was collected by cardiac puncture. The blood was left at room temperature for one hour to clot and the serum was recovered by centrifugation at 5000 rpm for 10 min. The serum was stored at -80ºC until needed.

2.16.6 Extraction of pneumococcal RNA from infected tissues:

Outbred 8-10-week-old female MF1 mice (Charles River, UK) were intranasally infected with 50 µl PBS containing $1x10^6$ D39 pneumococci, as described before (Yesilkaya et al., 2000, Manco et al., 2006). When the mice became lethargic they were anesthetized, and blood was obtained by cardiac puncture. Mice were killed by cervical dislocation; the lungs and nasopharynx were removed and homogenized on ice in 10 ml of sterile PBS using a tissue homogenizer. To remove host cells, lung homogenates and blood samples were centrifuged at 900 *g* for 6 min at 4ºC. Supernatants were subsequently centrifuged at 15,500 *g* for 2 min at 4ºC, and the bacterial pellet was stored at -80ºC until needed. Before pelleting, 20 µl of homogenate was removed, serially diluted in PBS, and plated onto blood agar in order to enumerate pneumococci

and to exclude the presence of contaminating micro flora. RNA extraction and purification were performed as described in sections 2.11.1, 2.11.2 and 2.11.3. qRT-PCR was performed as described in section 2.11.4.

Chapter Three

Results:

Section I: Construction of genetically modified strains

3.1 Mutation methods in *Streptococcus pneumoniae*:

In this study, two mutagenesis strategies were used to construct the isogenic mutants; mariner mutagenesis and gene splicing by overlap extension mutagenesis (SOEing). While mariner mutagenesis allows the insertion mutation to occur (Akerley et al., 1998, Yesilkaya et al., 2009), SOEing mutagenesis introduces insertion-deletion mutations (Horton, 1995). The mutation of esterase genes were done in two genetic backgrounds, D39 and TIGR4, both sequenced virulent strains (serotype 2 and serotype 4, respectively). The reason for constructing isogenic mutations in two backgrounds was to investigate whether the contribution of esterases to the pneumococcal biology is influenced by the strain background.

PCR technology allows mutation of target genes by deletion it and replacement with an antibiotic marker. Indeed, there is no need to deliver the foreign DNA using vectors as the target gene is mutated by homologous recombination. The upstream and downstream homology allows better recombination with genomic DNA. Here I used two mutagenesis methods: mariner and SOEing PCR mutagenesis (Yesilkaya et al., 2009, Horton et al., 1990, Martin et al., 2000).

Mutation can be introduced either through deletion of the target gene and insertion of an antibiotic marker, or through interruption of the target gene by inserting the antibiotic marker within coding sequence of the gene. Mariner mutagenesis was used in this study because it has been well-established method in Dr Hasan Yesilkaya's lab group (Yesilkaya et al., 2009). In this method, transposase inserts the antibiotic cassette within the target gene *in vitro*, and interrupts gene function. Mariner mutagenesis is dependent upon *in vitro* transposition, which allows antibiotic marker to disrupt the transcriptional process of target genes after transformation of the disrupted gene into wild type strain.

This technique allows the mutation of the target gene efficiently in a short period of time. As *S. pneumoniae* is naturally transformable microbe that can accept foreign DNA, it is more preferable that transformation is with a mutated target gene without plasmid as this process might cause a delay due to plasmid degradation by endonuclease. Endonuclease activity is relatively low in Gram-positive bacteria and this might not affect the linearized foreign DNA. It is noteworthy to mention here that the cost and speed of transformation with plasmid is relatively higher and slower than transformation with linear DNA. However, the insertion of the transposon is random, which disrupts the target gene randomly in different transcriptional orientations. Therefore, the mini-transposon might not disrupt the target gene and might insert upstream or downstream of the coding sequence of target gene. In addition, insertion of an antibiotic cassette into a target gene might be disadvantageous, as the random insertion of antibiotic cassette might not interrupt the target gene totally. There might be transcriptional sequence before or after the interruption site, and this could lead to translation of parts of the target gene.

The other mutagenesis technique that I used in this study was gene splicing by overlap extension PCR mutagenesis (SOEing PCR). This method of mutation was recently established in Dr. Hasan Yesilkaya's lab group. I started using this method to introduce a double mutation of *ΔestA-axe* isogenic mutant. I created kanamycin resistant *ΔSPD_1506* (*Δaxe*) using SOEing method of mutation. Then the mutated region was amplified, and transformed into spectinomycin resistant *ΔestA* isogenic mutant background. In addition, I used this method to introduce a point mutation at the serine within the active site of target enzymes. This allows replacement of the active site serine with alanine by using modified primers used for amplifying the flanks of the target gene. This method is a cost-effective and simple technique that allows recombination of DNA without a need for cloning. It totally depends on PCR to delete and replace a section or the whole target gene *in vitro*. The site directed mutagenesis using SOEing PCR is highly efficient as the recombination and mutagenesis can be performed efficiently, and the mutation occurs *in vitro*. Furthermore, there is no need to grow and maintain vectors.

Both mutagenesis techniques were successfully used to introduce isogenic mutants in *S. pneumoniae* to investigate the phenotypic function of target genes (Yesilkaya et al., 2009, Hajaj et al., 2012)

3.1.1 Construction of esterase mutants by mariner mutagenesis:

In vitro mariner transposon mutagenesis was used to generate four esterase isogenic mutants in D39 background as described in Materials and Methods (section 2.9.1). The plasmid pR412 carrying the spectinomycin mini-transposon cassette was used to interrupt the esterase genes by insertion into the target gene in the presence of *HimarI* transposase. For this, the antibiotic cassette, DNA fragment containing the region to be mutated and transposase were mixed together. Transposase allows the antibiotic cassette to insert into the target gene randomly. The gap repair step cured the nicks created by the insertion of the cassette. Finally, the *in vitro* mutagenized DNA fragments carrying the transposon mini-cassette were transformed into *S. pneumoniae*. The transformants were selected on blood agar plates supplemented with spectinomycin.

The successful construction of mutants was tested by PCR using different sets of primers as illustrated in Figure 3.1. The first set included primers to amplify the entire mutated region including the spectinomycin resistance cassette; the second set was prepared by mixing the forward chromosomal primers for each target gene with the antibiotic cassette based MP127 primer; the third set consisted of the antibiotic cassette based MP128 and the reverse chromosomal primers for each target gene. While the first primer set reveals the presence or absence of the mutation, the last two sets allow determination of the orientation of the cassette in the mutated region.

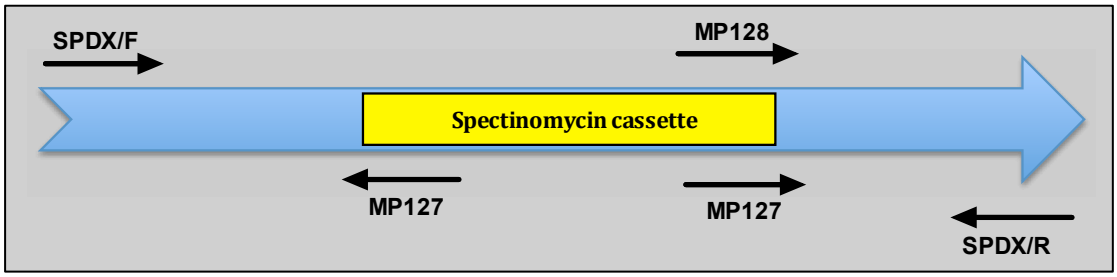

Figure 3.1: Schematic description of mutation confirmation strategy. SPXF and SPXR are chromosome based, and represent the forward and reverse primers which amplify entire mutated region. X represents the gene code. MP127 and MP128 are spectinomycin resistant mini cassette specific primers (Martin et al., 2000).

Figure 3.2 shows the PCR products amplified from transformants as well as D39 wild type DNA using target gene specific primers. As can be seen from the Figure, the PCR amplicons from wild type DNA was 2 kb for SPD_0534 (*estA*), SPD_1239 and SPD_0932, and 1.4 kb for SPD_1506 (*axe*) (L1, L23, L5 and L7, respectively, on Figure 3.2). The insertion of spectinomycin resistance cassette into the target genes increased the expected size of amplicons by approximately 1.15 kb. The size of amplicons from the mutation of SPD_0534, SPD_1239 and SPD_0932 were approximately 3.1 kb (L2, L4 and L6, respectively, in Figure 3.2), whereas the amplicon size from SPD_1506 was 2.5 kb (L8 in Figure 3.2), confirming a successful insertion of spectinomycin resistance cassette into the target genes (Figure 3.2).

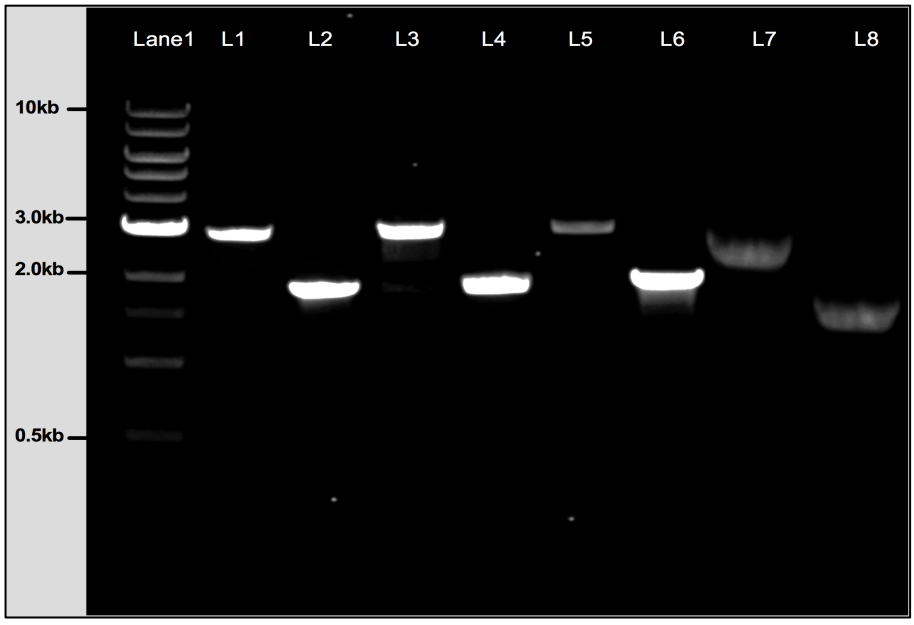

Figure 3.2: Agarose gel electrophoresis showing the amplicons from putative esterase mutants in D39 background. Lanes 1: 500 ng of 1kb DNA ladder (New England Biolabs); L1, L3, L5 and L7 represent the amplification products from *ΔSPD1239*, *ΔSPD0534* (*ΔestA)*, *ΔSPD0932*, and *ΔSPD1506* (*Δaxe)*, respectively; L2, L4, L6 and L8 indicate the products from the wild type DNA using the same primers.

Figure 3.3 shows agarose gel electrophoresis analysis of PCR products for representative transformants with the three sets of primers. The results are consistent with the successful transformation of the mutation into D39 wild type. The size of amplicons obtained with the spectinomycin resistance cassette-based primers for each mutation is different due to the differences in insertion sites of spectinomycin resistance cassette (Figure 3.3). For example, SPD534/F and MP127 primers produced amplicon length of 850 bp (L2 in Figure 3.3), indicating approximately that the spectinomycin cassette was about 850 bp away from the 5'-end of target gene. Similarly, MP128 and SPD534/R primers amplified amplicon length of 900 bp (L3 in Figure 3.3), indicating that the insertion of spectinomycin resistance cassette was about 900 bp away from 3'-end of target. A transformant from each mutation was designated as *ΔSPD1239*, *ΔSPD0534* (*ΔestA*), *ΔSPD0932*, and *ΔSPD1506* (*Δaxe*) in D39 background for further study.

To be able to identify the exact insertion site of the antibiotic cassette in the coding sequence, the mutated regions were amplified using MP128 primer with single primer PCR technique as described in Materials and Methods (section 2.9.4). The amplified products were sent for sequencing. The amplicons were sequenced using the MP127 primer. The sequence data was analysed using BLAST, which is available at the National Centre for Biotechnology Information (www.ncbi.nlm.nih.gov). Sequencing results of mutated regions revealed the precise insertion sites and the direction of spectinomycin resistance cassette in relation to the direction of transcription of target genes (Figure 3.4) (Appendix 1).

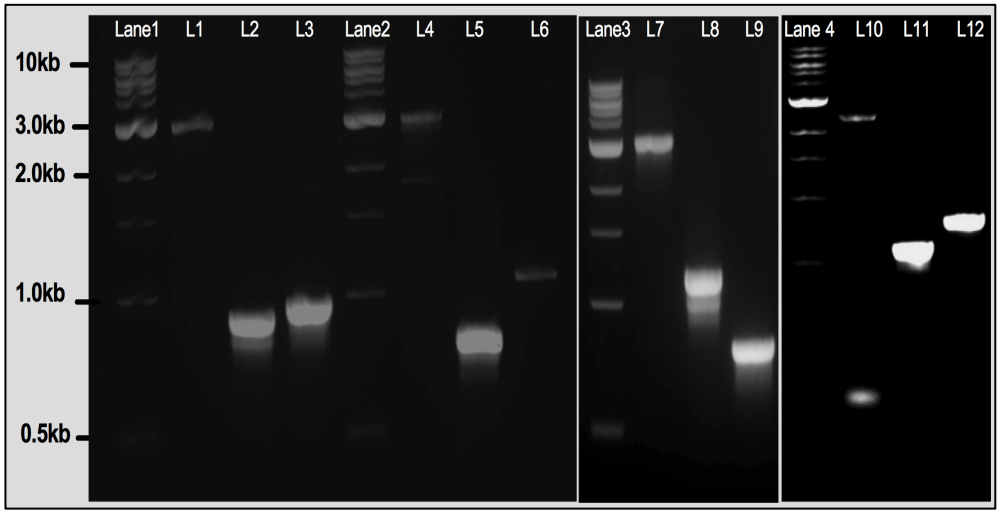

Figure 3.3: PCR analysis of esterase mutants in D39 background. Lanes1, 2, 3, 4: 500 ng of 1 kb DNA ladder (New England Biolabs); The amplicons from *ΔSPD1239* using SPD1239F/R, SPD1239F/MP127, and MP128/SPD1239R primers are shown in L1, L2, and L3, respectively. The amplicons from *ΔSPD0534* (*ΔestA*) using SPD053F/R, SPD0534F/MP127, and MP128/SPD0534R primers are shown in L4, L5 and L6, respectively. The amplicons from *ΔSPD0932* using SPD0932F/R, SPD0932F/MP127, and MP128/SPD0932R primers are shown in L7, L8 and L9, respectively. The amplicons from *ΔSPD1506* (*Δaxe*) using SPD1506F/R, SPD1506F/MP127 and MP128/SPD1506R primers are shown in L10, L11 and L12, respectively.

To transfer the mutations from D39 background to TIGR4 background, the genetic regions containing the mutations were amplified from esterase isogenic mutants using the SPDX/F and SPDX/R primer sets, and the amplicons were then transformed into *S. pneumoniae* TIGR4 after purification as described in Materials and Methods (section 2.6). The transformants were analysed for the successful integration of the mutated region into the TGR4 chromosome by PCR as described above. One transformant for each target was selected and designated as *ΔSP1408, ΔSP416 (ΔestA), ΔSP1052* and *ΔSP1695 (Δaxe)* (note that the mutants in TIGR4 background has 'SP' tag rather than 'SPD').

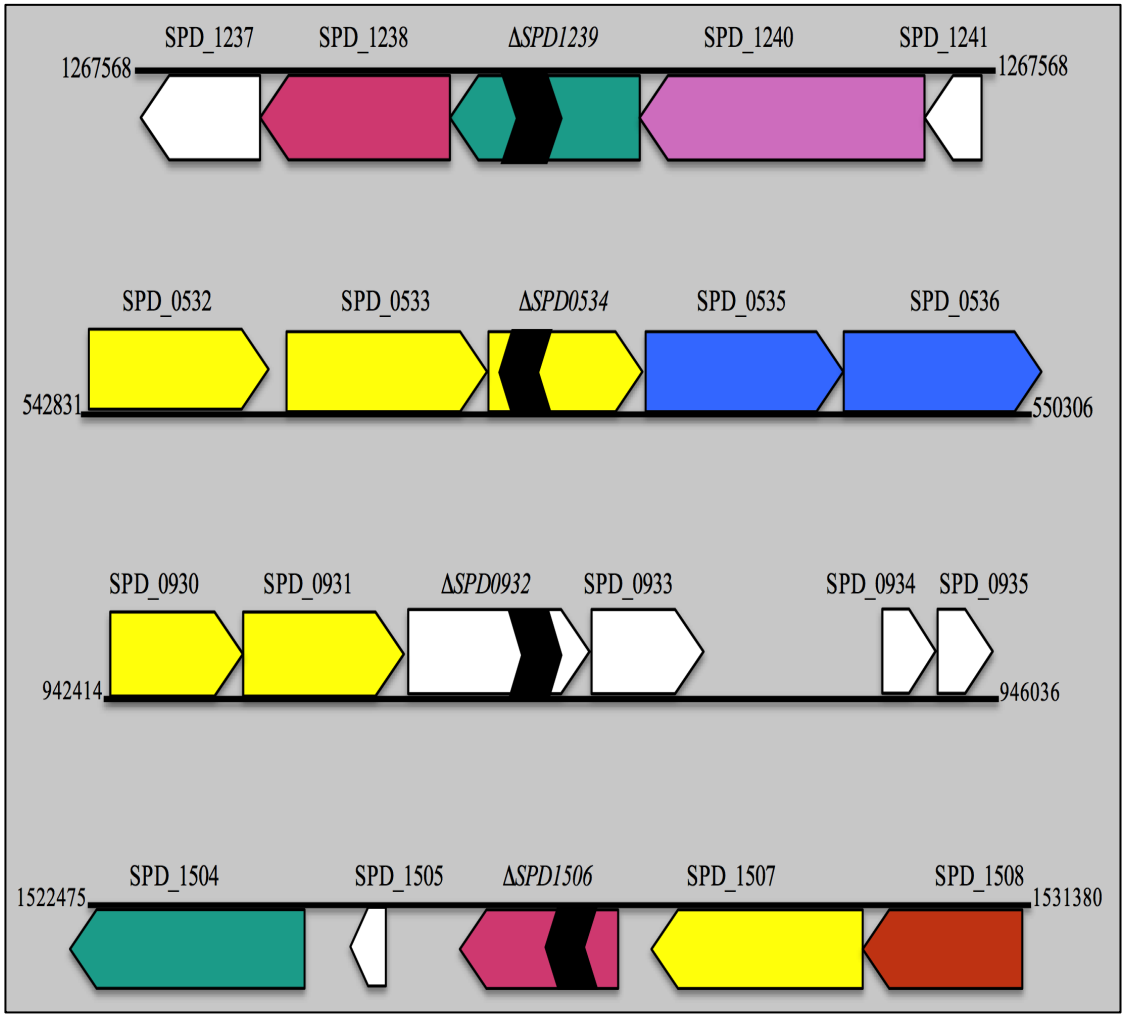

Figure 3.4: Schematic representation of spectinomycin resistance cassette insertion into target genes. The chromosome is represented with a solid line, and genes are shown with a block arrow with the direction of their transcription. The colour of each gene is given according to the colour codes for pathway categories of the Kyoto Encyclopaedia of Genes and Genomes (KEGG) (http://www.genome.jp/kegg/). Yellow: environmental information processing; Green: lipid metabolism; Blue: glycan biosynthesis and metabolism; White: unclassified; Orchid: metabolism of cofactors and vitamins; Deep pink: biosynthesis of other secondary metabolites; Orange red: metabolism of other amino acids. The chevrons inside genes represent the approximate positions of spectinomycin resistance cassette insertion, and the direction of chevrons represents the orientation of the spectinomycin resistance cassette.

3.1.2 Construction of double mutants:

Double esterase neuraminidase isogenic mutants were constructed to check whether esterases potentiate activity of neuraminidase A. To construct the double *ΔestAnanA*, and *ΔaxenanA* mutants in strain D39, the mutated regions in *ΔestA* and *Δaxe* were amplified using SPD0534/F and SPD0534/R, and SPD1506/F and SPD1506/R primers (Table 2.10 in Materials and Methods), respectively, and the amplicons were then transformed into *ΔnanA* isogenic mutant, which contains a kanamycin resistance cassette. *ΔnanA* was provided from the Dr. Hasan Yesilkaya's laboratory stocks bank. The transformants were selected on blood agar plates supplemented with kanamycin and spectinomycin.

Colony PCR was done to check the transformants. Figure 3.5 shows colony PCR results for putative *ΔestAnanA* and *ΔaxenanA* double isogenic mutants. As can be seen from the Figure, when the uninterrupted *estA* and *axe* genes were amplified from the wild type pneumococcal DNA, the size of amplicons is about 2 kb, whereas the insertion of spectinomycin cassette into the wild type genes generated a product of approximately 3 kb when *ΔestAnanA* and *ΔaxenanA* were used as template, indicating the mutagenesis of *estA* and *axe* genes in *ΔnanA*.

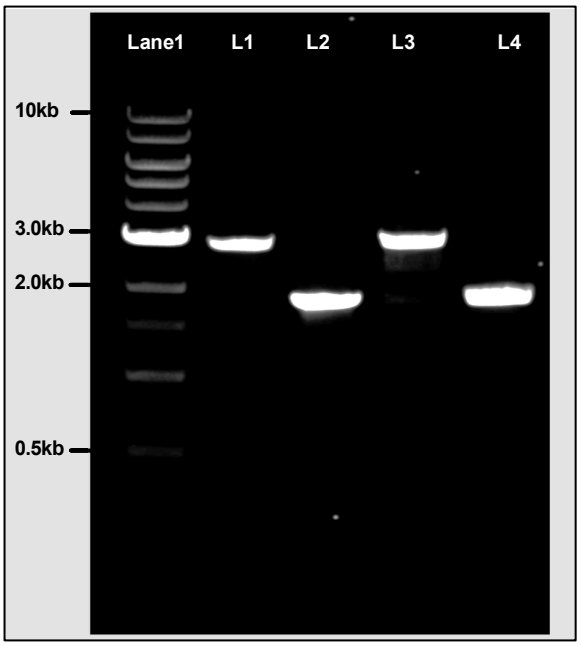

Figure 3.5: Agarose gel electrophoresis for confirmation of double esterase-neuraminidase A mutant construction. Lane 1: 500 ng of 1 kb DNA ladder (New England Biolabs); L1: the amplicons for *estA* from *ΔestAnanA*, obtained using SPD534/F and SPD534/R primer set; L2: the amplicons for *estA* from D39 wild type; L3: the amplicon for *axe* from *ΔaxenanA*, obtained using SPD1506/F and SPD1506/R primer set; L4: *axe* amplicon of for *axe* from D39 wild type using SPD1506/F and SPD1506/R primers.

3.1.3 Construction of a double *ΔestAaxe* isogenic mutant:

A double *axe* and *estA* isogenic mutant was constructed to study whether any synergistic effect exist between pneumococcal esterases. SOEing mutagenesis was used to create a double esterase isogenic mutant. This mutation strategy relies on the deletion of the coding region and insertion of an antibiotic resistance cassette. Figure 3.6 shows a schematic representation of SOEing PCR. This technique consists of two steps: first step amplifies upstream (920 bp), downstream (920 bp) of *axe* using D39 wild type DNA (L3 and L4, respectively, in Figure 3.7), as well as kanamycin resistant cassette (890 bp) (L1 and L2 in Figure 3.7) using pCEP plasmid as the template. These

fragments were purified and used as template for second PCR step as illustrated in Figure 3.6. The second step fuses upstream and downstream fragments to kanamycin resistant cassette. The chimeric nature of SPD1506UP/F and SPD1506D/F allows the required homology between the flanks and kanamycin resistant cassette for efficient fusion (Figure 3.6). The fused DNA fragment was then transformed into *ΔestA* isogenic mutant and the transformants were selected on blood agar plates supplemented with spectinomycin and kanamycin. The replacement of *axe* with kanamycin resistant cassette was confirmed by colony PCR as described in Materials and Methods (section 2.9.4). Figure 3.8 shows the amplicons amplified from *ΔestAaxe* double isogenic mutant. As can be seen from the Figure, the upstream (L1 and L2 in Figure 3.8), downstream (L3 and L4 in Figure 3.8) and kanamycin resistant cassette (L5 and L6 in Figure 3.8) flanks have been successfully amplified from *ΔestAaxe* DNA. Furthermore, amplicon of upstream flank with kanamycin resistant cassette (L7 in Figure 3.8), and downstream with kanamycin resistant cassette (L8 inFigure 3.8) was also successfully amplified from *ΔestAaxe* DNA.

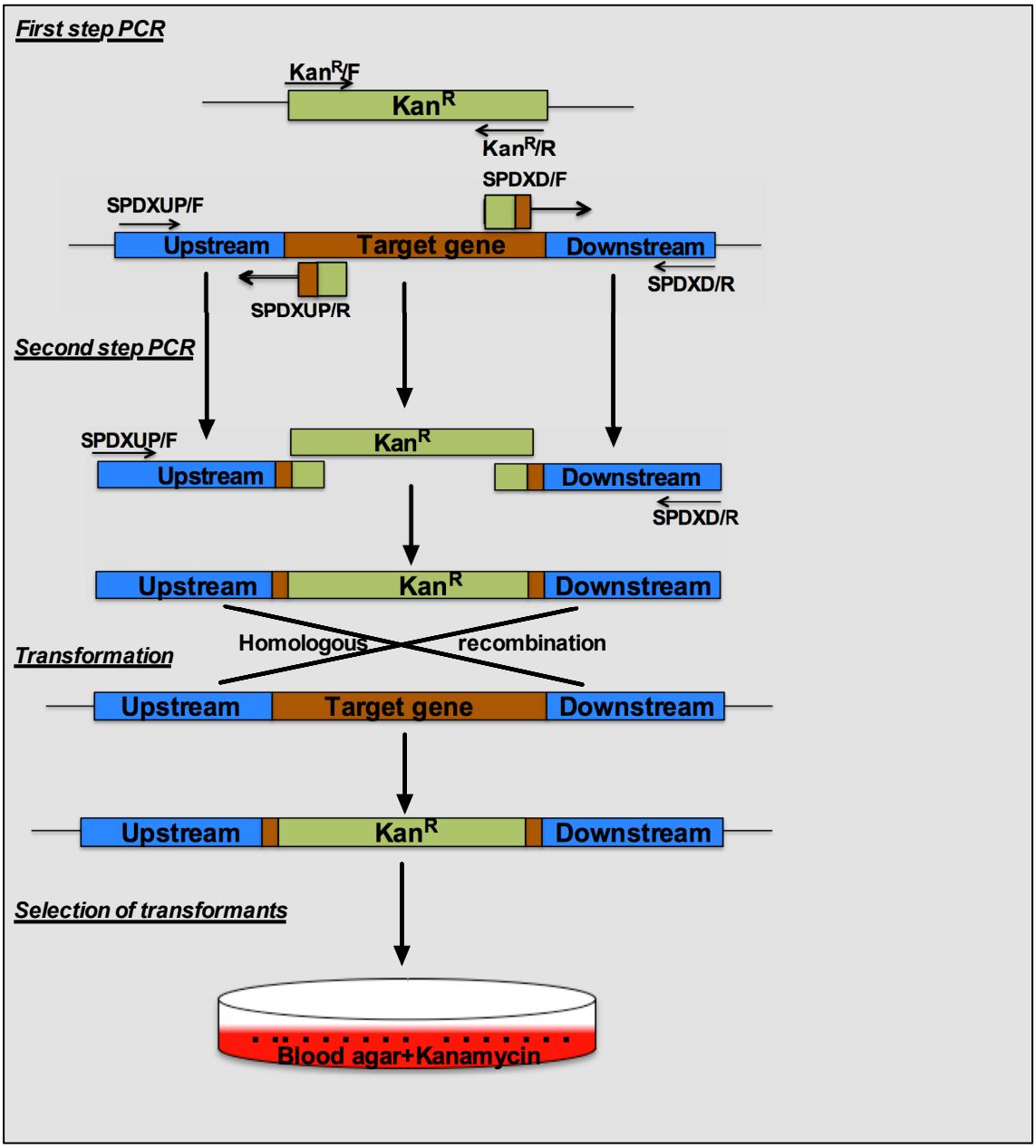

Figure 3.6: Illustration of SOEing PCR steps for allelic insertion deletion mutagenesis. Primer designations: SPDXUP/F: forward primer of upstream flank; SPDX/R: reverse primer of upstream flank; SPDXD/F: forward primer of downstream flank; SPDXD/R: reverse primer of downstream flank; Kan^R/F and Kan^R/R: forward and reverse primers of kanamycin resistance cassette. SPDX represents the gene designation number.

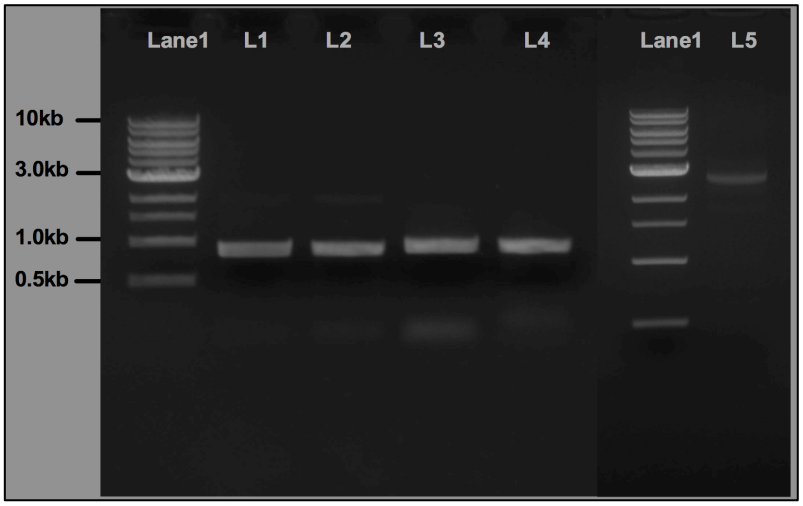

Figure 3.7: Agarose gel electrophoresis showing the amplicons representing kanamycin resistance cassette and flanking regions of *axe*. Lane1: 500 ng of 1 kb DNA ladder; L1 and L2: kanamycin resistance cassette (890 bp) amplified from the plasmid pCEP; L3: upstream region of *axe* gene (920 bp); L4: downstream region of *axe* gene (920 bp); L5: the fused SOEing product for *Δaxe* mutation.

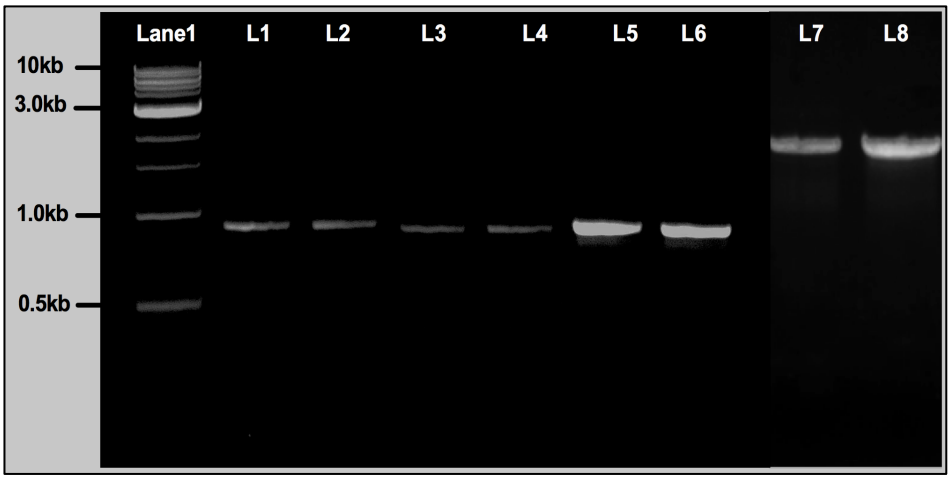

Figure 3.8: Agarose gel electrophoresis showing the amplified kanamycin resistance cassette and flanking regions of *axe* from a putative *ΔestAaxe* mutant. Lane1: 500 ng of 1 kb DNA ladder; L1 and L2: upstream fragment (920 bp) using SPD1506UP/F and SPD1506UP/R primers; L3 and L4: downstream fragment (920 bp) using SPD1506D/F and SPD1506D/R primers; L5 and L6: amplicon of kanamycin resistance cassette (890 bp) using Kan^R-F/R primers; L7: amplicon of upstream flank and kanamycin resistance cassette using SPD1506UP/F and KanR/R primers; L8: amplicon of downstream flank and kanamycin resistance cassette using KanR/F and SPD1506D/R primers.

3.1.4 Genetic complementation of isogenic mutants:

ΔestA and Δaxe isogenic mutants were complemented with an intact copy of each respective gene in order to rule out that the observed phenotypic changes were not due to polar effect of mutation. pCEP, which is a non-replicative plasmid in *S. pneumoniae*, was used for the introduction of an intact copy of the gene of interest into the transcriptionally silent site in the pneumococcal chromosome. The coding regions of *estA* and *axe* were amplified using SPD1506Comp/F and SPD1506Comp/R, and SPD0534Comp/F and SPD0534Comp/R primers (Table 2.17 in Materials and Methods), for *axe* and *estA*, respectively (Figure 3.9). These primers amplify the entire coding region of esterases gene with their putative promoter region, and they incorporate *NcoI*/*BamHI* sites, and *Bstz17I*/*BamHI* sites for *estA* and *axe*, respectively. The analysis of amplicons by agarose gel electrophoresis indicated the successful amplification of genome regions: 980 bp and 1300 bp for *estA* and *axe*, respectively) (L1 and L2 in Figure 3.9). Both pCEP, and the inserts for *axe* and *estA* were digested using respective restriction enzymes (Figure 3.10), and ligated as described in Materials and Methods (section 2.10.1).

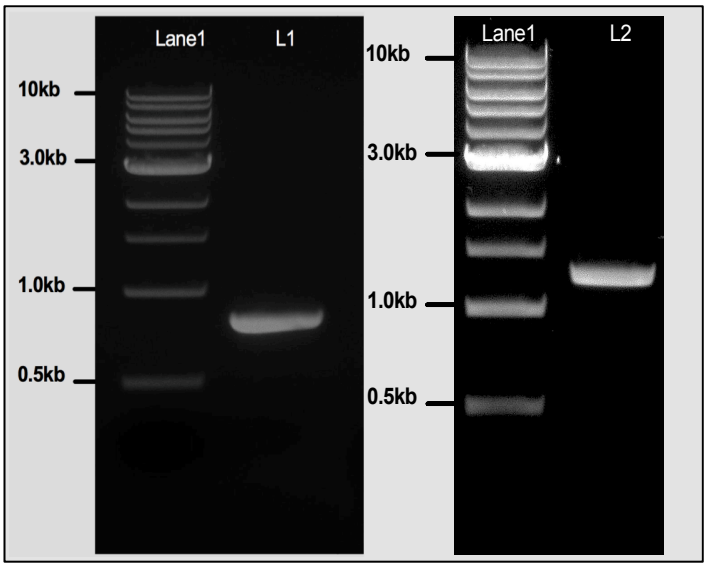

Figure 3.9: Agarose gel electrophoresis analysis of amplicons representing each of *estA* (L1) and *axe* (L2). Lane1 contains 500 ng 1 kb DNA ladder (New England Biolab).

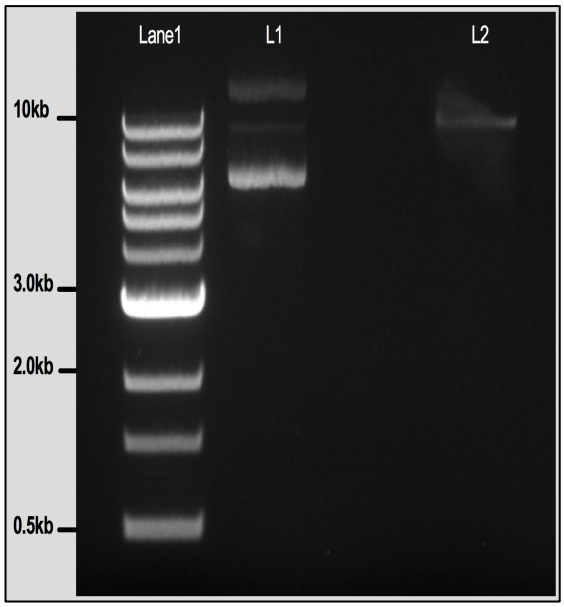

Figure 3.10: Agarose gel electrophoresis of restriction endonuclease treated pCEP. The plasmid was digested either with NcoI and BamHI for *estA*, or with BstZ17I and BamHI for *axe* ligation. Each digestion produces similar size of vector. Lane1: 500 ng 1kb DNA ladder (New England Biolab); L1: 100 ng of uncut pCEP; L2: 100 ng of cut pCEP.

A sample of ligation mixture was transformed into *E. coli* BL21 cells, and the transformants were selected in the presence of kanamycin. A few transformants were analysed by colony PCR using malF2 and pCEPR2 primers, whose recognition sites are located either side of the multiple cloning site, and amplify approximately 500 bp fragment from empty pCEP. Figure 3.11 shows gel electrophoresis of amplicons amplified from transformants of *E. coli* containing pCEP-*estA* and pCEP-*axe* using malF2 and pCEPR2 primers. As can be seen from Figure 3.11, when the transformants were used as template, the size of amplicons was 1480 bp (L1-L4 in Figure 3.11) and 1800 bp (L5-L8 in Figure 3.11) for *estA* and *axe*, respectively, whereas with the empty plasmid, the amplicon size was only about 500 bp (L9 in Figure 3.11) when empty pCEP was used as template. The recombinant plasmids were designated as pCEP-estA and pCEP-axe (Figure 3.12).

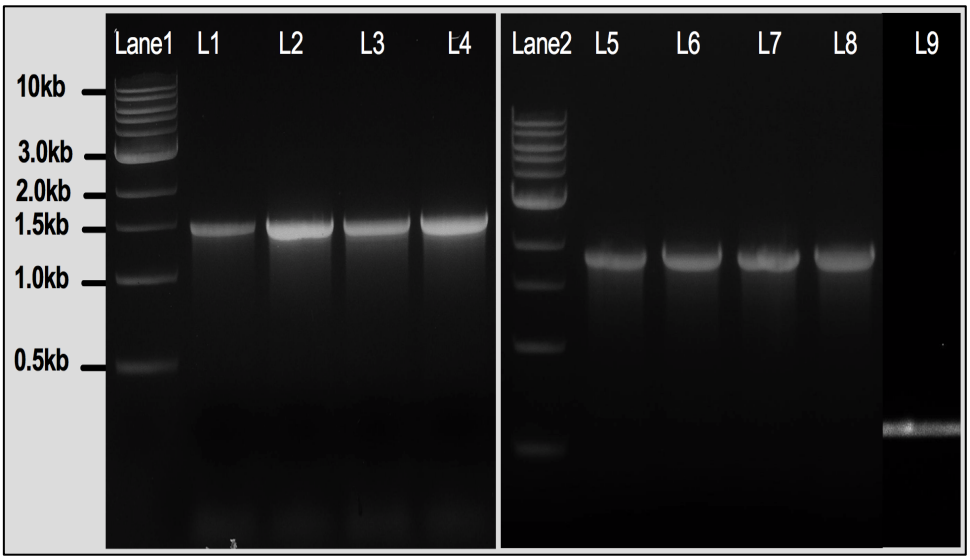

Figure 3.11: Agarose gel electrophoresis illustrating colony PCR of *E. coli* pCEP-*estA* and *E. coli* pCEP-*axe* transformants using malF2 and pCEPR2 primers. Lane 1 and Lane 2: 500 ng 1 kb DNA ladder (New England Biolab); L1, L2, L3 and L4: *estA* amplicons from *E. coli* pCEP-estA; L5, L6, L7 and L8: *axe* amplicons from *E. coli* pCEP-axe.

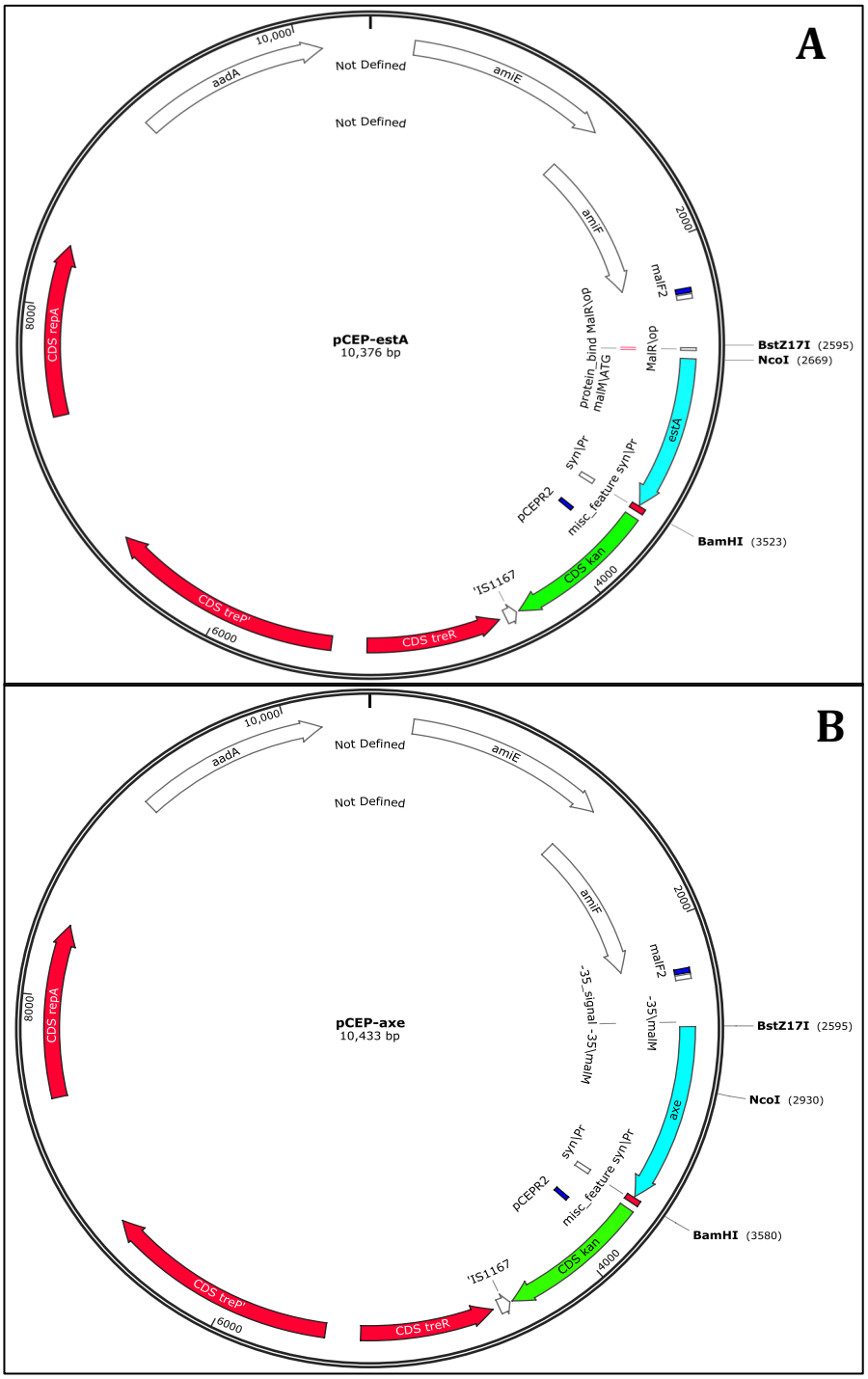

Figure 3.12: Schematic representation of pCEP plasmid illustrating the successful insertion of *estA* (**A**) and *axe* (**B**). The cloned esterase genes are shown in bright blue. The plasmid based malF2 and pCEPR2 primer recognition sites are shown in dark blue. *amiE*, *amiF*, *treR* and *treP* upstream and downstream cloning region are the homologous regions that recombine with the pneumococcal transcriptionally silent site.

The recombinant plasmids were then transformed into the *ΔestA* and *Δaxe* isogenic mutants, and the transformants were selected in the presence of spectinomycin and kanamycin. To confirm the successful integration of genes of interest into *ΔestA* and *Δaxe* chromosomes, colony PCR was done for selected colonies using malF2 and pCEPR2 primers. These primers amplify only about 500 bp DNA product when empty pCEP was used as template. However, the insertion of esterase genes increases the size of amplicon to 1480 (L1-L3) and 1800 bp (L4-L6) for *estA* and *axe*, respectively (Figure 3.13). In addition, one transformant for each genetic complementation was selected, designated as estAComp and axeComp, and the intact copies of the *axe* and *estA* were amplified using malF2 and pCEPR2 primers, and sent for sequencing. The sequence results showed a successful complementation of *ΔestA* and *Δaxe* with intact copies of *estA* and *axe.*

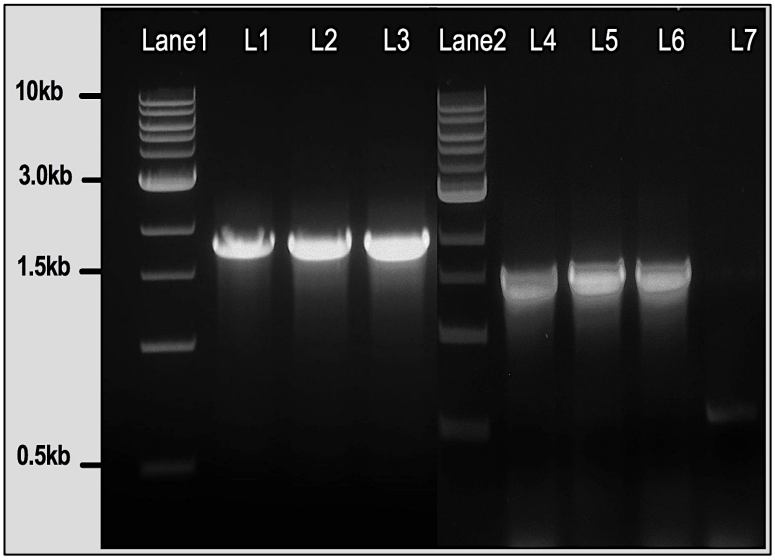

Figure 3.13: Agarose gel electrophoresis showing successful complementation of *Δaxe* and *ΔestA* by colony PCR of selected transformants. Lane1 and Lane2: 500 ng 1 kb DNA ladder (New England Biolab); L1, L2 and L3 contains amplicons representing *axe* from axeComp, and L4, L5 and L6 represent the *estA* amplicons from estAComp; L7 represent amplicons from empty pCEP. malF2 and pCEPR2 primers were used for amplifcations.

3.1.5 Site directed mutation for amino acid replacement by SOEing PCR:

The aim of this task was to introduce point mutation in putative serine active site (S^{121} for EstA and S^{181} for Axe) of esterases by replacing the nucleotide thiamine to guanine, and investigate whether serine residue is responsible for the catalytic activity of these enzymes. The modifications would allow assessment of importance of this residue using the recombinant protein, and through creation of pneumococcal strains expressing modified esterases by *cis* complementation of esterase mutants. The sequence analysis of EstA and Axe revealed that both esterases contain a serine active site when aligned with other known esterases in which the serine was shown to be important for catalytic activity. Figures 3.14 and 3.15 show amino acid sequence alignment of EstA and Axe with other known esterases. As can be seen from the Figure, EstA and Axe have a catalytic Ser-Asp-His triad, and the active site serine is located within the conserved G-X-S-X-G consensus sequence.

To introduce site directed mutations, two consecutive PCR were used: in the first reaction left- and right flank of target genes with their putative promoter regions were amplified as previously described section 2.9.2, except that the internal primers were modified to introduce the desired mutation as illustrated in Figure 3.16. The amplified flanking regions carrying the mutagenic site and desired restriction sites were analysed by agarose gel electrophoresis to check their size (Figure 3.17). The second PCR reaction used the products of the first step PCR as template to fuse the flanks. The fused PCR product for each target gene was analysed by agarose gel electrophoresis, and then gel purified as described in section 2.6 (Figure 3.17). The purified products were then digested and ligated with pCEP plasmid as described in section 2.10.1. The ligation products were transformed into *E.coli* BL21, and the transformants were selected on LB agar plates supplemented with kanamycin. Colony PCR using malF2 and pCEPR2 primers confirmed the insertion of gene of interest. One of the transformants containing the insert for each gene was selected for plasmid extraction as described in section 2.7. The purified recombinant plasmids were then designated as pCEP-estA^{S121A} and pCEP-axe^{S181A} for further work.

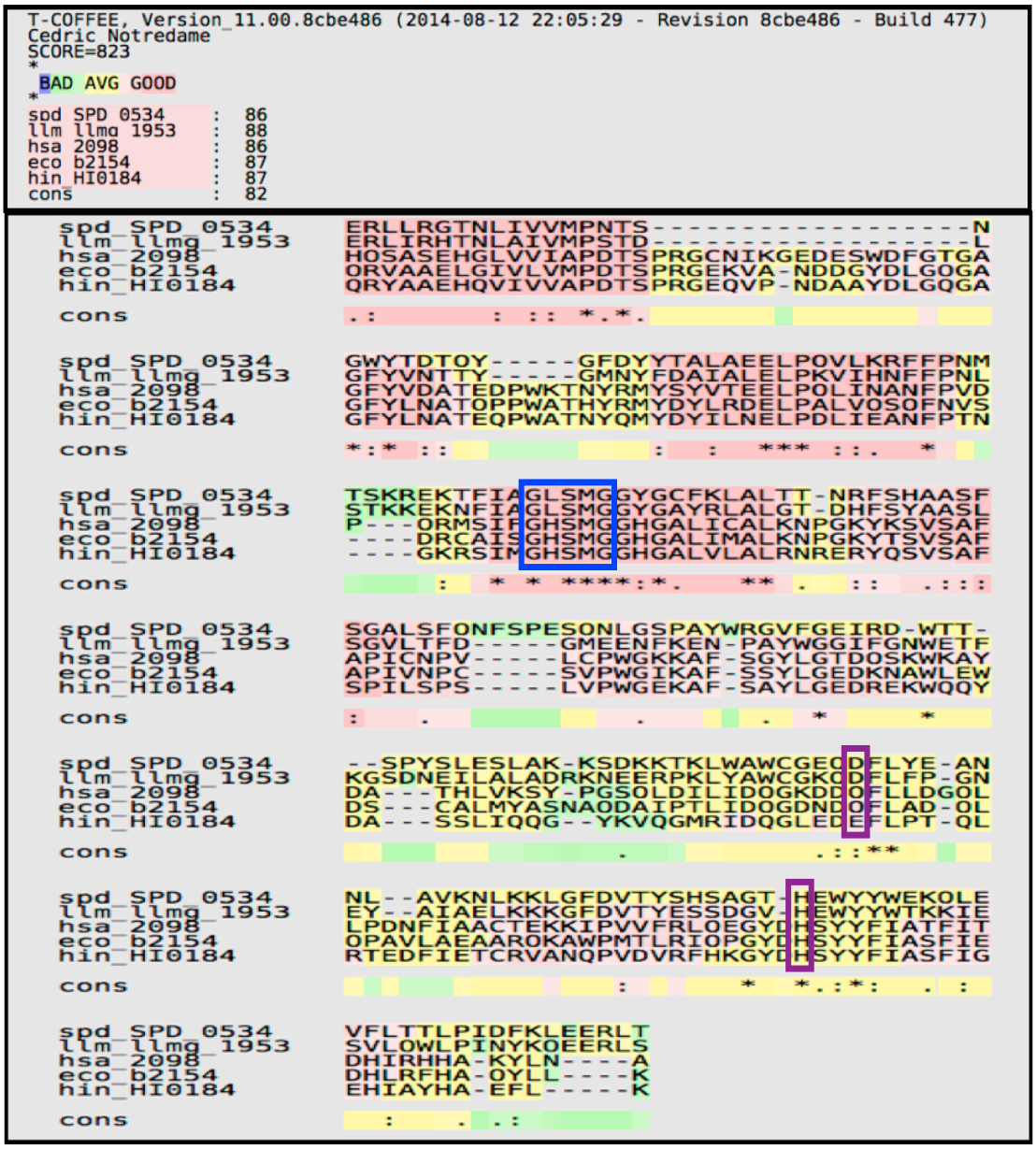

Figure 3.14: Amino acid sequence alignment of EstA with known esterases. The blue box represents the conserved consensus sequence and serine active site is located in the centre of the conserved sequence. The purple boxes represent the catalytic residues of Asp and His. The colour range represents the similarity range from high similarity (Red) to low similarity (Mountain blue). The black stars represent the identical residues. Biological sources and accession numbers for the sequences are as follow: SPD_0534: EstA from *S. pneumoniae*; llm_1953: tributyrin esterase from *Lactococcus. lactis*; hsa_2098: human esterase D; eco_b2154: S-formylglutathione esterase from *E. coli* K12 MG1655; hin_HI0184: S-formylglutathione esterase from *H. influenzae* Rd KW20. Sequence alignments were assembled using T-COFFEE software and visualized using ESPript software, both located on the ExPASy Proteomics Server (http://au.expasy.org/).

Figure 3.15: Amino acid sequence alignment of Axe with known esterases. The blue box represents the conserved consensus sequence and serine active site is located in the centre of the conserved sequence. The purple boxes represent the catalytic residues of Asp and His. The colour range represents the similarity range from high similarity (Red) to low similarity (Mountain blue). The black stars represent the identical residues. Biological sources and accession numbers for the sequences is as follows: SPD_0534: EstA from *S. pneumoniae*; llm_1953: tributyrin esterase from *L. lactis*; hsa_2098: human esterase D; eco_b2154: S-formylglutathione esterase from *E. coli* K12 MG1655; hin_HI0184: S-formylglutathione esterase from *H. influenzae* Rd KW20. Sequence alignments were assembled using T-COFFEE software and visualized using ESPript software, both located on the ExPASy Proteomics Server (http://au.expasy.org/).

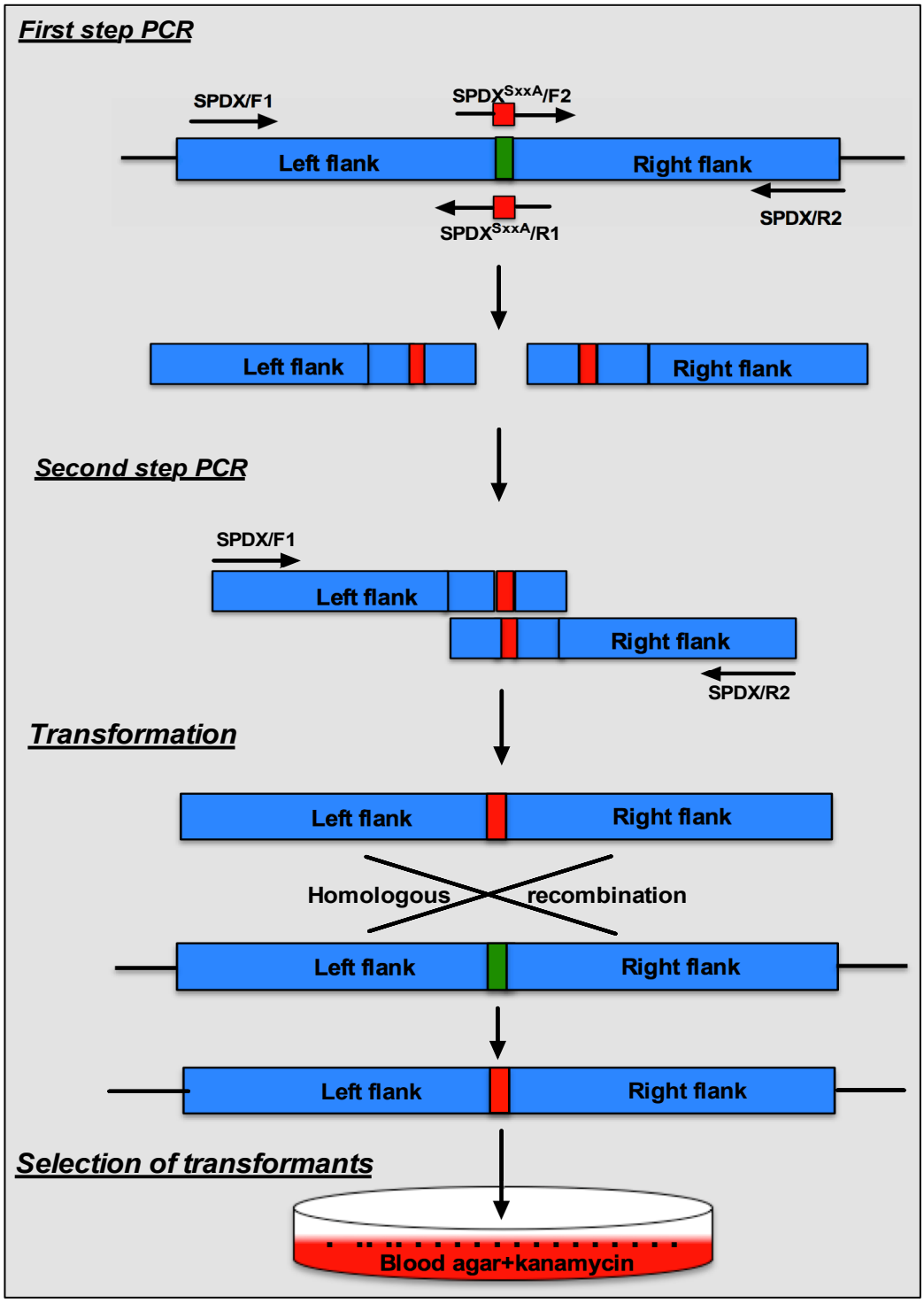

Figure 3.16: Schematic representation of site directed mutagenesis for amino acid replacement using Splicing Overlap Extension PCR (SOEing PCR). SPDX/F1 and SPDX/R2 refer to gene specific primers. SPDX^{SxxA}/F2 and SPDX^{SxxA}/R2 refer to mutagenic site primers that carry replaced nucleotide (red rectangle) where xx refers to the position of serine active site being replaced with alanine

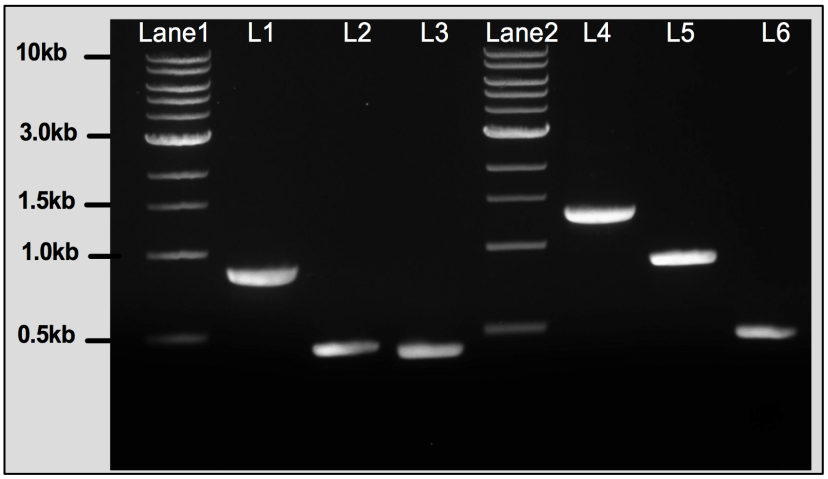

Figure 3.17: Agarose gel electrophoresis analysis of the amplicons representing the left and right flanks, and fused products of *estA* and *axe* for site directed mutagenesis. Lane 1 and Lane 2: 500 ng of 1 kb DNA ladder; L1: the joined product for $estA^{S121A}$; L2: the left flank of $estA^{S121A}$; L3: the right flank of $estA^{S121}$ mutation; L4: the joined product for axe^{S181A} mutation; L5: the left flank of axe^{S181A}: L6: the right flank of axe^{S181A}.

Figure 3.18 shows agarose gel electrophoresis of amplicons amplified from pCEP-estA^{S121A} and pCEP-axe^{S181A} recombinant plasmids using different sets of primers. As can be seen from the Figure, when primers annealing coding regions of esterases were used, the size of amplicons was 780 bp and 980 bp (L1 and L4 in Figure 3.18) for $estA^{S121A}$ and axe^{S181A}, respectively. However, when SPDXComp/F and SPDXComp/R primers were used, which amplify coding region and putative promoter sites, the size increased to 980 bp and 1300 bp (L2 and L5 in Figure 3.18) for $estA^{S121A}$ and axe^{S181A}, respectively. Similarly, the size of $estA^{S121A}$ and axe^{S181A} also increased to 1480 bp and 1800 bp (L3 and L6 in Figure 3.18), respectively, when malF2 and pCEPR2 primer set was used, as use of these primers will add 500 bp to the target gene.

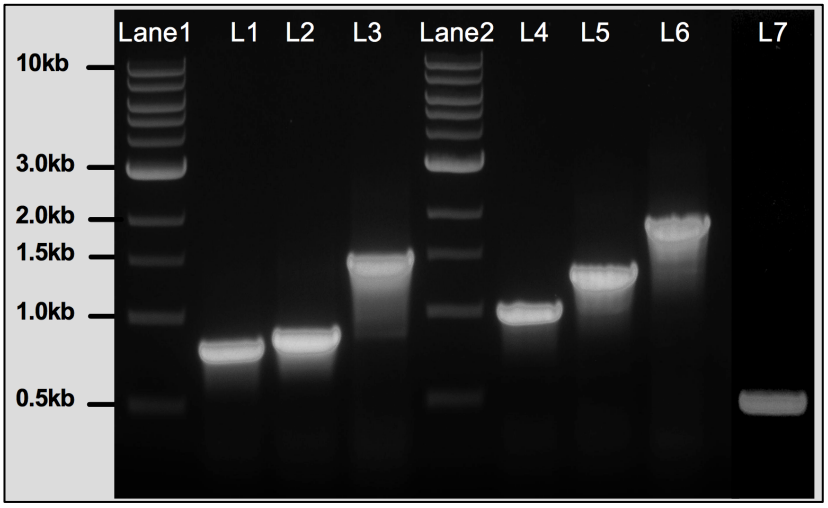

Figure 3.18: Agarose gel electrophoresis analysis showing the amplicons from *E. coli* carrying pCEP-estA^{S121A} and pCEP-axe^{S181A} recombinant plasmids. Lane 1 and Lane 2: 500 ng 1 kb DNA ladder (New England Biolab); L1: *estA*^{S121A} amplified using EstAF/R primers; L2: *estA*^{S121A} amplified using SPD0534CompF/R primers; L3: *estA*^{S121A} amplified using malF2 and pCEPR2 primers; L4: *axe*^{S181A} amplified using AxeF/R primers; L5: *axe*^{S181A} amplified using SPD1506CompF/R primers; L6: *axe*^{S181A} amplified using malF2 and pCEPR2 primers; L7: amplicon amplified from empty pCEP using malF2 and pCEPR2.

The recombinant plasmids, pCEP-estA^{S121A} and pCEP-axe^{S181A} were then transformed into *ΔestA* and *Δaxe* isogenic mutants as previously described (section 2.9.3). The Figure 3.19 shows the successful complementation of *ΔestA* and *Δaxe* with the modified *estA*^{S121A} and *axe*^{S181A}. As can be seen from the Figure, the size of amplicons was 1480 bp and 1800 bp, respectively, for *estA*^{S121A} and *axe*^{S181A} when amplified using malF2 and pCEPR2 primers (Figure 3.19), corresponding to the size of *estA* (980 bp) and *axe* (1300 bp) plus 500 bp of sequence represented in pCEP. This result shows a successful complementation of *ΔestA* and *Δaxe* with a modified copy of *estA*^{S121A} and *axe*^{S181A}, respectively.

Successful replacement of selected amino acid residues were further confirmed by DNA sequencing. The target genes were amplified from the recombinant plasmids using

malF2 and pCEPR2 primers. The amplified products were then column purified and sent for DNA sequencing at PNACL, University of Leicester. The gene specific primers, SPD0534^{S121A}/F and SPD0534^{S121A}/R for *estA*^{S121A} and SPD1506^{S181A}/F and SPD1506^{S181A}/R for *axe*^{S181A} were used for sequencing. The sequence results were analysed by BLAST hosted at National Centre for Biotechnology Information (NCBI) (Appendix 2). The results showed a successful replacement of thiamine to guanine in both sequences, which leads to the replacement of serine with alanine. The modified complemented strains were then designated as estAComp^{S121A} and axeComp^{S181A} for further analysis.

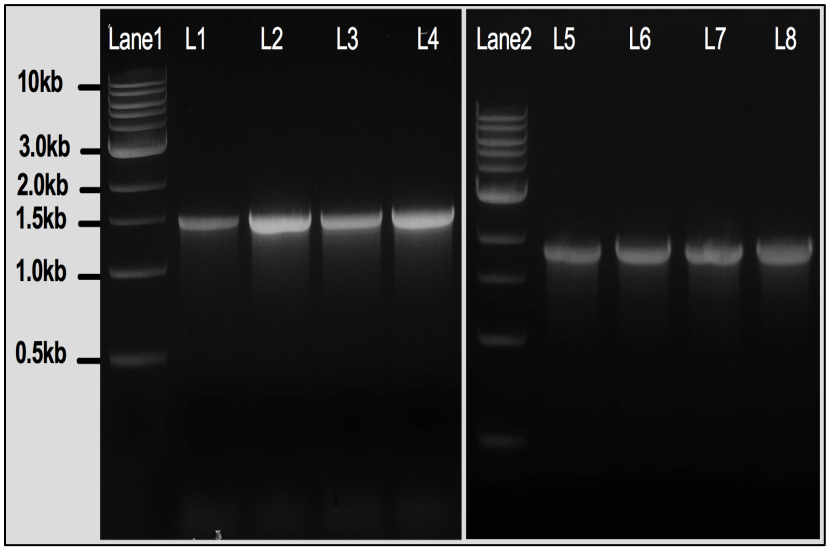

Figure 3.19: Agarose gel electrophoresis showing successful construction of pneumococcal strains carrying modified copies of *estA*^{S121A} and *axe*^{S181A}. Lane 1 and Lane 2: 500 ng 1 kb DNA ladder (New England Biolab); L1, L2, L3 and L4: *estA*^{S121A} amplicons from estAComp^{S121A} using; L5, L6, L7 and L8: *axe*^{S181A} amplicon amplified from axeComp^{S181A}. malF2 and pCEPR2 primers were used for amplification.

3.1.6 Total enzyme activity in isogenic esterase mutants:

Enzyme activity was assayed to detect total esterase activity both in wild type parental strains, D39 and TIGR4, and their isogenic esterase mutants as described in section 2.13. All strains were grown in BHI. When the $OD_{(600)}$ reached approximately between 0.9-1.0, corresponding to late exponential phase, the cultures were harvested by centrifugation, and the cell free lysates were prepared. Protein concentration of lysates was determined using the Bradford assay as described in Materials and Methods.

The results showed that all isogenic esterase mutants had significantly lower esterase activity than the respective wild types (Figure 3.20) when p-NPA was used as a substrate. For example, while the wild type D39 strain had 50.8mU±1.0 (n=6), the activity levels for the mutants were 41.5 (±1.6) mU, 18.9 mU (±0.7), 42.8mU±1.2 and 36.8mU±1.2 (n=6) for *ΔSPD1239*, *ΔSPD0534* (*ΔestA*), *ΔSPD0932* and *ΔSPD1506* (*Δaxe*), respectively. The difference in activity between the mutants and wild type was significant ($p<0.01$). With regard to TIGR4 background, esterase activity showed the same results as D39 strain (Figure 3.20). As can be seen from the Figure, the total esterase activity in TIGR4 wild type (52.09 mU±1.1, n=6) was significantly higher than *ΔSP1408* (38.83 mU±1.7, n=6), *ΔSP0614* (*ΔestA*) (17.6 mU ±0.32, n=6), *ΔSP1052* (39.12mU±1.6, n=6) and *ΔSP1695* (*Δaxe*) (30.93mU±1.4, n=6) ($p<0.01$). The largest reduction in activity was observed with *ΔSPD0534* (*ΔestA*) (*ΔSP0614* in TIGR4), indicating that SPD_0534 is the main gene coding for pneumococcal esterase specific for p-NPA.

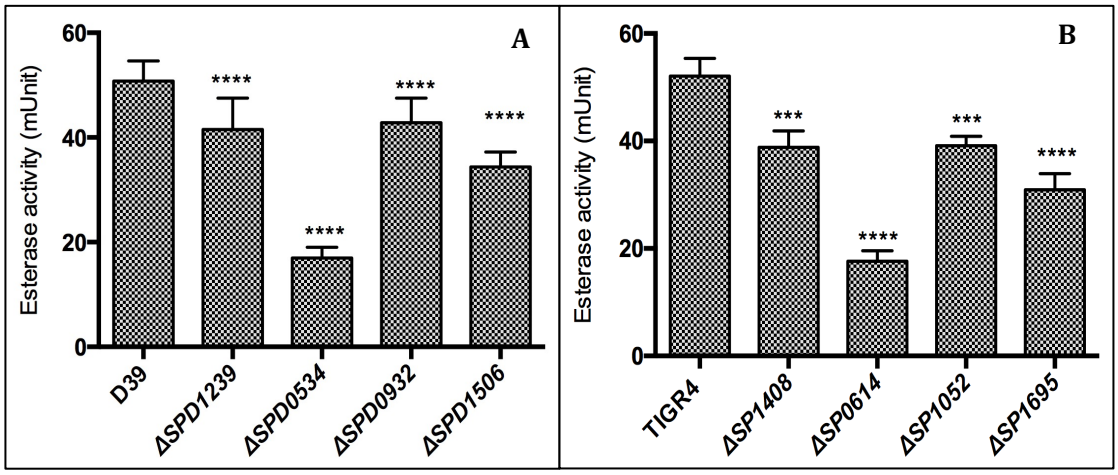

Figure 3.20: Esterase activity in isogenic pneumococcal esterase mutants in D39 (A) and TIGR4 (B) backgrounds using p-NPA as a substrate. The enzyme activity is expressed as micromoles of p-nitrophenol released from the substrate per milligram of protein per minute. ***: $p<0.001$; ****:$p<0.0001$ compared to wild type.

When p-NPB was used as a substrate, the isogenic mutants in D39 background, *ΔSPD1239* (19.86 mU±1.1, n=6) and *ΔSPD0932* (19.08 mU ±1.2, n=6) had similar activity as the wild type (21.16 mU ±0.4, n=6), whereas *ΔSPD0534* (*ΔestA*) (7.3 mU±0.64 n=6) and *ΔSPD1506* (*Δaxe*) (14.6 mU ±0.6, n=6) exhibited significantly lower activity than the wild type (22.4 mU ±1.3, n=6) ($p<0.01$) (Figure 3.21). Similarly, in TIGR4 background, the total esterase activity in wild type (20.2 mU ±0.11, n=6) was significantly higher than *ΔSP0614* (*ΔestA*) (6.03 mU ±0.32, n=6) and *ΔSP1695* (*Δaxe*) (17.9 mU ±0.48, n=6) ($p<0.01$). However, *ΔSP1239* (19.8 mU ±0.13, n=6) and *ΔSP1052* (19.8 mU ±0.48, n=6) did not show significant difference in activity when compared to TIGR4 wild type ($p>0.05$). These results show that esterase encoded by SPD_1239 and SPD_0932, are specific for short-chain acyl esters (Fig. 3.21), while esterases encoded by SPD_0534 and SPD_1506 are very likely active against 2- and 4-carbon acyl esters (Table 3.1).

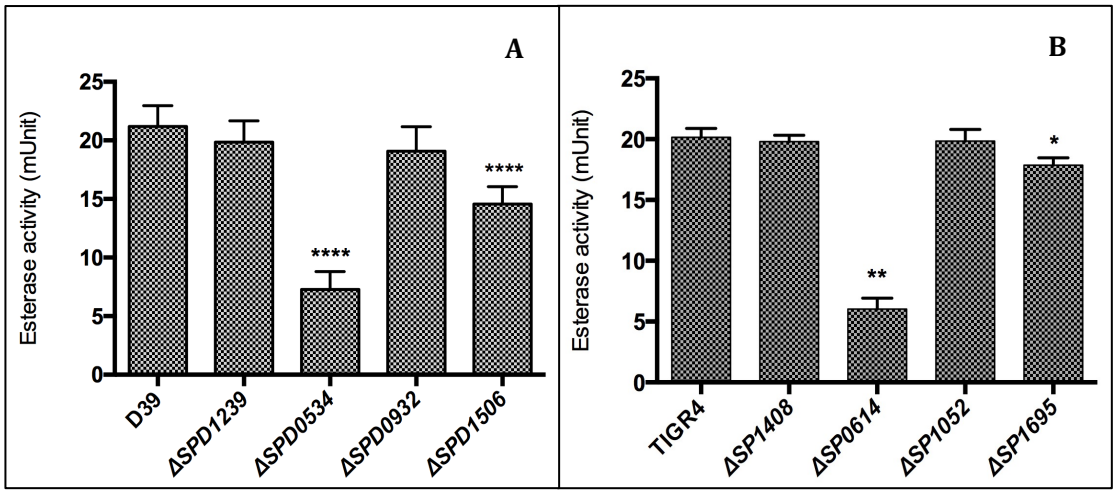

Figure 3.21: Esterase activity in isogenic pneumococcal esterase mutants in D39 (A) and TIGR4 (B) backgrounds using p-NPB as a substrate. The enzyme activity is expressed as micromoles of p-nitrophenol released from the substrate per milligram of protein per minute. *: $p<0.05$; **$p<0.01$; ****: $p<0.0001$ compared to D39 and TIGR4 wild types.

Table 3.1: Total esterase activity of isogenic pneumococcal esterases mutants in D39 and TIGR4 backgrounds.

	Total esterase activity (mU)			
	p-NPA		**p-NPB**	
Strains	**D39**	**TIGR4**	**D39**	**TIGR4**
Wild type	50.8±1	52.1±1.1	21.2±0.4	20.2±0.11
ΔSPD1239	41.5±1.6	38.8±1.7	19.9±1.1	19.8±0.13
ΔSPD0534	18.9±0.7	17.6±0.32	7.3±0.64	6.03±0.32
ΔSPD0932	42.8±1.2	39.12±1.6	19.08±1.2	19.8±0.48
ΔSPD1506	36.8±1.2	30.9±1.4	14.6±0.6	17.9±0.48

3.1.7 Total esterase activity of *ΔestAaxe* double mutant:

Having established the significant contribution of SPD_0534 and SPD_1506, total esterase activity in *ΔestAaxe* (*ΔSPD0534-SPD1506*) double isogenic mutant was also determined to find out whether the mutation of EstA and Axe together would further decrease the total esterase activity or whether there is any compensatory interaction among esterases. The whole cell lysates of *ΔestAaxe* double isogenic mutant and D39 wild type were prepared as described in section 2.13.1. The chromogenic substrate was used in this assay. Figure 3.22 shows total esterase activity in *ΔestAaxe* compared to *ΔestA, Δaxe* and parental strain. The activity in *ΔestAaxe* (4.1 mU±1.01, n=6) was significantly lower compared to wild type strain (51.1 mU±1.4, n=6) (p<0.01). Furthermore, the total esterase activity was significantly reduced in *ΔestAaxe* (4.1 mU±1.01, n=6) when compared to *ΔestA* (16.26mU±1.21, n=6) and *Δaxe* (33.84 mU±1.4, n=6) (p<0.01). This result indicates that SPD_0534 (estA) and SPD_1506 (axe) code for major esterases specific to p-NPA, and mutation of both genes does not totally abolish total esterase activity.

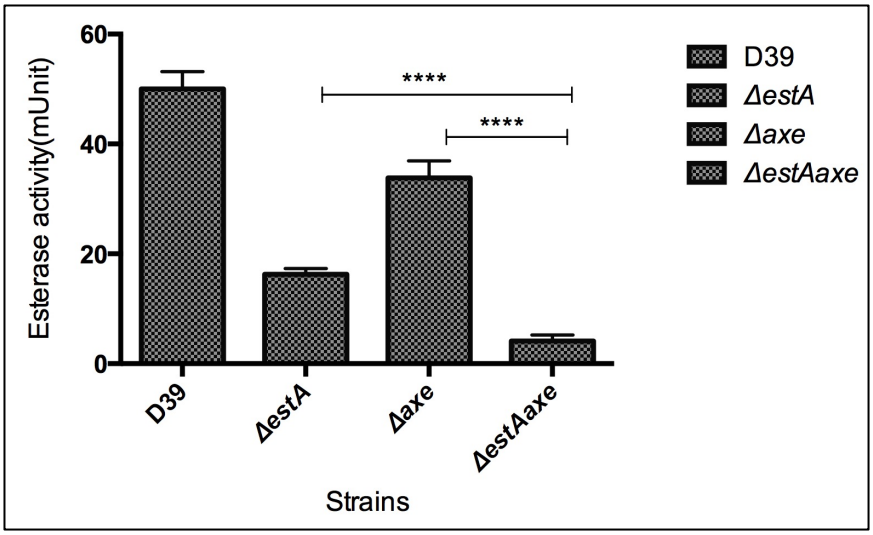

Figure 3.22: Esterase activity in isogenic double *ΔestAaxe* mutant in D39 background using p-NPA as a substrate. The enzyme activity is expressed as micromoles of p-nitrophenol released from the substrate per milligram of protein per minute. ****: p<0.001 compared to wild type.

3.1.8 Phenotypic analysis of complemented strains:

In order to test whether EstA and Axe activity could be restored in the genetically complemented strains of estAComp and axeComp, total esterase activity was determined in these strains using p-NPA as the substrate. The parental strains and the respective mutants were included as controls.

The results showed that the complemented strains with estAComp (32.9 ±0.4 mU, n=6) and axeComp (43.7 ±1.4 mU, n=6) had significantly higher esterase activity than the respective mutant strains (13.6 ±1.6 mU, n=6 for *ΔestA* and 28.9±1.75 mU, n=6 for *Δaxe*) ($p<0.01$) (Figure 3.23). Moreover, the total esterase activity in estAComp (32.9 ±0.4 mU, n=6 and axeComp (40.4 ±1.2 mU, n=6) was similar to the wild type (45.4 ±3.2 mU, n=6), indicating that the mutations did not cause polar effects.

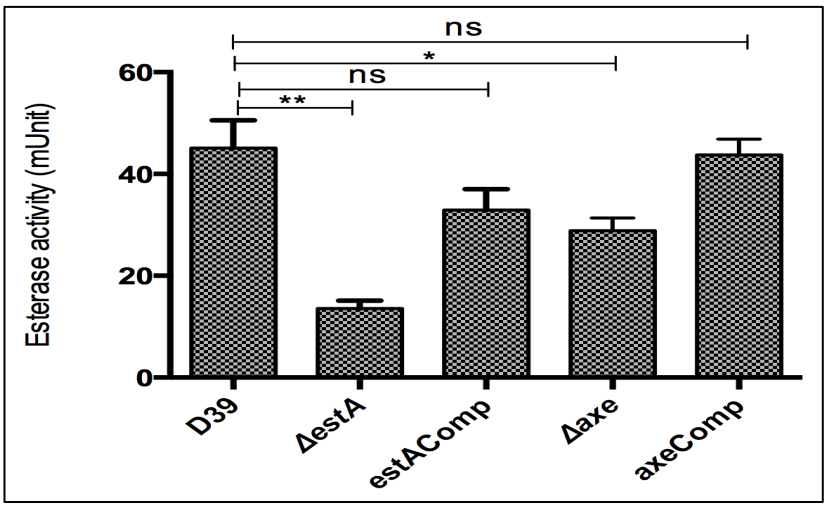

Figure 3.23: Esterase activity in pneumococcal esterase mutants complemented with intact copy of respective esterase genes in D39 background using p-NPA as a substrate. The enzyme activity is expressed as micromoles of p-nitrophenol released from the substrate per milligram of protein per minute. *: $p<0.05$; **: $p<0.01$ compared to wild type; ns: not significant.

3.1.9 Phenotypic analysis of strains modified with esterases':

The main reason for performing this assay was to investigate whether EstA and Axe rely on serine active site for catalytic activity. The amino acid sequence alignment revealed that both EstA and Axe contain a conserved putative catalytic serine active site. S^{121} and S^{181} in EstA and Axe, respectively, were replaced with alanine as described in section 3.1.5. The replacement of serine to alanine changes the shape of the binding pocket of the enzyme and abolishes the catalytic activity (Thamwiriyasati et al., 2010). The phenotypic analysis of strains complemented with intact copies of *estA* or *axe* indicated no polar effects in *ΔestA* and *Δaxe* (see section 3.3.3). If putative S^{121} and S^{181} in EstA and Axe are the active sites, therefore, it is expected that complementation with modified copy of $estA^{S121A}$ and axe^{S181A} would not restore the activity level.

Total esterase activity in modified complemented strains showed that the replacement of putative serine active sites abolished the esterase activity in $estAComp^{S121A}$ and $axeComp^{S181A}$ compared to wild type, indicating that S^{121} and S^{181} are responsible for catalytic activity of EstA and Axe, respectively (Figure 3.28). As can be seen from the Figure 3.24, total esterase activity for $estAComp^{S121A}$ (13.64±2.79mU, n=6) and $axeComp^{S181A}$ (27.16±3.2mU, n=6) was significantly lower than the respective intact complemented strains, estAComp (33.4 ±0.3 mU, n=6) and axeComp (41.7 ±1.7 mU, n=6) ($p<0.01$) as well as parental strain (47.7 ±1.4 mU, n=6) ($p<0.01$) (Figure 3.24). Furthermore, no significant difference between modified complemented strains ($estAComp^{S121A}$ and $axeComp^{S181A}$) and *ΔestA* and *Δaxe* could be seen ($p>0.05$), indicating that complementation with modified copies of esterases could not restore the wild type phenotype. These results show that S^{121} and S^{181} in EstA and Axe, respectively, are important residues for efficient catalytic activity of these enzymes.

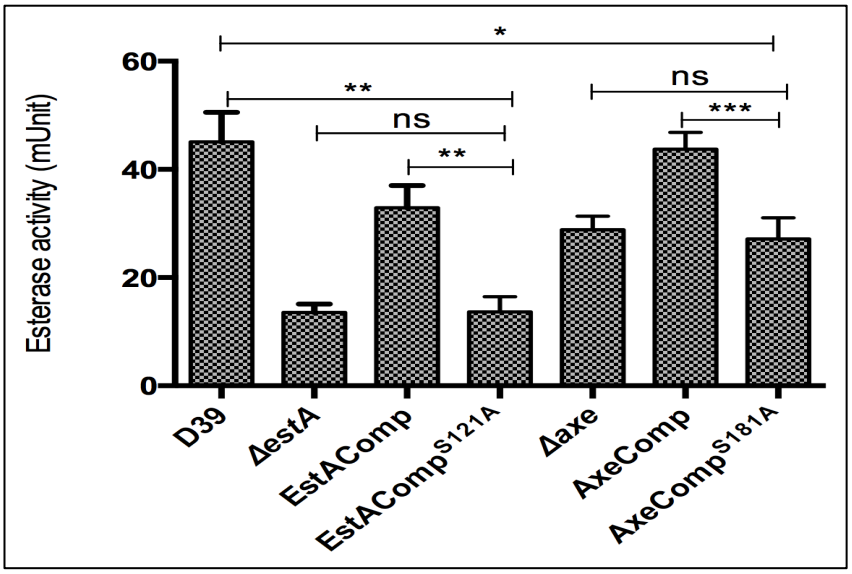

Figure 3.24: Esterase activity in esterase mutants complemented with modified *estA* and *axe*. p-NPA was used as a substrate. The enzyme activity is expressed as micromoles of p-nitrophenol released from the substrate per milligram of protein per minute. *: $p<0.05$; **$p<0.01$; ***:$p<0.001$ compared to wild type; ns: not significant.

Section II: Production of recombinant esterases

3.2: The choice of expression system for recombinant protein production

EstA and Axe have been shown to be major pneumococcal esterases in this study. Therefore, they were chosen for detailed characterisation, including kinetic analysis and substrate specificity. In order to investigate the involvement of esterases in NanA activity potentiation, the recombinant enzymes were needed for *in vitro* assays. Purified recombinant esterases were also used to find out whether they are able to degrade *O*-acetyl substituents from natural substrates such as BSM.

In this study, the pLEICES-01 plasmid, provided by the Protein Expression Laboratory (PROTEX)/Department of Molecular and Cell Biology/ University of Leicester, was used for production of recombinant esterases. This plasmid incorporates 6XHis (His-tagged) residues into the *N*-terminus of the protein, which allows the recombinant protein to anchor to TALON metal affinity column. The recombinant proteins are usually applied through Nickel affinity column that react only with proteins containing His-tag. Undesirable *E. coli* proteins were then washed away using appropriate buffers, and the bound recombinant proteins can be eluted with imidazole that can easily cleave the bond between metal affinity column and His-tag proteins. The vector also carries cleavable TEV site (Tobacco etch virus protease) that allows cleaving 6XHis tag from the protein if needed. The recombinant plasmid is usually transformed into *E. coli* BL21, which is genetically modified for protein expression purposes. It highly expresses T7 RNA polymerase, which is important for solubility of expressed proteins. This strain also has thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) knocked out. These genes are responsible for disulphate bond formation in bacteria, which is unfavourable in recombinant protein production.

3.2.1 Cloning and expression of esterases:

SPD_0534 (*estA*) and SPD_1506 (*axe*) were cloned and overexpressed as described in section 2.12; *estA* and *axe* were amplified from D39 wild type chromosomal DNA using primers carrying modifications to include homologous sequence to pLCECS-01 cloning site. Initially, the gradient PCR was done to determine the most optimum annealing temperature in which the highest yield of specific products could be obtained. The results revealed that 55˚C was the optimal temperature for cloning using gradient PCR

for *estA* and *axe* amplification (data not shown). In addition, PrimeSTAR HS premix polymerase was used to limit the likelihood of introducing mutations as this enzyme has a proof reading feature. PCR products were separated by agarose gel electrophoresis, and the specific band corresponding to *estA* and *axe* were gel purified. The Figure 3.25 shows agarose gel electrophoresis of *estA* and *axe* amplicons, and the expected bands sizes were 820 bp and 1020 bp, respectively. Amplicons representing *axe* and *estA* were used for cloning. The cloning procedure was done by Dr Xiaowen Yang, Department of Molecular and Cell Biology/ University of Leicester.

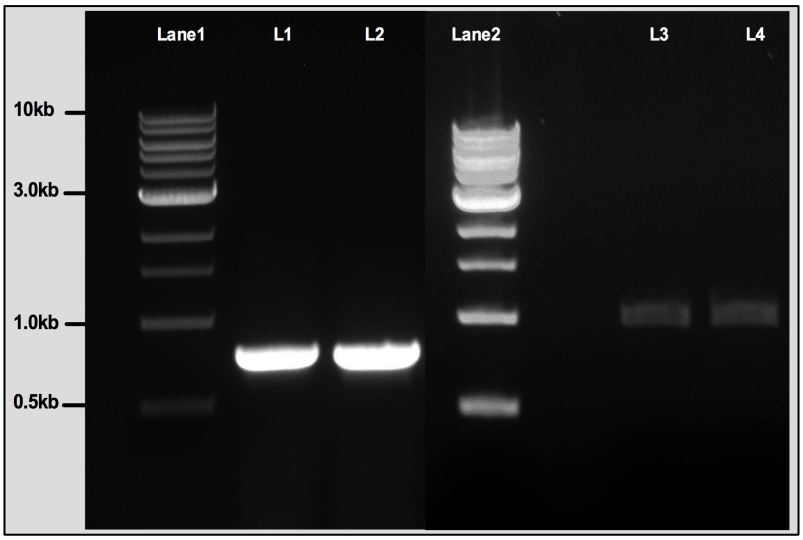

Figure 3.25: Agarose gel electrophoresis of PCR product for *estA* (820 bp) and *axe* (1020 bp). Lane 1 and Lane 2: 500 ng of 1kb DNA ladder (New England Biolabs); L1 and L2: *estA* amplicons. L3 and L4: *axe* amplicons.

After obtaining the recombinant plasmids, they were sequenced using plasmid based T7 primers (Table 2.22 in Materials and Methods). The sequence analysis confirmed that no mutation had occurred (Appendix 3). The recombinant plasmids were then transformed into *E. coli* BL21 DE for expression. The expression and purification protocol were provided in sections 2.12.3 and 2.12.4. The expression of *estA* and *axe* was induced using 0.5 mM IPTG at 25°C when the OD_{500} was between 0.4-0.6. The cultures were then allowed to grow for approximately 8-10 h until OD_{500} had reached 1.4-1.8. Finally, the cultures were harvested and re-suspended in binding buffer. The cell suspension was sonicated, and centrifuged to remove the pellet. The clear cell lysate containing His-tagged recombinant protein was applied through the TALON Metal Affinity resin. The undesirable *E. coli* proteins were then washed away with binding buffer. The successive elutions using 20, 40 and 100 mM imidazole buffer allowed the elution of the bound protein. The purified protein was analysed by SDS-PAGE, and the gel was stained with Coomassie Blue (Figure 3.26). The result showed the presence of protein bands with an approximate molecular weight of 31 kDa for EstA and 37 kDa for Axe, consistent with the expected hypothetical molecular weight of EstA and Axe (Figure 3.26).

Pneumococcal NanA was also expressed to investigate whether esterases could potentiate NanA activity. The construct for recombinant NanA protein was obtained from the laboratory stock, and it was expressed using the same protocol as EstA and Axe, as described in section 2.12.3. Figure 3.26 shows a clear band of the recombinant NanA with molecular weight of 108 kDa, which is consistent with the expected hypothetical molecular weight of NanA.

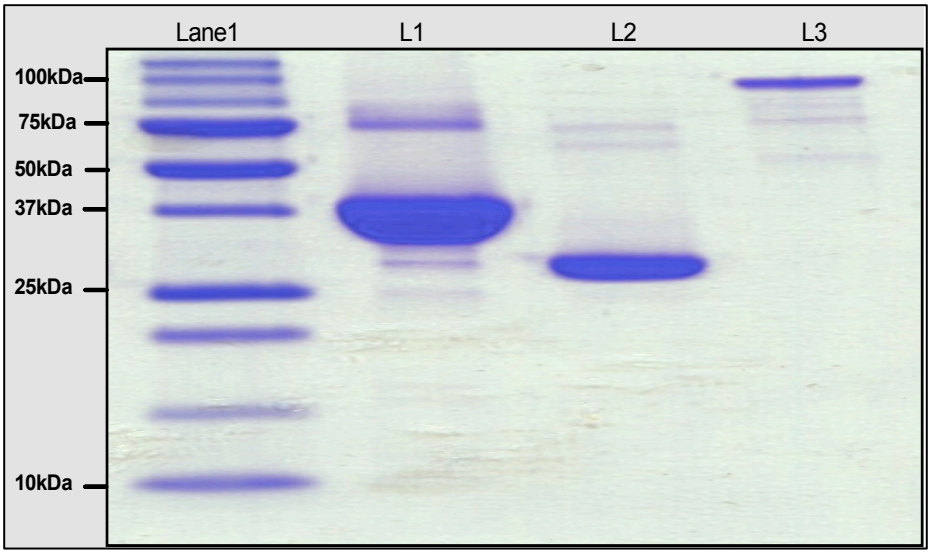

Figure 3.26: SDS-PAGE gel showing purified recombinant proteins eluted with 40 mM imidazole. Lane 1: Precision Plus protein standard ladder (Bio-Rad); L1: 50 μg of Axe (≅37kDa); L2: 25 μg of EstA (≅31kDa); L3: 15 μg of NanA (≅108kDa).

3.2.2 Production of genetically modified esterases:

In order to find out whether EstA and Axe are serine catalytic active enzymes, the recombinant mutant esterases EstA^{S121A} and Axe^{S181A} were cloned and expressed. As the complementation assay shown that modified copy of *estA*^{S121A} and *axe*^{S181A} could not restore wild type phenotype of esterases compared to complementation with intact copy of esterases, I concluded that the putative catalytic serine active site S^{121} and S^{181} for EstA and Axe, respectively, might be the catalytic active site. Therefore, the modified *estA*^{S121A} and *axe*^{S181A} were cloned and expressed for further study.

ΔestA^{S121A} and *Δaxe*^{S181A} were amplified from pCEP-estA^{S121A} and pCEP-axe^{S181A} (see section 3.1.5). The amplification of target genes utilised primers modified to include sites compatible with the pLEICS-01 expression vector to ease cloning (Table 2.22 in Materials and Methods). The amplicons were then cloned into pLEICS-01 plasmid and transformed into *E. coli* DH5α. The recombinant plasmids, pLEICS-01-estA and pLEICS-01-axe were purified and transformed into *E. coli* BL21 DE for protein expression. The recombinant modified esterases were expressed as described in sections

2.12.3 and 2.12.4. The purified proteins were then analysed by SDS-PAGE, and the gel was stained with Coomassie Blue. Figure 3.27 shows SDS-PAGE analysis of $EstA^{S121A}$ and Axe^{S181A}. As can be seen from the Figure, the size of recombinant esterases with an approximate molecular weight of 31 kDa for EstA, and 37 kDa for Axe was consistent with the expected hypothetical molecular weight of EstA and Axe (Figure 3.27).

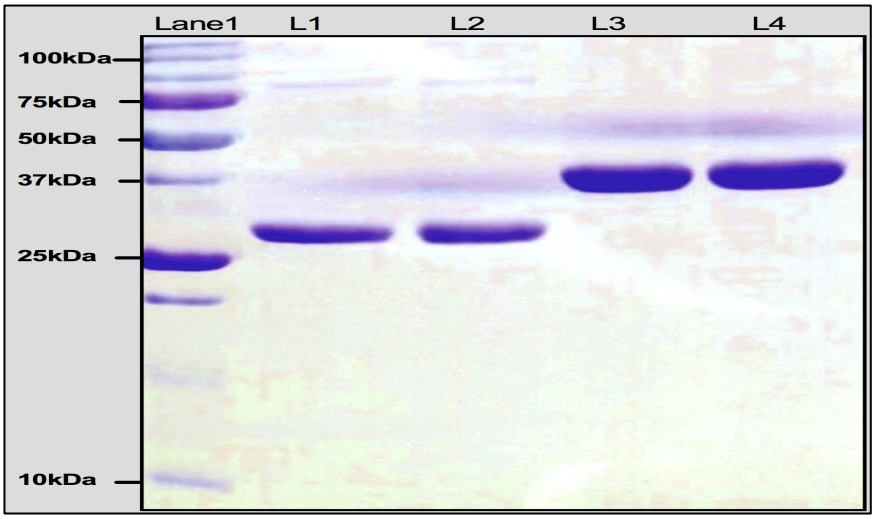

Figure 3.27: SDS-PAGE gel showing purified mutant recombinant proteins eluted with 40 mM imidazole. Lane 1: Precision Plus protein standard ladder (Bio-Rad); L1: 30 µg of $EstA^{S121A}$ (≅31kDa); L2: 40 µg of Axe^{S181A} (≅37kDa).

3.2.3 Quantification of recombinant proteins:

The quantification of recombinant proteins was achieved using the Bradford assay as described in Materials and Methods (section 2.13.2). The assay revealed that the purified recombinant proteins were at 7, 5.4, 5.5, 6.4 and 3.1 mg/ml, for EstA, Axe, $EstA^{S121A}$, Axe^{S181A} and NanA, respectively.

3.2.4 Western Blot assay for recombinant proteins:

In order to determine whether the purified recombinant proteins indeed contained the 6XHis-tag; Western blot analysis was done using anti-His tag antibody. 25 µg of purified recombinant EstA, $EstA^{S121A}$, Axe, Axe^{S181A}, and NanA were separated by

SDS-PAGE, and the proteins were electro-blotted onto nitrocellulose membrane. After overnight blocking, the membrane was incubated with anti-His tag antibody for 2 hours, and then with anti mouse IgG Fc specific alkaline phosphatase. The membrane was developed with BCIP/NBT for signal detection.

Figure 3.28 shows that the antibodies directed against His-tag reacted with the recombinant proteins, and the size of these bands were approximately 31 kDa for EstA and EstA^{S121A}, 37 kDa for Axe and Axe^{S181A}, and 108 kDa for NanA, indicating very strongly that these recombinant proteins had been successfully purified (Figure 3.28).

The definitive identity of the purified recombinant proteins were further verified using matrix-assisted laser desorption ionization-time of flight assay (MALDI-TOF). The results successfully showed a definitive match for all recombinant proteins (Appendix 4).

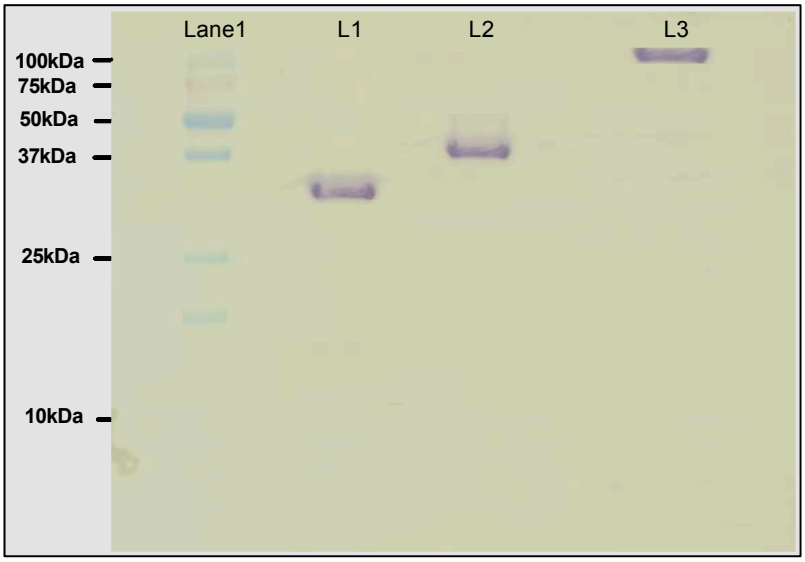

Figure 3.28: Western blot analysis of recombinant proteins. Lane 1: Precision Plus protein standard ladder (Bio-Rad); L1: EstA (≅31 kDa); L2: Axe (≅37 kDa); L3: NanA (≅108 kDa). All lanes contained approximately 10 μg of corresponding protein.

Chapter Four

Section I: Enzyme characterisation and kinetic assays:

4.1 Esterase activity on different synthetic substrates:

Esterases specifically hydrolyse substrates with a C_2-C_4 carbon chain length. However, the activity decreases upon increasing chain size, usually C_6 and above. The best examples of chromogenic substrates commonly used to assay esterase activity are p-nitrophenyl acetate (p-NPA) and p-nitrophenyl butyrate (p-NPB). While p-NPA is composed of a C_2 carbon chain ester, p-NPB has a C_4 carbon chain ester. These substrates have a phenolic compound called p-nitrophenol (p-NP), which is linked to the main carbon chain by ester bonds. p-NP is colourless when linked with the side ester chain. However, as soon as it is cleaved by esterases, it forms a yellow colour indicating that it is released from the substrate. Esterases are able to hydrolyse this linkage, and release p-NP. The amount of released p-NP can be measured spectrophotometrically, and is proportional to the enzyme activity present in the sample. One unit of esterase activity is defined as the amount of enzyme that produced 1 μmol p-NP per minute per milligram of protein under standard assay conditions.

4.1.1 Substrate specificity of recombinant esterases:

Esterases are able to hydrolyse short carbon chains of water-soluble substrates (Bornscheuer, 2002). Here, using cell free lysate, I demonstrated that the pneumococcal esterases could use p-NPA (C2) and p-NPB (C4) as substrates. Substrate specificity of recombinant EstA and Axe was also investigated to determine the full range of substrates that can be used by pneumococcal esterases.

The substrate specificity for EstA and Axe was determined using five chromogenic substrates, p-NPA (C2), p-NPB (C4), p-NPH (C6), p-NPO (C8) and p-NPD (C10) with different acyl chain length (Figure 4.1). The results showed a reverse correlation between the activity level and carbon chain length. The optimum activity of these esterases was against p-NPA, 426.1± 4.1 mU (n=6) and 164.3±3.19 mU (n=6) for EstA and Axe, respectively, whereas the minimum activity was against p-PND giving an activity level of 7.2±0.9mU (n=6) and 9.5±1.84mU (n=6) for EstA and Axe,

respectively. The difference in activity with these substrates relative to p-NPA was statistically significant (p<0.01) (Figure 4.1). These results show that EstA and Axe have activity for 2-6 carbon chain esters but the highest activity was obtained with p-NPA (2C length).

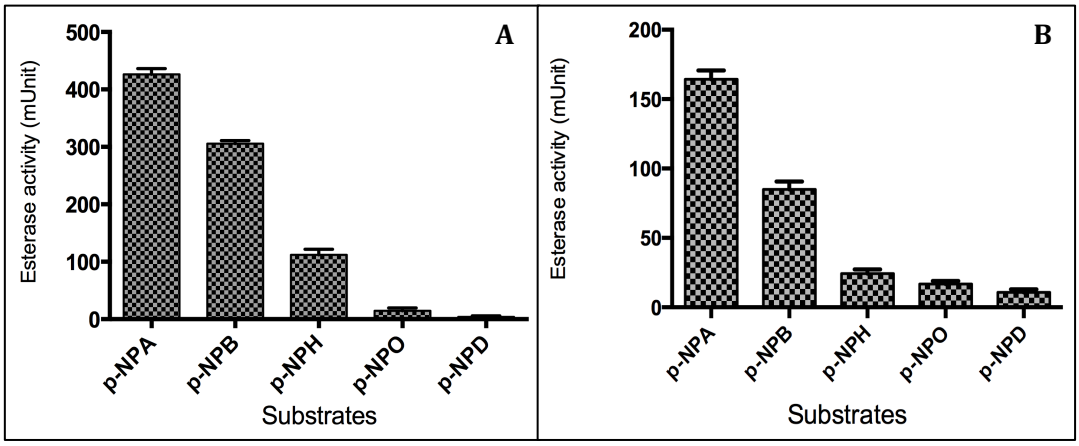

Figure 4.1: Activity of recombinant EstA (**A**) and Axe (**B**) against a range of chromogenic p-NP esters. One U of enzyme activity is expressed as micromole of p-nitrophenol released from the substrate per milligram of protein per minute. p-NPA: p-nitrophenyl acetate; p-NPB: p-nitrophenyl butyrate; p-NPH: p-nitrophenyl hexanoate; p-NPO: p-nitrophenyl octanoate; p-NPD: p-nitrophenyl decanoate.

4.1.2 Enzyme activity assays of modified recombinant esterases:

The esterase activity of modified recombinant esterases, $EstA^{S121A}$ and Axe^{S181A}, was also determined to investigate whether S^{121} and S^{181} are important for the catalytic activity of EstA and Axe, respectively. The modified recombinant enzymes were successfully expressed and purified as described in section 3.2.2. The putative serine active site of EstA and Axe was replaced by alanine using site directed mutagenesis as described in section 3.1.5. As before, the chromogenic substrate, p-NPA (C2), was used in this assay as EstA and Axe were shown to be most active against p-NPA.

The results showed that the replacement of S^{121} and S^{181} to alanine in EstA and Axe, respectively, abolished the catalytic activity. Figure 4.2 shows the activity of mutant recombinant esterases against p-NPA compared to wild type recombinant esterases. As can be seen from the Figure, when EstA and Axe were incubated with p-NPA, the total esterase activity was 433.5±7.4, (n=6) mU and 171.1±8.5, (n=6) mU, respectively. However, when $EstA^{S121A}$ or Axe^{S181A} were used, the total esterase activity was 13.62±1.5, (n=6) mU and 12.84±1.4, (n=6) mU, respectively. The difference was statistically significant (p<0.01). The activity of esterases was totally abolished in mutant recombinant esterases indicating that the putative serine active sites are responsible for EstA and Axe catalytic activity.

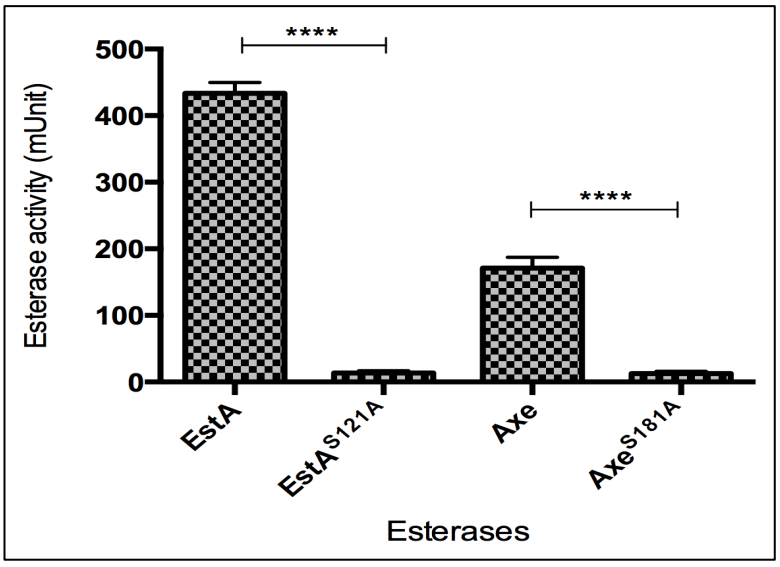

Figure 4.2: Total esterase activity of wild type and modified recombinant esterase using p-NPA as substrate. One U of enzyme activity is expressed as micromole of p-nitrophenol released from the substrate per milligram of protein per minute. ****:p<0.0001.

4.1.3 Kinetic characterisation of EstA and Axe:

This assay was done to determine whether these enzymes obey Michaelis Menten kinetics or not. Michaelis Menten kinetics are usually used to check enzyme affinity

dynamics and how fast the enzyme reacts with its substrate. It also indicates thesubstrate saturation point of an enzyme and the concentration of an enzyme required for optimal binding with its substrate. Two parameters can be determined in this assay: *Vmax* and *Km*. While *Vmax* is defined as the maximum velocity of an enzyme that can be achieved, *Km* is defined as the substrate concentration at which the reaction rate is half of *Vmax*.

The substrate p-NPA was identified to be the best substrate for esterase activity as demonstrated in section 3.3.5. Hence, it was selected for kinetic studies. Before doing enzyme kinetic assay, initially the best pH for EstA and Axe was determined. The enzyme was tested at a pH range from 3 to 8.5. The result showed that with increasing pH, the activity also gradually increased, peaking at pH 7.5, with a maximal activity of 443.6±1.0mU, (n=6) and 183.6±4.33mU, (n=6) for EstA and Axe, respectively. The activity then levelled off with further increase in pH, and then decreased at pH 8.5. The difference in activity at pH 7.5 and 8.5 was significantly different (p<0.01) (Figure 4.3). Hence, it was found that the best pH for recombinant esterases was 7.5.

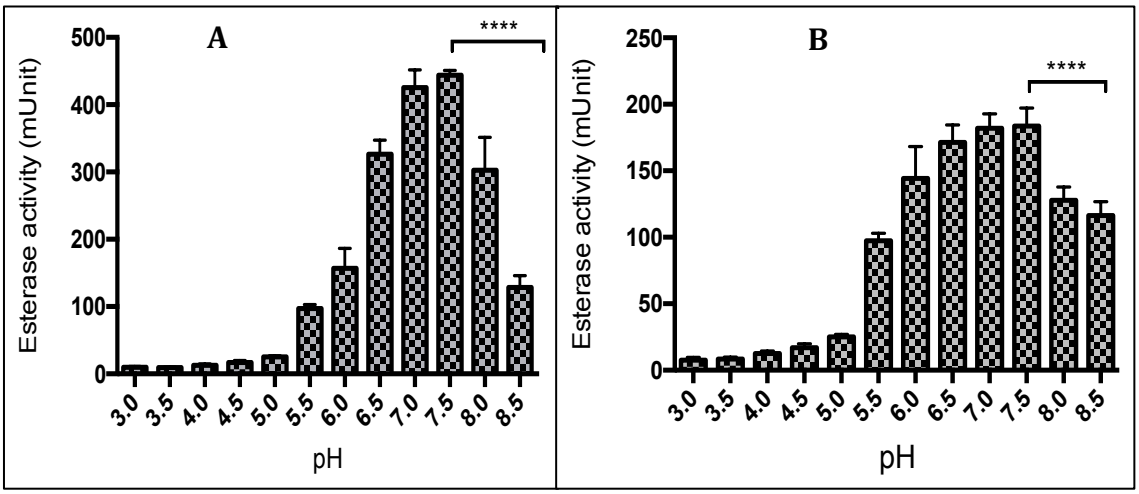

Figure 4.3: Determination of pH optima for esterases. Total esterase activity of EstA (**A**) and Axe (**B**) was determined at different pH. Two buffers were used: sodium citrate buffer with a pH range of 3.0 to 6.0, and sodium phosphate buffer with a pH range of 6.0 to 8.5 with 0.5 increments. ****: p<0.0001.

Next, the best enzyme concentration for the kinetic assay was determined. To do this, different concentrations of enzymes (15.625-, 31.25-, 62.5-, 125-, 500-, 750-, 1000 ng/ml) were tested at pH 7.5. The substrate concentration was constant at 0.01 M. 125 ng/ml of EstA and Axe were chosen as the best concentration for enzyme kinetics as above this concentration the activity of enzymes levelled off (data not shown).

Once the optimal pH and enzyme concentration were identified, *Vmax* and *Km* were determined as described in section 2.13.5. Calculations were performed using Graph Pad Prism software 6.0. Non-linear regression option was used to blot *Vmax* and *Km* for the enzyme (Figure 4.4). *Vmax* value of EstA was 364.1±13.9 mU and *Km* value was 4.7±0.63 mM, whereas *Vmax* value of Axe was 220±7.9 mU and *Km* value was 4.4±0.58 mM.

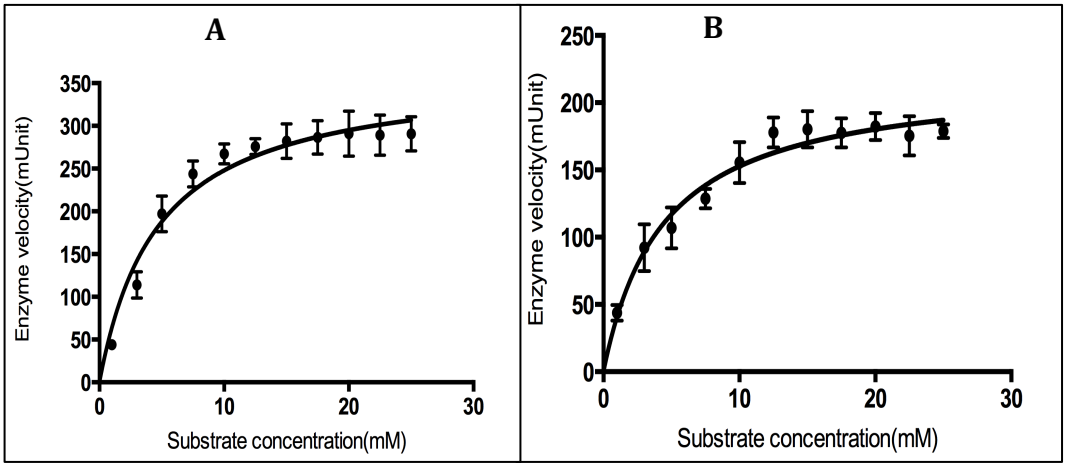

Figure 4.4: Determination of Michaelis Menten Kinetic parameters for EstA (**A**) and Axe (**B**) against p-NPA. Each datum point represents the mean of three independent experiments each with 5 replicates. *Km* and *Vmax* were calculated using non-linear regression using the program GraphPad Prism version 6.0 for Mac.

4.1.4 Enzyme activity assays using natural substrates:

The sequence database of *S. pneumoniae* annotated EstA (SPD_0534) and Axe (SPD_1506) as putative tributyrin esterase and acetyl xylan esterase according to conserved sequence, which aligned with known esterases. To determine whether these esterases are annotated correctly, I tried to test the activity of EstA and Axe using some natural substrates. These substrates contain ester bonds in different sites that can be specifically cleaved by certain esterases. To determine whether EstA and Axe could use tributyrin (triglyceride) and acetylated xylan as substrates, respectively, as the genome annotation suggested, the activities of EstA and Axe were determined against tributyrin and acetylated xylan. The tributyrin and acetylated xylan solutions were prepared as described in section 2.13.4. The qualitative and quantitative activity of esterases was determined by tributyrin plate assay, and spectrophotometric assay, respectively.

As shown qualitatively in Figure 4.5A, the recombinant EstA could use tributyrin as a substrate. A zone of clearance (24.5 mm) diameter, representing positive reaction, could be detected, and this was comparable to the zone of clearance, 26.8 mm, obtained with 50 µg of commercial lipase from *S. aureus*, which can also use tributyrin as a substrate. Figure 4.5B shows the reactivity of EstA in concentration dependent manner. The zone of clearance decreased when the concentration of EstA decreased. On the other hand, no activity with the $EstA^{S121A}$ or with Axe could be obtained against tributyrin. This result shows that EstA belongs to carboxylesterase family and that serine S^{121} is responsible for EstA catalytic activity. However, Axe could not use tributyrin as a substrate, thus it does not belong to carboxylesterase. Carboxylesterases are one of esterase family that can cleave ester bonds between fatty acids of triglycerides (Arpigny and Jaeger, 1999, Zhang et al., 2005).

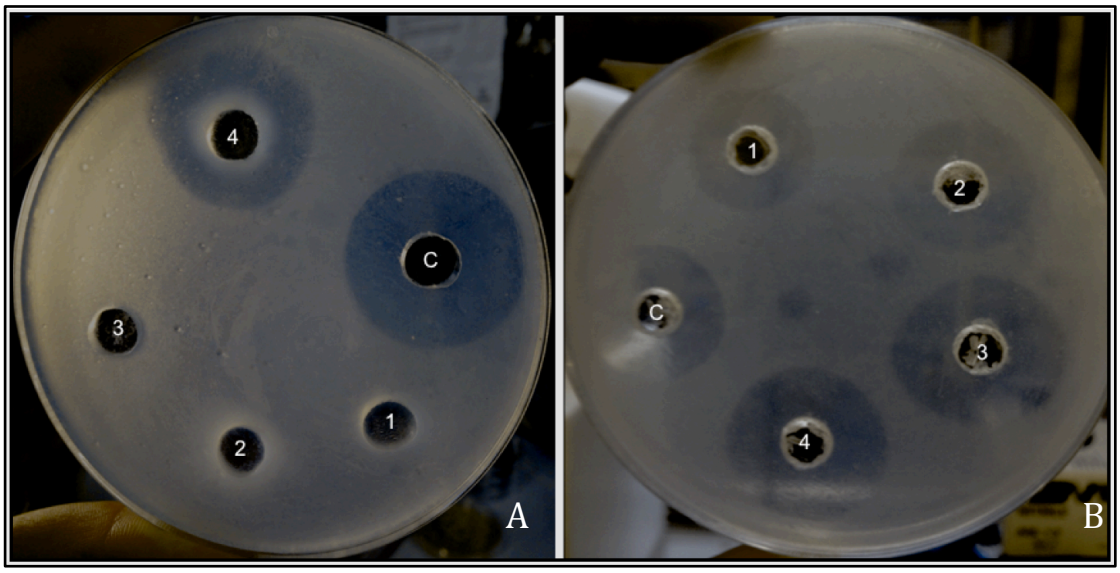

Figure 4.5: Reactivity of recombinant enzymes in tributyrin plate assay. **A**: 1: 50 µg EstA; 2: 50 µg EstA^{S121A}; 3: 50 µg Axe; 4: 50 µg Axe^{S181A}; C: 50 µg commercial lipase. **B**: Reactivity of recombinant EstA at different concentrations. 1: 20 µg; 2: 30 µg; 3: 40 µg; 4: 50 µg; C: 50 µg commercial lipase (Sigma). The assay was repeated 3 times and a representative image is shown.

The specific activity of Axe and EstA against tributyrin was also measured quantitatively using a spectrophotometric assay. The decrease in absorbance from cloudy emulsified to clear substrate indicated that enzymes are able to use emulsified tributyrin as substrate. The decrease in absorbance of reaction mixture was measured as esterase activity. Commercial lipase was included as positive control as it can use tributyrin as substrate (Martinez-Martinez et al., 2007). Figure 4.6 shows the decrease in absorbance at 450 nm for recombinant esterases-tributyrin reaction at different time points. The absorbance of the reaction mixture significantly decreased when tributyrin solution was incubated with EstA compared to negative control, in which no change in absorbance was detected (p<0.01). As before, Axe did not show any activity towards tributyrin (Figure 4.6).

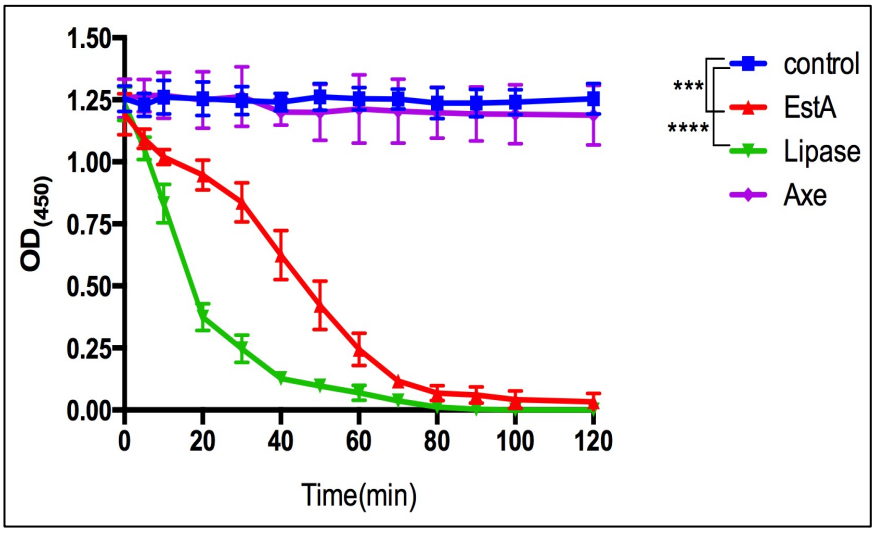

Figure 4.6: The activity of recombinant esterases on tributyrin. The substrate solution was prepared in 100 mM Tris pH 7.5, 25 mM $CaCl_2$, and 0.5% (v/v) tributyrin. $OD_{(450)}$ was recorded over 120 min with 20 min intervals. 50 µg of each enzyme was used. The control was set as no enzyme reaction. Each line represents the mean of data from 6 independent experiments each with 3 technical replicates. ***: $p<0.001$; ****: $p<0.0001$.

Enzyme activity was quantified in comparison to a standard curve generated with the known concentrations of commercial lipase (Figure 4.7). It was found that EstA had 198.3±1.7 U (n=6) against tributyrin, where one unit was defined as the amount of enzyme in µg that can liberate 1 µmol of glycerol from triglyceride per min. However, no activity could be seen when Axe was incubated with tributyrin solution.

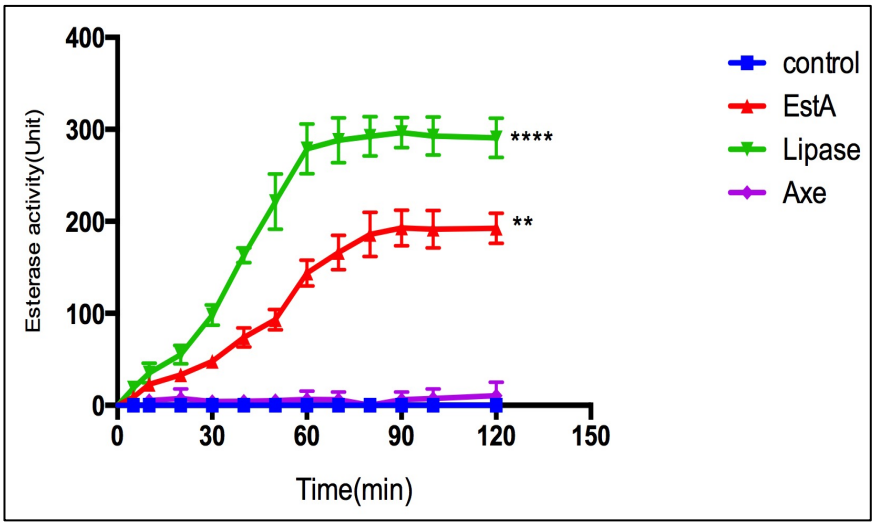

Figure 4.7: Esterase activity assay using tributyrin (triglyceride) as substrate. The enzyme activity is expressed as the amount of enzyme in µg that can liberate 1 µmol of glycerol from triglyceride per min. Each line represents the mean of data from 6 independent experiments each with 3 technical replicates. **: $p<0.01$; ***: $p<0.001$ compared to control (no enzyme).

The specific activity of EstA and Axe against acetylated xylan was also determined. The decrease in absorbance at $OD_{(616)}$ was recorded when the substrate solution containing BTB was incubated with either EstA or Axe. Figure 4.8A shows the change in absorbance of reaction mixture set up with different EstA and Axe concentrations. As can be seen from the Figure, there is an inverse relationship between the absorbance and the EstA and Axe concentration. The decrease in absorbance is clearly evident in Figure 4.8B. These results show that both esterases could release the *O*-acetyl group from acetylated xylan.

Furthermore, in order to find out whether S^{121} and S^{181} in EstA and Axe, respectively, are responsible for the catalytic activity of esterases to utilise acetylated xylan, the genetically modified recombinant esterases, $EstA^{S121A}$ and Axe^{S181A}, were incubated

with acetylated xylan substrate. It was noticed that replacement of serine active site of EstA and Axe with an alanine residue abolished the catalytic activity of both recombinant esterases (Figure 4.8A).

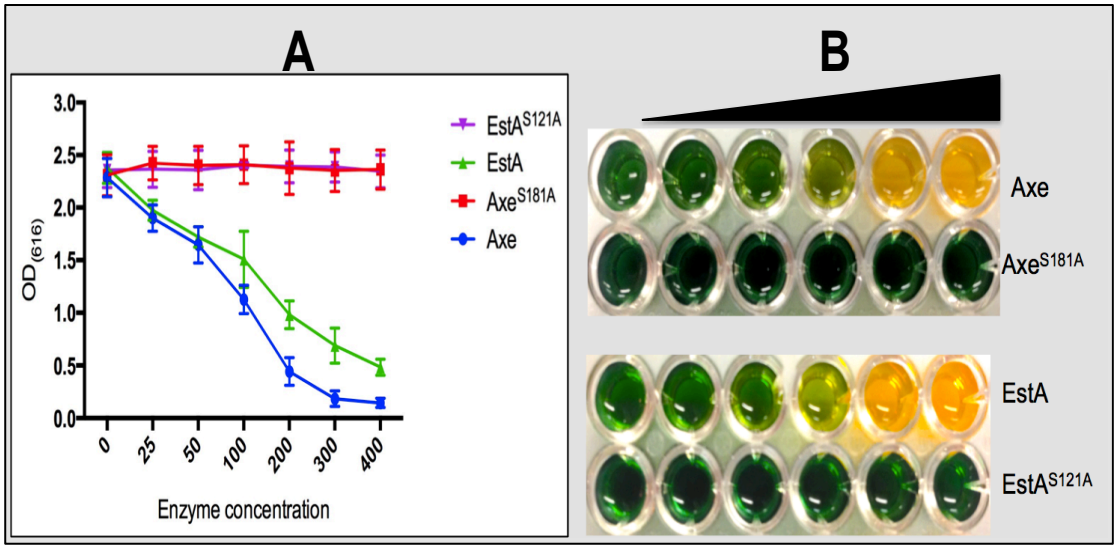

Figure 4.8: The catalytic activity of recombinant esterases on acetylated xylan as substrate. The substrate solution was prepared in 5 mM sodium phosphate buffer pH 7.3, 0.01% (w/v) BTB, and 30 mM acetylated xylan. Absorbance at $OD_{(616)}$ was recorded at regular intervals. 50 µg of each enzyme was used. **A:** the change in absorbance for different concentration of enzymes at 616 nm. **B:** reactivity of esterases and acetylated xylan. Each line represents the mean of data from 6 independent experiments each with 3 technical replicates.

To quantify the activity of EstA and Axe against acetylated xylan, the amount of released acetate from acetylated xylan, was plotted against the known concentration of acetate. The activity of esterases was measured as µmol of acetic acid produced per minute under the standard reaction conditions. Figure 4.9 shows total esterase activity of EstA and Axe for acetylated xylan as the substrate. As can be seen from the Figure,

there is a positive correlation between esterase concentration and the amount of acetate released. For example, when 400 mU of enzyme was used, the amount of released acetate was 15.5±0.67 µmol and 16±0.6 µmol for EstA and Axe, respectively. However, when 25 mU of the enzyme was used, the amount of released acetate was 2.4±0.23 µmol and 2.8±0.33 µmol for EstA and Axe, respectively (Figure 4.9).

The results show that EstA can use both tributyrin and acetylated xylan as substrate; therefore, it was designated as tributyrin esterase (EtsA). On the other hand, Axe could only use acetylated xylan as the annotation suggested, and so was designated as acetyl xylan esterase (Axe).

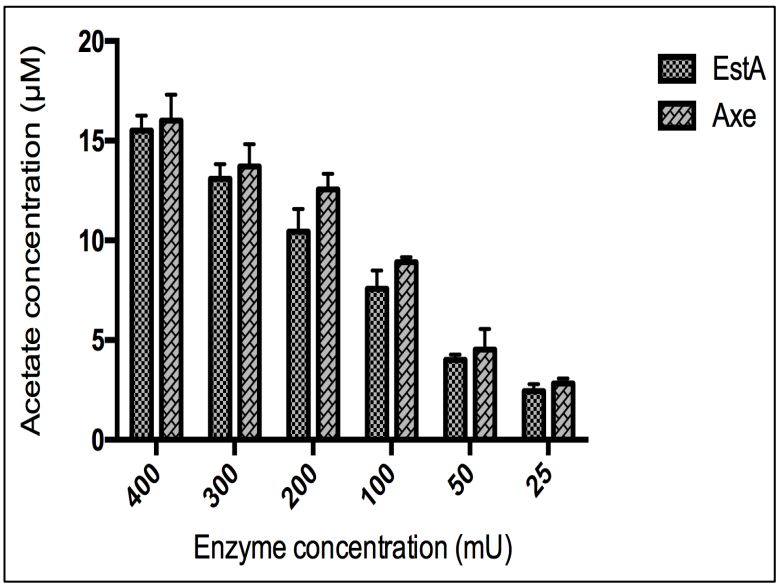

Figure 4.9: Concentration dependent esterase activity using acetylated xylan as the substrate. The assay was set up at pH 7.3 and different concentrations of enzyme used. The amount of released acetate in the reaction mixture was measured by using a standard curve generated with known concentrations of acetate. Each column represents the mean of data from 6 independent experiments each with 3 technical replicates.

4.1.5 Enzyme activity assays using BSM as an organic substrate:

O-acetylation of terminal sialic acids occurs naturally, and this may limit the cleavage rate of sialic acid from glycoconjugates by pneumococcal neuraminidases. It is the hypothesis of this thesis that esterases potentiate neuraminidase activity by removing O-acetylation. To test this hypothesis, bovine sub-maxillary mucin (BSM) was used as a substrate because BSM contains up to 17% sialic acid, and 22.5% of this is reported to be acetylated (Pal et al., 2000). Hence, BSM is a suitable organic substrate for esterases. *O*-acetyl groups are cleaved by esterases by removal of *O*-acetyl groups.

Initially, esterases' activity on BSM was assayed by detection of acetate released from the substrate. To determine time dependent acetate release by EstA and Axe, 50 μg of each recombinant enzyme was incubated with 20 mg/ml BSM for 30, 60, 90 and 120 min. In line with the recommendation of the acetate detection kit's manufacturer the longest incubation period in this assay was chosen as 120 min. The results showed that acetate release increased in a time dependent manner. Figure 4.10A and 4.10B show acetate released from BSM in the presence of EstA and Axe, respectively. It was found that with increasing incubation time, higher acetate, hence esterase activity, release could be detected. The released acetate concentration at 120 min was significantly higher at 120 min than 30, 60 and 90 min ($p<0.01$).

In addition, to find out whether S^{121} and S^{181} are responsible for the catalytic activity of EstA and Axe on BSM, respectively, the activity of mutant recombinant esterases, $EstA^{S121A}$ and Axe^{S181A}, was determined. The mutant esterases were incubated with BSM for 120 min and the released acetate was checked as above. The results showed that the replacement of S^{121} and S^{181} to alanine abolished the catalytic activity of EstA and Axe, respectively. Figure 4.11 shows the activity of mutant recombinant esterases on BSM compared to wild type recombinant esterases. As can be seen from the Figure, when EstA and Axe were incubated with BSM, the concentration of released acetate was 112.5±8.9 and 107±5.5 μg/ml, respectively. However, when $EstA^{S121A}$ and Axe^{S181A} were used for the assay, the amount of released acetate was 13±1.5 and 12.8±2.4 μg/ml, respectively. The difference was statistically significant ($p<0.01$).

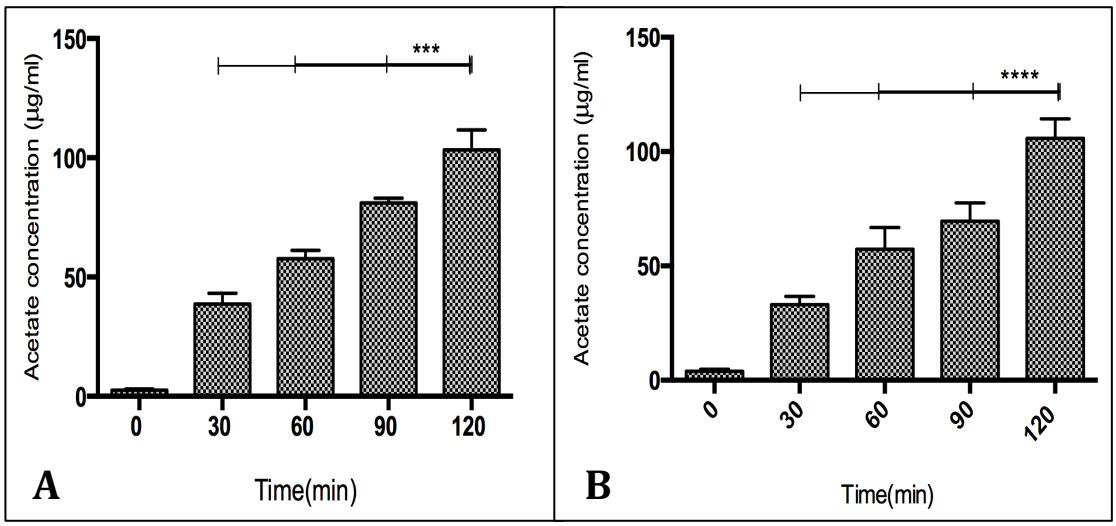

Figure 4.10: Time dependent EstA (**A**) and Axe (**B**) activity using BSM as the substrate. The assay was set up at pH 7.5, and 50 µg enzyme was used for each reactions. The amount of released acetate was measured using a commercial kit (Megazyme acetic acid detection kit, Ireland). Each column represents the mean of data from 6 independent experiments each with 3 technical replicates. ***: $p<0.001$; ****: $p<0.0001$.

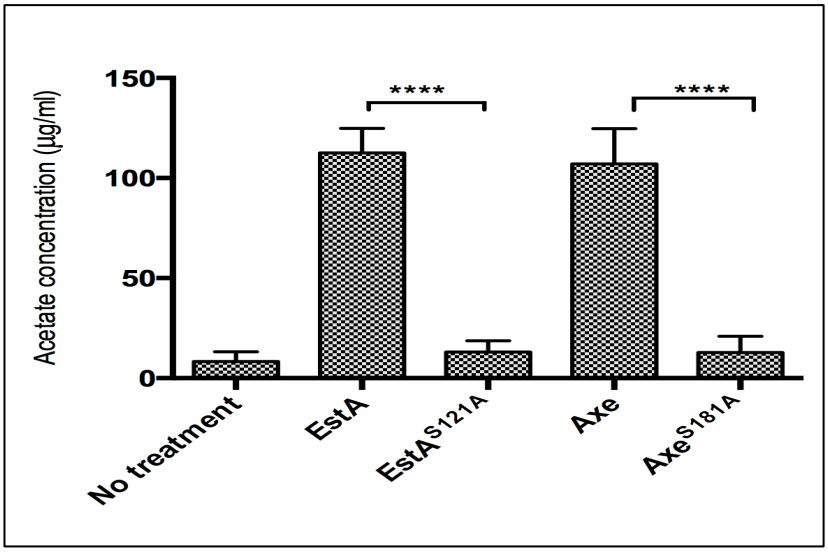

Figure 4.11: The activity of wild type and mutant recombinant esterase using BSM as the substrate. The assay was set up at pH 7.5 and 50 µg recombinant enzyme was used for each reaction. The amount of acetate was measured using a commercial kit (Megazyme acetic acid detection kit, Ireland). Each column represents the mean of data from 6 independent experiments each with 3 technical replicates. ****; $p<0.0001$.

4.1.6 Esterases potentiate neuraminidase activity:

After establishing the acetate release from BSM by EstA and Axe, I investigated whether the pre-treatment of BSM by esterases would increase the release of sialic acid by neuraminidase. As the removal of *O*-acetylation from terminal Sia unmasks the substrate and boosts the activity of neuraminidase A, the release of Sia from Bovine sub-maxillary mucin (BSM) is expected to be higher when the substrate is pre-incubated with esterases.

In this assay, BSM was pre-treated first with 50 µg of each recombinant esterase for different time intervals, and then incubated further with 50 µg of recombinant NanA for a same period as the incubation with recombinant esterase. The substrate solution was prepared according to the procedure described in section 2.13.7. Sialic acid released from BSM was measured as described in section 2.14 after centrifugation of the reaction mixture to separate free sialic acid from bound sialic acid (BSM).

This assay proved that esterases could potentiate the activity of NanA by cleaving higher level of Sia following pre-treatment of BSM with recombinant esterases. As can be seen in Figure 4.12, when BSM was pre-treated either with EstA or Axe and then treated with NanA, the level of sialic acid was significantly higher than NanA treatment alone ($p< 0.01$). As note, the treatment of BSM either with EstA or Axe alone did not release sialic acid, and the level of released sialic acid was the same as negative control that did not contain any enzyme (no treatment), indicating that increased release of sialic acid following pre-treatment with esterases was due to the removal of *O*-acetylation, and subsequent cleavage of sialic acid by NanA.

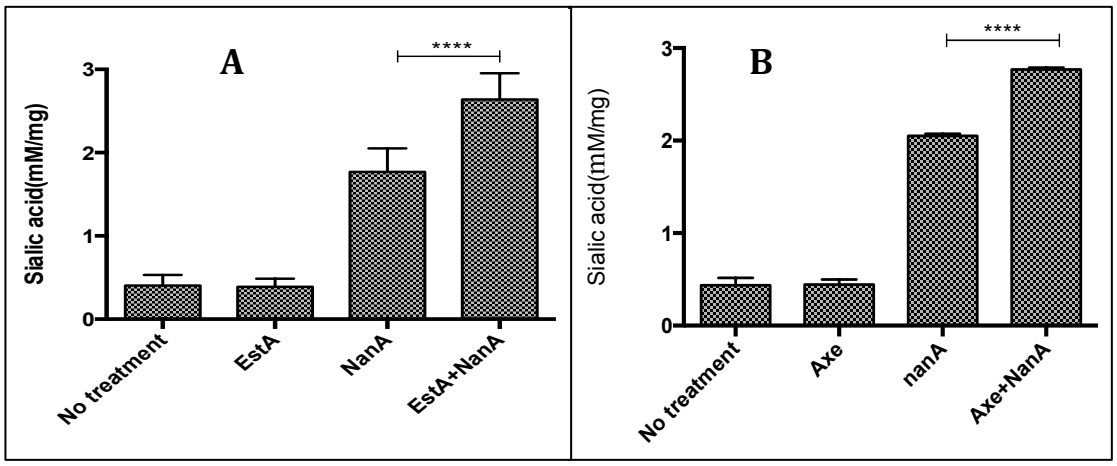

Figure 4.12: EstA (**A**) and Axe (**B**) potentiate NanA activity for cleavage of sialic acid from BSM. The substrate was pre-treated with 50 µg of recombinant esterases for 120 min, and then further incubated with 50 µg of recombinant NanA for 120 min. Each column represents the mean of data from 6 independent experiments each with 3 technical replicates. ****: $p<0.0001$ compared to NanA treatment. Sialic acid was measured as mmol of sialic acid per mg of BSM.

Figures 4.13A and 4.13B show the time dependent EstA and Axe potentiation of neuraminidase activity. When BSM was pretreated with EstA or Axe and then treated with NanA, the released sialic acid was significantly higher than sialic acid release with NanA alone in a time dependent manner. For example, after 120 min incubation (Figure 4.13), the amount of sialic acid released by NanA was 1.8±0.4 mmol/mg (n=6), whereas the sialic acid concentration was 2.9±0.3 mmol/mg and 2.7±0.4 mmol/mg (n=6) when BSM had been pre-treated either with EstA and Axe, respectively. The difference was clearly significant ($p<0.01$). The result was consistent with the hypothesis that the efficient removal of *O*-acetylation would potentiate NanA activity.

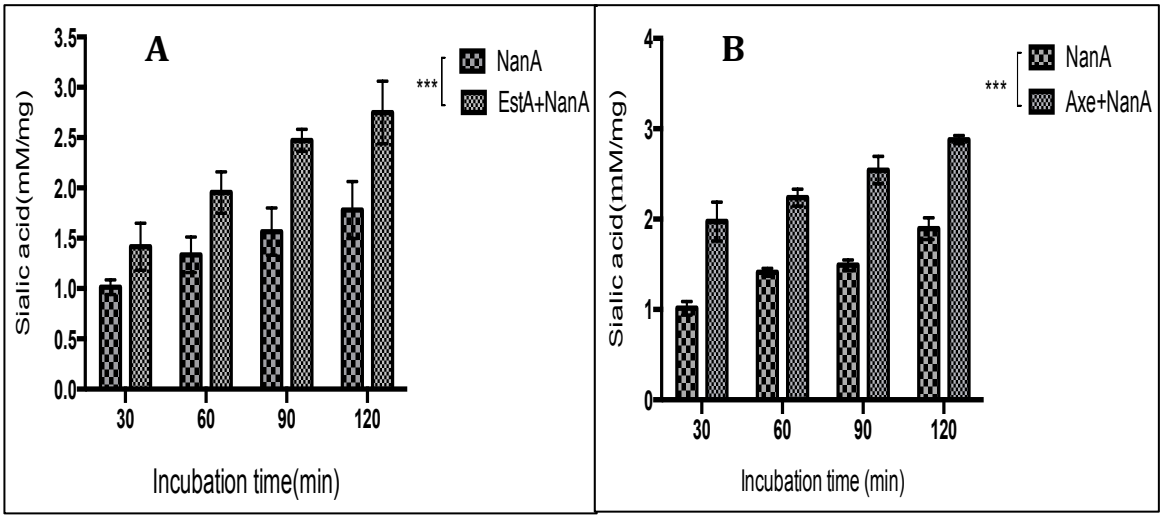

Figure 4.13: Pre-treatment of BSM with either EstA or Axe potentiates NanA activity. The substrate was pre-treated with 50 µg of each esterase in different time points, and then with 50 µg NanA for the same time as the esterase treatment. Each column represents the mean of data from 6 independent experiments each with 3 technical replicates. ***: p<0.001compared to NanA treatment. Sialic acid was measured as mmol of sialic acid per mg of BSM.

In addition, the role of S^{121} and S^{181} in esterases' augmentation of NanA activity was investigated. $EstA^{S121A}$ and Axe^{S181A} were pre-incubated with BSM for 120 min and then incubated subsequently with recombinant NanA for 120 min. Figure 4.14 shows the released sialic acid by NanA from BSM pre-incubated with genetically modified esterases compared to wild type recombinant esterases. As can be seen from the Figure, when BSM was pre-treated with genetically altered $EstA^{S121A}$ and Axe^{S181A}, the sialic acid release by NanA was same as that by NanA alone (p>0.05) indicating the importance of S^{121} and S^{181} residues in EstA and Axe, respectively (Figure 4.14).

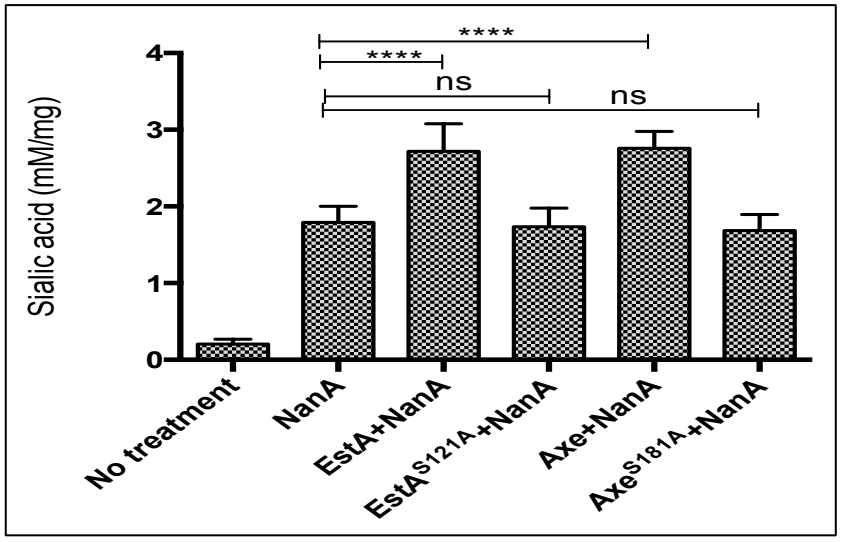

Figure 4.14: The role of serine residues in the catalytic activity in esterases' potentiating role in release of Sia by NanA from BSM. The substrate was pre-treated with 50 µg of esterases for 120 min and then with 50 µg of NanA for 120 min. Each column represents the mean of data from 6 independent experiments each with 3 technical replicates. ****: p<0.001 compared to NanA treatment alone. ns: not significant.

4.1.7 Identifying the subcellular localisation of esterases:

This assay was done to determine the subcellular localisation of EstA and Axe. D39 cellular fractions were prepared as described in section 2.16. The fractions were run on SDS-PAGE gel and electro-blotted on nitrocellulose membrane. After blocking, the membrane was incubated with polyclonal antibody raised against recombinant EstA or Axe as described in section 2.15. In this assay, recombinant EstA and Axe were used as control.

Figure 4.15 shows the blotted pneumococcal cell free fractions on nitrocellulose membrane hybridised with polyclonal EstA and Axe antibodies. As can be seen from the Figure, the polyclonal antibodies reacted strongly with the recombinant EstA and Axe. In addition, the antibodies reacted well with intracellular fractions, whereas there was very weak or no reaction between the antibody and cell membrane and cell wall

fraction. In addition, no reaction was detected with culture supernatant indicating the low concentration of esterases that can be secreted. It can be concluded that the likely localisation of EstA and Axe are intracellular, or in the interior cell membrane.

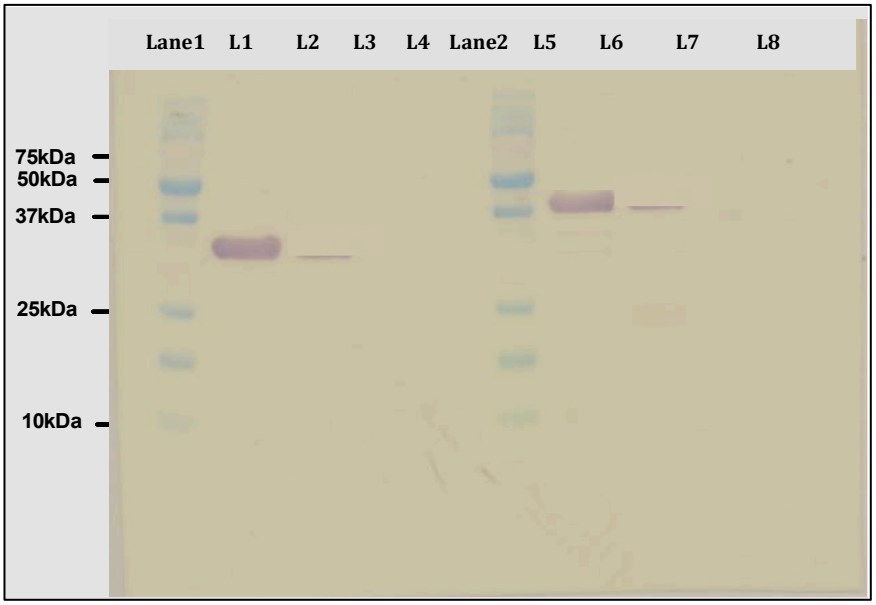

Figure 4.15: Determination of esterases' subcellular localisation in D39 using western blot. The membranes were incubated with polyclonal antibody, which had been raised against Axe and EstA in mice, for 1 h, and were incubated with secondary anti-FC mouse IgG antibody for 1 h. The bands were visualised by NCIP/NBT developing solution. Lane 1: Precision protein ladder; L1 and L5: 20 µg of recombinant EstA (≅31kDa) and Axe (≅37kDa), respectively; L2 and L6: intracellular fraction; L3 and L7: membrane fraction; L4 and L8: cell wall fraction.

Total esterase activity of subcellular fractions was determined to check the efficiency of cell fractionation. Figure 4.16 shows the total esterase activity of subcellular fractions. As can be seen from the Figure, total esterase activity of intracellular fraction was (51.9±2.5 mU, n=6) significantly higher than cell wall fraction (4.2±0.56 mU, n=6) and cytoplasmic membrane fraction (9.8±1.4 mU, n=6) (p<0.01). It was found that the

major activity of esterase was in intracellular cytoplasmic fraction, in line with the western blot results. In addition, esterase activity was also detected in culture supernatant of *S. pneumoniae* (18.1± 1.3 mU, n=5), indicating that pneumococcal esterases can be released outside cells (Figure 4.16).

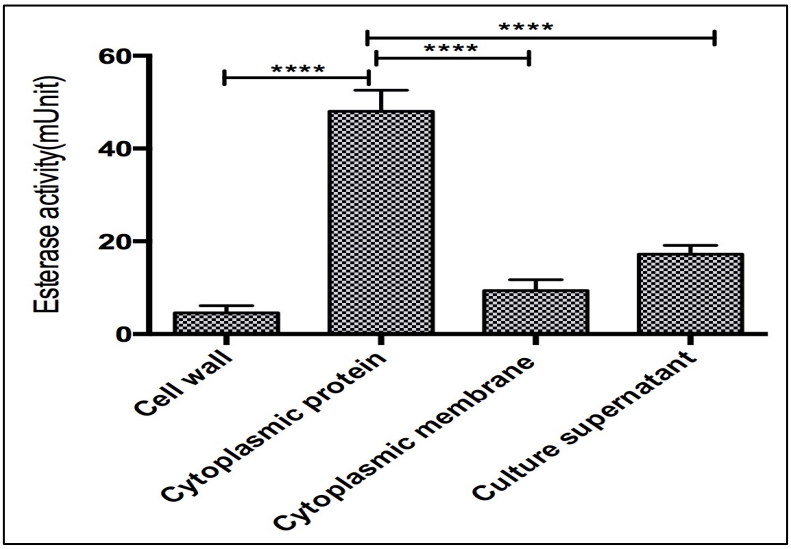

Figure 4.16: Total esterase activity in subcellular fractions of pneumococcal cell lysate and culture supernatant using p-NPA as substrate. The enzyme activity was expressed as micromoles of p-nitrophenol released from the substrate per microgram of protein per min. Each column represents the mean of data from 6 independent experiments each with 3 technical replicates.

Section II: *Ex vivo* assay and growth profiles

4.2 Growth profiles of isogenic mutants:

Having established the substrate specificity of esterases and their potentiating role for NanA through *O*-acetylation removal, next, I investigated whether esterases could potentiate NanA's activity in intact pneumococcal cells. For this, I used genetically a modified strains defective in EstA, Axe and their derivatives in combination with NanA mutant as well as parental strain. These strains were grown on CDM supplemented with BSM, and growth profiles and sialic acid release after growth were determined.

4.2.1 Growth profiles of esterase isogenic mutants on BHI:

To find out whether the deletion of esterase genes attenuate pneumococcal growth in BHI, esterase isogenic mutants were grown in BHI micro-aerobically, and the growth was recorded for 20 h at $OD_{(600)}$. Figure 4.17 shows the growth profiles of pneumococcal strains grown in BHI, which shows that the mutants could grow as well as the wild type, indicating that the mutation of esterase genes can be tolerated on rich medium.

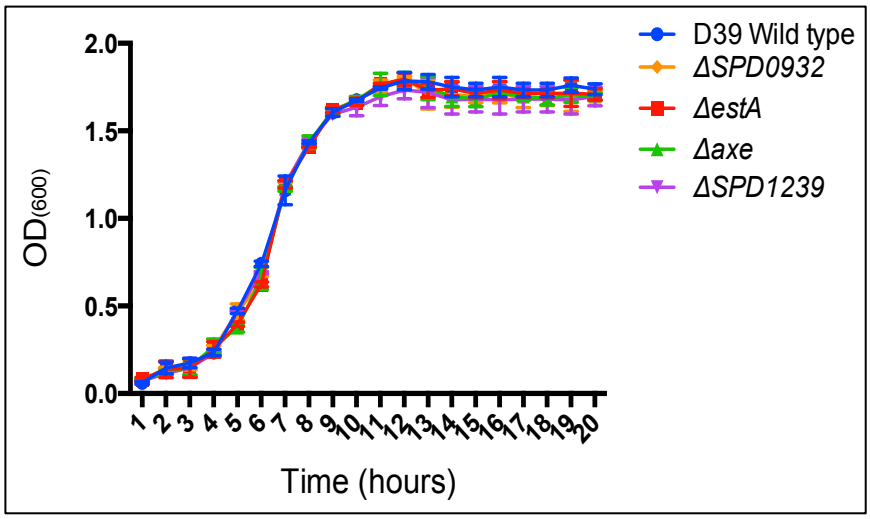

Figure 4.17: Growth profile of esterase isogenic mutants and D39 wild type on BHI. Each point is the mean of three independent tests in triplicates, and the vertical line represents the standard error of mean.

4.2.2 Analysis of BSM utilisation by the pneumococcus:

The pneumococcal ability to grow on chemical defined medium supplemented with minimal requirements of carbon source mimics the normal growth of the pneumococcus in the respiratory tract. The respiratory tract contains bound sugars within the mucin. The acquisition of these sugars as carbon source requires break down of host mucin.

As shown in *in vitro* enzyme assays, both esterases potentiate NanA activity as evidenced by increased release of sialic acid after pre-treatment of BSM with either EstA or Axe compared to NanA treatment alone. Therefore, the logical next step was to determine whether EstA and Axe would work in concert to enhance pneumococcal utilisation of BSM. I determined whether the interaction between esterases, EstA and Axe, and NanA is important for pneumococcal utilisation of BSM as the sole carbon source. For this, I compared the growth profiles of single mutants *ΔestA*, *Δaxe* and *ΔnanA* to that of double mutants, *ΔestAnanA* and *ΔaxenanA* on BSM as the sole carbon source. In addition to growth, the released sialic acid in culture supernates was determined. This was possible because D39 is defective in utilisation of sialic acid because the sialic acid transporter in D39 is naturally mutated (Bidossi et al., 2012, Hoskins et al., 2001); hence any released sialic acid remains in culture supernatant.

Initially, the growth profile of D39 strain was performed in CDM supplemented with different concentrations of BSM, 0.5%, 0.25%, 0.166% and 0.071% (w/v), to identify the best BSM concentration for the assay. The growth was measured by quantifying CFU after 0, 1, 3, 6, 12 and 24 h of growth. Figure 4.18 shows the growth profile of D39 wild type in different concentrations of BSM. As can be seen from the Figure, when 0.5% (w/v) and 0.25% (w/v) of BSM were used, the pneumococcus could not survive, and the CFU count decreased significantly, indicating that at these concentrations BSM is toxic to *S. pneumoniae*. On the other hand, at 0.166% (w/v) of BSM, the CFU count increased approximately 1 Log_{10} after 6 h growth. The highest increase in CFU count was on 0.071% (w/v) of BSM (3 Log_{10} after 6 h). Therefore, the best concentration of BSM was 0.071% (w/v) mucin for pneumococcal growth. It is worth mentioning that the CDM without BSM does not support pneumococcal growth, and the pneumococcus started to die gradually over 12 h incubation time.

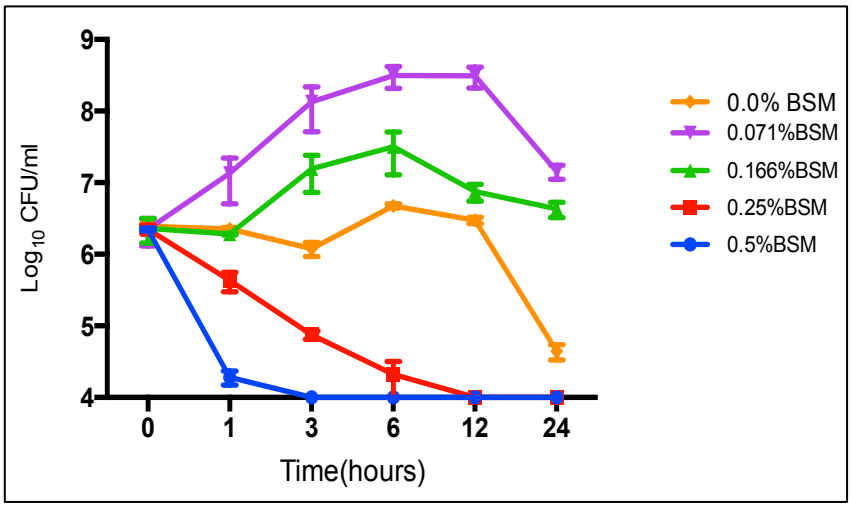

Figure 4.18: Growth profile of D39 wild type in CDM supplemented with different concentrations of BSM as carbon source. Each point is the mean of three independent tests in triplicates and the vertical line represents the standard error of mean.

The growth was then performed for the isogenic mutants using the optimal concentration of BSM as sole carbon source for the pneumococcal strains. *ΔestA*, *Δaxe*, *ΔnanA,* *ΔestAnanA*, *ΔaxenanA*, *ΔestAaxe*, estAComp, axeComp, estAComp^{S121A} and axeComp^{S181A} were included in the growth assay. Figures 4.19 and 4.20 show the growth of pneumococcal strains in CDM supplemented with 0.71% (w/v) of BSM. As can be seen from the Figure 4.19, after 6 h growth the colony counts for *ΔestA* (Log_{10} 7.2±0.27 CFU/ml) and estAComp^{S121A} (Log_{10} 7.7±0.55 CFU/ml) was significantly lower than that of wild type (Log_{10} 9.1±0.87 CFU/ml) and estAComp (Log_{10} 8.85±0.34 CFU/ml) (p<0.01). There was no significant difference in growth profiles of D39 and estAComp (p>0.05). The major attenuation in growth, however, was seen between D39 wild type and *ΔestAnanA* as the Log_{10} CFU/ml of double mutant decreased progressively during the time course of growth. Furthermore, the results have shown that the replacement of S^{121} to alanine for estAComp^{S121A} reduced pneumococcal ability to utilise BSM as sole carbon source. On the other hand, *Δaxe* grew as well as the wild type, and after 6 h growth; there was no difference in bacterial counts between wild type D39 and *Δaxe* (p>0.05).

Moreover, there was no difference in bacterial counts of *ΔaxenanA* and *ΔnanA* (Figure 4.20). The attenuation in *ΔaxenanA* was, therefore, due to the mutation of *nanA*. The results show that EstA has a role in pneumococcal growth, while Axe does not show any contribution in this experimental system.

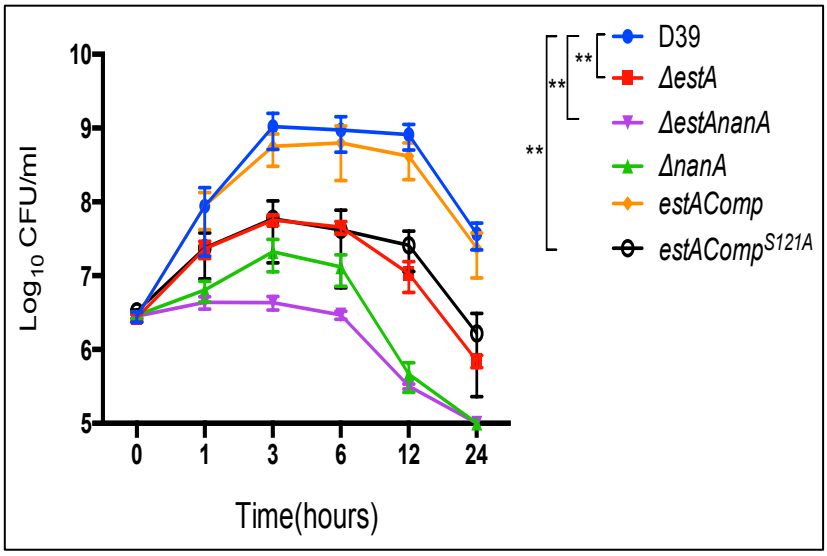

Figure 4.19: Growth curve of *ΔestA* and its derivatives in D39 background in CDM supplemented with 0.071% (w/v) of BSM as carbon source. Each point is the mean of three independent tests in replicates and the vertical line represents the standard error of mean. **: $p<0.001$.

The double *ΔestAaxe* mutant was also tested in CDM supplemented with BSM, and the growth was compared with *ΔestA* and *Δaxe* as well as the wild type. Figure 4.21 shows the growth profile of *ΔestAaxe* compared with either *ΔestA* or *Δaxe*. As can be seen from the Figure, at 6 h incubation time, the growth profile of *ΔestAaxe* (Log_{10} 6.84±0.91 CFU/ml) was not significantly lower than *ΔestA* (Log_{10} 7.42±0.31 CFU/ml) ($p>0.05$), indicating that Axe is not involved in pneumococcal growth on BSM.

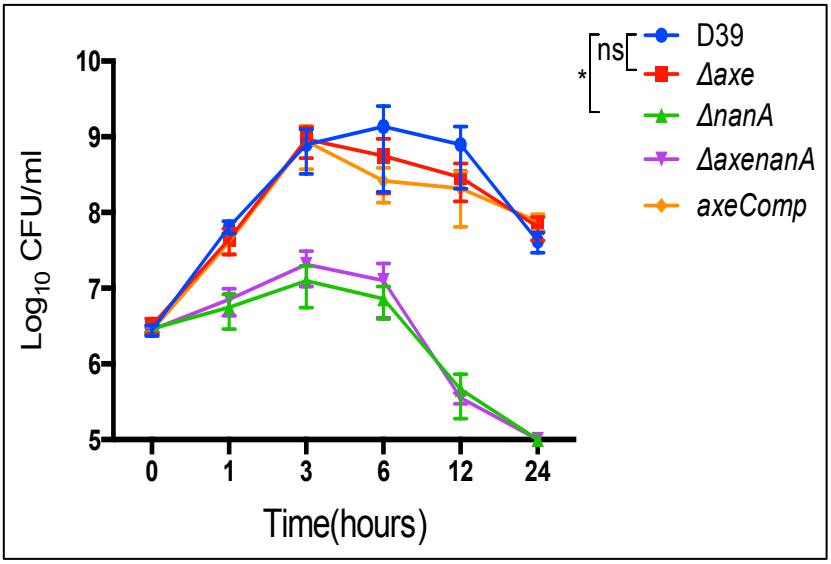

Figure 4.20: Growth curve of *Δaxe* and its derivatives in D39 background in CDM supplemented with 0.071% (w/v) of BSM as carbon source. Each datum point is the mean of three independent tests in replicates and the vertical line represents the standard error of mean. *: $p<0.01$. ns: not significant.

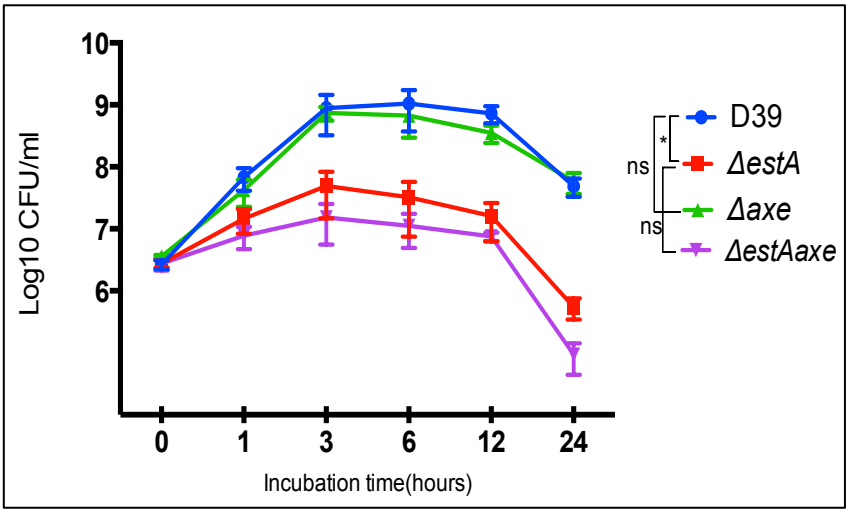

Figure 4.21: Growth profile of *ΔestAaxe* in D39 background grown on CDM supplemented with 0.071% (w/v) of BSM as carbon source. Each datum point is the mean of three independent tests in replicates and the vertical line represents the standard error of mean. *: $p<0.01$; ns: not significant.

4.2.3 Analysis of sialic acid release by pneumococcal strains:

The pneumococcal growth on CDM supplemented with BSM could be verified by measuring the released sialic acid from BSM after overnight growth. As mentioned before, D39 wild type strain is unable to utilise sialic acid due to a mutation in its neuraminate lyase, which is important for sialic acid utilisation (Hoskins et al., 2001). Hence, the released sialic acid will remain in the culture supernate, and could be easily measured. Pneumococcal strains were grown overnight on CDM supplemented with BSM. The culture was then centrifuged to remove pneumococci, and free sialic acid level in supernatant was checked as described in section 2.14.

Figures 4.22A and 4.22B show the level of sialic acid in BSM culture supernatant of pneumococcal strains. As can be seen from the Figures, the level of sialic acid was consistent with the growth profiles of pneumococcal strains. The highest level of sialic acid was released by D39 (212±10.3 µM/mg n=5) and estAComp (195.4±5.3 µM/mg, n=5), and the lowest from *ΔestAnanA* (25.3±1.3 µM/mg) (p<0.01) (Figure 4.22A). Moreover, the released sialic acid concentration in *ΔestA* (143.5±6.3 µmol/mg), estAComp^{S121A} (138.7±4.5 µmol/mg) and *ΔnanA* (65.8±7.5 µmol/mg) culture supernates was significantly lower than the wild type, but significantly higher than *ΔestAnanA* (p<0.01) (Figure 4.22A). There was no significant difference in released sialic acid concentration between *ΔestA* and estAComp^{S121A} (p>0.05).

On the other hand, the level of released sialic acid was not attenuated in *Δaxe* compared to parental strain (Figure 4.22B). As can be seen from the Figure, the level of sialic acid from *Δaxe* (209.23±8.3 µmol/mg) culture supernate was as high as the wild type. This result shows that either Axe is not involved in potentiating NanA for Sia release from BSM, or the absence of Axe in *Δaxe* might be compensated by other esterases such as EstA.

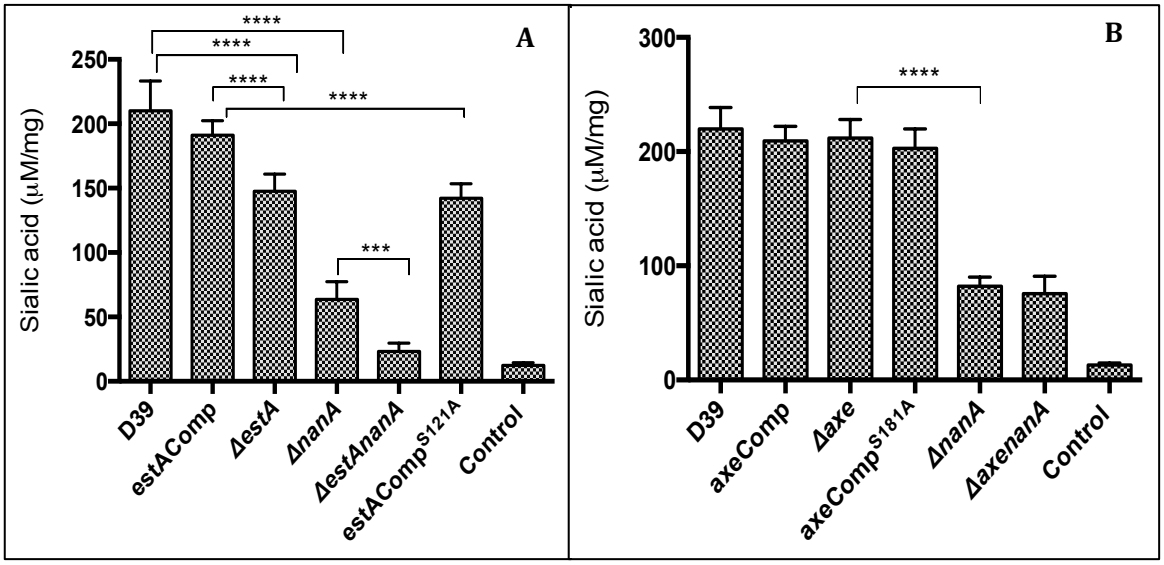

Figure 4.22: Release of Sia from BSM by pneumococcal strains. (**A**) *ΔestA* and (**B**) *Δaxe* and their derivatives were grown overnight on CDM supplemented with 0.071 % BSM as sole carbon source, and Sia levels in culture supernatant were measured. Data represent the mean of three independent tests in replicates and the vertical line represents the standard error of mean. ****: $p<0.0091$.

Section III: *In vivo* studies:

4.3 Esterases' contribution to pneumococcal colonisation and virulence:

In vitro results led me to investigate the role of pneumococcal esterases *In vivo*. The contribution of esterases in pneumococcal colonisation and virulence was evaluated using mouse models of colonisation and pneumonia. While the colonisation model allows evaluation of esterases function in nasopharyngeal survival, the pneumonia model evaluates the importance of pneumococcal proteins in invasion. Both models provide useful information for the involvement of the enzymes under study in pneumococcal physiology and virulence (Chiavolini et al., 2008).

Mouse models allow the understanding the mechanism of pneumococcal infection. The mouse is easy to maintain, and the pneumococcal infection in the mouse mimics that seen in humans (Kadioglu and Andrew, 2005). It has been reported that pneumococcal carriage in humans and mice lasts the same duration of time. The similarity of carriage dose has also been reported (McCool et al., 2002). The serotypes that can be carried in mice are able to colonise human nasopharynx (Wu et al., 1997b).

The success of pneumococcal infection in mice is dependent upon the mouse strain used. Gingles et al. (2001) identified mouse strains that do not respond to pneumococcal infection and colonisation. They found that BALB/c and C|57BL/6 strains did not respond to pneumococcal infection. These strains are naturally resistant to invasive serotype 2 (D39). For example, BALB/c mice could survive even 7 days post-infection, whereas MF1 mice starts showing signs of disease less then 24 h post-infection.

In this study, MF1 mouse strain was chosen as it is an outbred mouse, and mimics heterogeneous human population. They are sensitive to pneumococcal infections compared to other mice strains.

4.3.1 Assessment of esterases' contribution in pneumococcal colonisation:

In order to investigate the esterases' role in pneumococcal colonisation in the nasopharynx, the mouse model was used as described in section 2.18.3. *ΔestA* and

Δaxe isogenic mutants and their derivatives (*ΔestAnanA*, *ΔaxenanA* and parental strain) were included in this assay.

Figure 4.23A shows the numbers of pneumococci recovered from the nasopharyngeal tissues of mice infected with *ΔestA* and its derivatives. The CFU/mg of D39 recovered from nasopharyngeal tissues remained relatively constant over 7 days: Log_{10} CFU/mg for day 0, 3, and 7 days, respectively were 3.3 ±0.8, 3.4 ±0.4 and 3.2 ±0.54, n=5 for all). However, at 3 and 7 days post-infection, the numbers of *ΔestA* (Log_{10} 2.6±0.4 and 2.11±0.3 CFU/mg, n=5, respectively) and *ΔnanA* (Log_{10} 2.18±0.12 and Log_{10} 1.6±0.2 CFU/mg, n=5) were significantly lower than D39 (p<0.01). Though, the biggest attenuation in CFU/mg was detected with *ΔestAnanA* (Log_{10} 1.9±0.1 CFU/mg and 0.35 ±0.12 CFU/mg (n=5) at 3 and 7 days post-infection, respectively). The numbers of *ΔestAnanA* at 3 and 7 days post infection were significantly lower than those of *ΔestA* and *ΔnanA* (p<0.01).

On the other hand, Axe did not contribute to pneumococcal colonisation either independently or in combination with NanA in this model (Figure 4.23B). The numbers of *Δaxe* at 3 and 7 days post-infection (Log_{10} 3.33±0.3 and 3.1±0.1 CFU/mg, n=5, respectively) were similar to D39 (p>0.05). Moreover, the mutation of *axe* did not have any significant effect in colonisation of *ΔaxenanA*, as the numbers of double mutant recovered from nasopharynx was similar to that of *ΔnanA*, indicating that the reduction in CFU/mg is due to *nanA* deletion (Figure 4.23B).

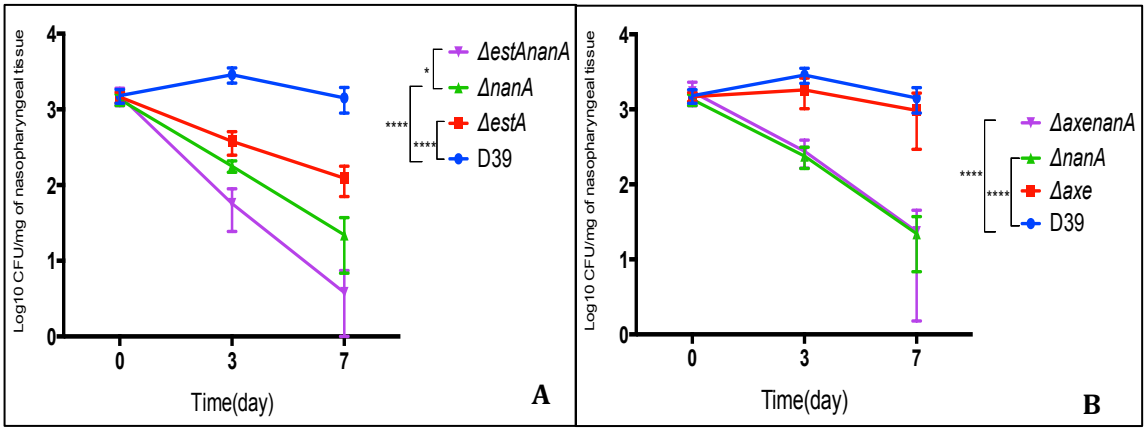

Figure 4.23: Assessment of esterases' role in nasopharyngeal colonisation of *S. pneumoniae*: (**A**) *ΔestA* and (**B**) *Δaxe* and their derivatives were tested for their ability to colonise the nasopharynx after intranasal infection. Each time point represents a mean of 5 individual CFU/mg of nasopharyngeal tissue. The vertical bars indicate the standard error of mean. *: $p<0.01$; ****: $p<0.0001$.

4.3.2 Determination of esterases' contribution in virulence:

The contribution of EstA and Axe to pneumococcal virulence was determined in a mouse model of pneumococcal pneumonia. *ΔestA* and *Δaxe* isogenic mutants, their derivatives (*ΔestAnanA*, *ΔaxenanA,* estAComp, axeComp) and the parental strain were included in this assay. Mice were infected intranasally as described in section 2.18.4. After infection, the mice were monitored regularly for sign of disease, and they were culled when they displayed any sign of lethargy, and time to reach this point was considered as 'survival time'. During the assay, the mice were tail bled in two time points, 24 and 36 h post-infection, and bacterial counts in blood were determined.

Figures 4.24A and 4.24B show the survival of mice infected with *ΔestA* and *Δaxe* isogenic mutants and their derivatives. As can be seen from the Figure 4.24A, the median survival time of cohort infected with *ΔestA* (55 h ±5.41, n=10) isogenic mutant was, significantly higher than the cohort infected with D39 wild type (32.5 h ±6.5, n=10) ($p<0.01$). Furthermore, the median survival of cohort infected with

ΔnanA (55 h ±5.21, n=10) isogenic mutant was the same as *ΔestA*. Moreover, the result show that the median survival time of cohort infected with *ΔestAnanA* (75 h ±5.1, n=10) was significantly higher than either *ΔestA* or *ΔnanA* isogenic mutants (p<0.01), indicating the synergistic impact of EstA and NanA in pneumococcal virulence. The complemented estAComp (31.8 h ±3.1, n=10) was as virulent as the wild type (p>0.05). The results showed a clear contribution of *estA* and *ΔnanA* in pneumococcal virulence.

On the other hand, the median survival time of cohort infected with *Δaxe* (37 h ±6.1, n=10) was similar to the median survival time of cohort infected with parental strain (34.5 h ±2.5, n=10), and the difference was not significant (p>0.05). There was no difference in median survival time of cohorts infected with *ΔnanA* (65 h ±6.6, n=10) and *ΔaxenanA* (67 h ±6.8, n=10) (p>0.05). The results show that Axe does not contribute to pneumococcal virulence in this model (Figure 4.24B).

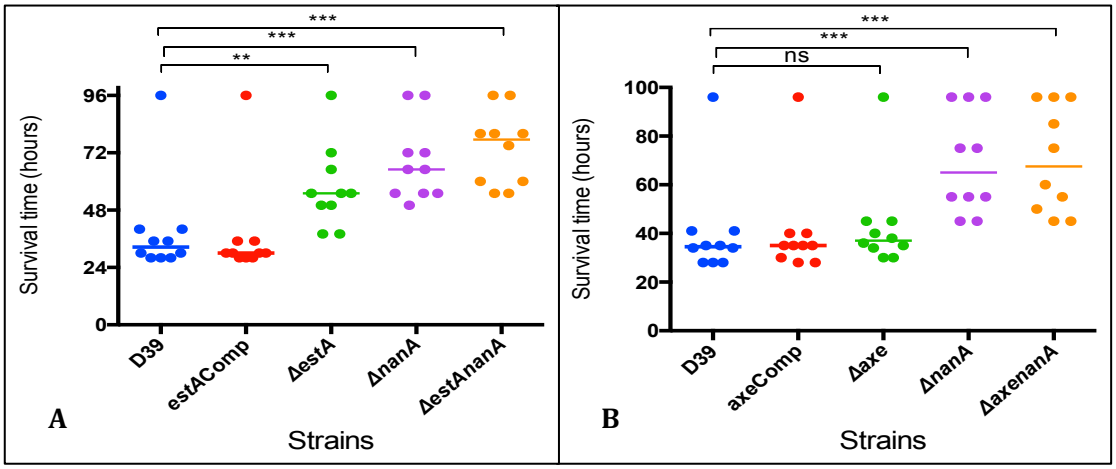

Figure 4.24: Virulence testing of pneumococcal strains. Survival of mice infected intranasally with *ΔestA* (**A**), *Δaxe* (**B**) and their derivatives. The vertical bars refer to the median survival. Each point corresponds to the survival time of an individual mouse. **: p<0.001; ***: p<0.0001; ns: not significant.

In addition, the progression of bacteraemia was determined after intranasal infection. Tail bleeding of mice was done at 24 and 36 h post-infection as described in section 2.4. The Figure 4.25A shows pneumococcal counts recovered from blood of mice infected intranasally with *ΔestA* isogenic mutant and its derivatives. The numbers of *ΔestA* isogenic mutant recovered from blood at 24 and 36 h post-infection (Log_{10} 5.6±0.3 CFU/ml and 7.9±0.1 CFU/ml, n=10, respectively) were significantly lower than that of D39 wild type for the same time point (Log_{10} 6.9±0.2 CFU/ml and 9.3±0.5 CFU/ml at 24 and 36 h, respectively) (p<0.01). There was no significant difference in the counts of estAComp and D39 wild type (p>0.05) indicating that the mutation of *estA* did not cause a polar effect. Moreover, the Log_{10} CFU/ml of *ΔestAnanA* double mutant (4.6±0.3 CFU/ml and 5.3±0.4 CFU/ml at 24 and 36 h post-infection, respectively) was significantly lower than the wild type (p<0.01) (Figure 4.25A). However, no significant difference could be seen in bacterial count between *ΔnanA* and *ΔestAnanA* isogenic mutants at 24 and 36 h post-infection (p>0.05).

With regard to the *Δaxe* isogenic mutant, the numbers of bacteria recovered from blood at 24 h (Log_{10} 6.3±0.5 CFU/ml) and 36 h (Log_{10} 8.8±0.71 CFU/ml) post-infection were similar to those of D39 (6.7±1.2 CFU/ml and 9.4±1.4 CFU/ml at 24 and 36 h, respectively) (p>0.05) (Figure 4.25B). The numbers of *ΔnanA* retrieved from blood (4.5±0.8 CFU/ml and 5.9±0.4 CFU/ml at 24 and 36 h, respectively) were significantly lower than D39 parental strain. Moreover, there was no significant difference in CFU/ml of *ΔnanA* and *ΔaxenanA* (p>0.05). The results show that *Δaxe* isogenic mutant was as virulent as the wild type, and the attenuation of *ΔaxenanA* double isogenic mutant was due to deletion of *nanA*.

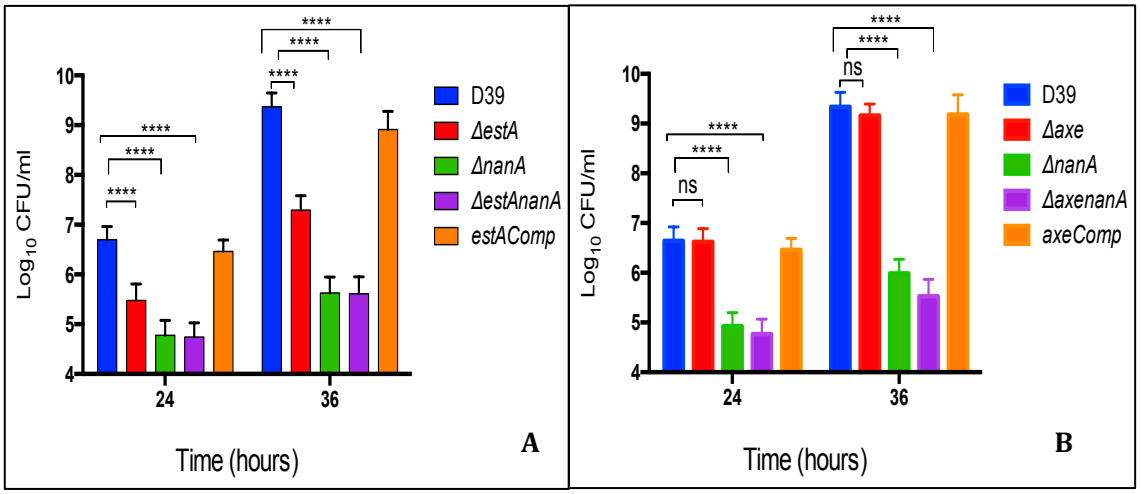

Figure 4.25: Progression of bacteraemia after intranasal infection with pneumococcal strains. The counts of pneumococcal strains recovered from blood of mice infected intranasally with *ΔestA* (A), *Δaxe* (B) and their derivatives. Each column represents a mean count of 10 individual CFU of blood samples with the standard error of mean. ****: $p<0.001$. ns: not significant.

4.3.3 Pneumococcal esterases and neuraminidase A are required for glycoconjugates sialylation or sialic acid secretion:

Ex vivo assay showed that EstA and NanA activities are required for pneumococcal cleavage of sialic acid from BSM. It was therefore hypothesised that the strains mutated in *estA*, *nanA* or both would release less sialic acid *In vivo* than the wild type strain. In order to investigate the mechanism of EstA's involvement in pneumococcal virulence, the level of free sialic acid from nasopharyngeal lavage of mice colonised with *ΔestA* and *ΔnanA* isogenic mutants was assessed. The mice were colonised with different strains of pneumococci, and were then culled 7 days post-infection. Nasopharyngeal lavage of mice was taken as described in section 2.18.3. The nasopharyngeal lavage samples were processed for free sialic acid level as described in section 2.14. The results show that the concentration of free sialic acid in nasopharyngeal lavage was below the detection limit. Hence, it was decided to check

the concentration of bound sialic acid after chemical release as described previously (Siegel et al., 2014).

The Figures 4.26A and 4.26B show bound sialic acid level of nasopharyngeal mucin of mice colonised with *ΔestA* and *Δaxe*, and their derivatives. As can be seen from Figure 4.26, the level of bound sialic acid in mice colonised with D39 wild type (35±3.7 µmol/mg, n=5) was significantly higher than non-colonised mice (6.09±4.1 µmol/mg, n=5) ($p<0.01$). This might have been due to stimulatory effect of pneumococcal colonisation on mucin secretion. On the other hand, the level of sialic acid of nasopharyngeal lavage of mice colonised with *ΔestA* and *ΔnanA* significantly decreased compared to wild type (23.1±8.1 µmol/mg, n=5 and 11.7±6.4 µmol/mg, n=5 for *ΔestA* and *ΔnanA*, respectively) ($p<0.01$). However, the concentration of the bound sialic acid in the lavages of mice infected with the double *ΔestAnanA* isogenic mutant did not show a significant difference than *ΔnanA* ($p>0.05$) (Figure 4.26). There was also no difference in the amount of bound sialic acid between the double *ΔestAaxe* mutant and *ΔestA* isogenic mutant ($p>0.05$).

In addition, the results did not show any difference in sialic acid level in nasopharyngeal lavage of mice colonised with *Δaxe* when compared to parental strain (Figure 4.26B). The level of bound sialic acid of nasopharyngeal lavage of mice colonised with *Δaxe* (32±4.9 µmol/mg, n=5) was not significantly different from parental strain (35±3.7 µmol/mg, n=5) ($p>0.05$). The results showed that Axe is not involved in sialic acid cleavage from nasopharyngeal mucin.

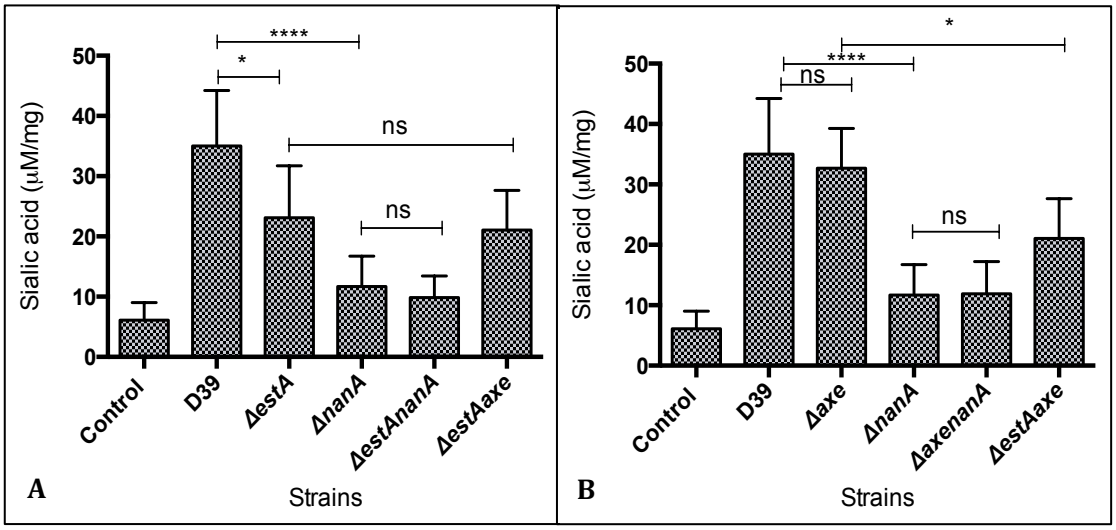

Figure 4.26: The role of EstA (**A**) and Axe (**B**) in sialic acid cleavage in nasopharyngeal tissues of mice colonised with different pneumococcal strains. The level of bound sialic acid in nasopharyngeal lavage of mice 7 days post-infection. Mice challenged with PBS served as negative control. Each column represents the mean value of bound sialic acid derived from 5 mice with their SEM. Sialic acid measured as μmol of sialic acid per mg of nasopharyngeal tissue. *: $p<0.01$; ****: $p<0.0001$; ns: not significant.

Section IV: Transcriptional analysis of esterase genes *in vitro* and *In vivo*:

Transcriptional analysis of esterases was determined *in vitro* and *In vivo*. The expression of esterase genes was quantified *in vitro* to determine, first, whether the exposure to BSM relative to glucose would induce the expression of these genes, and second, to assess whether inactivation of one esterase gene would be compensated by the other esterases. *In vivo* gene expression was determined to test the hypothesis that esterases are up regulated in respiratory tract relative to blood.

4.4.1 *In vitro* transcriptional analysis:

In vitro expression of esterase genes in D39 strain was determined initially by growing D39 until early exponential phase (OD_{600} to 0.2-0.3). At this point, pneumococci were pelleted and the supernatant was removed. The pellet was washed with PBS, and diluted with CDM containing either 0.071% of BSM, or 55 mM glucose and incubated for 1 h. The pneumococcal culture was then pelleted and mRNA extraction was processed as described in section 2.11.

Figure 4.27 shows the difference in *in vitro* expression level of esterase and *nanA* genes in D39 wild type exposed to BSM relative to the exposure to glucose. As can be seen from the Figure, the expression levels of *estA, axe* and *nanA* went up 4.5 ±0.66, 2.07±0.52 and 10.7±0.71 folds (n=3), respectively, relative to their expression on glucose, supporting their involvement in host derived sugar cleavage or utilisation. However, the *SPD1239* and *SPD0932* esterases genes showed a down regulation in their expression (-3.83±0.07 and -3.03±0.63 folds, respectively), indicating that they are very likely not involved in host glycoprotein degradation (Figure 4.27).

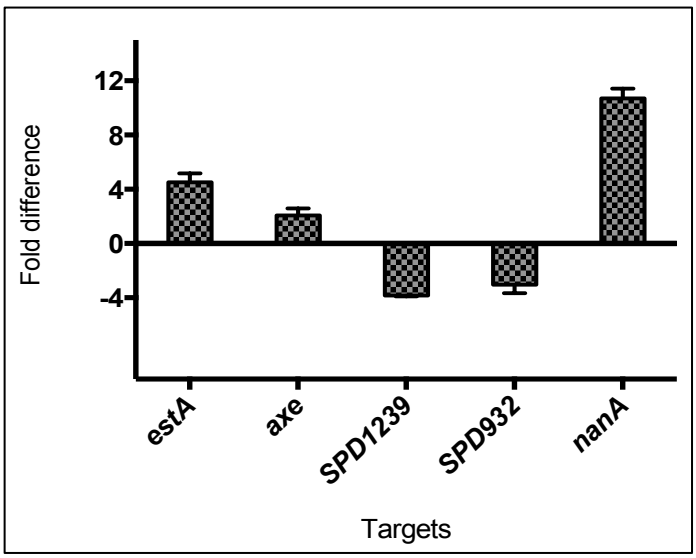

Figure 4.27: Gene expression of esterase and *nanA* genes in D39 wild type exposed to BSM. The expression levels on BSM were compared to exposure to glucose. Columns represent fold difference of target genes and vertical bars indicate standard error of mean. 1.5-fold difference was considered as significant. The expression levels represent the means of three independent experiments each in replicates with their SD.

4.4.1.1 Investigation of compensatory effect among pneumococcal esterases:

To investigate whether the absence of one esterase could be compensated by the other, the transcription level of esterase genes were analysed in *ΔestA* and *Δaxe* isogenic mutants relative to their expression in D39 after exposure to BSM. The analysis of both *ΔestA* and *Δaxe* isogenic mutants revealed that *estA* and *axe* could compensate each other's absence (Figures 4.28A and 4.28B). For example, the mean CT value for *estA* transcription in *Δaxe* isogenic mutant was 7.73±1.21 (n=3). On the other hand, the expression level of *axe* in *ΔestA* was 6.2±0.41 (n=3). This indicates that there is a compensatory effect between EstA and Axe. With regard to the expression of other esterases, *SPD1239* and *SPD0932*, the expression level went down (1.8±0.21 and 2.6±0.51, n=3 for *SPD1239* and *SPD0932*, respectively) in *ΔestA* of these genes comparing to D39 wild type indicating that the absence of Axe and EstA cannot be compensated by the esterases coded by these gene (Figures 4.28A and 4.28B).

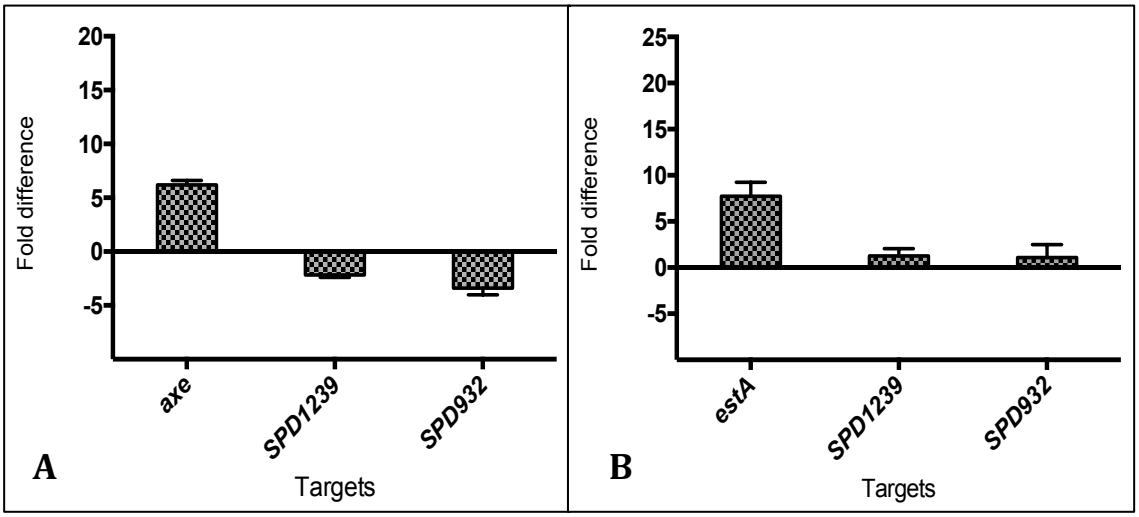

Figure 4.28: The expression of pneumococcal esterases in *ΔestA* (**A**) and *Δaxe* (**B**) isogenic mutants exposed to BSM. The expression levels are compared to D39 wild type exposed to BSM. Columns represent fold difference of target genes and vertical bars indicate standard error of mean. 1.5 fold difference in considered as significant difference. The test was done in three independent experiments in replicates.

4.4.2 *In vivo* transcriptional analysis:

Transcriptional analysis of *estA*, *axe* and *nanA* was done *In vivo*. The change in expression of target genes in pneumococci recovered from nasopharynx, lungs and blood was examined, and the fold difference in expression in respiratory tissues was given relative to expression of same genes in blood. The tissue samples were processed as described in section 2.17, and data was analysed as described in section 2.11.

Figures 4.29A and 4.29B show the fold difference in gene expression of pneumococcal esterase in nasopharynx and lungs, respectively, relative to blood. As can be seen from the Figure 3.57A, the expression of *estA*, *axe* and *nanA* increased (12.6±0.71, 6.5±0.53 and 27.7±0.20, n=3, respectively) in nasopharynx relative to blood (Figure 4.29B) (Table 4.1).

Figure 4.29B shows fold difference of *estA*, *axe* and *nanA* genes in the lungs relative to blood. As can be seen from the figure, the expression of *estA*, *axe* and *nanA* went up (6.32±0.71, 1.65±0.53 and 13.64±0.20, n=3, respectively) in the lungs relative to blood (Table 4.1).

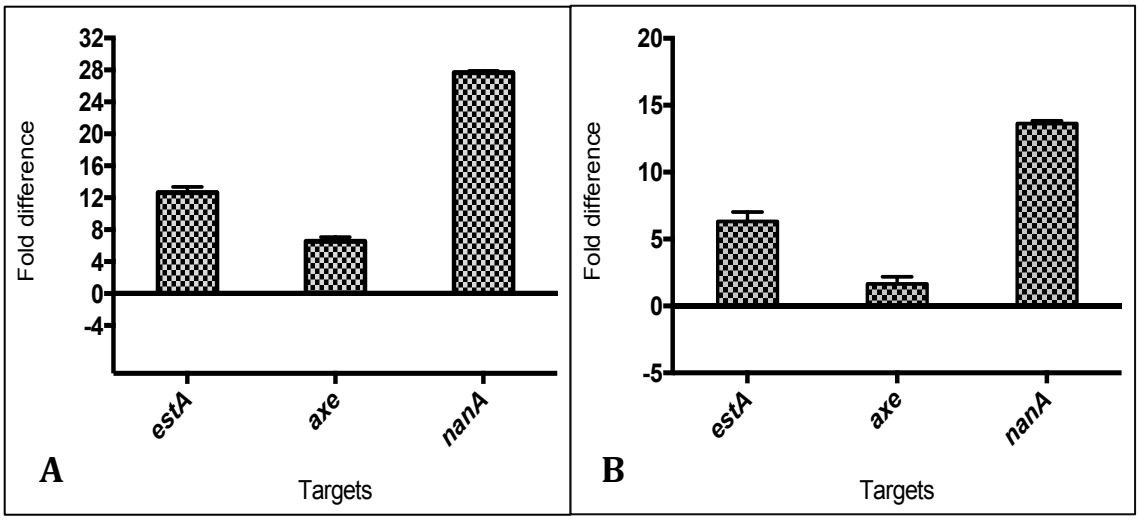

Figure 4.29: The expression of *estA*, *axe* and *nanA* genes of *S. pneumoniae* recovered from nasopharynx (A) and lungs (B) relative to their expression in blood. Columns represent fold difference in expression of target genes, and vertical bars indicate standard deviation. 1.5-fold difference in expression was considered as significant. The test was done in three independent experiments in replicates.

Table 4.1: *In vitro* and *In vivo* fold difference of pneumococcal esterases and NanA.

Target genes	Fold difference in expression		
	***In vitro* expression**	***In vivo* expression ****	
	In BSM*	**Nasopharynx**	**Lungs**
estA	+4.5 ±0.66	+12.64±0.71	+6.32±0.71
axe	+2.07±0.52	+6.54±0.53	+1.65±0.53
SPD1239	-3.83±0.07	-4.34±0.45	-14.34±0.45
SPD0932	-3.03±0.63	-6.25±0.92	-2.70±0.92
nanA	+10.7±0.71	+27.67±0.20	+13.64±0.20

+: Up regulated; -: Down regulated.

*: Fold difference change is relative to glucose grown pneumococci.

**: Fold difference change is relative to pneumococci recovered from blood.

Chapter Five

Discussion:

5. Overview:

The pneumococcus is considered to be one of the most dangerous pathogens of humans worldwide. It is the main cause of invasive infections in young and elderly populations (AlonsoDeVelasco et al., 1995). Although *S. pneumoniae* asymptomatically colonises the upper respiratory tract of healthy people, it can also cause a wide range of infections in different tissue sites including the lungs, blood, meninges and middle ear (CDC, 2012). This diversity of infection reflects the success of the pathogen to adapt in the host, and demonstrates its versatility to change its phenotype from commensal to invasive pathogen.

To figure out how the pneumococcus causes infection, a better understanding of the role of virulence factors is needed. *S. pneumoniae* has a large number of virulence factors that have been extensively studied (Kadioglu et al., 2008, AlonsoDeVelasco et al., 1995). Amongst these, some are important for attachment, and the others are involved in invasion, and escape from host immune system. Pneumococcal survival in host tissues is a prerequisite for its virulence, and the microbe requires a constant supply of nutrients. *S. pneumoniae* relies on host glycoproteins to get its carbon requirement. The direct acquisition of large molecular weight host sugars is not possible; hence several enzymes are needed to break down host glycoproteins to release sugar monomers. These enzymes include neuraminidases, β-galactosidases, β-*N*-acetyl hexoseaminidase and glycanase (King et al., 2006, Manco et al., 2006).

The sequential cleavage of host glycoproteins has a key role in pneumococcal attachment. The first sugar that needs to be cleaved from sialo-glycoconjugates is sialic acid, which can be released by pneumococcal neuraminidases (Corfield, 1992). This allows other exoglycosidases to cleave other sugars that are important for pneumococcal nutrition. Two features affect the initial cleavage of sugars from host glycoproteins by pneumococcal neuraminidases: first, the substrate specificity of

enzymes determined by the glycosidic linkages between sialic acid and other sugars, such as α2-3, α2-6 and α2-8 linkages (King, 2010), and the second potent effect is the occurrence of side chain hydroxyl groups at different sides such as 4-*O*-acetyl, 7-*O*-acetyl, 8-*O*-acetyl and 9-*O*-acetyl groups. It is well documented that bacterial and viral sialidases activity is reduced by high occurrence of *O*-acetylation of sialic acid (Schauer, 1982, Varki and Schauer, 2009). The ability to cleave sialic acid therefore is expected to have a major effect on the microbe's interaction with the respiratory mucin, consequently on its attachment and invasiveness. Therefore, investigation of the factors affecting the potency of neuraminidases is well justified.

Esterases play a key role in the removal of *O*-acetyl groups from glycoconjugates. They can act against free and bound sialic acid containing *O*-acetylation at 4, 7, and 9-*O*-acetyl groups (Schauer et al., 2011). These groups are known to be resistant to sialidase activity. Therefore, sialidase' ability to cleave the majority of sialic acid from glycoproteins is restricted by *O*-acetyl groups, which can be removed by esterases. Corfield et al. (1992) reported that esterases play an important role in bacterial colonisation and virulence. Therefore, esterase activity provides a novel mechanism to the pathogens in accessing host glycoconjugates potentiating the activity of bacterial neuraminidase (Corfield et al., 1991, Burnaugh et al., 2008).

The aim of this project was to characterise pneumococcal esterases and study their involvement in augmentation of neuraminidase activity. Four putative esterases have been studied, and two of which were extensively characterised. It has been shown in this study that EstA and Axe are involved in mucin de-*O*-acetylation *in vitro*. However, only EstA was shown explicitly to be involved in pneumococcal growth on mucin containing highly acetylated sialic acid, as well as in colonisation and invasiveness. In addition, I found that *S. pneumoniae* induces the expression of sialic acid containing host glycoconjugates, and NanA and EstA activities are required for this. The subcellular localisation assay revealed that EstA and Axe are intracellular proteins, but also can be found in the culture supernate. *In vitro* and *In vivo* analysis of gene expression revealed that *estA* and *axe* are significantly up regulated in respiratory tissues relative to blood, and in the presence of BSM relative to growth on glucose. I also showed that EstA and Axe are serine active site enzymes. The replacement of putative serine active site to alanine abolished the activity of these

enzymes.

5.1 Manipulation and complementation of pneumococcal esterases:

In order to investigate the functional role of putative esterase genes, the manipulation of these genes was needed. Different types of mutagenesis systems are available for *S. pneumoniae*. Mutagenesis could be deletion of the gene of interest or could be insertion of antibiotic resistance marker. The other method is insertion deletion mutagenesis. This method allows deletion of the gene of interest and insertion of antibiotic resistance marker instead. In this study, two types of mutagenesis systems were used: mariner mutagenesis and SOEing PCR mutagenesis (Horton et al., 1990, Akerley et al., 1998).

Mariner mutagenesis relies on transposase enzyme called *Himar1*. *Himar1* transposase consists of two domains: helix-turn-helix (HTH) motif at the terminal ends, which recognises the binding site at inverted repeats (IRs), and the catalytic motif responsible for cutting DNA elements (Munoz-Lopez and Garcia-Perez, 2010). This enzyme inserts antibiotic cassette into the gene of interest by simple cut-paste mechanism. It binds to the inverted repeat (IRs) sequence allowing the catalytic motif to cut the antibiotic cassette ends and the target gene insertion site. The ends of transposon are then joined with each other according to the recombination of AT dinucleotide to form paired-end complex. This complex is then 3'-OH tailed and then transferred into random TA dinucleotide sequences within the target gene. In the next step, the nicks created by the insertion of antibiotic marker are repaired with T4 DNA polymerase, which fills the gaps (Lampe et al., 1996, Sakai and Kleckner, 1997, Munoz-Lopez and Garcia-Perez, 2010). *In vitro* transposition allows mutation of gene of interest without a need for vector. Once optimised, several genes can be mutated in a short period. This method of mutagenesis has been widely used to study different aspects of pneumococcal biology (Lampe et al., 1996, Akerley et al., 1998)

SOEing PCR mutagenesis allows insertion-deletion mutagenesis by construction of mutant allele by several steps of PCR. SOEing PCR mutagenesis was also used for site directed mutagenesis. The replacement of one nucleotide within the gene of interest was done in two-step PCR. SOEing PCR mutagenesis is also very efficient mutagenesis method as it does not rely on cloning (Horton et al., 1990). The error

frequency of this method is relatively low.

The natural genetic transformation of *S. pneumoniae* allows the foreign DNA to be delivered with or without non-replicating vectors (Lee et al., 1998). The ability to acquire foreign genetic material, which is also known as the competence state, depends on cell density and is governed by a pathway mediated by two component regulatory system (Morrison and Baker, 1979). The competence state occurs in early log phase, at which point peptide signal for the competence state is released and induces the pathway for transformation. The pneumococcus can easily integrates the acquired DNA at a homologous site allowing the replication of the heterologous piece of DNA replicating within the genomic DNA (Lacks, 1997).

In order to rule out the polar effect of mutations, wild type phenotypic restoration of these genes was achieved by genetic complementation. The plasmid pCEP (Guiral et al., 2006) was used for complementation of *ΔestA* and *Δaxe*. The main aim of complementation was to determine whether the mutation effects the expression of the genes downstream of the mutated region, which is also known as polar effect. The complementation of *ΔestA* and *Δaxe* isogenic mutants was successfully achieved. The wild type phenotypic restoration of genes was checked by assessing the activity of esterases in the complemented strains compared to respective isogenic mutants as well as wild type. A further verification of successful genetic complementation was observed in the growth of complemented strains in CDM supplemented with BSM as sole carbon source.

The complementation strategy used in this study used a non-replicative plasmid pCEP (chromosomal expression platform plasmid) in *S. pneumoniae* but replicates in *E. coli*. The replication of pCEP in *E. coli* allows the propagation of recombinant plasmid before transformation into *S. pneumoniae.* However, the cloning plasmid can be transformed directly into *S. pneumoniae* without intermediate cloning step into *E. coli.* In this study, I propagated the recombinant pCEP for sequencing to rule out any possibility of mutation. A maltose-inducible promoter region drives the expression of gene of interest though in this study the putative native promoters of *axe* and *estA* were included in the complementation constructs pCEP integrates into pneumococcal genome at a transcriptionally silent site through homologous recombination because the multiple cloning site is surrounded by a 2 kb homologous sequence, located

downstream of the *amiA* region, in the pneumococcal genetic region where the intact copy is inserted.

5.2 Enzymatic characterisation of pneumococcal esterases:

In this study, I identified that the products of four genes under study contribute to the total pneumococcal esterase activity. Their activity against synthetic and natural substrates had not been demonstrated before. Mutation of these esterase genes was performed using mariner mutagenesis method. The effect of the mutations was determined using p-nitrophenyl esters as non-specific substrates. These substrates are cleaved by esterase, and p-nitrophenol is released as a result of this reaction, which changes the colour of reaction from a colourless solution to yellow colour (Figure 5.1). The results showed that the mutation of all putative esterase genes reduced the total esterase activity. The highest reduction in the activity could be recorded in *ΔestA* and *Δaxe*, indicating that tributyrin esterase (EstA) and acetyl xylan esterase (Axe) are the major pneumococcal esterases. This result is in agreement with previous studies (Brault et al., 2012, Kim et al., 2008b, Fernandez et al., 2000), which showed that esterases are able to use p-NP esters as synthetic substrate.

Two of the pneumococcal esterase genes and neuraminidase A were successfully cloned, expressed and purified using Talon Metal Affinity column. EstA and Axe were cloned and expressed because the mutation of these two esterases significantly attenuates the total activity of esterase in the pneumococcus. The identity of recombinant proteins was confirmed using western blot assay and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) assay. The substrate specificity test revealed that both recombinant esterases are specific to short chain carbon substrates (C2-C6), and the activity was significantly reduced when long chain (C8-C10) substrates were used. This was in line with a previous study, which showed that tributyrin esterase purified from *L. lactis* showed substrate specificity against short carbon chain substrates (Fernandez et al., 2000, Nardi et al., 2002). It is widely known that esterases can hydrolyse short chain substrates less than 10 carbons substituents (Faiz et al., 2007, Kim et al., 2006).

The substrate specificity can differ amongst esterases. Esterases could use a wide range of substrates depending on the acyl ester groups of these substrates. Having a

broad substrate range provides an evolutionary benefit in host survival for microbes during colonisation and disease, similar to that is observed for *S. pneumoniae* in this study. Bacterial genome mining study done by Wang et al. (2010) found that amongst 84 bacterial esterases, 46 were predicted to be specific for p-NPA. The true carboxylesterase from *Bacillus pseudofirmus* is found to be highly specific to middle and short carbon chain (less than C6) p-NP esters, which is in line with my findings (Rao et al., 2013). Intracellular esterase from *Streptomyces coelicolor* was found to be active against p-NP esters with C2-C12 carbon chain (Brault et al., 2012). The optimal activity of EstA and Axe against short acyl esters indicate that both esterases are similar to typical esterases (Bornscheuer, 2002).

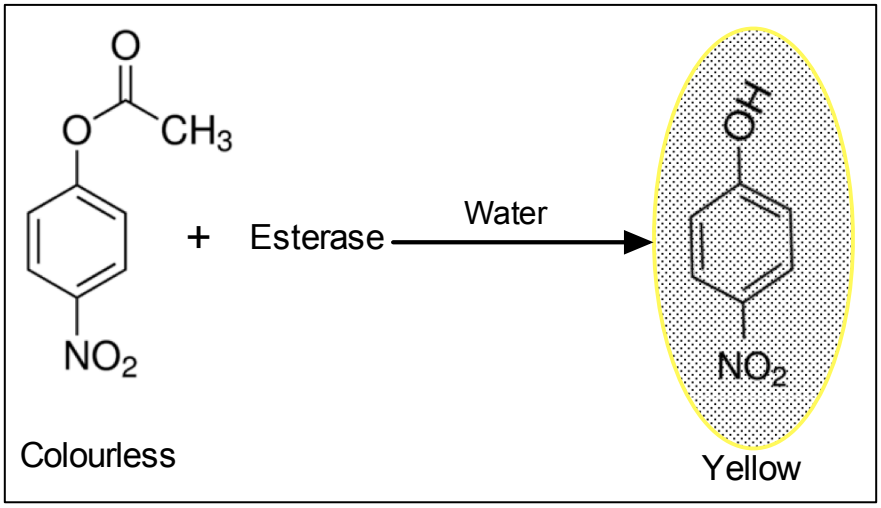

Figure 5.1: Hydrolysis of p-nitrophenyl acetate (colourless) by esterase. The reaction results p-nitrophenol (yellow).

Kinetic characterisation of EstA and Axe was done to identify the optimal condition of pneumococcal esterases. I first identified the optimal pH of EstA and Axe as pH plays a vital role in enzymatic process. The pH of the reaction mixture can affect the activity of esterases (Schomburg et al., 2004). The pH is more likely linked to the

proton activation and deactivation of titratable amino acid residues. In other words, the negative charge of active site residue is deprotonated after the cleavage of substrate (Neves Petersen et al., 2001). In order to reach optimal function, esterases require their catalytic residues to be protonated in certain pH range. Therefore, any change in pH affects the equilibrium of protonated and deprotonated forms of catalytic residues (Neves Petersen et al., 2001). The difference in pH values of esterase-substrate reaction should be stable at certain level. The level of pH might be changed due to the cleavage of ester bonds of the substrate. However, establishment of optimal pH would be confusing. The optimal pH of other pneumococcal esterases might be different and this interacts with total esterase activity. Determination of pH value of each esterase separately might solve this problematic issue.

Determination of optimal pH also allows better understanding of how pneumococcal esterases perform, and to determine the optimal condition of enzyme catalytic activity. The pH range in nasopharyngeal cavity is within the range of optimal activity of esterases (Brunworth et al., 2012). Therefore, all pneumococcal enzymes including esterases rely on pH of host nasopharynx. Determination of pH values may also reveal information about where esterases are involved in the pathogen-host interaction in different tissues. For example, the pH value in nasopharynx is different than that in blood (pH value in blood stream is 7.36) (Neves Petersen et al., 2001).

Here, I determined the optimal pH of EstA and Axe and found that optimal pH of both esterases was 7.5. Two buffers were prepared in this study: sodium phosphate buffer with a pH range of 6.0-8.5 and sodium citrate buffer with a pH range of 3.0-6.0. This range of pH allows better understanding of how esterases react in different pH values as optimal pH of esterases might be varied (Neves Petersen et al., 2001). Esterases are found to be active at neutral pH values (Neves Petersen et al., 2001). This neutral pH results in negative charge of serine catalytic active site. The negative charge of serine catalytic active site has a key role for esterase activity. This allows a repulsion of negatively charged fatty acids, which is released from triglycerides, away from serine active site. This repulsion optimises the catalytic active site of esterases allowing it to return the activity cycle (Neves Petersen et al., 2001). To link this with how pH affects esterases activity *In vivo*, it has recently been reported that the pH value of nasopharyngeal secretions is ranged between 6.1 to 7.9 (Brunworth et al.,

2012), which is within the optimal range of esterases activity. It is therefore reasonable to assume that pneumococcal esterases are active within the pH range of mucosal layer, consistent with the *in vitro* results obtained with EstA mutant.

Kinetic parameters were determined using p-NPA as a synthetic substrate as it is shown to be the optimal synthetic substrate for EstA and Axe. *Vmax* and *Km* values for EstA were found to be 364.1 mU and 4.7 mM, respectively, whereas *Vmax* and *Km* values for Axe were 220 mU and 4.4 mM. Determination of kinetic parameters reveals whether these enzymes obey Michaelis Menten kinetics. This allows determining the enzyme affinity dynamics and how fast the enzyme is reacting with its substrate in optimal concentration. It also allows indicating the saturation point of esterases. Enzyme affinity to synthetic substrates is determined by the *Km* value. This means high affinity of esterases is due to small *Km* values. EstA affinity was lower than Axe indicating that Axe might have other functions in pneumococcal physiology. They might be involved in different functions other than removal of *O*-acetylation of substrates. Supporting this idea, esterases have wide range of roles including esterification and catabolism of fatty acids (Bornscheuer et al., 1999). On the other hand, *Vmax* value indicates the saturation point of enzyme (the point which the concentration increase of enzyme does not affect the activity) (Bornscheuer et al., 1999). By knowing this point the mechanism of enzyme-substrate binding could be interpreted. Here, EstA activity was higher than Axe indicating that EstA could use substrates more efficiently.

Esterases can also be characterised by using natural substrates. Hydrolysis of natural substrates can be detected by either recording the decrease in tributyrin turbidity embedded in solidified agar or measuring the change of the acetylated xylan colour due to the release of acetate (Smeltzer et al., 1992, Martinez-Martinez et al., 2007). The tributyrin assay is more convenient with enzymes that can use water-soluble substrates (Smeltzer et al., 1992). Esterases can utilise tributyrin (triglycerides) and produce fatty acids that turns the turbidity of agar plates to clear, which can be measured spectrophotometrically. Tributyrin was chosen as natural substrate because it is the most relevant water-soluble substrate for carboxylesterases that can cleave ester bonds between fatty acids. This substrate can be used to distinguish some certain esterases with carboxylesterase family that are able to utilise the carboxylic acid ester

bonds within the substrates with short carbon chains (Smeltzer et al., 1992). Acetylated xylan was also chosen as acetylated substrate to find out whether EstA and Axe are able to cleave *O*-acetyl groups from the substrate (Martinez-Martinez et al., 2007).

Here, I used tributyrin (triglycerides) and acetylated xylan as natural substrates. I found that EstA is able to hydrolyse tributyrin and reduces the turbidity of the substrate. However, Axe is unable to hydrolyse tributyrin as substrate. The diversity of esterases' substrate range reflects the function of these enzymes (Arpigny and Jaeger, 1999). This means the function of esterases might differ depending upon the function of these esterases. Therefore, EstA differs from Axe in triglyceride utilisation. EstA belongs to carboxylesterase family since it can cleave ester bonds within repeating units of acyl chain of triglyceride (tributyrin), and can also remove *O*-acetyl groups from acetylated xylan and release acetate. It has been well established that carboxylesterases can only hydrolyse acyl glycerol with short carbon chain less than 10 such as tributyrin (Arpigny and Jaeger, 1999).

However, Axe is not able to hydrolyse tributyrin as substrate but is able to remove *O*-acetylation from hydroxyl groups of acetylated xylan. Therefore, it belongs to carbohydrate esterase family (CEs). Members of this family are highly specific against natural substrates that contain of *O*-acetyl groups. Axe is known to cleave *O*-acetyl side moieties from repeating units of hemicellulose and cellulose. Cellulose consists of repeating units of polysaccharides in plants such as xylose. This structure can be substituted to different residues such as acetyl, arabinosyl and glucuronyl. Cellulose is also can be *O*-acetylated in position 2- and 3- of xylose (Martinez-Martinez et al., 2007). The mammalian host does not have cellulose but it has repeating units of different carbohydrates in its extracellular matrix, which is analogous to cellulose (Martinez-Martinez et al., 2007). Therefore, it is plausible that EstA and Axe may be needed *In vivo* when the pneumococcus encounters such substrates.

5.3 EstA and Axe are serine active site esterases:

The active site of esterases is usually within the typical catalytic triad of the nucleophile serine, proton carrier histidine and aspartate/glutamate. This typical triad

forms a sophisticated pocket responsible for esterases' catalytic activity (Zhang et al., 2005). In the majority of esterases, serine and histidine residues are usually present, whereas aspartate/glutamate residues might be absent in some of them such as esterase from *Streptomyces scabies* that contains catalytic dyad of Ser-His (Wei et al., 1995).

In this study, I investigated whether pneumococcal esterases are serine catalytic enzymes. The sequence alignment of both EstA and Axe with other functionally characterised esterases showed that the catalytic triad of Ser-His-Asp is present, and the putative active site serine is conserved within the consensus G-X-S-X-G sequence motif (Figure 3.14 and 3.15). The putative serine active site in both Axe and EstA was mutated to find out their catalytic role. SOEing PCR mutagenesis was used to introduce point mutation in *estA* and *axe*. This method based on the replacement of serine to alanine. S^{121} and S^{181} were replaced with alanine in EstA and Axe, respectively. Alanine has been chosen to be replaced with serine because it is known as a neutral hydrophobic amino acid that normally buried within the core of protein structure unlike serine, which is known as polar amino acid that participate in hydrogen bond and intend to be on the surface of proteins (Ollis et al., 1992, Bornscheuer, 2002). The mutant recombinant $EstA^{S121A}$ and Axe^{S181A} were successfully expressed and purified. The enzyme activity of mutant esterases revealed that S^{121} and S^{181} are needed for catalytic activity of EstA and Axe, respectively, as the activity of mutant esterases was abolished. This result is in agreement with the previous studies that proved the importance of serine active site in enzyme catalytic activity in some microbes such as *Bordetella pertussis*, *N. gonorrheae* and *M. tuberculosis* (Thamwiriyasati et al., 2010, Zhang et al., 2005, Ollis et al., 1992, Bornscheuer, 2002, Weadge and Clarke, 2007).

The serine active site within G-X-S-X-G of serine protease esterases provides flexibility for substrate binding, and this might be a reason for the wide range of substrate specificity. The possible substrates for esterases might differ depending on the function of enzyme (Huang et al., 2001). Arpigny and Jaeger (1999) classified lipolytic enzymes according to their conserved consensus sequences and substrate specificity. Both EstA and Axe are found to have their conserved catalytic triad of Ser-Asp-His, and the active site Ser is found in conserved consensus sequence (Figure

3.14 and 3.15). These findings were in line with the findings of Rao et al. (2013), who showed that replacement of Ser at the active site to Ala abolished the catalytic activity of the carboxylesterase. Another study by Liu et al. (2007) found that extracellular esterase from group A streptococci has typical C-X-S-X-G conserved motif, and Ser^{178} is the main catalytic residue within the conserved sequence. Furthermore, the replacement of Ser^{215} by Ala completely abolished the catalytic activity of carboxylic esterase in *M. tuberculosis* (Lun and Bishai, 2007). The other pneumococcal esterases, acyl-ACP thioesterase and phosphoesterase, do not have a conserved consensus sequence of G-X-S-X-G when they are aligned with EstA and Axe. This suggests that acyl-ACP thioesterase and phosphoesterase belong to another family of esterases that do not have a conserved G-X-S-X-G consensus sequence (Arpigny and Jaeger, 1999). Further enzymatic and functional characterisation is needed for putative acyl-ACP thioesterase and phosphoesterase.

The sequence similarity analysis of amino acids of EstA and Axe using T-coffee multiple sequence alignment software showed a high similarity to esterases from other organisms. The analysis showed that EstA has 86% similarity score to other known esterases such as tributyrin esterase of *L. lactis* and S-formylglutathione esterase from *H. influenzae* (Figure 3.14). It was also found that the consensus amino acid sequence GLSLG is present in EstA. The overall identity score of EstA was nearly 64% when compared to the characterised esterases. This striking similarity proposes a typical α/β hydrolase structure of EstA because the structure of α helixs and β strands provides this sophisticated characteristic structure for esterases. However, the similarity score of Axe was lower than EstA. The overall similarity score of Axe was shown to be 57% similarity at amino acid sequence level when aligned with other known esterases. The consensus sequence of Axe was found to be GASOG, which is responsible for catalytic activity of serine active site, showing the distinct structure of the enzyme than EstA although the catalytic triad is found to be similar to known esterases (Figure 3.15). Similarly, the identity score of Axe was also relatively low (45%) compared to EstA. The difference in primary structure of Axe does not mean that it is different hydrolase enzyme. Therefore, the main characteristic of esterases is the catalytic residue similarity in 3D structure, which provides the exact form of hydrolase enzymes showing the position of catalytic residues and binding pockets. The catalytic triad of Ser-His-Asp forms the active pocket of

enzymes that is required for enzyme binding and hydrolysis. However, this catalytic triad is not present in some esterase families. For example, erythromycin esterase is a serine active protease but the catalytic triad is absent (Kim et al., 2002). In this case, other amino acids might form a typical catalytic activity of esterases. For example, His-Glu pair was proposed to be the catalytic active site of erythromycin esterase A and B family (Morar et al., 2012)

5.4 EstA and Axe are able to remove *O*-acetylation from BSM:

The role of pneumococcal esterases in *O*-acetyl cleavage was studied. The most suitable substrate for this assay is BSM (Pal et al., 2000). BSM is highly acetylated at 4-, 7- and 9-*O*-acetyl side chain. It is commercially available, and the methodologies are available to prepare them for enzymatic studies (Schauer et al., 2011). *O*-acetylation of animal glycoproteins differs among different species. For example, 4-*O*-acetylation occurs frequently in guinea pig and horse glycoconjugates (Iwersen et al., 2003). In human, it was found that 20% of nasal mucin is *O*-acetylated. Therefore, it is speculated that people with highly acetylated nasal mucin are less susceptible to viral infections (Schauer et al., 2011). As it is widely known that viruses are able to remove *O*-acetylation from nasal mucin (Heimer and Meyer, 1956), it is proposed that the bacterial secondary infections are mediated by this mechanism. Indeed, pneumococcal esterases might also be involved in the *O*-acetyl group removal process from airway cavity mucin. Supporting this point, a study found that co-infection of mice with viruses does not increase the level of secondary infection indicating that *S. pneumoniae* is able to cause infection even without primary infection with viruses (Narayana Moorthy et al., 2013). This means that the pneumococcus encounters all host substrates to cause infection and uses all enzymes, including EstA and Axe, which allows the pneumococcus to cause infection.

Lipolytic enzymes such as esterases were shown to remove *O*-acetylation of sialic acid. My finding here is that EstA and Axe are able to cleave *O*-acetyl groups from BSM in agreement with previous studies. A study conducted by Corfield et al. (1992) reported that esterases such as sialate-4-*O*-acetylesterase could remove *O*-acetylation from glycoproteins. Viral esterases could cleave *O*-acetyl groups from glycoproteins (Regl et al., 1999) and hemagglutinin esterase (Vlasak et al., 1987, Zhang et al., 1992, Cornelissen et al., 1997, Sugiyama et al., 1998). Sugiyama et al. (1998) found that

hemagglutinin esterase from Mouse Hepatitis Virus is able to cleave the majority of *O*-acetyl groups from BSM. However, some publications reported that hemagglutinin esterase purified from Puffinosis Virus could not hydrolyse *O*-acetyl groups from BSM (Klausegger et al., 1999). Viral esterases have very limited specificity against *O*-acetyl substituents. For example, esterase purified from Murine Coronavirus is specific to 4-*O*-acetyl group than 9-*O*-acetyl. As a result, it can liberate acetate due to the cleavage of 4-*O*-acetyl group than 9-*O*-acetyl (Sugiyama et al., 1998, Regl et al., 1999).

Although EstA and Axe were found to be able to remove *O*-acetylation from terminal sialic acid *in vitro*, other functions should also be taken into consideration. Esterases can be classified into two functional groups: firstly, esterases that cleave *O*-acetyl substituents from carbohydrate repeating units of polysaccharides such as xylan, galactoglucomannan and conjugated sialic acid (Corfield et al., 1986, Tenkanen et al., 1995). This process is required as part of microbe's acquisition of carbon source, as the cleavage of *O*-acetylation allows sequential cleavage of sugar rich substrates. The second functional class of esterases are those involved in microbial virulence related and regulatory functions. These esterases enhance the pathogens lipid metabolism. For example, carboxyl esterase from *M. tuberculosis* is found to be a novel enzyme required for mycobacterial lipid/ester metabolism (Guo et al., 2010). Esterase can also convert inactive toxins into active form as found in *B. pertusisis* (Westrop et al., 1996). In both forms, esterases are required for cleavage of *O*-acetyl groups from substrates. Therefore, as *In vivo* study could not show any attenuation of an Axe mutant in pneumococcal colonisation and virulence, it might be involved in pneumococcal fatty acid pathways or other unknown functions that need to be investigated.

EstA and Axe are capable of de-*O*-acetylating BSM *in vitro*; a phenomenon that has been studied extensively in other microorganisms supporting my findings in this study. For example, 9-*O*-acetylesterase, which is also known as receptor-destroying enzyme, from influenza virus is found to be vital in viral infections (Herrler et al., 1987). They found that this viral esterase is able to utilise 9-*O*-acetyl substituents of host glycoproteins. Similarly, Group A streptococci (GAS) can use bovine sub-maxillary mucin less than porcine sub-maxillary mucin for nutritional requirements as

the latter is less *O*-acetylated mucin (Davis et al., 1979). Supporting this, a study found that human faecal bacteria could utilise highly acetylated sialic acid less efficiently than non-acetylated sialic acid (Corfield et al., 1992). They found that *O*-acetylation prevents bacterial utilisation of gastric glycoprotein, as 50% of sialic acid within gastric mucin is *O*-acetylated sialic acid (Corfield et al., 1995).

Here, I showed that recombinant EstA and Axe could cleave *O*-acetyl groups from Bovine sub maxillary mucin (BSM) *in vitro*. It was found that both EstA and Axe are able to cleave *O*-acetyl substituents from BSM. The cleavage was dependent on incubation time, and enzyme concentration. Although it was shown here that EstA and Axe could use BSM as substrate and release acetate as a result of *O*-acetyl hydrolysis, my results did not indicate whether pneumococcal esterases are specific to particular acetylation, as esterases are known to be specific to certain *O*-acetyl side chains (Regl et al., 1999). Currently, the site specificity of pneumococcal esterases is not known. The unavailability of specific *O*-acetyl substrates makes the investigation of specificity of pneumococcal esterases problematic. Synthesis of defined substrates might be useful to understand the specificity of pneumococcal esterases and how EstA and Axe utilise *O*-acetyl groups at certain acyl groups. As the chemical structure of BSM shows that *O*-acetylation occurs at positions 7-, 8- and 9-*O*-acyl groups (Diaz et al., 1989, Schauer, 1982), it is clear that EstA and Axe are specific to either of these acyl groups.

As the aim of this study was to investigate the pneumococcal esterases in terms of sialic acid de-*O*-acetylation, it is noteworthy to check whether de-*O*-acetylation mechanism occurs in other organisms. *O*-acetylation and de-*O*-acetylation commonly occurs in nature. A wide range of enzymes are involved in removal of *O*-acetylation from carbohydrates and glycoproteins. For example, the removal of *O*-acetyl groups is well established in plant polysaccharides such as pectin and xylan. Plant cellulose consists of repeating units of xylose, which is a type of carbohydrate that is linked with each other to form polysaccharides. These repeating units of sugars can be modified by substituents such as *O*-acetyl groups. The removal of repeating tandems of xylose by bacterial xylanase is restricted by *O*-acetylation of polysaccharides unless *O*-acetyl groups are removed by esterases (Martinez-Martinez et al., 2007). Therefore, the complete hydrolysis of plant cellulose by bacteria needs the release of

O-acetyl modifications allowing better hydrolysis of xylose by xylanase. Viral esterases are another example of *O*-acetyl removal. It has been well known that viral esterases are able to cleave *O*-acetyl substituents from substrates and release acetate as end product. Most of human faecal bacteria are able to de-*O*-acetylate of gastric mucin by using sialate-*O*-acetylesterase (Corfield et al., 1992). Therefore, these findings support the results achieved in this study.

5.5 Neuraminidase activity is potentiated by pneumococcal esterases:

To investigate the synergetic role of esterases with neuraminidase, BSM was incubated with recombinant EstA and Axe for different lengths of time, and then incubated with recombinant NanA. Sialic acid release from the substrate was measured. The results showed a clear synergism between esterases and NanA as more sialic was released by NanA after incubation with either esterase. The synergism was time and concentration dependent. This proves that pneumococcal esterases enhance NanA activity *in vitro*. However, the total sialic acid release was not confirmed as the composition of BSM before and after incubation was not assayed although it is reported that BSM contains 17% sialic acid and 22% of this sialic acid is *O*-acetylated (Pal et al., 2000).

The results in this study found that sialic acid release is limited by *O*-acetylation as not all sialic acids are released by pneumococcal NanA. It has also been proved here that pneumococcal EstA and Axe enhance the release of sialic acid from BSM. These findings are in agreement with studies that found *O*-acetylation could limit sialic acid release. For example, *O*-acetylation of terminal sialic acid prevents the total release of sialic acid from glycoprotein by the periodontal pathogen *Tannerella pasteurellosis* (Stafford et al., 2012). It has also been found that NanH produced by the pathogen can not cleave all sialic acid bonds except α-2,3-sialyl and α-2,6-sialyl bonds. Sialate-*O*-acetylesterase produced by human faecal bacteria especially *Bacteriodes* genus can cleave the *O*-acetyl groups from gastric glycoprotein (Corfield et al., 1992). In addition, it was shown that Group B *Streptococcus* could release up to 40% of sialic acid from BSM, and the rest is blocked due to the *O*-acetylation of sialic acid (Davis et al., 1979). This phenomenon is also clearly noticed in viral esterases, which could release *O*-acetyl groups from glycoproteins. The release is dependent upon the type of *O*-acetylation. Influenza C virus could release 9-*O*-acetyl substituent in sialic acid but

not 4- and 7-*O*-acetyl groups (Zimmer et al., 1992). However, some microorganisms do not need to cleave *O*-acetyl groups from sialic acid. Varki and Diaz (1983) showed that *Streptococcus sanguis* could cleave the majority of *O*-acetylated sialic acid, and *O*-acetyl substituents could not hinder the cleavage of sialic acid by microbial sialidase. This point may be linked to the environmental niche of the microbes and the abundance of *O*-acetylated substrates in these niches, and the importance of sugars for the survival and virulence of different microbial species.

I also determined whether EstA and Axe are able to release sialic acid from BSM. Esterases could not release sialic acid from BSM. This means that esterases are specific enzymes against *O*-acetyl substituents, whereas they could not cleave α-ketosidic linkage, α-2,3, α-2,6 and α-2,8 linkages, which link sialic acid to galactose substituent in glycoconjugates, and they can be cleaved by pneumococcal sialidases, NanA and NanB (Newstead et al., 2008, Camara et al., 1994, Gut et al., 2008, Xu et al., 2008). Conversely, although NanA is able to release sialic acid from BSM, it could not cleave ester bonds of *O*-acetyl substituents. Further assays for pneumococcal esterase specificity are needed. Using defined sialic acid with acetylation at certain acyl side chains is required to investigate substrate specificity of pneumococcal esterases.

5.6 Pneumococcal esterases are important for pneumococcal growth on mucin:

The aim of this project was to investigate the involvement of esterases in the cleavage of sialic acid *O*-acetylation. To do that, *ΔestA*, *Δaxe*, *ΔnanA*, *ΔestAnanA*, *ΔaxenanA* and their complemented strains were used to find out whether these mutants were attenuated in utilisation of mucin. Neuraminidase A (NanA) isogenic mutant was included to check whether it could potentiate NanA with esterases in the cleavage of Sia. The involvement of these genes in mucin utilisation was assessed by growing the isogenic mutants in chemically defined medium (CDM) supplemented with bovine sub-maxillary mucin (BSM).

Previously, it had been shown that *S. pneumoniae* (Yesilkaya et al., 2008) and *S. oralis* (Byers et al., 1999) could grow in media supplemented with porcine gastric mucin (PGM) (Yesilkaya et al., 2008) and α-acid-glycoprotein (Byers et al., 1999) as a sole carbon source. I found that BSM is toxic to the pneumococcus at high

concentration. Toxicity of mucin was reported previously (Heimer and Meyer, 1956, Chen et al., 1993, Yolken et al., 1994, Kawakubo et al., 2004, Gururaja et al., 1999). The secreted mucin provides antimicrobial molecules such as α-defensins, cathlecidins, lysozymes, collectins and lectins (Ouellette, 2010). These compounds are antimocrobicidals that interact with pathogens cell membranes and disrupt it. In addition, the mucin glycoprotein itself has antimicrobial characteristics, which restrict bacterial growth within mucus layer. For example, monosaccharide α(1,4)-linked *N*-acetylglucosamine is found to inhibit the cell wall synthesis of *H. pylori* (Kawakubo et al., 2004). It has also been reported that MUC7 has antimicrobial activity against *Candida* spp. and inhibits the yeast growth in saliva (Gururaja et al., 1999). Therefore, optimisation of BSM concentration that allows the pneumococcus to grow on mucin was the striking stage in this study.

The inability of pneumococcal *ΔestA* isogenic mutant and its derivatives to grow in CDM supplemented with BSM compared to wild type provides evidence for the involvement of these genes in exploitation of host glycoproteins for nutritional requirements. The growth assay showed that the single *ΔestA* and *ΔnanA* isogenic mutants exhibited a significant decrease in growth when compared to their parental strain, whereas the double *ΔestAnanA* isogenic mutant could not grow in CDM supplemented with BSM. This inability of growth might be related to inability of isogenic mutants to cleave *O*-acetyl groups from BSM respective to *ΔestA* (Herrler et al., 1985, Corfield et al., 1992), and inability to efficiently degrade terminal sialic acid (Burnaugh et al., 2008, Corfield, 1992, Yesilkaya et al., 2008, Manco et al., 2006). On the other hand, *Δaxe* isogenic mutant could utilise BSM as well as the wild type, despite the use of BSM as a substrate by Axe *in vitro*. This suggests that either Axe has no direct involvement in mucin utilisation or the absence of Axe enzyme is compensated by the activity of EstA or other esterases. This hypothesis was consistent with the increase in the transcriptional level of *estA* in *Δaxe* isogenic mutant exposed to BSM relative to wild type. Similarly, *In vivo* transcriptional analysis of pneumococcal esterases revealed that *estA* and *axe* are up regulated in pneumococci recovered from the nasopharynx and lungs of mice compared to blood, indicating potential involvement of Axe in pneumococcal survival in mucin rich respiratory tissues. Other reason for *Δaxe* not showing any attenuation in pneumococcal growth on BSM might be taken into consideration is the experimental conditions I used may

not be optimal for Axe, which might be influenced by temperature, presence of inhibitors, and the substrate specificity.

Previously, the sequential cleavage of host glycoconjugates was attributed to the action of exoglycosidases, and esterases' role has not been mentioned. Here, it is first study investigating the role of putative pneumococcal esterases and how their function affects the pneumococcal physiology and virulence in terms of sialic acid de-*O*-acetylation. A general figure of sequential cleavage of host sugars by *S. pneumonia*, incorporating esterases's role is shown in Figure 4.2. The sequential cleavage of host glycoproteins is dependent upon pneumococcal exo-glycosidases such as neuraminidases, β-galactosidases, N-acetylglucosaminidase and mannosidase (King et al., 2006). However, the initial cleavage of terminal sialic acid is restricted by *O*-acetylation (Schauer et al., 2011, Corfield et al., 1986). It has been reported that esterases can cleave *O*-acetyl substituents of sialic acid. It could be concluded that pneumococcal EstA and Axe are involved in sequential cleavage of host glycoconjugates. The cleavage of *O*-acetyl groups allows better sequential cleavage of sugars underlying.

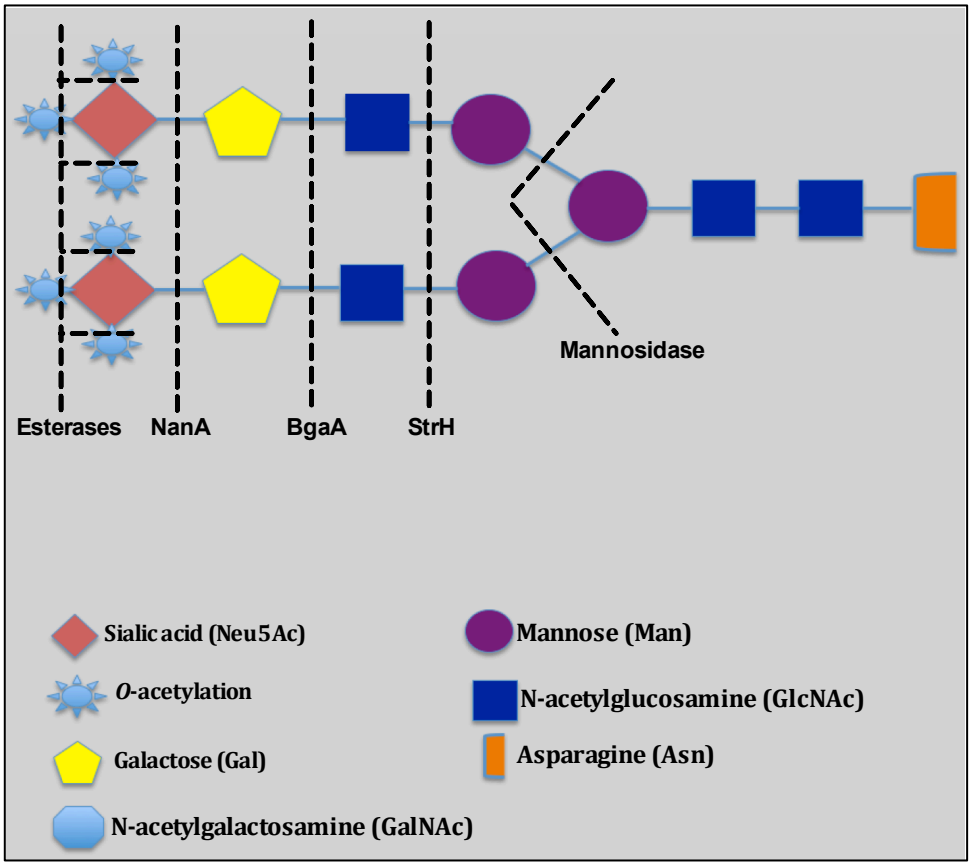

Figure 5.2: Schematic representation of host glycoconjugates sequential cleavage by pneumococcal enzymes. Terminal sialic acid, galactose, *N*-acetylglucosamine, and mannose are cleaved by neuraminidase (NanA), β-galactosidases, *N*-acetylglucosaminidase (StrH), and mannosidase, respectively (Yesilkaya et al., 2008, Manco et al., 2006, King et al., 2006). The sequential cleavage of host sugars is restricted by *O*-acetylation of terminal sialic acid, and pneumococcal esterases are involved in de-*O*-acetylation of terminal sialic acid.

Subcellular localisation assay identified EstA and Axe as intracellular proteins though esterase activity could be detected in the spent culture supernates as well. Detection of esterase activity in extracellular milieu is consistent with the reduced growth yield and sialic acid concentration of *ΔestA ΔestAnanA* on BSM. The mechanism of esterase release is not known. However, our data indicate the presence of active export mechanism rather than spontaneous cell lysis for esterase release as the level of

esterase activity in culture supernatant was higher than the activity of lactate dehydrogenase, an intracellular enzyme, relative to their concentration in intracellular fraction. It has been reported that certain pneumococcal intracellular proteins, such as α-enolase and pneumolysin, which do not have a signal peptide similar to EstA and Axe, can have a cell surface localization (Bergmann et al., 2001, Canvin et al., 1995). Therefore, future studies should investigate the mechanism of esterase release.

5.7 Pneumococcal esterases enhance sialylation of host glycoconjugates:

Here, I investigated whether pneumococcal esterases are involved in host mucin degradation during colonisation. To do that, mice were colonised with different pneumococcal strains including *ΔestA*, *ΔnanA* and *Δaxe* isogenic mutants and their derivatives, and the nasopharyngeal lavage of infected mice were processed for bound and free sialic acid. The expected outcome of this assay was that if pneumococcal esterases were involved in host mucin degradation, the level of free sialic acid would be higher in nasopharyngeal lavage of mice infected with the wild type but lower in the cohorts infected with *ΔestA*, *Δaxe, ΔnanA, ΔestnanA, and ΔaxenanA* isogenic mutants. Unfortunately, I could not substantiate my hypothesis as the level of free sialic acid was below the detection limit. Therefore, I decided to determine the level of bound sialic acid. I found that the pneumococcus induces the secretion or sialylation of mucin, which was in line with the study done by (Siegel et al., 2014). In mice colonised with D39 wild type, the level of bound sialic acid was significantly higher than the control group that received PBS. Interestingly, the level of bound sialic acid was significantly decreased in mice colonised with *ΔestA* and *ΔnanA, ΔestAnanA* isogenic mutants. However, no difference in bound sialic acid level could be noticed in *Δaxe* isogenic mutant compared to wild type. The plausible explanation for why the pneumococcus induces host mucin expression is that as a consequence of pneumococcal colonisation, the host mucin genes are expressed due to constant stimuli by the pneumococcus. It is well established that pneumococcal products stimulate airway mucin gene expression (Manco et al., 2006, Siegel et al., 2014). Indeed, the mucus layer dynamically responds to bacterial infections (McGuckin et al., 2011). The host mucin is highly sialylated at terminal sialic acid when the mice infected with either virus or pneumococci (Angata and Varki, 2002, Siegel et al., 2014).

The secretion or sialylation of respiratory mucin is not induced when mice were colonised with *ΔnanA* isogenic mutant, as the level of bound sialic acid was significantly lower than parental strain. This may indicate that cleavage of sialic acid is recognised as a signal for secretion of respiratory mucin secretion (Dahiya et al., 1992, Yesilkaya et al., 2008). The excessive secretion or sialylation of mucin in nasopharynx is directly linked with pneumococcal colonisation in infants and children under the age of 5 years old (Rodrigues et al., 2013). The sialylation of host mucin allows better substrate for pneumococcal neuraminidases and, as a result, increases the colonisation density. It was found that exogenous administration of sialic acid enhances pneumococcal colonisation in nasopharynx (Trappetti et al., 2009). This might occur due to mucin induction by the released sialic acid as a result of viral or bacterial infections (Siegel et al., 2014). To conclude, as a result of pneumococcal infection, the sialic acid cleavage would induce the secretion or the sialylation of mucin in respiratory tract. To investigate whether *S. pneumoniae* induces the secretion or the sialylation of mucin, testing of host mucin expression before and after pneumococcal colonisation and infection is needed.

5.8 Pneumococcal colonisation and virulence:

In vivo evaluation of pneumococcal esterases involvement in colonisation and acute infection in mouse model showed that EstA, but not Axe, is involved in pneumococcal colonisation and virulence. It was observed that *ΔestA* and *ΔestAnanA* isogenic mutants displayed a significant attenuation in pneumococcal colonisation and acute infection model compared to the parental strain. In colonisation model, *ΔestA*, *ΔnanA* and *ΔestAnanA* isogenic mutants could not survive in the nasopharynx as well as the wild type. The number of pneumococci retrieved from nasopharyngeal tissues was significantly lower than the wild type at 7 days post-infection, and the double *ΔestAnanA* isogenic mutant was cleared from the nasopharynx 7 days post-infection. The direct involvement of EstA alone or in combination with NanA was clear both in colonisation and invasion. On the other hand, Axe contributed neither the pneumococcal colonisation nor to virulence. Although *in vitro* results revealed a clear involvement of Axe in de-*O*-acetylation of sialic acid, the *In vivo* results did not reflect *in vitro* results. This may be due to compensatory effect of other genes coding for proteins that are involved in host glycoprotein degradation. For example, the

expression of EstA, and NanA is up regulated in the absence of Axe, which are known to have a role in degradation of host glycoproteins (King et al., 2005, Manco et al., 2006). Other explanation is that the dose of pneumococci used for intranasal challenge was high, which may mask any subtle contribution Axe might have. Therefore, lowering the challenge dose can test this point.

It is curious that a human pathogen like *S. pneumoniae* has acetyl xylan esterase (Axe) activity. As far as it is known this enzyme uses xylan as a substrate. Xylan is found in plant tissues, and is composed of repeating xylose residues, which are highly acetylated, and can be utilised by microbial pathogens of plants (Gilbert, 2010). Esterases from plant pathogens can cleave *O*-acetyl groups from cellulose allowing other enzymes, such as xylanases, β-xylosidases, α-arabinofuranosidases and methylglucuronidases, to cleave cellulose for carbon source requirements, an analogous situation to sequential cleavage of mammalian host glycans. The activity of xylanases is restricted by *O*-acetyl groups of xylan. Acetyl xylan esterase activity makes the depolymerisation of xylan easier (Lee et al., 1986). The structural characteristics of plant cellulose are significantly similar to glycoproteins of mammalian host. They share the same repeating tandems of carbohydrates forming polysaccharides. These polysaccharides are also modified with different final modifications such as *O*-acetylation. Therefore, it could be speculated here that pneumococcal acetyl xylan esterase may be using an unknown substrate (s) in host. Therefore, future studies using different substrates should be able to identify the extent function of this enzyme.

Data gathered in this study allows us to link EstA to sialic acid cleavage *in vitro* and *In vivo*. However, it cannot be ruled out that esterases may have wider role in pneumococcal biology, which requires further investigation. A wide range of published data can support this postulation. For example, the deletion of cell membrane-associated carboxylic esterase from *M. tuberculosis* attenuates the virulence (Lun and Bishai, 2007). The authors linked the decrease in *M. tuberculosis* cell load in lungs and blood to the absence of esterase. Indeed, esterases could hydrolyse the host fatty acid, providing the nutrition to *M. tuberculosis*. It has also been found that secreted esterase from GAS is an important virulence factor in subcutaneous infections. Streptococcal Secreted Esterase (Sse) plays a critical role in

soft tissue damage and systematic spread from skin to bloodstream and organs (Zhu et al., 2009).

The function of the lipolytic enzymes in pathogenesis and virulence is not well annotated. The limited available evidence suggests their importance in microbial virulence. For example, esterases and lipases were shown to be important for the virulence of *Streptococcus pyogenes*, *Staphylococcus aureus* (Jaeger et al., 1994), *Burkholderia cepacia* (Mullen et al., 2007), and *M. tuberculosis* (Lun and Bishai, 2007). It has been reported that esterases are involved in bacterial sporulation (Higerd, 1977). The contribution of lipases/esterases to microbial virulence was linked to their ability to modify host cell lipids (Mumy et al., 2008). Carboxyl esterase coded by Rv3487c has been characterised from *M. tuberculosis* and linked to mycobacterial ability to cause lung infection in mice (Zhang et al., 2005, Camacho et al., 1999). It has been found that *Pseudomonas aeroginosa*, a cystic fibrosis agent, has membrane-associated esterase (Wilhelm et al., 1999), which is important for *P. aeruginosa* lipid production, cell attachment and biofilm formation (Wilhelm et al., 2007). Furthermore, it has also been reported that esterases are able to convert some inactive bacterial toxins into active toxins. For example, esterase (CyaC) from *Bordetella pertussis* converts inactive adenylate cyclase-hemolysin toxin (CyaA), which is a key virulence factor in this pathogen, into active phase (Westrop et al., 1996).

Other point to consider regarding esterases' role in the pathogenesis of *S. pneumoniae*, is related to *S. pneumoniae*-virus interaction, and the effect of this interaction on attachment. It is well-established that an initial viral infection predisposes individuals to secondary pneumococcal infections (McCullers (2006). Respiratory viruses are detected in the nasopharyngeal cavity of 90% of children under the age of 5 years, which is the most vulnerable age group for pneumococcal infections (McCullers, 2006). Pneumococcal loads in nasopharynx is directly linked with influenza infections in adults and children (Bogaert et al., 2004a). It is widely known that influenza viruses contain esterase genes (Herrler et al., 1985). Viral esterases are directly involved in viral attachment and virulence. It can be speculated that these esterases might mediate *S. pneumoniae* attachment by unmasking the terminal sialic acid of glycoprotein. However, co-infection with virus does not

increase the level of pneumococcal secondary infection of mice (Narayana Moorthy et al., 2013), indicating that *S. pneumoniae* is also able to cause infection by using its endogenous enzymes that are involved in glycoprotein degradation such as EstA, Axe and NanA.

Concluding remarks:

S. pneumoniae is one of the most extensively studied pathogens. However, an understanding of how this pathogen colonises and causes infection is still not obvious. Pneumococcal utilisation of host glycoconjugates for nutritional requirements is an important determinant of *S. pneumoniae* survival and persistence in the host during colonisation and invasive disease. As the pneumococcus relies on the sugar compounds in host glycoprotein, it needs different mechanisms to unmask the host glycoprotein and break through underlying components. In this process, one third of pneumococcal genome is involved including the attachment to host glycoprotein, sequential cleavage of sugars and transportation of these sugars into pneumococcal fermentation pathways.

In this study, I investigated the role of pneumococcal esterases in terms of sequential cleavage of host sugars. EstA is found to be involved in pneumococcal utilisation of mucin *in vitro* and *In vivo*. EstA was shown to be the major pneumococcal esterase, and able to use BSM as a substrate. Furthermore, EstA deletion attenuates pneumococcal colonisation and infection. However, although Axe was able to use BSM as substrate *in vitro*, its *In vivo* role remains to be investigated. The deletion of *axe* did not affect pneumococcal colonisation and infection. Transcriptional analysis of pneumococcal esterases showed that EstA and Axe are up regulated when *S. pneumoniae* is exposed to BSM. Further investigation of pneumococcal enzymes involved in sugar utilisation and their cooperative interaction can reveal how *S. pneumoniae* utilises host carbohydrates for carbon source requirements.

Future work:

In order to understand how *S. pneumoniae* survives and causes infection, further study is needed. The pneumococcus uses exoglycosidases for sequential cleavage of host sugars. In this study, I focused on pneumococcal esterases and how EstA and Axe are involved in pneumococcal utilisation of host glycoprotein. However, the other two esterases, acyl-ACP thioesterase and phosphoesterase, have not been studied in detail. It might be noteworthy to investigate their role in pneumococcal physiology.

To investigate the function of pneumococcal esterases universally, it is vital to check whether these esterases are found in pneumococcal population and whether the activity of esterases is strain dependent. This can also be linked with how these esterases activity affect pneumococcal virulence within virulent and non-virulent strains. Bioinformatics analysis of pneumococcal esterases can be done to investigate the existence of pneumococcal esterases within different serotypes.

A new class of bacterial esterase has been discovered. These esterases are able to use a wide range of substrates that are highly *O*-acetylated. Some bacterial esterases are found to be involved in the final modification of cell wall peptidoglycan (Weadge et al., 2005). These esterases are known as *O*-acetylpeptidoglycan esterases (Apes). For example, some of major human pathogens such as *Campylobacter jejuni*, *H. pylori*, *N. gonorrhoeae*, *N. meningitidis* and *S. aureus* are found to express Apes (Weadge and Clarke, 2006, Clarke and Dupont, 1992). The main function of these esterases is to control the *O*-acetylation of bacterial cell wall peptidoglycan (Clarke and Dupont, 1992). Bacterial peptidoglycan is composed of repeating units of N-acetylglucosamine linked with N-acetylmuramic acid. These repeating units are then modified with *O*-acetylation at C6 hydroxyl group. Regulation of bacterial peptidoglycan *O*-acetylation helps pathogens to escape from host lysozymes and phagocytosis. It has also been found that *O*-acetylation of bacterial peptidoglycan is de-*O*-acetylated by esterases to limit bacterial autolysins as well as host innate immune enzymes (Moynihan and Clarke, 2011). The de-*O*-acetylation of peptidoglycan is vital to maintain the biosynthesis of bacterial peptidoglycan (Moynihan and Clarke, 2011). It was also found that *O*-acetylation of peptidoglycan is required for cell division and separation in *Bacillus anthracis* (Laaberki et al., 2011). This suggests that esterases are protease enzymes that can catalyse a wide

range of substrates. To control bacterial peptidoglycan de-*O*-acetylation, esterases are needed. It is plausible therefore that other pneumococcal esterases that could not show any role in glycoprotein degradation, may have roles other than what has been described in this study.

The involvement of Axe in glycoprotein utilisation, colonisation and virulence could not be shown. This might be because BSM is not the best substrate for Axe or the mouse model of infection could not demonstrate its involvement. It might be interesting to define a better substrate for Axe, and modify the *In vivo* model for further study of Axe. The adjustment of bacterial CFU challenged might be useful to check any minor involvement of Axe. In addition, investigation of Axe role in other animal model might be noteworthy as the mucin composition varies upon the animal species (Varki and Sharon, 2009). Identifying suitable defined acetylated substrates that mimic host mucin or other glycoproteins would be very valuable. The defined acetylated substrate can provide information about enzyme site specificity that allows better understanding of the enzyme function.

Another follow up study would be to identify the target for EstA and Axe. As mentioned earlier, glycoproteins are *O*-acetylated in 4-, 7- and 9-*O*-acetyl substituent. Therefore, it would be interesting to identify which acyl group is targeted by pneumococcal esterases. To do that, synthesis of defined acetylated sialic acid substrates would be needed.

Kinetic characterisation of EstA and Axe using natural substrate is another interesting future study. This can provide the catalytic characterisation and affinity of pneumococcal esterases. The catalytic activity of esterase with natural substrates might be different than synthetic substrates. The chromogenic substrates are synthesised in known structure and the target of enzymes is already known. These sorts of substrates contain the terminal bond specific to esterases that allows simple enzyme-substrate reaction process. On the other hand, natural substrates can provide more precise information of the reaction process. The natural substrates are compact substrates, and this would mimic the exact process of enzyme kinetics.

It is also noteworthy to investigate whether pneumococcal esterases can be used as a potential target to develop anti-infective compounds against *S. pneumoniae*. For this

initially recombinant esterases can be used to immunise mice as described previously (Liu et al., 2007). Subsequently mice would be challenged with the lethal dose of *S. pneumoniae*, and the survival of animals would be determined. Furthermore, the serum antibody level before and after vaccination can be determined to see if immunisation with esterases somehow induce protective antibody level. The available data supports the view that esterases can be used as immunogenic agent against bacterial infections. Liu et al. (2007) showed that carboxylic esterase protects mice against subcutaneous infection caused by Group A streptococci (GAS). They found that active and passive immunisation by streptococcal esterase decreased the ability of pathogen to cause infection. Their data suggest that esterase involved in streptococcal invasion of the connective tissues of skin. It was also reported that GAS secretes esterase that is able to hydrolyse platelet-activating factor (Liu et al., 2012), inhibits neutrophil recruitment and inactivates the PAF's ability to activate neutrophils. This allows the pathogen disseminate deep into host tissues (Zhu et al., 2009, Liu et al., 2007). It has also been found that patients with pharyngitis infections have antibodies specific to esterases.

It is also noteworthy to investigate the role of esterases in pneumococcal attachment to mammalian cells. The percentage of pneumococcal attachment might be assessed using wild type and mutant strains. It is known that NanA is responsible for initial attachment of *S. pneumoniae* during colonisation and invasion (King et al., 2006, King, 2010). The specific question therefore would be whether esterases play role in attachment alone or in combination with NanA. To check pneumococcal attachment to mammalian cells, epithelial cell line CCL-23 (also referred to Hep-2 cells) can be used as monolayer cells. Three steps are involved in this assay: 1- preparation of cells and bacterial suspension, 2- mixing the bacterial suspension with monolayer cells, and 3- determination of the level of attachment using viable counts of attached cells.

Appendix 1

A: SPD_1239 alignment with *ΔSPD_1239:*

```
SPD_1239        GACCATTCATATCCAAGTCGTAAAAACGAACATGGTAATCCTTGCTGATCGGCTCTTCCA  62
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1269192  GACCATTCATATCCAAGTCGTAAAAACGAACATGGTAATCCTTGCTGATCGGCTCTTCCA  1269251
SPD_1239        AGGATTCATACTTCGGTCCACGGATAAGTTTTTTATCAAAATCAGACTGGTAAGGAGCCA  122
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1269252  AGGATTCATACTTCGGTCCACGGATAAGTTTTTTATCAAAATCAGACTGGTAAGGAGCCA  1269311
SPD_1239        CAATCTCAGGTTCAACAGCATGGACTTTTCGACTGTCGCGGTCCATGAGAACAAAGGTCG  182
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1269312  CAATCTCAGGTTCAACAGCATGGACTTTTCGACTGTCGCGGTCCATGAGAACAAAGGTCG  1269371
SPD_1239        CCATCATATGGATGAGCTCCTGCCCCGCTTCATTATAAATAGTAAAGCGACGGTAGCAAA  242
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1269372  CCATCATATGGATGAGCTCCTGCCCCGCTTCATTATAAATAGTAAAGCGACGGTAGCAAA  1269431
SPD_1239        AAAGTCGATTGTAGCTCAAGGCTTCCGTTTCGATGGTAATTTCTTCCGCAAAACGAGGCA  302
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1269432  AAAGTCGATTGTAGCTCAAGGCTTCCGTTTCGATGGTAATTTCTTCCGCAAAACGAGGCA  1269491
SPD_1239        AACGAACCACCTCAATATCATATTCTGCGATAATCCAGACCAGATTATAGTCTTCCAAAA  362
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1269492  AACGAACCACCTCAATATCATATTCTGCGATAATCCAGACCAGATTATAGTCTTCCAAAA  1269551
SPD_1239        TGGCCTTATCACTAACTCCCAGTTCAATCGATTGCATCCCTGAAACTTGCAGTGACAGCA  422
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1269552  TGGCCTTATCACTAACTCCCAGTTCAATCGATTGCATCCCTGAAACTTGCAGTGACAGCA  1269611
SPD_1239        AAATCACATCTGGAAGTTTGATATGACCGTTCATATCAGCCATATCAAAAGGAATTTTCA  482
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1269612  AAATCACATCTGGAAGTTTGATATGACCGTTCATATCAGCCATATCAAAAGGAATTTTCA  1269671
SPD_1239        TTTTCATTTGATAAGTTAAGCCCAT
                |||||||||||||||||||||||||
Sbjct  1269672  TTTTCATTTGATAAGTTAAGCCCAT
```

Insertion site of spectinomycin cassette into SPD_1239 gene sequence:

CTAAACCTTTTTTATTTCTTGCCAAGTAATGATTGCTTGGGCATTGATAACCCCATCACTTGTAATTTCATGCTTGCTTTCCAGTC
CAGTCCGTTCAACAGCCGATGTAATCACCCCACCTGGTCGAACTTCCTTGACATACTTGAGGTTGATTTTCTTGGGAATATAGTGG
GTCAAAAAATCCGCTCCCATGACCTCAAAAATCCAGTCCAAGTATTTACTGTTATTGACATspectinomycin.cassetteGAC
CATTCATATCCAAGTCGTAAAAACGAACATGGTAATCCTTGCTGATCGGCTCTTCCAAGGATTCATACTTCGGTCCACGGATAAGT
TTTTTATCAAAATCAGACTGGTAAGGAGCCACAATCTCAGGTTCAACAGCATGGACTTTTCGACTGTCGCGGTCCATGAGAACAAA
GGTCGCCATCATATGGATGAGCTCCTGCCCCGCTTCATTATAAATAGTAAAGCGACGGTAGCAAAAAAGTCGATTGTAGCTCAAGG
CTTCCGTTTCGATGGTAATTTCTTCCGCAAAACGAGGCAAACGAACCACCTCAATATCATATTCTGCGATAATCCAGACCAGATTA
TAGTCTTCCAAAATGGCCTTATCACTAACTCCCAGTTCAATCGATTGCATCCCTGAAACTTGCAGTGACAGCAAAATCACATCTGG
AAGTTTGATATGACCGTTCATATCAGCCATATCAAAAGGAATTTTCATTTTCATTTGATAAGTTAAGCCCAT

B: SPD_0534 (*estA*) alignment with *ΔSPD0534* (*ΔestA*):

```
SPD_0534       AAAGGTACAAGACGGGAATATCTTCACACTCTGGTTCTTCCACTCGATTGGCATCAGGGT  70

               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct  547084  AAAGGTACAAGACGGGAATATCTTCACACTCTGGTTCTTCCACTCGATTGGCATCAGGGT  547025

SPD_0534       AGAGGACATTCACCCCCCACTCCATATCCAATACTTGTGAGTAATACTCGATTTTCATTA  130

               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct  547024  AGAGGACATTCACCCCCCACTCCATATCCAATACTTGTGAGTAATACTCGATTTTCATTA  546965

SPD_0534       CTGCCAT

               |||||||

Sbjct  546964  CTGCCAT
```

Insertion site of spectinomycin cassette into SPD_0534 gene sequence:

CTAAGTCAGTCTCTCTTCTAATTTGAAATCAATTGGTAGGGTTGTTAAAAAAACTTCCAATTGTTTTTCCCAGTAGTACCACTCGT
GAGTTCCAGCGCTATGGCTATAGGTCACATCAAAACCTAGTTTTTTGAGATTTTTCACTGCGAGATTATTGGCTTCGTACAAGAAA
TCCTGTTCGCCACACCACGCCCAAAGTTTGGTCTTTTTATCCGATTTTTTAGCCAGACTTTCAAGAGAATAGGGACTAGTTGTCCA
GTCTCTAATCTCTCCAAAAACACCTCTCCAGTAGGCTGGACTTCCCAGATTTTGACTTTCAGGAGAAAAGTTTTGAAAGCTGAGGG
CACCTGAAAAACTAGCTGCATGAGAAAAACGATTTGTCGTAAGAGCCAGTTTGAAGCAGCCGTAGCCTCCCATAGAAAGACCAGCG
ATAAAGGTCTTTTCACGCTTGCTCGTCATATTAGGGAAGAAGCGTTTCAGAACCTGTGGCAATTCCTCTGCTAGAGCCGTGTAGTA
GTCAAAACCATACTGGGTATCGGTGTACCAACCATTGCTGGTATTGGGCATAACAACGATGAGATTAGTTCCTCGAAGCAAGCGTT
CTACATTGGTCCGCTTAAGCCAACTATTATGATTTCCAGACATCCCGTGCASpectinomycin.cassetteAAAGGTACAAGAC
GGGAATATCTTCACACTCTGGTTCTTCCACTCGATTGGCATCAGGGTAGAGGACATTCACCCCCCACTCCATATCCAATACTTGTG
AGTAATACTCGATTTTCATTACTGCCAT

C: SPD_0932 alignment with *ΔSPD0932*:

```
SPD_0932       TCTCACTTATCAGGTGCATATAAACGAAAAATTTTTTCTAAAGAAAATACACGATTTTGG  73

               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct  989965  TCTCACTTATCAGGTGCATATAAACGAAAAATTTTTTCTAAAGAAAATACACGATTTTGG  990024

SPD_0932       AGTTTAATATTAACTCGAAAGAATCTTCGCAACAACTTGATATTCTCTATAAAGAAGTTG  133

               ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||

Sbjct  990025  AGTTTAATATTAACTCGAAAGAATCTTCGCAACAACTTGATATTCTCTATAAAGAAGTTG  990084

SPD_0932       GTGTATTAA

               |||||||||

Sbjct  990085  GTGTATTAA  990093
```

```
Sbjct  990085  GTGTATTAA
```

Insertion site of spectinomycin cassette into SPD_0932 gene sequence:

ATGAGAGGGTTTAATAACAAGATAAAGTCTGTTTATCAAGAACTAACAAATTCCAAAGAGAAATTCGGTAGCTTTCACAAGACTTT
AATTCATTTGCATACACCTGTTTCTTATGATTACAAGCTATTTTCTAATTGGACTGCAACGAAATATAGAAAAATTACTGAAGATG
AACTATATGATATATTTTTTGAAAATAAGAAAATAAAAGTTGATAAGACAATTTTTTTTAGTAATTTTGATAAGGTTGTTTTTTCT
AGTTCAAAAGAATATATTAGTTTTCTTATGTTAGCAGAGGCAATCATAAAAAATGGAATAGAAATAGTTGTAGTAACTGATCATAA
TACTACCAAAGGTATTAAAAAGTTACAAATGGCAGTCTCAATCATAATGAAAAATTATCCGATTTATGATATACATCCTCATATTT
TACATGGAGTAGAAATTAGTGCAGCAGATAAATTGCATATTGTATGTATATATGATTATGAACAAGAATCATGGGTTAATCAATGG
TTAAGTGAAAATATTATAAGTGAGAAAGATGGAAGTTATCAACATTCACTGACTATAATGAAGGATTTCAATAATCAAAAAATAGT
TAACTATATTGCTCATTTCAATAGTTATGACATTTTGAAAAAAGGT**Spectinomycin.cassette**TCTCACTTATCAGGTGCA
TATAAACGAAAAATTTTTTCTAAAGAAAATACACGATTTTGGAGTTTAATATTAACTCGAAAGAATCTTCGCAACAACTTGATATT
CTCTATAAAGAAGTTGGTGTATTAA

D: SPD_1506 (*axe*) alignment with *ΔSPD1506* (*Δaxe*):

```
SPD_1506        TTTTCCGTTATTTCAAGTTTTACGACCCCTTCCATGAAACAGAGGAGGAAATCATGGCGA  76
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527251  TTTTCCGTTATTTCAAGTTTTACGACCCCTTCCATGAAACAGAGGAGGAAATCATGGCGA  1527192
SPD_1506        CCCTTGCCTATATCGATGTCAAAAATCTTGCCCATCGTATCCAAGGTGAGGTTAAGATGA  136
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527191  CCCTTGCCTATATCGATGTCAAAAATCTTGCCCATCGTATCCAAGGTGAGGTTAAGATGA  1527132
SPD_1506       TTACGGGCTTGGACGACGATGTTTGCTATCCCATTACCCAGTTTGCGATTTATAATCGTC  196
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527131  TTACGGGCTTGGACGACGATGTTTGCTATCCCATTACCCAGTTTGCGATTTATAATCGTC  1527072
SPD_1506        TGAC
                ||||
Sbjct  1527071  TGAC
```

Insertion site of spectinomycin cassette into SPD_1506 gene sequence:

ATGAAAAATCCAGCTTTGCTAGAAGAAATTAAGACCTATAGAGGAAGGGATGAGGTTCCGGAAGACTTTGATGATTTCTGGGATG
GGGAAGTGAAAAATGTTTCCACGCTTCCATCCTACCACTTGGAGGAAAGAGATTTCCACATTCCTCAAGTCAAGTGCTATGAGTT
AACATTTGAAGGAAGCAAGGAAGGAAAGGTCTATGCACGCATTGTTCTTCCAAAGAGTGAGGAGAAGGTCCCATTAATCTTCCAT
TTTCATGGTTATATGGGACGTGGCTGGGACTGGGCCGACATGCTGGGCTTCACCGTAGCTGGTTACGGTGTTGTTTCCATGGATG
TGCGGGGCCAGTCAGGTTACTCACAAGACGGCTTGCGTTCTCCTTTAGGAAATACCGTGAAGGGGCATATTATCCGTGGTGCTGT
GGAAGGTCGGGACCACCTCTTTTATAAGGATGTTTATCTGGATATTTACCAGTTGGTCGAAATTGTTGCTAGTCTGTCTCAGGTT
GATGAGAAGCGTCTTTCTAGCTATGGTGCCTCACAAGGAGGGGCTCTAGCTCTAGTTGCAGCAGCGCTCAATCCTCGAATTCAGA
AAACAGTTGCCATTTATCCCTTCTTGTCAGACTTCAGACGGGTGATTGAGATTGGTAATACTAGCGAGGCTTACGACGAAC**Spec**
tinomycin.cassetteTTTTCCGTTATTTCAAGTTTCACGACCCCTTCCATGAAACAGAGGAGGAAATCATGGCGACCCTTGC
CTATATCGATGTCAAAAATCTTGCCCATCGTATCCAAGGTGAGGTTAAGATGATTACGGGCTTGGACGACGATGTTTGCTATCCC
ATTACCCAGTTTGCGATTTATAATCGTCTGACCTGCGATAAAACCTATCGCATCATGCCTGAGTATGCTCACGAAGCCATGAATG
TATTTGTCAATGACCAAGTCTACAACTGGCTCTGTGGAAGTGAGATTCCTTTTAAATATCTAAAATAA

Appendix 2

A: Alignment of SPD_534 (*estA*) with SPD0534Comp^{S121A} (estAComp^{S121A}): The nucleotide in red bold colour and green highlight shows the replacement of serine amino acid to alanine.

```
SPD_534Comp^S121A CATGGCAGTAATGAAAATCGAGTATTACTCACAAGTATTGGATATGGAGTGGGGGGTGAA  185
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  546957  CATGGCAGTAATGAAAATCGAGTATTACTCACAAGTATTGGATATGGAGTGGGGGGTGAA  547016

SPD_534Comp^S121A TGTCCTCTACCCTGATGCCAATCGAGTGGAAGAACCAGAGTGTGAAGATATTCCCGTCTT  245
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547017  TGTCCTCTACCCTGATGCCAATCGAGTGGAAGAACCAGAGTGTGAAGATATTCCCGTCTT  547076

SPD_534Comp^S121A GTACCTTTTGCACGGGATGTCTGGAAATCATAATAGTTGGCTTAAGCGGACCAATGTAGA  305
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547077  GTACCTTTTGCACGGGATGTCTGGAAATCATAATAGTTGGCTTAAGCGGACCAATGTAGA  547136

SPD_534Comp^S121A ACGCTTGCTTCGAGGAACTAATCTCATCGTTGTTATGCCCAATACCAGCAATGGTTGGTA  365
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547137  ACGCTTGCTTCGAGGAACTAATCTCATCGTTGTTATGCCCAATACCAGCAATGGTTGGTA  547196

SPD_534Comp^S121A CACCGATACCCAGTATGGTTTTGACTACTACACGGCTCTAGCAGAGGAATTGCCACAGGT  425
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547197  CACCGATACCCAGTATGGTTTTGACTACTACACGGCTCTAGCAGAGGAATTGCCACAGGT  547256

SPD_534Comp^S121A TCTGAAACGCTTCTTCCCTAATATGACGAGCAAGCGTGAAAAGACCTTTATCGCTGGTCT  485
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547257  TCTGAAACGCTTCTTCCCTAATATGACGAGCAAGCGTGAAAAGACCTTTATCGCTGGTCT  547316

SPD_534Comp^S121A TGCTATGGGAGGCTACGGCTGCTTCAAACTGGCTCTTACGACAAATCGTTTTTCTCATGC  545
                  |=||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547317  TTCTATGGGAGGCTACGGCTGCTTCAAACTGGCTCTTACGACAAATCGTTTTTCTCATGC  547376

SPD_534Comp^S121A AGCTAGTTTTTCAGGTGCCCTCAGCTTTCAAAACTTTTCTCCTGAAAGTCAAAATCTGGG  605
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547377  AGCTAGTTTTTCAGGTGCCCTCAGCTTTCAAAACTTTTCTCCTGAAAGTCAAAATCTGGG  547436

SPD_534Comp^S121A AAGTCCAGCCTACTGGAGAGGTGTTTTTGGAGAGATTAGAGACTGGACAACTAGTCCCTA  665
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547437  AAGTCCAGCCTACTGGAGAGGTGTTTTTGGAGAGATTAGAGACTGGACAACTAGTCCCTA  547496

SPD_534Comp^S121A TTCTCTTGAAAGTCTGGCTAAAAAATCGGATAAAAAGACCAAACTTTGGGCGTGGTGTGG  725
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547497  TTCTCTTGAAAGTCTGGCTAAAAAATCGGATAAAAAGACCAAACTTTGGGCGTGGTGTGG  547556
```

```
SPD_534CompS121A CGAACAGGATTTCTTGTACGAAGCCAATAATCTCGCAGTGAAAAATCTCAAAAAACTAGG  785
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547557  CGAACAGGATTTCTTGTACGAAGCCAATAATCTCGCAGTGAAAAATCTCAAAAAACTAGG  547616

SPD_534CompS121A TTTTGATGTGACCTATAGCCATAGCGCTGGAACTCACGAGTGGTACTACTGGGAAAAACA  845
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547617  TTTTGATGTGACCTATAGCCATAGCGCTGGAACTCACGAGTGGTACTACTGGGAAAAACA  547676
SPD_534CompS121A ATTGGAAGTTTTTTTTAACAACCCTACCAATTGATTTCAAATTANAAGAGAGACTGACTTAG  906
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547677  ATTGGAAGTTTTTTTAACAACCCTACCAATTGATTTCAAATTAGAAGAGAGACTGACTTAG  547737
```

B: Alignment of SPD_1506 (*axe*) with SPD1506Comp^{S181A} (axeComp^{S181A}): The nucleotide in red bold colour and green highlight shows the replacement of serine amino acid to alanine.

```
SPD_1506CompS181A ATGAAAAATCCAGCTTTGCTAGAAGAAATTAAGACCTATAGAGGAAGGGATGAGGTTCCG  110
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527927  ATGAAAAATCCAGCTTTGCTAGAAGAAATTAAGACCTATAGAGGAAGGGATGAGGTTCCG  1527868
SPD_1506CompS181A GAAGACTTTGATGCTTTCTGGGATGGGGAAGTGAAAAATGTTTCCACGCTTCCATCCTAC  170
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527867  GAAGACTTTGATGCTTTCTGGGATGGGGAAGTGAAAAATGTTTCCACGCTTCCATCCTAC  1527808
SPD_1506CompS181A CACTTGGAGGAAAGAGATTTCCACATTCCTCAAGTCAAGTGCTATGAGTTAACATTTGAA  230
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527807  CACTTGGAGGAAAGAGATTTCCACATTCCTCAAGTCAAGTGCTATGAGTTAACATTTGAA  1527748
SPD_1506CompS181A GGAAGCAAGGAAGGAAAGGTCTATGCACGCATTGTTCTTCCAAAGAGTGAGGAGAAGGTT  290
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527747  GGAAGCAAGGAAGGAAAGGTCTATGCACGCATTGTTCTTCCAAAGAGTGAGGAGAAGGTT  1527688
SPD_1506CompS181A CCATTAATCTTCCATTTTCATGGTTATATGGGACGTGGCTGGGACTGGGCCGACATGCTG  350
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527687  CCATTAATCTTCCATTTTCATGGTTATATGGGACGTGGCTGGGACTGGGCCGACATGCTG  1527628
SPD_1506CompS181A GGCTTCACCGTAGCTGGTTACGGTGTTGTTTCCATGGATGTGCGGGGCCAGTCAGGTTAC  410
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527627  GGCTTCACCGTAGCTGGTTACGGTGTTGTTTCCATGGATGTGCGGGGCCAGTCAGGTTAC  1527568
SPD_1506CompS181A TCACAAGACGGCTTGCGTTCTCCTTTAGGAAATACCGTGAAGGGGCATATTATCCGTGGT  470
                  ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527567  TCACAAGACGGCTTGCGTTCTCCTTTAGGAAATACCGTGAAGGGGCATATTATCCGTGGT  1527508
```

```
SPD_1506Comp^S181A GCTGTGGAAGGTCGGGACCACCTCTTTTATAAGGATGTTTATCTGGATATTTACCAGTTG  530
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527507  GCTGTGGAAGGTCGGGACCACCTCTTTTATAAGGATGTTTATCTGGATATTTACCAGTTG  1527448
SPD_1506Comp^S181A GTCGAAATTGTTGCTAGTCTGTCTCAGGTTGATGAGAAGCGTCTTTCTAGCTATGGTGCC  590
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527447  GTCGAAATTGTTGCTAGTCTGTCTCAGGTTGATGAGAAGCGTCTTTCTAGCTATGGTGCC  1527388
SPD_1506Comp^S181A GCACAAGGAGGGGCTCTAGCCCTAGTTGCAGCAGCGCTCAATCCTCGAATTCAGAAAACA  750
                   =||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527387  TCACAAGGAGGGGCTCTAGCCCTAGTTGCAGCAGCGCTCAATCCTCGAATTCAGAAAACA  1527328
SPD_1506Comp^S181A GTTGCCATTTATCCCTTCTTGTCAGACTTCAGACGGGTGATTGAGATTGGTAATACTAGC  810
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527327  GTTGCCATTTATCCCTTCTTGTCAGACTTCAGACGGGTGATTGAGATTGGTAATACTAGC  1527268
SPD_1506Comp^S181A GAGGCTTACGACGAACTTTTCCGTTATTTCAAGTTTTACGACCCCTTCCATGAAACAGAG  870
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527267  GAGGCTTACGACGAACTTTTCCGTTATTTCAAGTTTTACGACCCCTTCCATGAAACAGAG  1527208
SPD_1506Comp^S181A GAGGAAATCATGGCGACCCTTGCCTATATCGATGTCAAAAATCTTGCCCATCGTATCCAA  930
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527207  GAGGAAATCATGGCGACCCTTGCCTATATCGATGTCAAAAATCTTGCCCATCGTATCCAA  1527148
SPD_1506Comp^S181A GGTGAGGTTAAGATGATTACGGGCTTGGACGACGATGTTTGCTATCCCATTACCCAGTTT  990
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527147  GGTGAGGTTAAGATGATTACGGGCTTGGACGACGATGTTTGCTATCCCATTACCCAGTTT  1527088
SPD_1506Comp^S181A GCGATTTATAATCGTCTGACCTGCGATAAAACCTATCGTATCATGCCTGAGTATGCTCAC  1050
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527087  GCGATTTATAATCGTCTGACCTGCGATAAAACCTATCGTATCATGCCTGAGTATGCTCAC  1527028
SPD_1506Comp^S181A GAAGCCATGAATGTATTTGTTAATGACCAAGTCTACAACTGGCTCTGTGGAAGTGAGATT  1110
                   ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527027  GAAGCCATGAATGTATTTGTTAATGACCAAGTCTACAACTGGCTCTGTGGAAGTGAGATT  1526968
SPD_1506Comp^S181A CCTTTTAAATATCTAAAATAA  1131
                   ||||||||||||||||||||
Sbjct  1526967  CCTTTTAAATATCTAAAATAA  1526947
```

Appendix 3

A: Alignment of SPD_0534 (estA) with SPD0534 cloned into pLEICES-01:

```
pLEICES-01-estA  CATGGCAGTAATGAAAATCGAGTATTACTCACAAGTATTGGATATGGAGTGGGGGGTGAA  185
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  546957    CATGGCAGTAATGAAAATCGAGTATTACTCACAAGTATTGGATATGGAGTGGGGGGTGAA  547016

pLEICES-01-estA  TGTCCTCTACCCTGATGCCAATCGAGTGGAAGAACCAGAGTGTGAAGATATTCCCGTCTT  245
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547017    TGTCCTCTACCCTGATGCCAATCGAGTGGAAGAACCAGAGTGTGAAGATATTCCCGTCTT  547076

pLEICES-01-estA  GTACCTTTTGCACGGGATGTCTGGAAATCATAATAGTTGGCTTAAGCGGACCAATGTAGA  305
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547077    GTACCTTTTGCACGGGATGTCTGGAAATCATAATAGTTGGCTTAAGCGGACCAATGTAGA  547136

pLEICES-01-estA  ACGCTTGCTTCGAGGAACTAATCTCATCGTTGTTATGCCCAATACCAGCAATGGTTGGTA  365
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547137    ACGCTTGCTTCGAGGAACTAATCTCATCGTTGTTATGCCCAATACCAGCAATGGTTGGTA  547196

pLEICES-01-estA CACCGATACCCAGTATGGTTTTGACTACTACACGGCTCTAGCAGAGGAATTGCCACAGGT  425
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547197   CACCGATACCCAGTATGGTTTTGACTACTACACGGCTCTAGCAGAGGAATTGCCACAGGT  547256

pLEICES-01-estA TCTGAAACGCTTCTTCCCTAATATGACGAGCAAGCGTGAAAAGACCTTTATCGCTGGTCT  485
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547257   TCTGAAACGCTTCTTCCCTAATATGACGAGCAAGCGTGAAAAGACCTTTATCGCTGGTCT  547316

pLEICES-01-estA TTCTATGGGAGGCTACGGCTGCTTCAAACTGGCTCTTACGACAAATCGTTTTTCTCATGC  545
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547317   TTCTATGGGAGGCTACGGCTGCTTCAAACTGGCTCTTACGACAAATCGTTTTTCTCATGC  547376

pLEICES-01-estA AGCTAGTTTTTCAGGTGCCCTCAGCTTTCAAAACTTTTCTCCTGAAAGTCAAAATCTGGG  605
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547377   AGCTAGTTTTTCAGGTGCCCTCAGCTTTCAAAACTTTTCTCCTGAAAGTCAAAATCTGGG  547436

pLEICES-01-estA AAGTCCAGCCTACTGGAGAGGTGTTTTTGGAGAGATTAGAGACTGGACAACTAGTCCCTA  665
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547437   AAGTCCAGCCTACTGGAGAGGTGTTTTTGGAGAGATTAGAGACTGGACAACTAGTCCCTA  547496

pLEICES-01-estA TTCTCTTGAAAGTCTGGCTAAAAAATCGGATAAAAAGACCAAACTTTGGGCGTGGTGTGG  725
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547497   TTCTCTTGAAAGTCTGGCTAAAAAATCGGATAAAAAGACCAAACTTTGGGCGTGGTGTGG  547556
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pLEICES-01-estA  CGAACAGGATTTCTTGTACGAAGCCAATAATCTCGCAGTGAAAAATCTCAAAAAACTAGG  785
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547557    CGAACAGGATTTCTTGTACGAAGCCAATAATCTCGCAGTGAAAAATCTCAAAAAACTAGG  547616
pLEICES-01-estA  TTTTGATGTGACCTATAGCCATAGCGCTGGAACTCACGAGTGGTACTACTGGGAAAAACA  845
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547617    TTTTGATGTGACCTATAGCCATAGCGCTGGAACTCACGAGTGGTACTACTGGGAAAAACA  547676
pLEICES-01-estA  ATTGGAAGTTTTTTTAACAACCCTACCAATTGATTTCAAATTANAAGAGAGACTGACTTAG  906
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547677    ATTGGAAGTTTTTTTAACAACCCTACCAATTGATTTCAAATTAGAAGAGAGACTGACTTAG  547737
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B: Alignment of SPD_1506 (*axe*) with SPD1506 cloned into pLEICES-01:

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pLEICS-01-axe    ATGAAAAATCCAGCTTTGCTAGAAGAAATTAAGACCTATAGAGGAAGGGATGAGGTTCCG  110
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527927   ATGAAAAATCCAGCTTTGCTAGAAGAAATTAAGACCTATAGAGGAAGGGATGAGGTTCCG  1527868
pLEICS-01-axe    GAAGACTTTGATGCTTTCTGGGATGGGGAAGTGAAAAATGTTTCCACGCTTCCATCCTAC  170
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527867   GAAGACTTTGATGCTTTCTGGGATGGGGAAGTGAAAAATGTTTCCACGCTTCCATCCTAC  1527808
pLEICS-01-axe    CACTTGGAGGAAAGAGATTTCCACATTCCTCAAGTCAAGTGCTATGAGTTAACATTTGAA  230
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527807   CACTTGGAGGAAAGAGATTTCCACATTCCTCAAGTCAAGTGCTATGAGTTAACATTTGAA  1527748
pLEICS-01-axe    GGAAGCAAGGAAGGAAAGGTCTATGCACGCATTGTTCTTCCAAAGAGTGAGGAGAAGGTT  290
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527747   GGAAGCAAGGAAGGAAAGGTCTATGCACGCATTGTTCTTCCAAAGAGTGAGGAGAAGGTT  1527688
pLEICS-01-axe    CCATTAATCTTCCATTTTCATGGTTATATGGGACGTGGCTGGGACTGGGCCGACATGCTG  350
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527687   CCATTAATCTTCCATTTTCATGGTTATATGGGACGTGGCTGGGACTGGGCCGACATGCTG  1527628
pLEICS-01-axe    GGCTTCACCGTAGCTGGTTACGGTGTTGTTTCCATGGATGTGCGGGGCCAGTCAGGTTAC  410
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527627   GGCTTCACCGTAGCTGGTTACGGTGTTGTTTCCATGGATGTGCGGGGCCAGTCAGGTTAC  1527568
pLEICS-01-axe    TCACAAGACGGCTTGCGTTCTCCTTTAGGAAATACCGTGAAGGGGCATATTATCCGTGGT  470
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527567   TCACAAGACGGCTTGCGTTCTCCTTTAGGAAATACCGTGAAGGGGCATATTATCCGTGGT  1527508
pLEICS-01-axe    GCTGTGGAAGGTCGGGACCACCTCTTTTATAAGGATGTTTATCTGGATATTTACCAGTTG  530
                 ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527507   GCTGTGGAAGGTCGGGACCACCTCTTTTATAAGGATGTTTATCTGGATATTTACCAGTTG  1527448
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pLEICS-01-axe   GTCGAAATTGTTGCTAGTCTGTCTCAGGTTGATGAGAAGCGTCTTTCTAGCTATGGTGCC  590
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527447  GTCGAAATTGTTGCTAGTCTGTCTCAGGTTGATGAGAAGCGTCTTTCTAGCTATGGTGCC  1527388
pLEICS-01-axe   TCACAAGGAGGGGCTCTAGCCCTAGTTGCAGCAGCGCTCAATCCTCGAATTCAGAAAACA  750
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527387  TCACAAGGAGGGGCTCTAGCCCTAGTTGCAGCAGCGCTCAATCCTCGAATTCAGAAAACA  1527328
pLEICS-01-axe   GTTGCCATTTATCCCTTCTTGTCAGACTTCAGACGGGTGATTGAGATTGGTAATACTAGC  810
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527327  GTTGCCATTTATCCCTTCTTGTCAGACTTCAGACGGGTGATTGAGATTGGTAATACTAGC  1527268
pLEICS-01-axe   GAGGCTTACGACGAACTTTTCCGTTATTTCAAGTTTTACGACCCCTTCCATGAAACAGAG  870
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527267  GAGGCTTACGACGAACTTTTCCGTTATTTCAAGTTTTACGACCCCTTCCATGAAACAGAG  1527208
pLEICS-01-axe   GAGGAAATCATGGCGACCCTTGCCTATATCGATGTCAAAAATCTTGCCCATCGTATCCAA  930
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527207  GAGGAAATCATGGCGACCCTTGCCTATATCGATGTCAAAAATCTTGCCCATCGTATCCAA  1527148
pLEICS-01-axe   GGTGAGGTTAAGATGATTACGGGCTTGGACGACGATGTTTGCTATCCCATTACCCAGTTT  990
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527147  GGTGAGGTTAAGATGATTACGGGCTTGGACGACGATGTTTGCTATCCCATTACCCAGTTT  1527088
pLEICS-01-axe   GCGATTTATAATCGTCTGACCTGCGATAAAACCTATCGTATCATGCCTGAGTATGCTCAC  1050
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527087  GCGATTTATAATCGTCTGACCTGCGATAAAACCTATCGTATCATGCCTGAGTATGCTCAC  1527028
pLEICS-01-axe   GAAGCCATGAATGTATTTGTTAATGACCAAGTCTACAACTGGCTCTGTGGAAGTGAGATT  1110
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1527027  GAAGCCATGAATGTATTTGTTAATGACCAAGTCTACAACTGGCTCTGTGGAAGTGAGATT  1526968
pLEICS-01-axe   CCTTTTAAATATCTAAAATAA  1131
                ||||||||||||||||||||||
Sbjct  1526967  CCTTTTAAATATCTAAAATAA  1526947
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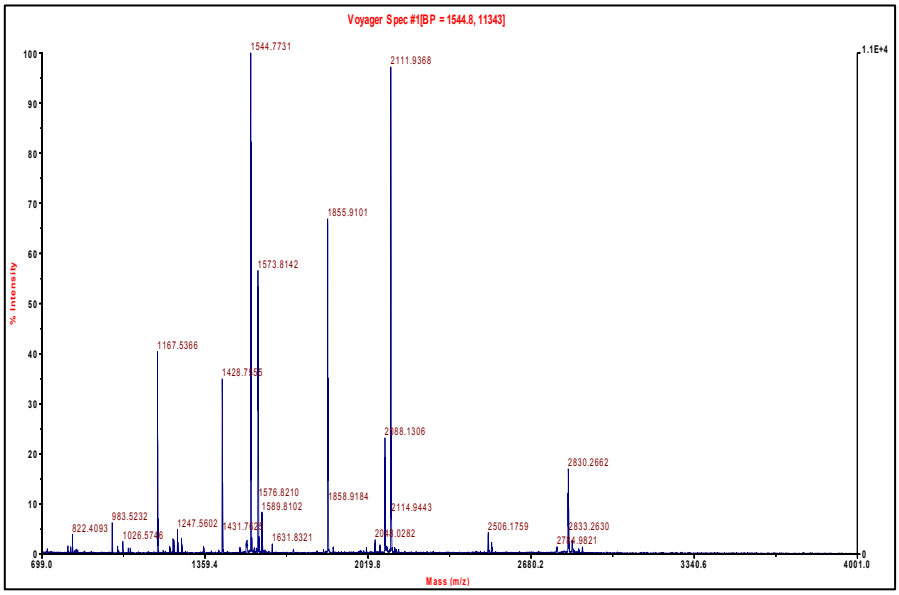
Appendix 4

Mass spectrometry confirming the identity of recombinant proteins (MALDI-TOF)

A: Acetyl xylan esterase (Axe)

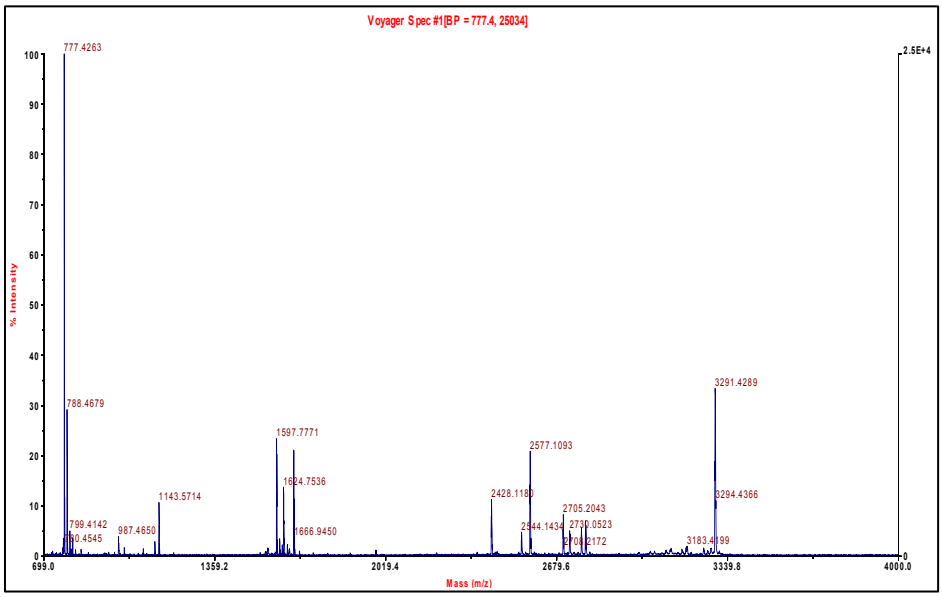


Axe amino acid alignment:

```
Hasan   13   MKNPALLEEIKTYRGRDEVPEDFDAFWDGEVKNVSTLPSYHLEERDFHIPQVKCYELTFE   72
             MKNPALLEEIKTYRGRDEVPEDFDAFWDGEVKNVSTLPSYHLEERDFHIPQVKCYELTFE
Sbjct   1    MKNPALLEEIKTYRGRDEVPEDFDAFWDGEVKNVSTLPSYHLEERDFHIPQVKCYELTFE   60
Hasan 73     GSKEGKVYARIVLPKSEEKVPLIFHFHGYMGRGWDWADMLGFTVAGYGVVSMDVRGQSGY   132
             GSKEGKVYARIVLPKSEEKVPLIFHFHGYMGRGWDWADMLGFTVAGYGVVSMDVRGQSGY
Sbjct 61     GSKEGKVYARIVLPKSEEKVPLIFHFHGYMGRGWDWADMLGFTVAGYGVVSMDVRGQSGY   120
Hasan 133    SQDGLRSPLGNTVKGHIIRGAVEGRDHLFYKDVYLDIYQLVEIVASLSQVDEKRLSSYGA   192
             SQDGLRSPLGNTVKGHIIRGAVEGRDHLFYKDVYLDIYQLVEIVASLSQVDEKRLSSYGA
Sbjct 121    SQDGLRSPLGNTVKGHIIRGAVEGRDHLFYKDVYLDIYQLVEIVASLSQVDEKRLSSYGA   180
Hasan 193    SQGGALALVAAALNPRIQKTVAIYPFLSDFRRVIEIGNTSEAYDELFRYFKFYDPFHETE   252
             SQGGALALVAAALNPRIQKTVAIYPFLSDFRRVIEIGNTSEAYDELFRYFKFYDPFHETE
Sbjct 181    SQGGALALVAAALNPRIQKTVAIYPFLSDFRRVIEIGNTSEAYDELFRYFKFYDPFHETE   240
Hasan 253    EEIMATLAYIDVKNLAHRIQGEVKMITGLDDDVCYPITQFAIYNRLTCDKTYRIMPEYAH   312
             EEIMATLAYIDVKNLAHRIQGEVKMITGLDDDVCYPITQFAIYNRLTCDKTYRIMPEYAH
Sbjct 241    EEIMATLAYIDVKNLAHRIQGEVKMITGLDDDVCYPITQFAIYNRLTCDKTYRIMPEYAH   300
Hasan 313    EAMNVFVNDQVYNWLCGSEIPFKYLK  338
             EAMNVFVNDQVYNWLCGSEIPFKYLK
```

Sbjct 301 EAMNVFVNDQVYNWLCGSEIPFKYLK 326

B: Tributyrin esterase (EstA):



EstA amino acid alignment:

Hasan 1 MAVMKIEYYSQVLDMEWGVNVLYPDANRVEEPECEDIPVLYLLHGMSGNHNSWLKRTNVE 60

MAVMKIEYYSQVLDMEWGVNVLYPDANRVEEPECEDIPVLYLLHGMSGNHNSWLKRTNVE

Sbjct 1 MAVMKIEYYSQVLDMEWGVNVLYPDANRVEEPECEDIPVLYLLHGMSGNHNSWLKRTNVE 60

Hasan 61 RLLRGTNLIVVMPNTSNGWYTDTQYGFDYYTALAEELPQVLKRFFPNMTSKREKTFIAGL 120

RLLRGTNLIVVMPNTSNGWYTDTQYGFDYYTALAEELPQVLKRFFPNMTSKREKTFIAGL

Sbjct 61 RLLRGTNLIVVMPNTSNGWYTDTQYGFDYYTALAEELPQVLKRFFPNMTSKREKTFIAGL 120

Hasan 121 SMGGYGCFKLALTTNRFSHAASFSGALSFQNFSPESQNLGSPAYWRGVFGEIRDWTTSPY 180

SMGGYGCFKLALTTNRFSHAASFSGALSFQNFSPESQNLGSPAYWRGVFGEIRDWTTSPY

Sbjct 121 SMGGYGCFKLALTTNRFSHAASFSGALSFQNFSPESQNLGSPAYWRGVFGEIRDWTTSPY 180

Hasan 181 SLESLAKKSDKKTKLWAWCGEQDFLYEANNLAVKNLKKLGFDVTYSHSAGTHEWYYWEKQ 240

SLESLAKKSDKKTKLWAWCGEQDFLYEANNLAVKNLKKLGFDVTYSHSAGTHEWYYWEKQ

Sbjct 181 SLESLAKKSDKKTKLWAWCGEQDFLYEANNLAVKNLKKLGFDVTYSHSAGTHEWYYWEKQ 240

Hasan 241 LEVFLTTLPIDFKLEERLT 259

LEVFLTTLPIDFKLEERLT

Sbjct 241 LEVFLTTLPIDFKLEERLT 259

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