Functional Characterisation of TprA/PhrA Quorum Sensing System in *Streptococcus pneumoniae*

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by

Anfal Shakir Motib

Department of Infection, Immunity and Inflammation
University of Leicester

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Statement of Originality

This accompanying thesis submitted for the degree of PhD entitled "**Functional Characterisation of TprA/PhrA Quorum Sensing System in *Streptococcus pneumoniae***" is based on work conducted by the author in the Department of Infection, Immunity and Inflammation of the University of Leicester during the period between January 2014 and December 2016.

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

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

Signed …… Date ……

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Abstract

Quorum-sensing (QS) mechanisms are pivotal for microbial adaptation to host environments, and often required for pathogenesis without affecting bacterial vitality. Hence targeting QS diminish the fitness cost of inhibition, and the emergence and spread of antibiotic resistance. I characterized the TprA/PhrA QS system in the important human pathogen *Streptococcus pneumoniae* with a view to target its operation using novel soluble linear molecularly imprinted polymers (LMIP). I found that TprA/PhrA system is commonly found in pneumococcal strains, and is required for mucin, galactose and mannose utilisation. On galactose, TprA is an activator of the virulence determinant neuraminidase (*nanA),* and controls the expression of nine different operons on galactose and mannose. *tprA* and *phrA* mutants are highly attenuated in the mouse model of pneumonia and septicemia, and in the chinchilla model of otitis media, indicating that the TprA/PhrA system is a major virulence determinant and a highly relevant anti-infective target. To interfere with the operation of TprA/PhrA, I used, for the first time, highly homogenous soluble LMIP specific to the PhrA peptide. LMIP decreased PhrA-induction in a dose-dependent and sequence-specific manner, and possessed no visible toxicity in the murine model. It was also shown that LMIP were protective against lethal pneumococcal challenge. This study sets the stage for studies on a novel class of drugs to target Gram positive pathogens.

Publications from this work

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Abbreviation

APS	Ammonium persulphate	**µg**	Microgram
BAB	Blood agar base	**µl**	Microlitre
AHL	Acyl homoserine lactone	**µM**	Micromolar
BHI	Brain heart infusion	**mM**	Millimolar
bp	Base pair	**ml**	Millilitre
BSA	Bovine serum albumin	**NADH**	Nicotinamide adenine dinucleotide
CDM	Chemically defined medium	**ng**	Nanogram
CFU	Colony forming unit	**nM**	Nanomolar
CSP	Competence stimulating peptide	**ONPG**	2-Nitrophenyl β-Dgalactopyranoside
dH2O	Distilled water	**OD**	Optical density
DNA	Deoxyribonucleic acid	**PBS**	Phosphate buffered saline
DTT	Dithiothreitol	**PCR**	Polymerase chain reaction
EDTA	Ethylenediaminetetraacetic acid	QS	Quorum sensing
EMSA	Electrophoretic mobility shift assay	**PFL**	Pyruvate formate lyase
g	Gram	**RNA**	Ribonucleic acid
Gal	Galactose	**RNAP**	RNA polymerase
GlcNAc	*N*-acetyl glucosamine	**SDS**	Sodium dodecyl sulphate
Glu	Glucose	**TAE**	Tris acetic acid EDTA
His	Histidine	**TCS**	Two component system
IPTG	Isopropyl β-D-1-thiogalactopyranoside	**TB**	Tris boric acid
kb	Kilobase	**TEMED**	Tetramethylethylenediamine
kDa	Kilodalton	**UV**	Ultraviolet
LA	Luria-Bertani agar	**v/v**	Volume per volume
LB	Luria-Bertani broth	**w/v**	Weight per volume
LMIP	Linear molecularly imprinted polymer	**x g**	Gravity force
mg	Milligram		

Table of contents

Chapter 1. Introduction

1.1. General description of *Streptococcus pneumoniae*

Streptococcus pneumoniae is a Gram positive, facultative anaerobic diplococcus, existing singly or in small chains. It is α-hemolytic on blood agar and grows well in the presence of 5% CO_2. The suitable pH range for this microbe is between 7.0-7.8, and it can grow in temperature range between 25-42 ºC (Rotta, 1986). One of the most significant features for its identification includes the sensitivity to ethylhydrocupreine HCl (optochin), which differentiates the bacterium from other α-hemolytic streptococci. Moreover, the pneumococcus cannot form haem compounds, thus it is catalase negative. Metabolically, *S. pneumoniae* ferments glucose and produces a large amount of lactic acid in the broth cultures (Brooks *et al.*, 2013). This microbe depends on fermentative metabolism because it does not have the required genes for respiration (Yesilkaya *et al.*, 2009). Thus, it obtains its energy by fermentative break down of host polysaccharides (Marion *et al.*, 2012). The pneumococcal extracellular enzyme systems for the polysaccharides and hexosamines facilitate the release of essential carbon sources (Tettelin *et al.*, 2001).

Streptococcus pneumoniae has a circular genome, which contains more than 2 million base pairs with 39.7 % mol GC content (Lanie *et al.*, 2007). Hoskins *et al.* (2001) reported that the genome of *S. pneumoniae* contains 2,038,615 bp and it is easy to transform with exogenous homologous DNA, and it has a high percentage of genes related to sugar uptake and metabolism (Iyer *et al.*, 2005). Devotion of high percentage genome capacity for sugar metabolism reflects the importance of sugar metabolism for the microbe. The cell wall of this bacterium consists of peptidoglycan with teichoic acid. In addition, it has a capsular polysaccharide, which is linked to the peptidoglycan and cell wall polysaccharides. Based on capsule types, there are over 91 different serotypes of pneumococci (Brooks *et al.*, 2013). Capsule is an essential component of pneumococcal virulence, and its synthesis is linked to central sugar metabolism. Thus *S. pneumoniae* requires activated carbohydrates to form its capsule. Most of the precursors required for capsule synthesis are generated during catabolism of sugars (Iannelli *et al.*, 1999; Bentley *et al.*, 2006), and the synthesis of capsule is connected strongly with the synthesis of peptidoglycan (Yother, 2011).

1.2. Epidemiology of pneumococci

Over the past century there has been a dramatic increase in the incidence of pneumococcal diseases around the world. *S. pneumoniae* colonises the nasopharynx of healthy individuals but also it can cause invasive diseases including pneumonia, meningitis, otitis media, and bacteremia (Korona-Glowniak *et al.*, 2016; Gillespie and Balakrishnan, 2000). It is currently not known in detail what causes transition from colonisation to invasive disease.

It causes more deaths than other invasive bacterial species (World Health Organization, 2014). Obaro and Adegbola (2002) reported that pneumococcal infections caused 40000 deaths in the US alone each year. The immune deficient people and children are more likely to be affected from pneumococcal diseases (Klugman *et al.*, 2009). World Health Organization (WHO) reported that *S. pneumoniae* causes approximately 1.6 million deaths every year, among which 0.7-1 million are children under 5 years of age especially in developing countries (World Health Organization, 2007). *Streptococcus pneumoniae* is responsible for 40,000 cases of lobar pneumonia, and over 15,000 cases of otitis media annually in England and Wales (Melegaro *et al.*, 2006). The mortality rate of pneumococcal meningitis is up to 20 % in developed countries, and in developing countries, the mortality can be as high as 50 % (Muley *et al.*, 2017).

It has been reported that 50 % of community acquired pneumonia (CAP) cases are due to *S. pneumoniae* (Sharma *et al.*, 2007; Anevlavis and Bouros, 2010). The common symptoms of pneumococcal pneumonia are fever, cough, and chest pain, and it can lead to respiratory failure by causing acute respiratory distress syndrome (ARDS). The lungs fill with fluid which leads to severe difficulties in breathing (Broaddus *et al.*, 2015). It was reported that the environmental factors play a role on increasing the rate of incidents of CAP, including the weather conditions and gender (Almirall *et al.*, 1999). More CAP has been reported during winter months than other seasons during the year, and the prevalence of CAP is higher among men than women (Almirall *et al.*, 1999). Being a tobacco smoker and alcohol abuse are also indicated as risk factors for acquiring pneumococcal infections (Almirall *et al.*, 1999; Muley *et al.*, 2017). In addition, ethnic background and host genetics have been known to impact on susceptibility to invasive pneumococcal infections. For example, in New York, pneumococcal bacteremia has been

reported to be more among black people than white. The rate of infections was 49.3 per 100,000 for African Americans while it was 13.8 per 100,000 for Caucasians (Bennett *et al.*, 1992; Robinson *et al.*, 2001). It seems likely that economic factors and medical conditions both play a key role on incidence rates.

1.3. Antibiotic resistance in pneumococcal population

The incidents of pneumococcal infections are increasing due to resistance of pneumococci to the common antibiotics hence there is an urgent need to find new effective drugs to treat pneumococcal diseases. Antibiotic resistance emerges chiefly due to innate microbial ability to evolve under selective pressure. Most traditional antibiotics interfere with bacterial growth or viability through their adverse effects on essential cell functions, such as cell wall synthesis, DNA replication, RNA transcription and protein synthesis (Clatworthy *et al.*, 2007). Consequently, in order to survive, bacteria have evolved mechanisms to destruct antibiotics by enzymes, to modify antibiotic targets, or to restrict drug penetration and stimulate drug efflux (Blair *et al.*, 2015).

Penicillin is used to treat pneumococcal diseases for many years. However, the pneumococcal resistance to penicillin appeared in 1940s and since then, the problem exacerbated in the 20th century (Brooks *et al.*, 2013). Many other bacterial species are resistant to β-lactams, secrete beta-lactamase enzymes. However, pneumococcal resistance to beta-lactams does not emerge due to β-lactamases but due to emergence of multiple mutations in the penicillin binding proteins (PBPs), which modify the structure of penicillin binding domain in the penicillin binding protein to prevent the binding of β-lactams (Zighelboim and Tomasz, 1980; Coffey *et al.*, 1995). The PBPs are enzymes which play a role in the murein synthesis of the pneumococcal cell wall and there are six types of PBPs in *S. pneumoniae* each with different molecular weight, and the resistance to specific β-lactam was occurred by the mutation in one or several forms of PBPs. It was shown that high rates of penicillin resistance require alterations of PBP2b, while resistance to third-generation cephalosporins has occurred firstly by the development of altered forms of PBP1a and 2x (Tomasz, 1979; Coffey *et al.*, 1995).

Other antibiotics are also used to treat pneumococcal infections such as macrolides, fluoroquinolones, and other non-beta-lactam antibiotics. However, the pneumococcus

developed resistance against these antibiotics too due to rapid rise of antibiotic use (Hyde *et al.*, 2001). The mechanism of pneumococcal resistance to macrolides consists of modification of target site by producing a methylase enzyme that incorporates adenine residue to a CH3- group on the 23S ribosomal RNA, or by acquiring point mutations in the 23S ribosomal RNA gene, which prevent the macrolides's inhibitory effect on the protein synthesis (McGee *et al.*, 2001). Similarly, the fluoroquinolone resistant pneumococci appeared due to mutations in the genes which code for DNA gyrase and topoisomerase IV in the quinolone resistance determining regions (QRDR) (Pletz *et al.*, 2007). Given the scale of pneumococcal infections and rising antibiotic resistance, therefore, there is an urgent need to develop new anti-infectives that are effective, and less prone to the development of drug resistance. One way of developing new antibiotics rely on identifying suitable microbial targets and subsequent modulation of these microbial targets. This study aims to identify pneumococcal targets that can be utilised to develop efficient anti-infective in future.

1.4. Vaccination against pneumococci

Vaccination protects 90% of people against pneumococcal diseases. There are 23-valent vaccine (PPSV-23), containing the capsules of 23 common serotypes, which is used to immunise adults efficiently, but it is inefficient for children under 2 years of age because carbohydrates are poor immunogens for children because they do not induce memory (Paradiso, 2011; Brooks *et al.*, 2013). To overcome this problem, a 7-valent conjugate vaccine (PCV-7) has been developed by conjugation of the pneumococcal capsular polysaccharides with diphtheria CRM197 protein, which can be used to immunise children against pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F (Paradiso, 2011). After PCV-7, 10-valent pneumococcal conjugate vaccine, PCV10, was introduced and it contains polysaccharides from PCV7 plus three extra serotypes that can protect children under 2 years old from invasive pneumococcal disease (Balsells *et al.*, 2017). To increase serotype coverage further, the 13-valent pneumococcal conjugate vaccine, PCV13, was introduced for prevention of IPD and otitis media. This vaccine was approved for use among children aged 6 weeks to 71 months (Centers for Disease Control and Prevention (CDC), 2010). However, the protection conferred by these vaccines is still serotype dependent, and commonly after vaccination, there is a rise in the incidence of infections with non-vaccine types. This is known as serotype replacement (Kadioglu *et*

al., 2008; Balsells *et al.*, 2017). Therefore, there is still a need for vaccines that can provide protection against all serotypes (Geng *et al.*, 2014).

1.5. Virulence determinants of *Streptococcus pneumoniae*

Streptococcus pneumoniae colonises the nasopharynx without causing disease. However, the pneumococcus can also spread to the lower respiratory tract to cause pneumonia. In addition, the microbe can get into blood or central nervous system to cause septicemia and meningitis, respectively, while its presence in middle ear causes otitis media. In these different tissues, this bacterium produces several virulence factors that are important for colonisation and invasive diseases (Barthelson *et al.*, 1998; Kadioglu *et al.*, 2002). The most important pneumococcal virulence factors include: the capsular polysaccharide, pneumolysin (Ply), hyaluronate lyase (Hyl) also known as hyaluronidase, neuraminidases (NanA and NanB), choline binding proteins (CbpA) also known as pneumococcal surface protein C (PspC), pneumococcal surface protein A (PspA), pneumococcal surface antigen A (PsaA), enolase (Eno), and autolysin (LytA) (Figure 1.1) (Kadioglu *et al.*, 2008). While some of the virulence factors are involved in nasopharyngeal carriage, such as neuraminidases, others contribute to invasive disease, including capsular polysaccharide and pneumolysin (Ply). While each virulence determinant has a specific role, they work in concert to increase the pathogenicity of *S. pneumoniae* (Kadioglu *et al.*, 2002; Kadioglu *et al.*, 2008).

Neuraminidases, also known as sialidases, degrade terminal sialic acid from glycoproteins and glycolipids (King *et al.*, 2004). There are at least 3 genes encode for neuraminidase activity in *S. pneumoniae*: *nanA, nanB* and *nanC*. All strains of pneumococci have *nanA* and most of them have *nanB* while half of the strains have *nanC* (Pettigrew *et al.*, 2006). In reference *S. pneumoniae* serotype 2 strain D39, there are two neuraminidases, NanA and NanB. NanA plays an essential role for pneumococcal colonisation and survival in the animal tissues (Manco *et al.*, 2006). It was shown that the mutant of neuraminidase A was cleared from the nasopharynx and lungs within 12 h post infection. In addition, the NanB mutant could not grow in the animal tissues and the animals did not develop neither pneumonia nor sepsis (Manco *et al.*, 2006). Furthermore, it was reported that neuraminidase plays an important role in pneumococcal biofilm formation since it was shown that a small molecule inhibitor of NanA blocks biofilm

formation using an *in vitro* model of biofilm formation with human airway epithelial cells, and this may occur probably by targeting bacterial sialylated glycoconjugates (Parker *et al.*, 2009; Soong *et al.*, 2006). Sialic acid (*N*-acetylneuraminic acid, NeuNAc) is one of the most important carbon sources for *S. pneumoniae*, and it plays an essential role for pneumococcal adhesion and invasion, and it is considered as molecular signal for enhancing biofilm formation (Gualdi *et al.*, 2012).

The polysaccharide capsule (CPS) is located in the most outer layer of *S. pneumoniae* cell wall and is covalently linked to the outer surface of the peptidoglycan (Kadioglu *et al.*, 2008). It is one of the most important virulence factors in *S. pneumoniae*. AlonsoDeVelasco *et al.* (1995) showed that non-capsulated strains of *S. pneumoniae* are approximately 10^5-fold less virulent than the strains that have capsule. It tends to protect the organism against the mucus and phagocytosis of the immune cells (Kadioglu *et al.*, 2008). Capsule helps the bacterium to transit via the luminal mucus of the epithelial surface (Nelson *et al.*, 2007). The thickness of pneumococcal capsule is approximately 200-400 nm (Sørensen *et al.*, 1990). *S. pneumoniae* needs capsule for colonisation in the animal tissue and the thickness of capsule is important to cause disease (Nelson *et al.*, 2007). In addition, the capsule expression is essential for bacterial survival in the lungs, ear, blood, and cerebral spinal fluid (Park *et al.*, 2007; Calix *et al.*, 2012).

Pneumolysin (Ply) is an important virulence factor of *S. pneumoniae*. It is a cholesterol dependent cytolysin (CDC) that causes a large transmembrane pore in cholesterol containing membranes (Kadioglu *et al.*, 2008; Marriott *et al.*, 2008; Mitchell and Mitchell, 2010). A series of structural alterations takes place in Ply structure when it is transformed from a soluble monomer to oligomer, which leads to the cytolytic activity of the pneumolysin and increases the inhibition of the phagocytic cells (Tilley *et al.*, 2005; Hirst *et al.*, 2004; Marshall *et al.*, 2015). Pneumolysin binds and activates the classical pathway of complement and diverts it from cell surface of *S. pneumoniae* (Mitchell *et al.*, 1991). Kadioglu *et al.* (2002) reported that pneumolysin plays an important role for pneumococcal survival in the mice that have acute pneumonia. In addition, Berry *et al.* (1999) demonstrated that pneumolysin is essential for dissemination of the pneumococcus from the lungs to blood stream. It was observed that loss of pneumolysin reduced brain cell apoptosis as the purified pneumolysin induced microglial and neuronal apoptosis *in vitro* by translocation of apoptosis inducing factor dependent (AIF-dependent) (Braun *et*

al., 2002). In addition, it was shown that pneumolysin has a role in biofilm formation. Inactivation of pneumolysin in *S. pneumoniae* D39 and TIGR4 led to significantly reduced biofilm formation than the wild type strains on plastic and human cell substrates under static or continuous flow conditions (Shak *et al.*, 2013). Despite the involvement of sugar metabolism in virulence, the role of pneumolysin in host derived sugar metabolism remain undiscovered.

Bacteriocin is a small antimicrobial peptide produced by pneumococcal species to inhibit the growth of other members in the same species which do not produce the cognate immunity protein during competition in the upper respiratory tract (Dawid *et al.*, 2007). The bacteriocin producer strain encodes an immunity protein to protect itself from its own bacteriocin during colonisation (Dawid *et al.*, 2007). These antimicrobial peptides are expressed by the *blp* locus (bacteriocin like peptide) and are under the control of a quorum sensing pheromone. It was shown that TCS13 (Blp) is a peptide sensing system that control a regulon including genes encoding for Blps, which appears to be, in certain respect, similar to the competence stimulating peptide (CSP) controlled *com* regulon, including density dependent gene regulation, requirement for peptides, and its binding region (de Saizieu *et al.*, 2000). It was shown that the Blp (bacteriocin like peptide) TCS (486 hk/rr pair) involved in virulence in a respiratory tract mouse model (Throup *et al.*, 2000).

Hyaluronidase (Hyl) is an enzyme whose activity degrades hyaluronic acid in to disaccharide units (Berry *et al.*, 1994). This enzyme helps the pneumococcus to invade the connective tissue by facilitating the breakdown of hyaluronan component, which leads to increase the permeability of tissue (Kostyukova *et al.*, 1995; Zwijnenburg *et al.*, 2001). Hyaluronidase was shown to increase the pathogenicity of pneumococci by helping the microbe to pass through the connective tissue, thus it leads the organism to translocate from the respiratory system into the blood (Berry *et al.*, 1994).

Choline-Binding Proteins (CBPs) are a group of cell wall-anchored proteins that interact with the teichoic acid component in the pneumococcal cell wall (Kadioglu *et al.*, 2008; Mitchell and Mitchell, 2010). There are 10-15 choline binding proteins in *S. pneumoniae* and most of them play a role in virulence, including a hydrolytic enzyme (LytA), pneumococcal surface protein A (PspA), and pneumococcal surface protein C (PspC)

(Bergmann and Hammerschmidt, 2006). LytA is one of the most important hydrolytic enzymes of pneumococcal cell wall. It was demonstrated that LytA has a role in virulence and required for pneumonia and bacteraemia in a mouse model of pneumococcal infection (Canvin *et al.*, 1995). This role is attributed to the activity of this enzyme to lyse the bacterial cells and release the pneumolysin from the cytoplasm (Kadioglu *et al.*, 2008).

Other pneumococcal cell surface virulence factors, include pneumococcal antigen A (PsaA), which is a cell surface lipoprotein and part of ABC transporter (Kadioglu *et al.*, 2008), and enolase, which is involved in glycolysis (Ghosh and Jacobs-Lorena, 2011). PsaA plays a role in pneumococcal adhesion, virulence, and enhance pneumococcal oxidative stress resistance (Briles *et al.*, 2000; Ogunniyi *et al.*, 2000). Enolase binds to plasminogen to help the bacterium to spread via the basement membrane (Eberhard *et al.*, 1999; Ghosh and Jacobs-Lorena, 2011). Hence, there are many virulence factors involved in pneumococcal adaptation and survival in the host tissues. However, the regulation of these factors is not known. It is very likely that the expression of these virulence determinants is influenced by changing environmental conditions, such as oxygen, temperature, pH, and the changes in composition and quantity of nutrients, especially those of sugars.

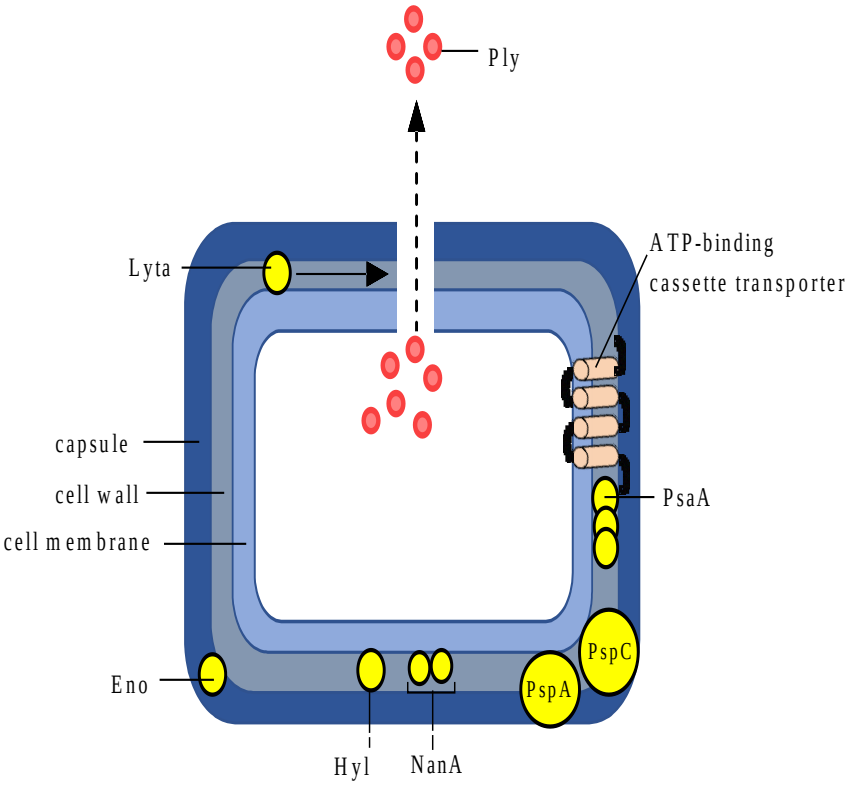


Figure 1.1. Schematic representation of selected virulence factors in *S. pneumoniae*. Abbreviations: LytA, autolysin A; Ply, pneumolysin; PspA and PspC, pneumococcal surface proteins A and C respectively; NanA, neuraminidase A; Hyl, hyaluronidase; PsaA, pneumococcal surface antigen A; Eno, enolase. Figure is constructed based on Kadioglu *et al.* (2008).

1.6. Sugar metabolism is involved in virulence

A virulence factor is a substance that is produced by pathogenic bacteria and cause damage in the host tissue directly (Wassenaar and Gaastra, 2001). This definition has excluded the genes involved in metabolism, such as the genes that are required for metabolism and energetics (Smith, 2007). Thus, in recent years, many researchers have focused on the study of the genes involved in metabolism (Shelburne *et al.*, 2008a).

For Gram negative bacteria, the carbohydrates are considered to be signalling substances, which significantly influence the expression of virulence genes of enteric pathogens in the intestine (Pacheco *et al.*, 2012). The genome wide study of *Salmonella* shows that this

bacterium uses different carbon sources (glucose, glucose-6-phosphate (G-6-P), and gluconate) during the infection (Hautefort *et al.*, 2008). In addition, the transcriptional factors involved in modulation of metabolic processes have also been shown to be important for virulence gene expression, such as the transcription factor cAMP receptor protein (CRP), which plays a role in the carbon catabolite repression (CCR). Mutation in the CRP or decrease in cAMP levels can lead to a reduced virulence in *Salmonella enterica* (Curtiss and Kelly, 1987; Teplitski *et al.*, 2006; Görke and Stülke, 2008). Many carbohydrate transporter systems in *Brucella* could be crucial for survival of the bacterium in the host tissues during the infection to provide the pathogen with carbohydrates as a carbon source or energy. Two quorum sensing systems, VjbR and BabR, regulate several genes that are responsible for central metabolic steps in *Brucella*. Mutation of *vjbR* and *babR* caused a significant difference in the growth of *Brucella* in defined medium. However, no differences in the growth profile of these mutants could be seen during growth in a rich medium (Barbier *et al.*, 2011).

In Gram positive bacteria, for example in *Clostridium perfringens*, CcpA is important for regulation and the production of collagenase and sporulation (Varga *et al.*, 2004). In addition, the exoenzymes of *Streptococcus mutants*, such as glucosyltransferase (Gtfs) produces the polysaccharides (soluble glucans from sucrose and starch). These exopolysaccharides are the major components of the cariogenic plague biofilm matrix, and are considered as essential virulence factors in dental caries (Bowen and Koo, 2011). Moreover, the addition of glucose, sucrose, or trehalose to the semisynthetic growth media activates the M protein synthesis in *Streptococcus pyogenes* (Pine and Reeves, 1978), that is encoded by essential virulence determinant *emm,* which protects the bacterium against phagocytosis and aids its adherence to different host tissues (Navarre and Schneewind, 1999). In addition, the processes of the carbon catabolite repression (CCR) can control the expression of *mga* for multiple gene regulator of group A *Streptococcus* (Poncet *et al.*, 2009). Mga is important for M protein synthesis, cell adhesion and invasion, and protects the bacterium against the onslaught of immune system (Hondorp and McIver, 2007).

In *Streptococcus pneumoniae*, the genome wide studies, including signature tagged mutagenesis (STM) and microarray analysis, have shown that a large percentage of genes involved in sugar metabolism is linked to the virulence (Hava and Camilli, 2002;

Ogunniyi *et al.*, 2012). The pneumococcus is a strictly fermentative bacterium for energy production (Paixão *et al.*, 2015). Carbohydrate metabolism plays an essential role in pneumococcal pathogenesis as it provides the bacterium with crucial nutrients (Tettelin *et al.*, 2001; Yesilkaya *et al.*, 2009). Glucose is the preferred carbon source for energy (Carvalho *et al.*, 2011). However, the concentration of glucose in the human airway is below 1 mM (Philips *et al.*, 2003). In the nasopharynx, the pneumococcus modifies the glycoconjugates, which consist of a variety of monosaccharides that can be used as nutrients by the microbe (Yesilkaya *et al.*, 2007; Paixão *et al.*, 2015). The mucin glycoprotein has several sugars including sialic acid, fucose, and glucosamines. However, the most abundant sugar in mucin is known to be galactose and galactosamines (Terra *et al.*, 2010). Thus, it seems that galactose is one of the main carbon sources for pneumococcal energy requirement in the respiratory tract.

Galactose metabolism in *S. pneumoniae* is thought to have a key role in colonisation and progression to invasive state (Paixão *et al.*, 2015). This is because inactivation of either galactose catabolic pathways, Leloir or tagatose, leads to a significant reduction in pneumococcal ability to colonise the nasopharynx and cause invasive disease. Interstingly, in the same mouse model the pneumococcal strains mutated in mannose or in *N*-acetylglucosamine catabolism, did not have any significant attenuation on either colonisation or in virulence (Paixão *et al.*, 2015). This indicates that the pneumococcus relies heavily on galactose catabolism in the respiratory tract. Unlike glucose, galactose is fed into glycolysis initially through tagatose or Leloir pathways (Figure 1.2) (Von Wright and Axelsson, 2011; Paixão *et al.*, 2015), and catabolism of galactose leads to mixed acid fermentation. In the tagatose pathway, galactose is converted to dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate (GAP), whereas in the Leloir pathway, galactose is converted to glucose-6-phosphate (G6P), before entering into glycolysis (Price *et al.*, 2012; Paixão *et al.*, 2015). It was reported that inactivation of essential enzymes of Leloir and tagatose pathways, GalK (galactokinase) and LacD (tagatose 1,6-diphosphate aldolase), respectively, renders the pneumococcus unable to grow in the presence of galactose *in vitro* (Paixão *et al.*, 2015). In addition, galactose metabolism in *S. pneumoniae* is also involved in Wzy-dependant pathway for capsular polysaccharide synthesis. For example, α–glucose-1-phosphate is the primary source for capsular polysaccharide synthesis and it originates exclusively from galactose catabolism (Hardy *et al.*, 2000; James *et al.*, 2013). Thus, the study of galactose

metabolism may help to identify targets which are linked to virulence, and which can be used for developing new drugs or vaccines.

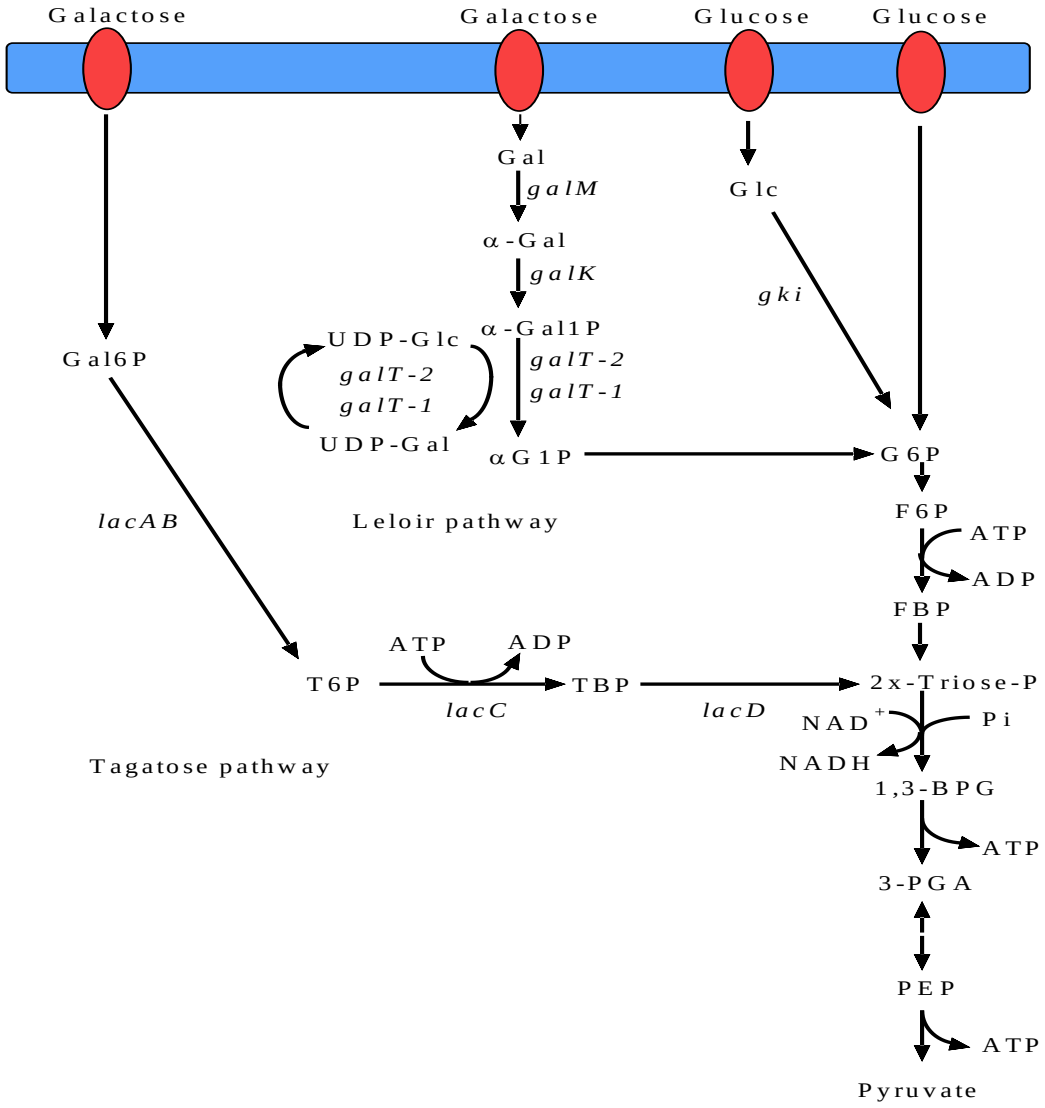


Figure 1.2. Schematic representation of the conversion of galactose and glucose to pyruvate in *S. pneumoniae*. The Leloir and tagatose 6-phosphate pathways for galactose dissimilation are shown on the left. Abbreviations for gene names: *lacA* and *lacB*, galactose 6-phosphate isomerase units; *lacC*, tagatose 6-phosphate kinase; *lacD*, tagatose 1,6-bisphospahte aldolase; *galM*, aldose1-epimerase; *GalK*, galactokinase; *GalT*, galactose-1-phosphate uridylyltransferase; *GalE*, UDP-glucose 4-epimerase. Abbreviations: T6P, tagatose-6-phosphate; TDP, tagatose 1,6-disphosphate; Gal6P, galactose-6-phosphate isomerase; α-Gal1P, α-galactose-1-phosphate; α-G1P, α-glucose-1-phosphate; G6P, glucose-6-phosphate, F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; 1,3-BPG, 1,3-bisphosphoglycerate; 3-PGA, 3-phosphoglycerate; PEP, phosphoenolpyruvate; Glu, glucose; and Gal, galactose. Figure is constructed based on Price *et al.* (2012); Von Wright and Axelsson (2011); Paixão *et al.* (2015).

It was shown that other additional enzymes that are involved in pneumococcal sugar metabolism, such as pyruvate formate lyase (PFL), lactate dehydrogenase (LDH), and pyruvate oxidase (PO/SpxB), also have an important role in pneumococcal survival and virulence (Yesilkaya *et al.*, 2009; Spellerberg *et al.*, 1996; Gaspar *et al.*, 2014). All these enzymes can use pyruvate as a substrate. The presence of multiple enzymes for pyruvate dissimilation clearly indicates the importance of pyruvate metabolism for *S. pneumoniae*. Indeed, it was reported that PFL is important for galactose catabolism and survival *in vivo* (Yesilkaya *et al.*, 2009). The importance of PFL has been linked to its role in ATP generation for energy requirements in the nutrient limited regions of the host. The knockout *pflB* strain appeared to have decrease in ATP synthesis level, and its mutation led to a reduction in the amount of acetyl-CoA, which is the precursor for pneumococcal fatty acid and choline synthesis (Yesilkaya *et al.*, 2009). It was shown that several regulators are involved in active PFL synthesis, including CcpA and GlnR, both of which contribute to the virulence (Al-Bayati *et al.*, 2017).

Another enzyme that uses pyruvate as a substrate is LDH, which is a key pneumococcal enzyme for homolactic fermentation (Gaspar *et al.*, 2014). Mutation of *ldh* was reported to decrease virulence in a mouse model of pneumonia that develops after intranasal infection (Gaspar *et al.*, 2014). Similarly, the inactivation of *spxB,* encodes for pyruvate oxidase and is responsible for conversion of pyruvate to acetyl phosphate and CO_2 (PO/SpxB), caused a significant reduction in pneumococcal growth in host tissues (Spellerberg *et al.*, 1996). The contribution of SpxB in virulence was attributed to its role in generation of acetyl phosphate, which is important for synthesis of adhesion proteins and capsule production (Spellerberg *et al.*, 1996).

In addition to the involvement of effector proteins, the regulation of sugar metabolism plays a key role in pneumococcal virulence. CcpA is a transcriptional regulator, which regulates several genes that are responsible for pneumococcal metabolism and contributes to pneumococcal colonisation and survival in the lung. It has been reported that CcpA activates the glycolytic enzymes, including enolase, which is required for pneumococcal replication and survival in the host tissues (Iyer *et al.*, 2005; Bergmann *et al.*, 2001).
Another evidence that supports the involvement of sugar metabolism in pneumococcal virulence comes from the studies of pneumococcal sugar transporters. The pneumococcus has a large number of transporters devoted to sugar uptake, including classical

Phosphotransferase Systems (PTS), ATP- binding cassettes (ABC), and ion gradient driven transporters (Tettelin *et al.*, 2001; Bidossi *et al.*, 2012). It was reported that both ABC and PTS transporters are important in pneumococcal virulence (Polissi *et al.*, 1998; Lau *et al.*, 2001; Hava and Camilli, 2002; Chimalapati *et al.*, 2012). For example, the transporters for sucrose, Sus and Scr have influence on pneumococcal colonisation and pneumonia, these systems are regulated by SusR and ScrR, respectively (Iyer and Camilli, 2007). Moreover, the sucrose ATP binding cassette (ABC) transport system affected the pneumococcal survival in the lung tissues, while a sucrose PTS contributed to the pneumococcal ability to colonise in the nasopharynx (Iyer *et al.*, 2005). In addition, it was shown that the pneumococcal sialic acid transporter system (SatABC) also plays an important role in colonisation of upper respiratory tract. The inactivation of sialic acid transporter (SatABC), led to significant attenuation in pneumococcal colonisation (Marion *et al.*, 2011).

It is clear from the review of literature that the sugar metabolism is an important facet of pneumococcal virulence. However, while the role of effector proteins is well studied, the knowledge on how sugar metabolism is regulated in *S. pneumoniae* is sparse. Hence, it is essential to study the regulation of sugar metabolism to identify the pneumococcal mechanisms important for survival in the host tissues and virulence.

1.7. *In vivo* nutrients sources for *S. pneumoniae*

Streptococcus pneumoniae relies on acquisition of the host sugars for its energy requirement since this bacterium, as far as it is known, cannot survive outside human nasopharynx. It was found that one third of all pneumococcal transporters are devoted to carbohydrate uptake (Tettelin *et al.*, 2001; Marion *et al.*, 2011; Bidossi *et al.*, 2012). The pneumococcus can utilise 32 different types of carbohydrates *in vitro*, including glucosides, galactosides, and polysaccharides (Bidossi *et al.*, 2012; Terra *et al.*, 2015). Most of these carbohydrates, however, are sparse or not found in the respiratory tract, such that the concentration of glucose is very low or does not exist in the nasopharynx, but the blood is rich in glucose and its concentration can be between 4-10 mM (Philips *et al.*, 2003).

In respiratory tract, the major host glycoprotein is mucin (Yesilkaya *et al.*, 2007). Mucins are a major component of the mucus, which cover the surface of respiratory epithelial cells. Mucin glycoproteins are large macromolecular glycoproteins that has 2-20×10^5 Da molecular weight, containing up to 85 % carbohydrates, include *O*-glycans and high quantity of tandem repeats (TR) in the protein backbone (Wiggins *et al.*, 2001; Rose and Voynow, 2006). The *O*-glycans of mucins have 1-20 residues that are found as linear or branched structures (Linden *et al.*, 2008). The peptide backbone of mucin has serine, threonine, and proline residues (Valle and Todisco, 2009). The initial binding between peptide and carbohydrate is formed by glycosidic bond between *N*-acetylgalactosamine and the hydroxyl groups of serine or threonine residues (Linden *et al.*, 2008). It was found that the carbohydrate side chain of most mucins consists of *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc), *N*-acetylneuraminic acid (NeuNAc), galactose (Gal), and fucose (Fuc). The sugar residues are attached to the protein core through a *N*-acetylgalactosamine moiety (Sheehan *et al.*, 1991; Rose and Voynow, 2006; Terra *et al.*, 2010). The pneumococcus can utilise these sugars from mucin to enhance its ability for colonisation, adherence and biofilm formation, and for growth as illustrated in Figure 1.3 (King, 2010).

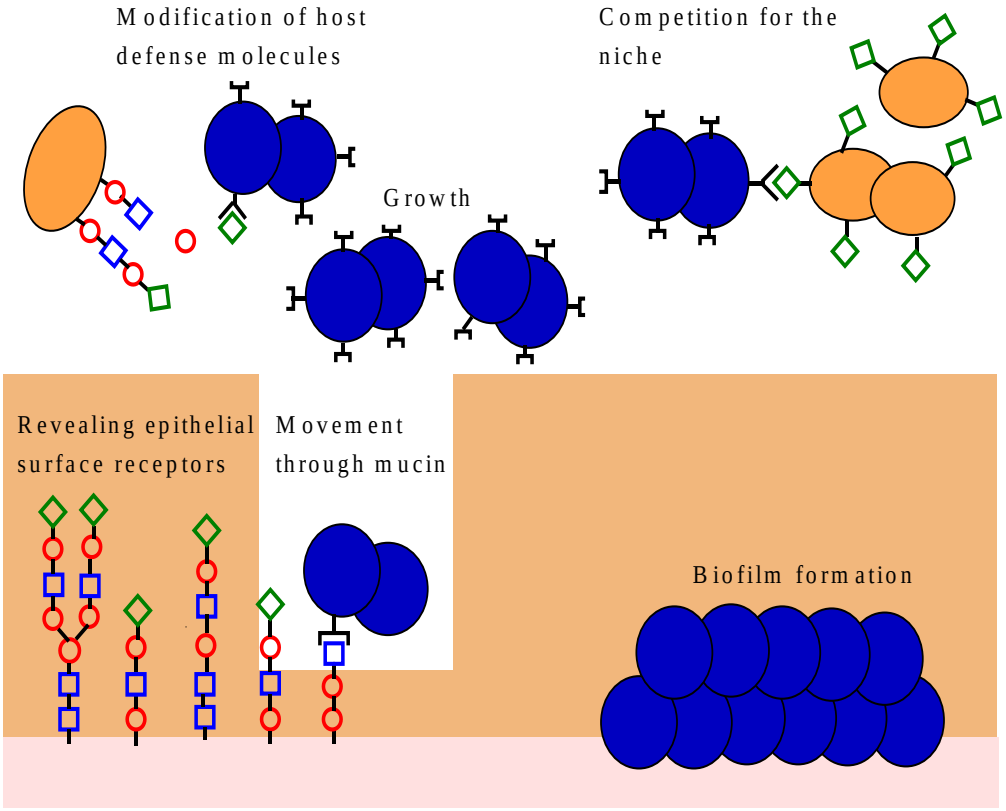


Figure 1.3. Schematic representation of potential roles of pneumococcal modification of airway glycans to bacterial colonisation, competition, nutrient acquisition, and invasion. Exoglycosidase enzymes including neuraminidases (NanA), β-galactosidase (BgaA), BgaC, and *N*-acetylglucosaminidase (StrH) work together to sequentially remove sialic acid, galactose, and *N*-acetylglucosamine from glycoproteins and exposes mannose residues, which play an important role in pneumococcal nutrient metabolism, pathogenesis via its role in expose the surface receptor of the host cells, allowing pneumococcal adhesion, biofilm production, and immune protection. The figure is constructed based on King (2010).

The complex host glycans can be modified by pneumococcal exoglycosidase enzymes, including neuraminidases (NanA), β-galactosidase (BgaA), BgaC, and *N*-acetylglucosaminidase (StrH). It is believed that these three enzymes work together to sequentially remove sialic acid, galactose, and *N*-acetylglucosamine from glycoproteins of the host tissues and exposes mannose residues, which provide additional carbon source and mediate attachment (King *et al.*, 2006). The transcriptome analysis of *S. pneumoniae* grown in the presence of mucin showed differential expression of genes mainly involved in deglycosylation, transport and catabolism of galactose, mannose, and *N*-acetylglucosamine (Paixão *et al.*, 2015). Therefore, given the importance of host derived-

sugars in pneumococcal virulence, it is important to understand the metabolic regulation of these sugars.

Neuraminidase is the key enzyme for deglycosylation of host glycans, and the pneumococcus has three genes for neuraminidase: *nanA, nanB,* and *nanC*. NanA codes for the main pneumococcal neuraminidase activity. Neuraminidase activity is required for cleavage of terminal sialic acid residues from glycoproteins and mucins. The initial cleavage of sialic acid is a prerequisite for sequential deglycosylation of host glycans (Gualdi *et al.*, 2012). It was shown that neuraminidase A plays an essential role for mucin utilisation as the *nanA* mutant could not grow on medium containing mucin as the sole carbon source (Yesilkaya *et al.*, 2007). The role of neuraminidase in virulence is attributed to its role in decreasing the viscosity of mucus, which may help the microbe to colonise in the host tissues (Tong *et al.*, 2000). Moreover, both neuraminidase enzymes, NanA and NanB, can cleave sialic acid from glycans to increase the ability of pneumococcal adherence and colonisation in the upper respiratory tract (Paton *et al.*, 1993). Therefore, it is important to study the regulation of neuraminidase which has a wide-ranging effect on pneumococcal survival *in vivo* and virulence.

In addition to its role in pneumococcal nutrient metabolism, neuraminidase contributes to pneumococcal pathogenesis via its role in biofilm production, adhesion, and immune protection (Parker *et al*., 2009; Burnaugh *et al.*, 2008). It was observed that the expression of *nanA* was up regulated in the pneumococcus during growth under biofilm conditions (Oggioni *et al.*, 2006). The neuraminidase activity may expose the surface receptor of the host cells, allowing pneumococcal adhesion (Paton *et al.*, 1993; Manco *et al.*, 2006). Furthermore, it was shown that sialic acid can induce the expression of NanA and NanB and considered as a signalling molecule to activate the neuraminidase gene expression, and this specificity leads to increase pneumococcal ability for adherence to the host cell surfaces and biofilm formation (Trappetti *et al.*, 2009). In addition, it was reported that the expression of *nanA* is repressed in the presence of glucose (Afzal *et al.*, 2015; Manco *et al.*, 2006). Therefore, it is essential to understand the pneumococcal regulatory systems that modulate pneumococcal neuraminidase activity in the presence of different sugars.

Streptococcus pneumoniae expresses two surfaces associated exoglycosidases a β-galactosidase A (BgaA) and BgaC. BgaA has a high specificity for cleavage of terminal

galactose β (1-4) linked to *N*-acetylglucosamine (GlcNAc). This specify indicates that BgaA is not involved in lactose metabolism unlike most other microbial β-galactosidases (Zeleny *et al.*, 1997; Zahner and Hakenbeck, 2000; King *et al.*, 2006). On the other hand, BgaC has specificity for galactose β (1-3) linked to *N*-acetylglucosamine (GlcNAc) (Jeong *et al.*, 2009). Both BgaA and BgaC are important for pneumococcal colonisation (Kaufman and Yother, 2007; Jeong *et al.*, 2009). Inactivation of both galactosidases decreases total galactosidase activity but does not abolish it (Terra *et al.*, 2010), showing that there may be other enzymes with galactosidase activity.

Pneumococcus also codes for other surface associated enzymes such as β-*N*-acetylglucosaminidase (StrH) which cleaves terminal β1-linked *N*-acetylglucosamine (GlNAc) attached to mannose (King *et al.*, 2006), and glycanase (Endo-α-*N*-acetylgalactosaminidase, Eng), which is an *O*-glycosidase required for cleavage of sialylated core-1-*O* linked glycans (Marion *et al.*, 2009). Thus, in the nasopharynx, *S. pneumoniae* can deglycosylate both *N*- and *O*- linked glycans from mucin and mucin like proteins for energy requirements (Burnaugh *et al.*, 2008; Marion *et al.*, 2012). Despite the key role of complex host glycans and glycan derived sugars in the pneumococcal survival in the respiratory tract, the regulation of genes that are involved in host-derived sugars such as galactose, and those involved in regulation of enzymes responsible for cleavage of sugars, such as *nanA*, remains to be discovered.

1.8. Transcriptional regulation in bacteria

Bacteria regulate their gene expression very tightly to produce the required products at the suitable time and niche. These processes allow bacteria to adapt and survive rapidly under environmental stimuli (Seshasayee *et al.*, 2011). The regulation of transcription is the most critical event for microbial adaptation. The main component in all transcriptional regulation in bacteria is the multi-subunit DNA dependent RNA polymerase (Browning and Busby, 2004). The binding site of the RNA polymerase which has the active region for the binding to both the DNA template and the RNA product during transcription consists of two α-subunits. Each one of these units is composed of two independently folded domains, including the amino-terminal domain (αNTD) and carboxy-terminal domain (αCTD) which are linked together with an approximately 20 amino acid flexible region (Linker) (Korzheva *et al.*, 2000; Blatter *et al.*, 1994). The αNTD is responsible for

the assembly of the core subunits, while the αCTD is a DNA binding module which has an essential role at specific promoters (Gourse *et al.*, 2000). At the beginning of transcription, the RNA polymerase should be interacting with σ-subunit to form the holoenzyme. The main function of σ-factor is to position the RNA polymerase holoenzyme at a specific promoter sequence and to provide unwinding of the DNA duplex near the transcription start site (Gross *et al.*, 1998; Wösten, 1998). Most bacteria have several σ-factors, which enable recognition of different promoters, (Browning and Busby, 2004).

The transcription of genes is controlled by promoters and the initiation of transcription requires interaction of the RNA polymerase with a specific promoter sequence to form an open complex, where the duplex DNA near the transcription start site is unwound. The synthesis of RNA chain directly from the template DNA begins by the formation of phosphodiester bond between the initiating and adjacent nucleotide triphosphates. After this, the RNA polymerase is moved into the elongation complex that is responsible for extension of RNA chain (Browning and Busby, 2004).

There are four different sequence elements which are responsible for promoter recognition by RNA polymerase, including -10, -35, extended -10, and UP elements (Figure 1.4). The -10 hexamer and -35 hexamer are located 10 and 35 bp, respectively up stream of transcription start site and recognised by domain 2 and domain 4 of the RNA polymerase σ-subunit, respectively. The other two elements of promoter, the extended -10 element is consisted of 3-4 bp sequence which is located immediately up stream of -10 hexamer and recognised by domain 3 of the RNA polymerase σ-factor, while the UP element consists of approximately 20 bp located up stream of the -35 hexamer and recognised by C-terminal domain of RNA polymerase α-subunits (Sanderson *et al.*, 2003; Browning and Busby, 2004).

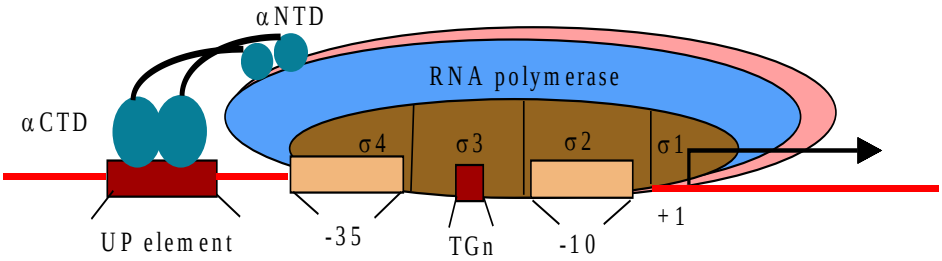


Figure 1.4. Schematic representation of RNA polymerase and its interactions at promoters for illustrating the different interactions between promoter elements and the RNA polymerase. The consensus sequences for the -35, -10, extended –10 (TGn), and UP elements are shown. Figure is constructed based on Browning and Busby (2004).

Transcription factors (TFs) are proteins that bind with promoters for activation or repression of the transcription. Many of these TFs have specific DNA binding domains and these interact with the specific sequence in target promoters (Seshasayee *et al.*, 2011). There are several steps for involvement of TFs in modulation of transcription. First, the TF binds to specific sequence which is located up stream of -35 element of promoter and directs RNA polymerase to the promoter via binding directly with RNA polymerase. Second, the activator interacts with a specific sequence overlaping -35 element of promoter and interacts with the domain 4 of the σ-subunit of RNA polymerase. This contact recruits the RNA polymerase to the promoter. Finally, simple activation takes place when the TF changes the conformation of the target promoter sequence to enable the binding of RNA polymerase with -10 and/or -35 elements of promoter. This activation needs the TF to interact at/or very close to the elements of promoter as illustrated in detail in Figure 1.5 (Browning and Busby, 2004; Sheridan *et al.*, 2001). The repressor TR decreases the initiation of transcription at target promoters by various ways, and this has been illustrated in Figure 1.6 in detail. For example, by preventing the RNA polymerase to bind with promoter DNA by occupying core promoter elements, or through DNA looping (Figure 1.6) (Browning and Busby, 2004).

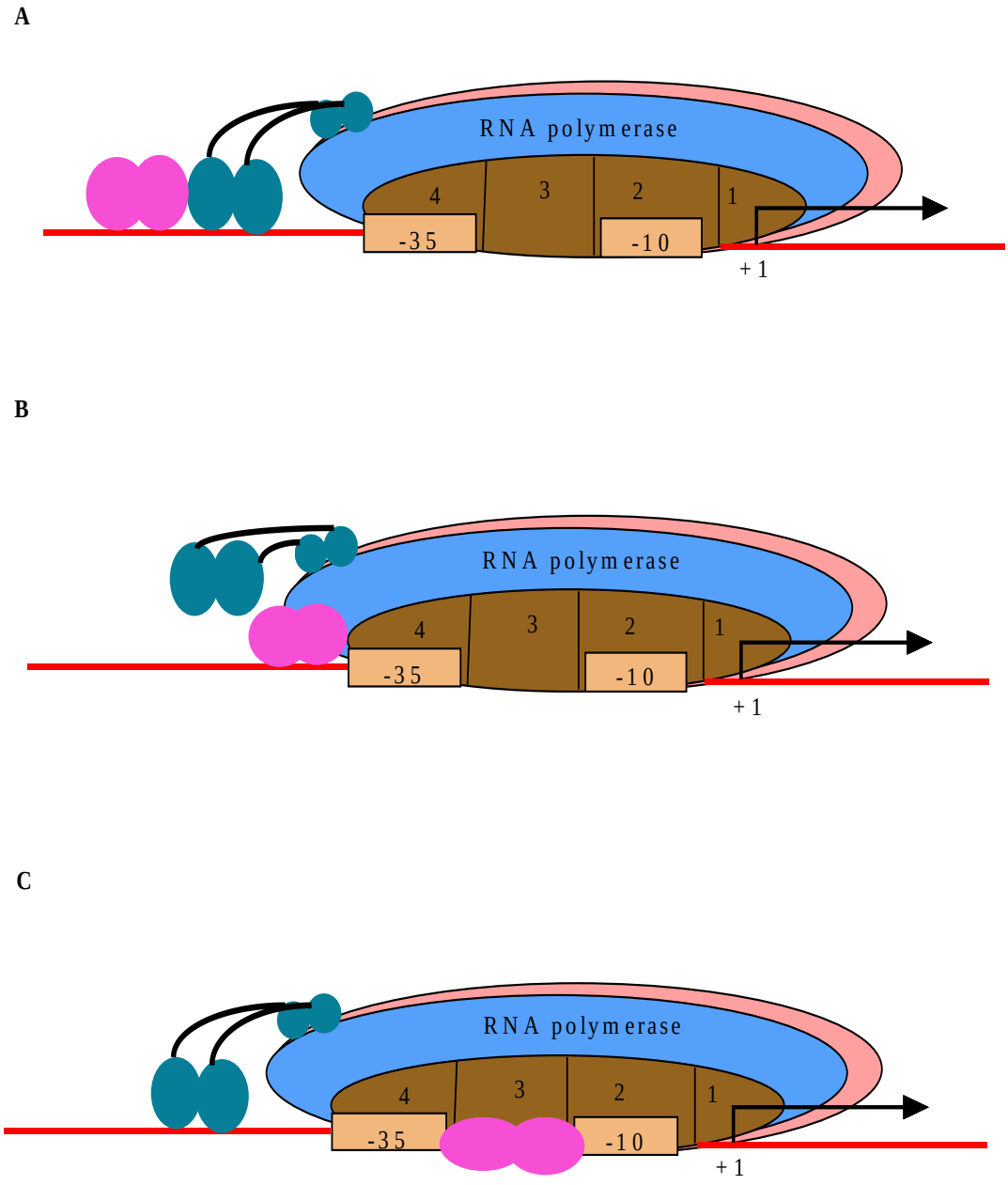


Figure 1.5. Schematic representation of activation at simple promoters. The figure shows the organisation of RNA polymerase and activator subunits during activation at simple promoters. **A:** Class I activation. The activator is bound to an upstream site and contacts the αCTD of RNA polymerase, thereby recruiting the polymerase to the promoter. **B:** Class II activation. The activator binds to a target that is adjacent to the promoter –35 element, and the bound activator interacts with domain 4. **C:** Activation by conformation changes. The activator (shown in pink) binds at, or near to, the promoter elements and realigns the –10 element and the –35 element so that the RNA polymerase holoenzyme can bind to the promoter. Figure is constructed based on Browning and Busby (2004).

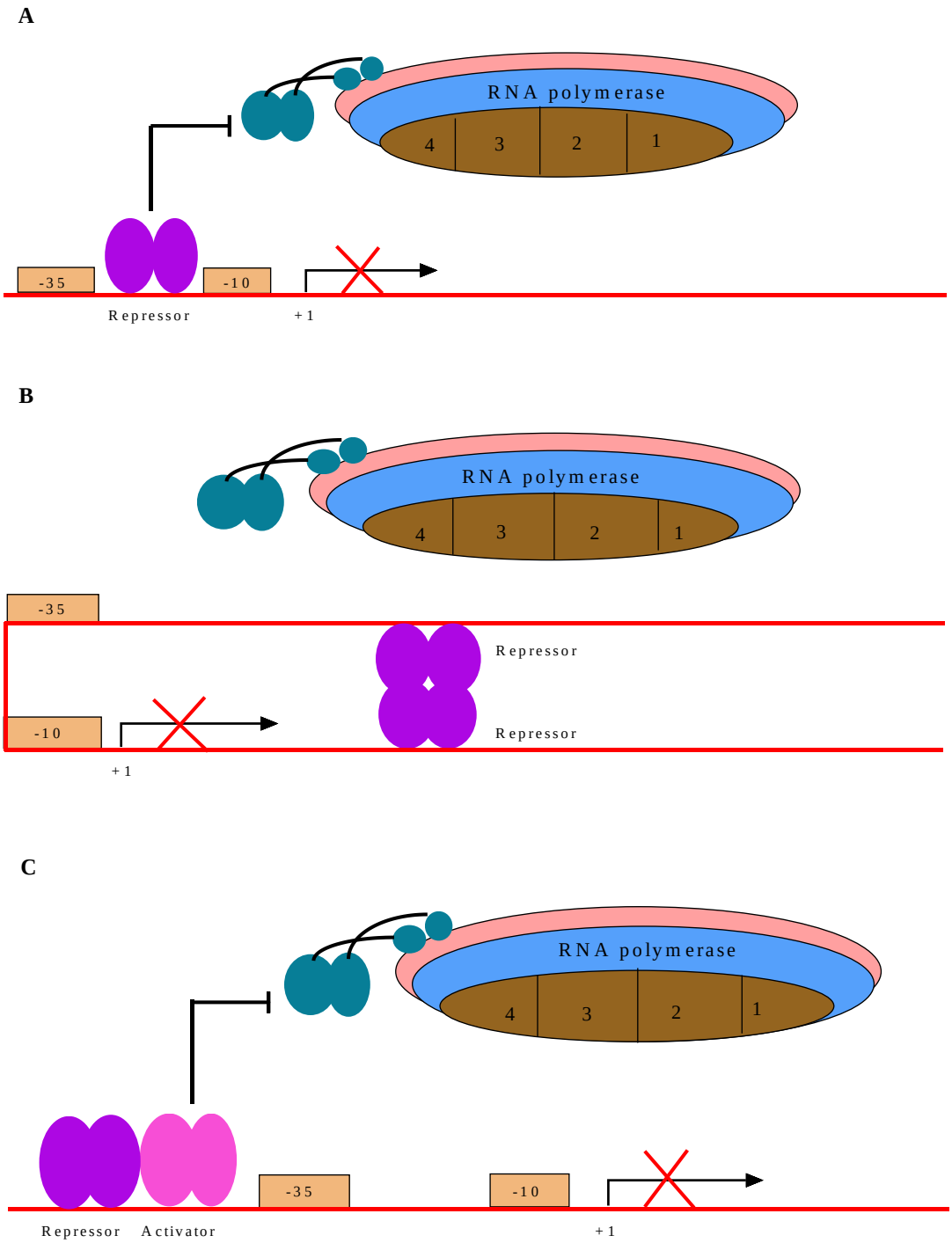


Figure 1.6. Schematic representation of mechanisms of repression. **A**: Repression by steric hindrance. The repressor binding site overlaps core promoter elements and blocks recognition of the promoter by the RNA polymerase holoenzyme. **B**: Repression by looping. Repressors bind to distal sites and interact by looping, repressing the intervening promoter. **C**: Repression by the modulation of an activator protein. The repressor binds to an activator and prevents the activator from functioning by blocking promoter recognition by the RNA polymerase holoenzyme. The repressor is shown in purple and the activator in pink. Figure is constructed based on Browning and Busby (2004).

1.9. Transcriptional regulatory systems in *Streptococcus pneumoniae*

The coordinated expression of pneumococcal genes during the infection is crucial. *S. pneumoniae* colonises firstly the nasopharynx and then can spread to the lower respiratory tract to cause various diseases at different host tissues such as pneumonia, bacteraemia, otitis media, and meningitis (Barthelson *et al.*, 1998; Kadioglu *et al.*, 2002). In each tissue, the pneumococcus is exposed to different temperature, sugar composition and concentration, and oxygen concentration. Thus, adaptation to changing environmental conditions is crucial for virulence and bacterial survival *in vivo* (Charpentier *et al.*, 2000; Konkel and Tilly, 2000; Rosch *et al.*, 2009; Porcheron *et al.*, 2016). Transcriptional regulators allow pneumococcus to adapt to a multitude of environments at different host sites (Hava *et al.*, 2003). Broadly, *S. pneumoniae* encodes two kinds of regulatory systems: stand-alone regulators and two component regulatory systems (TCS) (Gohar *et al.*, 2008). Some TCS differ from those relying on relay of signal from a membrane-bound histidine kinase that senses a specific environmental stimulus and a corresponding response regulator that mediates the cellular response, and require the production and process of peptide pheromones in Gram positive bacteria (Kleerebezem *et al.*, 1997). These TCS are known as quorum sensing systems and they operate in a cell density dependent manner as described below in detail. It has been shown that different response regulatory networks can affect each other's operation and this regulatory response allows the bacterium to survive in different host niches (McIver, 2009).

1.9.1. Stand-alone regulators

Stand-alone response regulators control the expression of essential regulons for colonisation, immune evasion, metabolism, virulence and response to stressors in the presence of inducing condition (McIver, 2009; Shelburne *et al.*, 2010). They are called stand-alone regulators because they do not have a cognate sensor kinase or has not been identified yet. These regulators respond to specific environmental signals that modulate bacterial physiological responses for adaptation. It was shown that the transcriptional regulator MgrA has a role in activation of virulence gene expression as it is required for pneumococcal colonisation and pneumonia in experimental animal model (Hemsley *et al.*, 2003), while the pneumococcal stand-alone Rgg regulator plays a role in response to oxidative stress, biofilm formation, and survival in oxygenated animal tissues (Bortoni *et*

al., 2009). The nutritional regulator CodY plays a role in the pneumococcal colonisation in the nasopharynx and the regulon of this regulator consists of genes involved in amino acid and carbon metabolism, and iron uptake (Hendriksen *et al.*, 2008). In addition, it was observed that inactivation of transcriptional regulator GlnR, which regulates glutamine and glutamate metabolism, attenuates pneumococcal colonisation in animal tissues (Hendriksen *et al.*, 2008). Despite the study of few stand-alone transcriptional regulators, the role of many regulators in pneumococcal biology remains to be investigated.

Among stand-alone regulators CcpA is worthy of a special emphasise due to its relevance to the hypothesis of this thesis. CcpA is the major transcriptional regulator of carbon catabolite repression (CCR) in many Gram-positive bacteria (Iyer *et al.*, 2005). This regulator belongs to the LacI/GalR transcriptional regulator family and modulates the expression of many catabolic operons in *B. subtilis* and various species of *Streptococcus* (Belitsky *et al.*, 2004; Asanuma *et al.*, 2004; Willenborg *et al.*, 2014). In *S. pneumoniae*, it was shown that CcpA regulated CCR of lactose-inducible β-galactosidase activity (Iyer *et al.*, 2005). CCR is a regulatory process that allows bacteria to utilise preferred carbon source in the presence of multiple sugar sources since it is waste of energy and inefficient metabolically to use all the carbon sources, preferred and non-preferred sugars, at the same time (Deutscher, 2008). Thus, CCR represses the expression of genes which are responsible for utilisation of non-preferred sugars until the cell has finished utilisation of preferred sugar (Deutscher, 2008; Carvalho *et al.*, 2011).

The affinity of CcpA to bind with an operator sequence, known as the catabolite repressible element (*cre*), is enhanced in the presence of histidine phosphoprotein (HPr). HPr is an integral component of the phosphoenolpyruvate dependent phosphotransferase system (PTS). The function of this system is to transfer high energy phosphate from phosphoenolpyruvate to the enzyme II complex during sugar uptake (Postma and Lengeler, 1985; Reizer *et al.*, 1999). In a media containing the preferred sugar, such as glucose, phosphorylation of HPr is activated on a conserved serine residue by the HPr kinase, which activates itself by metabolites such as the high energy glycolytic intermediate fructose-1,6-bisphosphate (FBP) to form CcpA complexed with the HPr-[Ser-P] (Thevenot *et al.*, 1995; Brochu and Vadeboncoeur, 1999; Frey *et al.*, 2003). This complex interaction increases the affinity of CcpA to bind with a specific DNA target sequence which is known as catabolite responsive element (*cre*) located within or before

the target genes or operons (Fujita *et al.*, 1995; Jones *et al.*, 1997; Servant *et al.*, 2005; Asanuma *et al.*, 2004). The binding of this dimeric complex causes repression of promoters, enhancing CCR (Asanuma *et al.*, 2004; Deutscher *et al.*, 1995; Deutscher *et al.*, 1994). It was reported that CcpA is not the only activator of CCR in *S. pneumoniae* (Iyer *et al.*, 2015). Thus, it is essential to identify additional regulators to understand the full complexity of sugar regulation, hence *in vivo* survival, in *S. pneumoniae*.

1.9.2. Two Component Regulatory Systems

The two component systems (TCS) or also known as two component signal transduction systems sense the external stimuli and enable the bacterium to respond and adapt to different environmental conditions (Hemsley *et al.*, 2003; Stock *et al.*, 2000). This system is present in most bacterial species and it is essential for survival and adaptation to the changes in environmental conditions. TCS are composed of a histidine kinase signal sensor and a response regulator. A membrane associated sensor histidine kinase (HK) transfers the phosphate group to the aspartate residue in the response regulator (RR). This process of phosphorylation of a response regulator alters the biochemical feature of its end product which contributes to transcriptional control of effector target genes under specific environmental conditions (Figure 1.7) (Gao *et al.*, 2007; Mitrophanov and Groisman, 2008). The functional aspect of TCS relies on phosphorelay system. This event happens when a histidine kinase removes the phosphoryl group to aspartate of a response regulator (Perraud *et al.*, 1999). After this the phosphoryl group is transferred from the response regulator to the histidine that contains a phosphor transfer protein, this process leads to produce an output that contributes in a cellular response (Appleby *et al.*, 1996). Therefore, any changes in the processes of phosphorylation will affect the ability of response regulator to regulate the biological properties. In addition, these processes are not determined only by the detection of signal by histidine kinase but also by the gene products, which can inhibit or activate these processes through feedback inhibition or activation (Mitrophanov and Groisman, 2008).

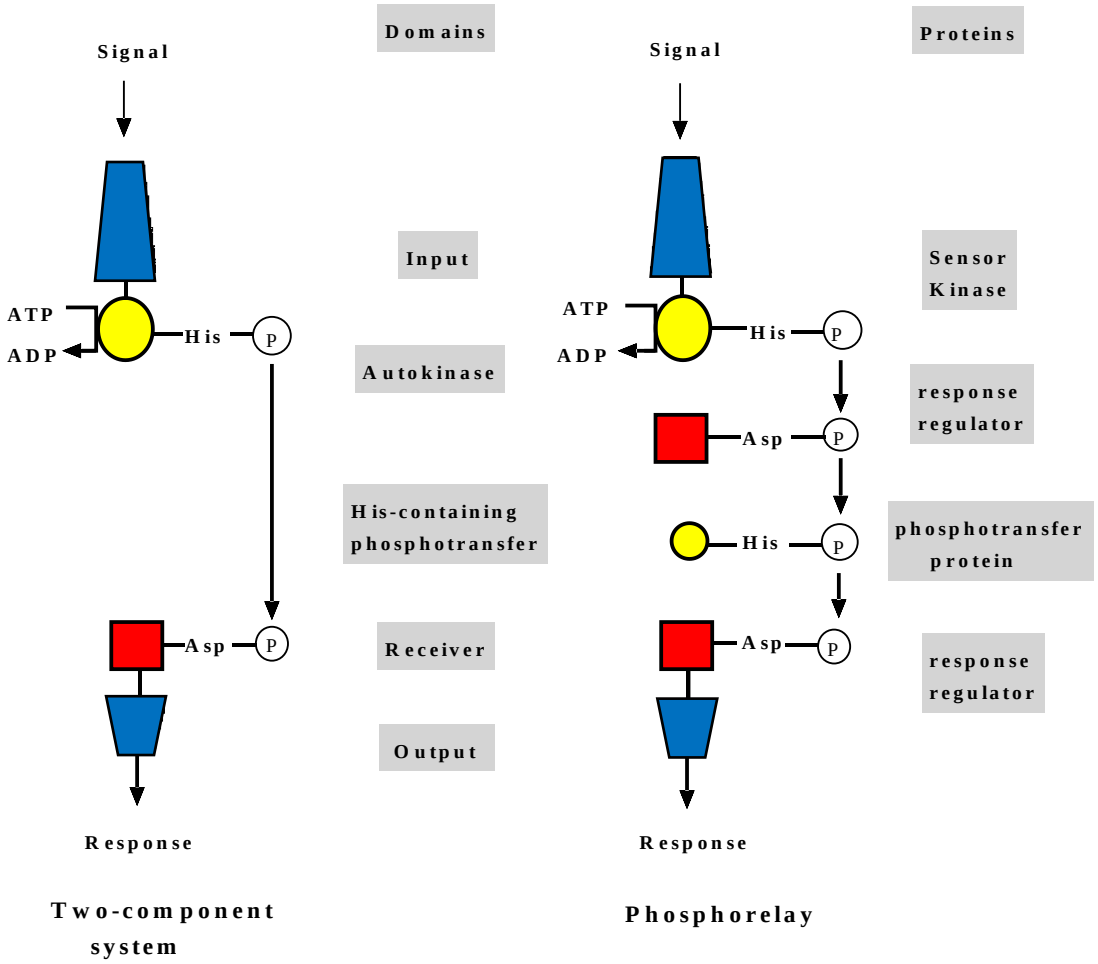


Figure 1.7. Schematic representation of mechanism of two component system and phosphorelay. In two component system, upon sensing an environmental stimulus, the histidine kinase (HK) autophosphorylates itself at a conserved histidine residue, then the phosphoryl group is transferred from the HK to the aspartate of response regulator (RR), which leads to transcriptional control of target genes. In phosphorelay, the histidine kinase removes the phosphoryl group to aspartate of a response regulator, then the phosphoryl group is transferred from the response regulator to the histidine that contains a phosphor transfer protein, after this the phosphoryl group is transferred to aspartate of final response regulator this process leads to cellular response. Figure is constructed based on Mitrophanov and Groisman (2008).

The genome of *S. pneumoniae* type 2 D39 strain contains 13 histidine kinase (HK) that are paired with response regulators (RR), plus one unpaired RR known as the orphan response regulator (Throup *et al.*, 2000; Lange *et al.*, 1999). Paterson *et al.* (2006) reviewed all available data for pneumococcal TCS and concluded that most of these TCSs are essential in the virulence and survival of pneumococcus in the animal tissues (Table 1.1). It was also noted that the function of pneumococcal TCS vary depending on genetic background of strains, and their contribution to the virulence was dependent on the site

of infection (Lange *et al.*, 1999). For example, TCS02 has a role in the regulation of cell wall and fatty acid biosynthesis, and in the expression of the virulence factor *pspA* (Ng *et al.*, 2003; Mohedano *et al.*, 2005). In addition, Ng *et al.* (2005) reported that the VicRK (YycFG) (TCS) is required for pneumococcal virulence. It was shown that the VicR (YycF) response regulator (RR) positively regulates the transcription of several genes encoding for important surface proteins that mediates murein biosynthesis and virulence factor expression, including PspA, and proteins (spr0096 and spr1875) involved in cell wall synthesis.

TCS05 or CiaRH is the first pneumococcal TCS identified (Guenzi *et al.*, 1994). CiaRH has a role in protecting the cells against the stress of competence development (Dagkessamanskaia *et al.*, 2004). In addition, Ibrahim *et al.* (2004) showed that the CiaRH regulates the expression of *htrA* that encodes a protein called high temperature requirement A protein which influence the ability of *S. pneumoniae* to colonise in the host tissues and grow at high temperatures. One of the most noteworthy TCS is TCS12, which modulates pneumococcal competence (Paterson *et al.*, 2006). This TCS responds to competence stimulating peptide (CSP), which is encoded by *comC,* and secreted through the ComA/B ABC transporter. ComD and the response regulator ComE form a functional TCS that involves in the regulation of genes important for competence (de Saizieu *et al.*, 2000). The role of competence regulation in virulence was shown by using a *comD* (*hk12*) mutant in D39 strain, which was found to be attenuated in pneumonia and bacteremia models (Bartilson *et al.*, 2001; Paterson *et al.*, 2006). It was suggested that due to the important roles they play in the viability and virulence of *S. pneumoniae,* TCS are considered to be good anti-infective targets (Barrett and Hoch, 1998; Paterson *et al.*, 2006). Despite the essential role of sugar acquisition in pneumococcal virulence and survival in the host tissues, the roles of these TCSs in sugar metabolism needs to be investigated in further detail.

Table 1.1. Represents two components systems in *S. pneumoniae* and their roles in virulence. Table is constructed baced on Paterson *et al.* (2006).

TCS	Alternative names	Showed role in virulence	References for role in virulence
01	480	Yes	Throup *et al.* (2000); Hava and Camilli (2002)
02	*vic, micAB, yyFG*, 492	Yes	Wanger *et al.* (2002); Kadiologlu *et al.* (2003)
03	474	No	
04	*pnpRS*, 481	Yes	McCluskey *et al.* (2004)
05	*ciaRH*, 494	Yes	Throup *et al.* (2000); Marra *et al.* (2002); Ibrahim *et al.* (2004)
06	478	Yes	Throup *et al.* (2000); Standish *et al.* (2005)
07	539	Yes	Throup *et al.* (2000); Hava and Camilli (2002)
08	484	Yes	Throup *et al.* (2000)
09	*zmpSR*, 488	Yes	Lau *et al.* (2001); Throup *et al.* (2000); Hava and Camilli (2002); Blue and Mitchell (2003)
10	*vncRS*, 491	No	
11	479	No	
12	*comDE*, 498	Yes	Lau *et al.* (2001); Throup *et al.* (2000); Bartilson *et al.* (2001); Hava and Camilli (2002)
13	*blpRH*, 486	Yes	Throup *et al.* (2000)
Orphan RR	*ritR*, 489	Yes	Throup *et al.* (2000); Ulijasz *et al.* (2004)

1.10. Quorum sensing systems

Bacterial populations can communicate with each other using signaling molecules to support their growth, biochemical activities and survival under different environmental conditions in a cell density dependent manner (Rocha-Estrada *et al.*, 2010). This is known as quorum sensing system. This method of communication relies on formation, excretion and differentiation of the molecules that are transported through bacterial cell membrane. Thus, when the concentration of bacterial cells rises, the recipient response regulator or sensor kinase will activate or inhibit the expression of genes that depend on quorum sensing system (Kleerebezem and Quadri, 2001; Rocha-Estrada *et al.*, 2010). Operation of quorum sensing system differs from that of 'traditional' TCS as the former relies on cell density or growth phase, and the presence of pheromone like peptides in Gram positive bacteria, which is secreted outside the cell and then internalised to interact with an intracellular factor (Lazazzera *et al.*, 1997; Lazazzera and Grossman, 1998). However, it is important to mention that there are also certain quorum sensing systems whose operation also relies on peptide pheromones but not necessarily peptide is imported back into the cell, rather, pheromone interacts with sensor histidine kinase. An example of such quorum sensing system is competence regulon in *S. pneumoniae* (Havarstein and Morrison, 1999; de Saizieu *et al.*, 2000). In these systems detection of pheromone peptides involves interaction of the peptide with the extracellular portion of a membrane bound histidine kinase, and leads to the autophosphorylation of the kinase and subsequent activation to phosphorylation of a cognate response regulator that mediates changes in gene expression (de Saizieu *et al.*, 2000). Thus, these peptides are recognized by the input domain of a typical sensor component of a two-component signal transduction system similar to other 'traditional' two-component regulatory system, consisting of a sensor and response regulatory protein, which use phosphorylation to transfer the information (Kleerebezem *et al.*, 1997).

1.10.1. Gram positive quorum sensing systems

Different cellular processes are regulated by quorum sensing system such as virulence, biofilm formation and excretion of proteolytic enzymes (Chang *et al.*, 2011). In Gram negative bacteria, the quorum sensing system is often modulated by acyl-homoserine lactone (acyl-HSL) molecules when its binds to the target cytoplasmic protein (Ng and

Bassler, 2009). Bacterial population produces acyl-homoserine lactone (acyl-HSL) signal, which is secreted through the cell membranes. When the concentration of acyl-HSL reaches a sufficient level in the environment, it binds to its receptor to activate quorum sensing dependent genes (Scholz and Greenberg, 2017). Depending on the bacterial species, QS regulates several physiological processes such as bioluminescence and swarming motility (Fuqua *et al.*, 2001).

On the other hand, Gram positive bacteria exploits small peptides known as pheromones as signal molecules to activate quorum sensing system. The pheromones are recognised by the histidine sensor kinase, cellular membrane, or the response regulator within the responder cell (Pottathil and Lazazzera, 2003). There are three families of QS systems in Gram positive bacteria according to characteristics of pheromones and type of their receptors: (1) Agr type cyclical oligo peptides; (2) double- glycine peptides (Gly- Gly); and (3) the RRNPP family QS systems. It has been shown that Gram-positive bacteria can use different types of QS mechanisms within a species to control the cellular processes (Cook and Federle, 2014; Fleuchot *et al.*, 2011; Mashburn-Warren *et al.*, 2010).

The Agr-family is referred to as accessory gene regulator that consists of four genes. In *Staphylococcus aureus, agrABCD* plays a role in regulation of several virulence factors (Saenz *et al.*, 2000). The cyclical peptide pheromone was encoded by *agrD* in *S. aureus*, and then it proposed to be exported and processed through a dedicated transport protein AgrB (Saenz *et al.*, 2000; Nakayama *et al.*, 2001). The AgrB has a putative cysteine endopeptidase domain and cyclization of the peptide is suggested to be required for transportation (Qiu *et al.*, 2005; Cook and Federle, 2014). The differentiation of pheromone occurs by a two-component signal transduction system (TCSTS) through binding the mature pheromone with sensor kinase AgrC on the cell surface and this leads to phosphorylation of the response regulator AgrA that interacts with the target promoters to regulate gene expression (Sturme *et al.*, 2002; Del Papa and Perego, 2011).

The Gly-Gly type peptide family includes a double glycine motif in the leader sequence (LSX_2ELX_2IXGG). This peptide contains an accessory domain, which proteolytically processes the leader sequence at the polypeptide site directly following the conserved Gly-Gly motif (Havarstein *et al.*, 1995). The pheromones of this family are detected by a two-component signal transduction system (TCSTS) which transmits a signal internally

by phosphorylation of cognate response regulators similar to Agr family. It was found that competence stimulating peptides (CSPs) of streptococci and bacteriocins class II belong to this family (Håvarstein *et al.*, 1994; Cook and Federle, 2014).

The RRNPP family is composed of Rgg (regulator gene of glucosyltransferases), Rap (aspartyl phosphate phosphatases), NPrR (neutral protease regulator), PlcR (Phospholipase C regulator) and PrgX (pheromone responsive gene regulator) family regulators. The genes that often encode for the RRNPP family proteins and their signaling pro-peptides are located in the chromosomes of bacteria or in the plasmids. The pro-signaling peptides are exported out of bacterial cell and processed to the mature signaling peptide by proteases. Then this mature peptide is transported in to the cell via oligopeptide permease (OPP) and subsequently interacts with RRNPP receptor protein (Gohar *et al.*, 2008; Pottathil and Lazazzera, 2003).

RRNPP family proteins are involved in wide-ranging biological processes. It was demonstrated that Rgg is a global transcriptional regulator that plays an important role in virulence and controls non-glucose carbohydrates metabolism in several Gram-positive bacteria (Zheng *et al.*, 2011). Inactivation of the *rgg* attenuated virulence of *Streptococcus suis* serotype 2 (SS2) significantly in a piglet infection model, and reduced the utilisation of non-glucose carbohydrates, such as lactose and maltose, and increased its adhesion and hemolytic activity *in vitro* (Zheng *et al.*, 2011). Rgg of *Streptococcus pyogenes* strain NZ131 (serotype M49) controls the expression of virulence factors and metabolic enzymes that may be crucial for the pathogen's adaptation to different metabolic substrates. Loss of *rgg* resulted in significant differences in catabolic substrate preference during growth in complex and defined media compared to the wild type strain. The Rgg regulators interact directly with their signal peptide. The pheromones are exported outside the cells by an enhanced expression of pheromone protein (Eep) and processed to mature peptides that are internalised through oligopeptide permease (OPP) to then bind with Rggs (Chang *et al.*, 2011; Cook *et al.*, 2013). Recently, it has been shown that the Rgg0939/SHP (short hydrophobic peptide) system in *S. pneumoniae* D39 play a role in surface polysaccharide synthesis (Junges *et al.*, 2017). The Rgg0939/SHP system induced the transcription of a *shp* and capsule gene locus, and over expression of the Rgg system increased capsule size and reduced the biofilm formation on lung epithelial cells. In addition, it has been shown that the Rgg family and its cognate SHP regulate virulence

peptide 1 (*vp1*) which is a highly expressed Gly-Gly peptide encoding gene in chinchilla middle ear effusions (Cuevas *et al.*, 2017). VP1 enhances the thickness and biomass of biofilm grown on chinchilla middle ear epithelial cells, hence, it is considered as a novel regulatory peptide for biofilm formation and pneumococcal pathogenesis (Cuevas *et al.*, 2017).

Core and Perego (2003) showed that there are 11 Rap phosphatases in *B. subtilis* which have six tetratricopeptide repeat (TPR) domains. These domains promote protein-protein interactions. Rap phosphatases in *B. subtilis* play a role in the sporulation of *B. subtilis* (Jiang *et al.*, 2000). The Rap proteins consist of approximately 375 amino acids in length and they appear to have greater than 25% identity among them (Pottathil and Lazazzera, 2003). It was shown that RapA, RapB, and RapE act as negative regulators of the phosphorelay signal transduction system that leads to start of sporulation by enhancing dephosphorylation of the response regulator SpoOF, whereas RapC controls competence development via modulating the activity of response regulator and transcriptional factor ComA (Pottathil and Lazazzera, 2003). The Phr peptide, required for activation of Rap phosphatases, has a signal sequence for export. The secreted peptide is processed by proteolysis to mature signaling peptide that can be imported by the oligopeptide permease (Opp) (Perego and Hoch, 1996; Perego *et al.*, 1991). At low cell density, Rap binds to the response regulator for inactivation but at high cell density, the mature Phr peptide binds to Rap to prevent its binding to response SpoOF regulator, which controls gene expression (Rocha-Estrada *et al.*, 2010).

The sex pheromone receptor (PrgX) in *E. faecalis* regulates antibiotic resistance plasmid pCF10 and it contributes in the regulation of conjugative transfer gene expression encoded in the plasmid. In this quorum sensing system, signaling happens between two types of cells, one with the plasmid (donor) and the other without plasmid (recipient) to permit the plasmid donor cells to regulate the expression of conjugation ability in response the density of the recipient cell. By using this system *E. faecalis* controls the conjugation process of the plasmid between donor and recipient cells (Kozlowicz *et al.*, 2006). The transcriptional regulator PrgX binds to two different signaling peptides, cCF10 (codified in the bacterial chromosome) which serves as a specific inducer of pCF10 conjugation genes, and the inhibitor iCF10 (encoded in the plasmid pCF10 in the donor cells) which function as repressor of pCF10 conjugation genes expression

(Kozlowicz *et al.*, 2006). Both donor and recipient cells synthesize cCF10, and most of cCF10 from the donor cell is sequestered by PrgY in its membrane, but the free peptide and iCF10 are transported to the cytoplasm via Opp. Then PrgX dimer specifically binds to pCF10 at two promoters in plasmid pCF10. cCF10 competes with iCF10 for binding to PrgX. When cCF10 binds to PrgX, it induces the gene expression while iCF10 binding causes inhibition of conjugation gene expression (Rocha-Estrada *et al.*, 2010).

Another quorum sensing system of RRNPP family is NprR, which regulates the neutral protease in *B. subtilis*. There are two alleles of this gene in different *B. subtilis* strains, *nprR1* and *nprR2*. Bacilli possessing *nprR1* produces 20 to 50 times less neutral proteases than others carrying *nprR2* (Toma *et al.*, 1986; Uehara *et al.*, 1979). Neutral protease is one of the essential enzymes for textile industry, and for detergents (Koetje *et al.*, 2003; Rocha-Estrada *et al.*, 2010).

PlcR regulators are another member of RRNPP family quorum sensing systems. PlcR was first described in *B. thuringiensis* as a positive transcriptional regulator of phosphatidylinositol specific phospholipase C gene (Lereclus *et al.*, 1996). In the *B. cereus* group, PlcR controls most of extracellular virulence factors. PlcR positively regulates 45 genes coding to produce extracellular proteins such as enterotoxins, haemolysins phospholipases and proteases (Agaisse *et al.*, 1999; Gohar *et al.*, 2008). The PlcR regulates the cellular processes such as biofilm formation and virulence in *B. cereus*, *B. anthracis*, and *B. thuringiensis*. It was demonstrated that the PlcR mutants of *B. cereus* and *B. thuringiensis* lost their virulence in an endophthalmitis infection in rabbit infection model (Callegan *et al.*, 2003; Gohar *et al.*, 2008). Agaisse *et al.* (1999) showed that PlcR in *B. thuringiensis* regulate the production of several virulence factors. These include degradative enzymes such as proteases, cell surface proteins, and toxins, such as haemolytic enterotoxins, non haemolytic enterotoxins, and phosphatidylinositol specific phospholipase (PlcA). Thus, these enzymes facilitate the protection of the bacterium against immune response and adhesion in the host tissue.

The function of PlcR relies on the existence of signaling peptide PapR, which is located about 70 bp downstream from *plcR* (Gominet *et al.*, 2001). *papR* encodes for a 48-aa peptide which is excreted and remain intracellular and then transported to the extracellular milieu through Opp system. When the processed PapR is imported back, it binds with

PlcR. Then 'activated' PlcR binds to *plcR* box, located in the promoter regions of PlcR regulated genes (Gominet *et al.*, 2001; Rocha-Estrada *et al.*, 2010), controlling their transcription (Figure 1.8). It was observed that there is a specific binding sequence for PlcR, known as PlcR box (TATGNAN$_4$TNCATA) in promoter regions of the genes regulated by PlcR in *Bacillus cereus* group (Agaisse *et al.*, 1999).

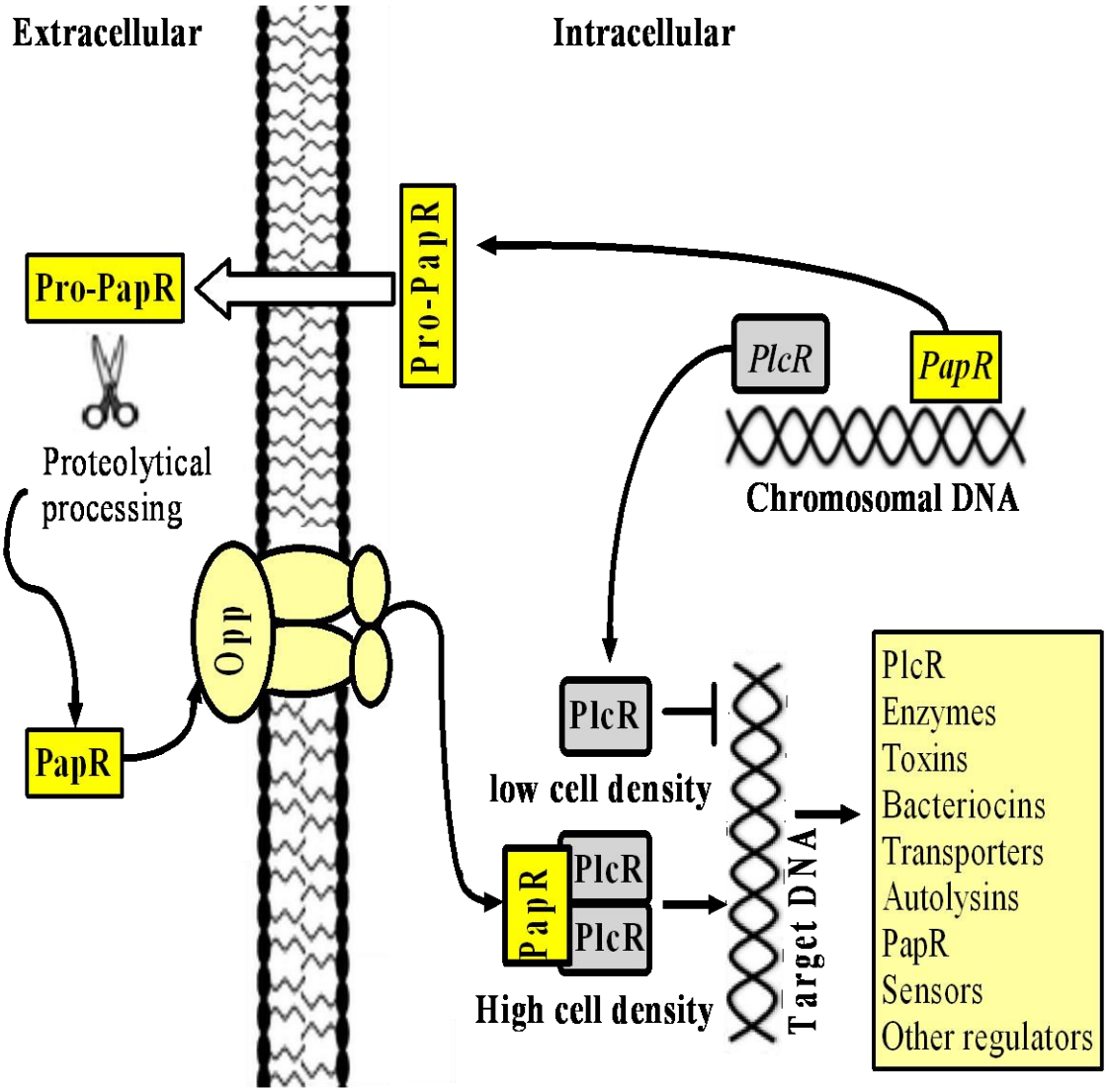


Figure 1.8. Diagram illustrating the functioning of PlcR/PapR QS system. PapR is synthesized and exported, after proteolytical processing the mature peptide is reinternalized via Opp system. At high cell density, PapR binds to PlcR, promoting a conformational change, oligomerization, and DNA binding capacity. The polymers bind to DNA in PlcR boxes stimulating transcription of the PlcR virulence regulon. Figure is constructed based on Rocha-Estrada *et al.* (2010).

1.11. PlcR transcriptional regulators in *Streptococcus pneumoniae*

In pneumococcus, the competence (*com*) locus is the best-studied QS system (Claverys *et al.*, 2007). However, recently, the homologs of several other QS systems have been identified in *S. pneumoniae* (Hoover *et al.*, 2015; Cuevas *et al.*, 2017; Junges *et al.*, 2017). In *S. pneumoniae* D39 strain, there are two homologs of PlcR: SPD_1745 and SPD_1786. The size of SPD_1786 is 900 bp, codes for a putative protein with 299 amino acids, and is located in 1782062-1783036 nucleotides in type 2 D39 strain genome. However, SPD_1745 is 864 bp, codes for a protein with 287 amino acids, and is located in 1738236-1739099 nucleotides in D39 genome. The amino acid identity between these two putative PlcR is 36%.

SPD_1745 has been established to be part of a peptide quorum-sensing system (TprA/PhrA) while this study was underway (Hoover *et al.*, 2015). The small PhrA peptide is analogous to PapR and encodes a 46-aa peptide, predicted to be secreted via the Sec pathway, and then re-imported through the Opp system. PhrA can activate the expression of the TprA regulon and contributes in autoregulation. However, the binding sequence for TprA is unidentified in *S. pneumoniae*, thus it is important to determine any conserved binding site in the putative promoter regions of the TprA regulon. In addition, it has been shown that in certain lineages of *S. pneumoniae*, such as PMEN1, characterized by penicillin resistance, there is an additional TprA/PhrA homolog, annotated as TprA/PhrA2. Similar to TprA/PhrA, the TprA/PhrA2 activity is modulated by its signaling peptide PhrA2 in a cell density dependent manner (Kadam *et al.*, 2017). Furthermore, in collaboration with others, it was demonstrated that PhrA2 regulates the TprA/PhrA system in *S. pneumoniae* strain D39 (Kadam *et al.*, 2017).

It was reported by Hoover *et al.* (2015) that the TprA/PhrA system controls the expression of a lantibiotic gene cluster that encodes members of the bacteriocin family antimicrobial peptides. In addition, the PhrA level is shown to be activated in the presence of galactose and repressed by glucose (Hoover *et al.*, 2015). Thus, this quorum sensing system is predicted to be active in the upper respiratory tract where the galactose is an important source of nutrient. Therefore, TprA/PhrA can help *S. pneumoniae* to colonise the nasopharynx or can be linked to virulence. Despite this prediction, Hoover *et al*. (2015) were unable to establish this system's importance in invasive pneumococcal disease.

Therefore, it is essential to study the role of TprA/PhrA in pneumococcal sugar metabolism, survival, and virulence to carry out detailed genetic, functional, and regulatory analysis in order to reveal its detailed operation and contribution to pneumococcal virulence.

1.12. Quorum sensing systems as anti-infective targets

Antibiotics have been instrumental in the fight against infectious diseases (Brooks and Brooks, 2014), but their effective utility is in danger due to the rapid rise of antibiotic resistant bacteria, indicating an urgent need to develop new anti-infectives that are effective, and less prone to the development of drug resistance. In light of the failure of traditional antibiotics and limited number of novel bacterial broad-spectrum targets, focus has been on alternative targets to develop new generation anti-infectives, such as those not essential for growth but important for infection, include virulence factors, which are required to cause host damage and disease (Brooks and Brooks, 2014). This approach has several potential advantages over targeting essential metabolic pathways, which include expanding the repertoire of bacterial targets, conserving the host endogenous microbiome, and decreasing the likelihood of drug resistance by exerting less selective pressure (Clatworthy *et al.*, 2007).

Interfering with bacterial communication systems is considered to be an effective strategy to develop novel anti-infectives (Brooks and Brooks, 2014; Clatworthy *et al.*, 2007). Bacteria are able to communicate with each other to coordinate their collective behaviour by detection and processing of chemical signals through a mechanism called quorum sensing (QS). Different microbial phenotypes are modulated by QS systems, including growth, biofilm formation, oxidative stress resistance, microbial competition and virulence expression (Rutherford and Bassler, 2012; Zhu *et al.*, 2002).

QS systems were manipulated in different bacterial species but particularly in the Gram-negative *Pseudomonas aeruginosa*, including the use of lactonases and amidases to inhibit enzyme synthesis, and methods to quench the autoinducer using peptides and antibody (Huang *et al.*, 2003; Gonzalez and Keshavan, 2006). While a degree of success in modulation of QS systems has been reported in *in vitro* and *in vivo* models (O'Loughlin *et al.*, 2013), more work needs to be done to improve compound stability, and large-scale

production of these compounds. These problems have led to the search for alternative tools to manipulate QS systems, such as biomimetic linear molecularly imprinted polymers (LMIP) (Vasapollo *et al.*, 2011). Molecular imprinting creates an artificial molecular recognition sites within a synthetic polymer in the presence of a template, in this case a QS signal molecule. Cross-linked MIPs were used successfully to suppress QS in *Vibrio fischeri* and *P. aeruginosa* (Piletska *et al.*, 2011; Piletska *et al.*, 2010). Cross-linked polymers unfortunately have limitations as drug candidates for the reason that it is next to impossible to elucidate their structure, and this might have implications for their approval by drug regulatory agencies. Non-imprinted linear polymers were used recently for suppression of QS in Gram negative bacteria (Cavaleiro *et al.*, 2015). The effect demonstrated by non-specific linear polymers was moderate, and it is expected that their specificity can be further improved by molecular imprinting. Unlike antibodies, aptamers and other molecules of biological origin targeting QS systems, LMIP capable of recognizing their target species in the template-derived sites will not be digested by enzymes, hence they will be preferred to the molecules of biological origin. Therefore, it is essential to illustrate the potential utility of LMIP as a novel antimicrobial therapy. In this study it was employed, for the first time, a soluble linear molecularly imprinted polymer (LMIP) prepared by solid-phase synthesis to interfere with the operation of a TprA/PhrA QS system in a Gram-positive pathogen (Motib *et al.*, 2017). The TprA/PhrA QS system was targeted since it is a member of an emerging and widespread superfamily of peptide-regulated QS systems, and this study presented data demonstrating that it is essential for pneumococcal colonisation and virulence in models of invasive, lung, and middle ear disease. Highly homogenous LMIP was specific to the PhrA peptide, decreased PhrA-induction in a dose-dependent and sequence-specific manner, and possessed no visible toxicity after intranasal or intraperitoneal administration in the murine model. The findings of this study provided proof of principle that LMIP can be used to block Gram-positive quorum-sensing peptides, setting the stage for studies on a novel class of drugs to target Gram positive pathogens.

1.13. Aims and objectives

I hypothesised that TprA/PhrA system plays an important role in pneumococcal sugar metabolism, survival, and virulence, and its activity can be modulated by LMIP *in vitro* and *in vivo*. Therefore, the main objective of this study was to identify the role of TprA/PhrA QS system in pneumococcal sugar metabolism, survival, and virulence, and test the modulation of TprA/PhrA by LMIP *in vitro* and *in vivo*. I aimed to study the role of TprA/PhrA QS system in pneumococcal survival and virulence *in vitro* and *in vivo*. I aimed also to identify TprA/PhrA regulon by microarray analysis on CDM supplemented with 55mM galactose or mannose to determine the environmental conditions important for induction of *tprA* and *phrA* and investigate the regulatory role between the components of TprA/PhrA system by construction and analysis of transcriptional *lacZ*-fusions to the promoter of *tprA*, *phrA*, and *nanA* in the wild type and mutant backgrounds. The aim of this study detected the putative binding sequence of TprA and study the direct interaction of this protein with the selected genetic loci using EMSA. LMIP was developed and tested for quenching peptide QS signal molecules of pneumococcal TprA/PhrA QS system in collaboration with Prof Sergey Piletsky's research group, Chemistry Department, University of Leicester.

Chapter 2. Materials and Methods

2.1. Biological and chemical materials

The bacterial strains and plasmids used in this study are listed in Table 2.1, and all the chemicals used in this study were obtained from Sigma unless otherwise stated.

Table 2.1. Bacterial strains and plasmids used in this study.

Strains/Plasmids	**Description/Use**	**Source**
S. pneumoniae		
D39	Serotype 2 strain	Laboratory stock
Δ*tprA*	D39; SPD1745:SpecR	Dr H. Yesilkaya
Δ*tprA*Comp	D39; *tprA* + Δ*tprA*:Spec^R; Kan^R	This study
Δ*phrA*	D39; SPD1746:Spec^R	This study
Δ*phrA*Comp	D39; *phrA*+ Δ*phrA*:Spec^R; Kan^R	This study
pPP1::*lacZ*-wt	D39; Δ*bgaA*::pPP1-*lacZ*; Tet^R	Dr H. Yesilkaya
P*tprA*::*lacZ*-wt	D39; Δ*bgaA*::P*tprA-lacZ*; Tet^R	Dr H. Yesilkaya
P*phrA*::*lacZ*-wt	D39; Δ*bgaA*::P*phrA-lacZ*; Tet^R	This study
P*nanA*::*lacZ*-wt	D39; Δ*bgaA*::P*nanA-lacZ*; Tet^R	This study
P*tprA-lacZ*-Δ*tprA*	Δ*tprA*:Spec^R; Δ*bgaA*::P*tprA-lacZ*; Tet^R	Dr H. Yesilkaya
P*tprA-lacZ*-Δ*phrA*	Δ*phrA*:Spec^R; Δ*bgaA*::P*tprA-lacZ*; Tet^R	This study
P*phrA-lacZ*-Δ*tprA*	Δ*tprA*:Spec^R; Δ*bgaA*::P*phrA-lacZ*; Tet^R	This study
P*phrA-lacZ*-Δ*phrA*	Δ*phrA*:Spec^R; Δ*bgaA*::P*phrA-lacZ*;Tet^R	This study
P*nanA-lacZ*-Δ*tprA*	Δ*tprA*:Spec^R; Δ*bgaA*::P*nanA-lacZ*;Tet^R	This study
P*nanA-lacZ*-Δ*phrA*	Δ*phrA*:Spec^R; Δ*bgaA*::P*nanA-lacZ*;Tet^R	This study
Escherichia coli		
One Shot® TOP10	Plasmid propagation	Invitrogen, UK
SPD1745-OneShot®TOP10	Plasmid propagation; Kan^R	This study
SPD1746-OneShot®TOP10	Plasmid propagation; Kan^R	This study

P*tprA*-OneShot® TOP10	Plasmid propagation; Amp^R	Dr H. Yesilkaya
P*phrA*-OneShot® TOP10	Plasmid propagation; Amp^R	This study
P*nanA*-OneShot® TOP10	Plasmid propagation; Amp^R	This study
SPD1745-pLEICS-01-One Shot® TOP10	Plasmid propagation; Amp^R	Dr H. Yesilkaya
BL21 (DE3) pLysS	Protein expression	Agilent Tech, USA
SPD1745-BL21(DE3) pLysS	Protein expression; Amp^R	This study
Plasmids		
pDL278	Amplification of $Spec^R$ (*aadA*)	Yesilkaya, 1999
pLEICS-01	6His-Tag for protein expression; Amp^R	PROTEX, UK
pCEP	Genetic complementation; Kan^R	Guiral *et al.*, 2006
pPP1	Promoterless *lacZ* for transcriptional fusions; Amp^R Tet^R	Halfmann *et al.*, 2007

2.2. Bacterial growth conditions

Streptococcus pneumoniae was grown either in brain heart infusion broth (BHI) or in blood agar base (Oxoid, UK) supplemented with 5% v/v defibrinated horse blood in the presence of 5% CO_2 at 37°C. All the media were prepared according to manufacturer's instructions and sterilised using the autoclave at 121°C for 15 min. Where appropriate, the culture medium was supplemented with different antibiotics: kanamycin 250 µg/ml, spectinomycin 100 µg/ml, or tetracycline 15 µg/ml.

Chemically defined medium (CDM) (Table 2.2) supplemented either with 55 mM of galactose, mannose, *N*-acetylglucosamine or glucose was used also to grow the pneumococcal strains microaerobically. This media was prepared by mixing 870 ml Basal solution, 80 ml Amino acids, 10 ml Micronutrients, 10 ml Nitrogenous bases, 1 ml Pyruvate, 4 ml Choline-HCl, and 10 ml Vitamins as previously described (Kloosterman *et al.*, 2006a). Then, CDM was filter sterilised and stored at 4°C until used.

When required, CDM supplemented with 55 mM galactose or glucose was also added with varying concentration (5-500 nM) of synthetic peptide (PhrA10) and/or with differing concentration (25, 50, 100 nM) of linear molecularly imprinted polymers (LMIP) which was developed and tested for quenching peptide QS signal molecules of pneumococcal TprA/PhrA quorum sensing system in collaboration with Prof Sergey Piletsky's research group, Dept Chemistry, University of Leicester. PhrA10 is the active processed signal peptide with capability to induce PhrA expression (Hoover *et al.*, 2015). This processed signal peptide (SNGLDVGKAD) was used as a template for LMIP preparation as described in detail in my recent publication (Motib *et al.*, 2017). The synthetic peptide (PhrA10) (Covalab R and D in biotechnology, France) was resuspended in Dimethyl sulfoxide (DMSO) to a concentration of 5 mM, and the stock solution of the synthetic PhrA was stored in -20°C until used.

Sicard's defined medium was prepared according to the instructions provided by Sicard (1964), and was used to grow the pneumococcal strains in the presence of mucin as described previously (Yesilkaya *et al.*, 2007). Firstly, minerals and buffer solution were prepared in 1 L nH_2O, pH 7.55. After this, all the amino acids (except glutamine and cysteine) were dissolved in the minerals and buffer solution, and then were autoclaved. The glutamine, cysteine and pyruvate were added to the vitamin solution and filter sterilised. Subsequently, this solution was added into the autoclaved minerals and buffer solution, and stored in the fridge at 4ºC until needed.

When required, Sicard's defined medium (2X) was supplemented with pork gastric mucin (PGM) (Sigma, United Kingdom). PGM was prepared as previously described (Yesilkaya *et al.*, 2007; Terra *et al.*, 2010) with some minor modifications. PGM was dissolved in distilled water and dialyzed against distilled water overnight at 4°C using a snakeskin dialysis membrane (Molecular mass cut off, 10 KDa, Pierce, Hoddesdon, United Kingdom). After this, PGM was lyophilized and was dissolved in 10 mM potassium phosphate buffer, pH 7.0 (1 M potassium phosphate buffer: 61.5 ml of 1 M K_2HPO_4 and 38.5 ml of 1 M KH_2PO_4, then it was diluted to 10 mM by dH_2O) at 8 mg/ml, and sterilised by autoclaving. This was mixed with double strength Sicard's defined medium at a concentration of 4 mg/ml.

Escherichia coli strains were grown in Luria broth (LB) (10 g/l tryptone, 10 g/l NaCl and 5 g/l yeast extract) with shaking at 37°C, or Luria agar (LA) plates (LB with 1.5 % w/v bacteriological agar). Where appropriate, ampicillin (100 µg/mL) or kanamycin (50 µg/mL) were added to the culture medium.

Table 2.2. The composition of CDM used for growth of *Streptococcus pneumoniae*.

Components		**Components**	
Basal solution	**g l^{-1}**	**Amino acids**	**g l^{-1}**
Na_2-β-glycerophosphate	26	Alanine	0.24
KH_2PO_4	1.0	Arginine	0.124
$(NH_4)_3$ citrate	0.6	Asparagine	0.352
Choline-HCl	0.4	Aspartate	0.4
Na-acetate	1.0	Glutamate	0.5
Vitamins	**mg l^{-1}**	Glutamine	0.392
Na-p-aminobenzoate	5.0	Glycine	0.176
D-Biotin	2.5	Histidine	0.152
Folic acid	1.0	Isoleucine	0.212
Nicotinic acid	1.0	Leucine	0.456
Ca (D^+) Pantothenate	1.0	Lysine	0.44
Pyridoxamine-HCL	2.5	Methionine	0.124
Pyridoxine-HCl	2.0	Phenylalanine	0.276
Riboflavin	1.0	Proline	0.676
Thiamine-HCl	1.0	Serine	0.34
DL-6,8-Thioctic acid	1.5	Threonine	0.224
Vitamin B_{12}	1.0	Tryptophane	0.052
Nitrogenous bases	**mg l^{-1}**	Valine	0.324
Adenine	10	**Micronutrients**	**mg l^{-1}**
Uracil	10	$MgCl_2$	200
Xanthine	10	$CaCl_2$	38
Guanine	10	$ZnSO_4$	5
Others			
Choline-HCl	10 **mg l^{-1}**	Pyruvate	0.1 **g l^{-1}**

Table 2.3. The composition of 2X Sicard's defined medium

Components		**Components**	
Minerals and Buffers	**g l^{-1}**	**Amino acids**	**mg**
NaCl	10	L-Arginine	400
NH_4Cl	4	L-Aspargine	20
KCl	0.8	L-Cysteine-HCl	200
Na_2HPO_4	0.24	L-Glutamine	40
$MgSO_4$	0.048	Glycine	240
$CaCl_2$	0.02	L-Histidine	300
FeSO4 $7H_2O$	0.00110	L-Isoleucine	13.1
Tris	9.68	L-leucine	13.1
Vitamins	**mg 10ml^{-1}**	L-Lysine	840
Biotin	0.030	L-Methionine	360
Choline	10	L-Threonine	350
Nicotinamide	1.2	L-Valine	11.7
Pantothenate	4.8	**Others**	
Pyridoxal HCl	1.2	Pyruvate, sodium	1.6 g
Riboflavine	0.6	Uracil	2 mg
Thiamine	1.2		

2.3. Viable counts of the bacterial aliquots

The viabilities of the bacterial cultures were determined according to Miles and Misra method (Miles *et al*., 1938). Routinely, microtiter plate was used to make serial dilutions of 20 µl bacterial suspension in 180 µl of sterile PBS, pH 7.0. Then 60 µl of each dilution was plated out. After drying the spots, plates were incubated in a candle jar overnight at 37°C. Then the colonies were counted in the sector that had 30-300 colonies. Lastly, the number of colony forming units (CFU) was calculated by the following equation and the mean was taken from the duplicate readings.

CFU= Number of colonies in sector × dilution factor × (1000/60).

2.4. Growth studies in BHI, CDM and Sicard's defined medium

Growth studies in BHI, and CDM supplemented with 55 mM of different sugars with or without LMIP were done using Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, UK). Flat bottom microtitre plate was used for growth studies. To initiate growth 2 µl of bacterial suspension containing ~5 x 10^9 CFU/ml was added into 198 µl of BHI or CDM. Then the microtitre plate was put in the Multiskan™ GO Microplate Spectrophotometer, which is programmed to run for 24 hours at 37°C by taking a reading every hour at OD600.

Sicard's defined medium was also used to evaluate the pneumococcal growth in the presence of mucin as described previously by Terra *et al.* (2010). Briefly, 150 µl of overnight pneumococcal culture was transferred into 10 ml Sicard's defined medium supplemented with PGM at a concentration 4 mg/ml. The bacterial growth was measured by colony counting on BAB using the protocol as described in section 2.3 by measuring the viable count of the bacterial aliquots at different times (0, 5, 7, 10, and 24). The bacterial growth rates (μ) were calculated by using the exponential phase of growth with the following equation:

$$(\mu)\ \mathrm{h}^{-1} = \ln OD_2 \text{or CFU2} - \ln OD_1 \text{or CFU1} \ / \ t_2 - t_1$$

Where: ln is the natural logarithm of a number, *t*= time; OD_2 and OD_1 are the cell densities at t_2 and t_1, respectively. CFU2 and CFU1 are the colony forming unit at t_2 and t_1, respectively. In addition, the growth yield was calculated by measuring the highest optical density during the bacterial growth or via measuring the highest cell forming unit during the bacterial growth.

2.5. Extraction of chromosomal DNA

Chromosomal DNA was extracted according to Saito and Miura (1963). The pneumococcal pellet obtained from overnight growth was resuspended in 400 µl TE buffer containing 25% w/v sucrose and incubated at 37ºC for 2 hours to acquire a clear

lysate. After this, the sample was centrifuged at 13000 rpm for 5 minutes in a bench top centrifuge (Sigma). The supernatant was transferred to a fresh tube, and added with an equal volume of liquidified phenol (Sigma). Then the tube was centrifuged at 13000 rpm for 10 minutes and the upper aqueous phase was transferred to a fresh tube. This process was repeated twice, and 500 µl of clear aqueous phase was mixed with 1 ml of 100% ethanol and 25 µl of 3 M sodium acetate, pH 5.2. Nucleic acids were pelleted by centrifugation at 13000 rpm for 5 minutes. The pellet was added with 500 µl of 70% ethanol, and centrifuged at 13000 rpm for 5 minutes. Ethanol was removed, and the pellet was dried. Then the pellet was resuspended in 250 µl of TE buffer and stored at 20ºC until needed.

2.6. Extraction of *E. coli* plasmid DNA

Plasmid DNA was extracted by using mini prep kit according to the manufacturer's instructions (Qiagen). The bacterial pellet was taken from 10 ml bacterial broth, which was grown at 37ºC for 12-16 hours with shaking incubator, by centrifugation at 4000 rpm (Sorvall legend T, Thermo Scientific) for 10 min at room temperature, and was resuspended in 250 µl buffer P1 containing RNase A. After this 250 µl of buffer P2 was added and mixed by inverting to obtain a clear lysate. Then 350 µl of buffer N3 was added and mixed immediately. The sample was centrifuged for 10 min at 13000 rpm (Microfuge, Sigma). After this process, the supernatant was transferred to the QIA prep spin column and centrifuged at 13000 rpm for 30-60 seconds. Then 0.5 ml of buffer PB was added to the QIA prep spin column and centrifuged for 30-60 seconds at 13000 rpm. Subsequently, 0.75 ml of buffer PE was used to wash the QIA prep spin column, and the column was centrifuged for 30-60 seconds. The QIA prep column was transferred to the collection tube, 50 µl of buffer EB was added, and it was centrifuged for 1 min at 13000 rpm to elute plasmid DNA. Then the eluted DNA was stored at -20ºC.

2.7. Agarose gel electrophoresis

Gel electrophoresis was done as described previously (Sambrook *et al.*, 1989). Agarose (Bioline, UK) 1% w/v was used to separate the DNA fragments. Agarose was solubilized in 1X TAE buffer (40 mM Tris-acetate and 1 mM EDTA, pH 8.0) (Sigma). Agarose mixture was solidified in a microwave, and when cooled, 0.2 µg/mL Ethidium Bromide

was added for DNA staining. DNA samples were mixed with 6X gel loading dye (New England Biolab) and were loaded into the wells. A 1 kb or 100 bp DNA ladder (New England Biolab, UK) was also loaded as a control to measure the approximate size of the DNA samples. The electrophoresis was carried out at 80-90 volts for approximately 1 h, Long wave UV transilluminator was used to visualise DNA fragments.

2.8. DNA purification from agarose slabs

DNA purification from agarose slabs was done according to the manufacturer's instructions (Promega). This method was used to purify and concentrate the DNA in solution and in gel slice. Purification of DNA from the gel was done by cutting the required DNA band from the gel and putting it into a 1.5 ml micro centrifuge tube. Then, 10 µl membrane binding solution was added per 10 mg of gel slice. After this, the solution was incubated at 50-65ºC to dissolve the gel slice and an equal volume of membrane binding solution was added to the DNA solution before transferring to the minicolumn assembly. Then, centrifugation was used at 13000 rpm for 1 min to discard flow through. The DNA was washed twice by adding first 700 µl, and then 500 µl of membrane wash solution and centrifugation at 13000 rpm for 1 min. The minicolumn was transferred carefully to a new 1.5 ml micro centrifuge tube, 30 µl of nuclease free water was added to the center of minicolumn, and incubated at room temperature for 1 min. Finally, centrifugation was used to elute the DNA at 13000 rpm for 1 min and DNA was stored at -20ºC.

2.9. Polymerase Chain Reaction (PCR)

Polymerase chain reaction was done by using a thermal cycler (Biometra, Germany). HotStarTaq *Plus* Master Mix (Qiagen, UK) or PrimeSTAR HS premix (Clontech, USA) were used for PCR. HotStarTaq *Plus* Master Mix was used for routine confirmation of cloning. This enzyme mix has a high product yield, and eliminates a nonspecific amplification of DNA by inactivating the Taq polymerase at lower temperatures, hence reducing nonspecific priming, and primer dimmers. The PCR reaction was done in a total volume of 20 µl containing 2 µl of DNA (15 ng/µl), 2 µl of primers (10 pmol each/reaction), 10 µl HotStarTaq *Plus* Master Mix, and 6 µl of DNase-RNase free water. After initial enzyme activation (10 min at 95ºC), DNA was amplified through 30 cycles

consisting of 45 seconds of denaturation at 95ºC, 45 seconds of annealing at temperature specific for each primer pairs, and elongation at 72ºC for 1 min/1000 bp according to the product size, followed by a final elongation step at 72ºC for 10 min, and hold at 4°C.

PrimeSTAR HS premix was used for insertion deletion mutations to get high accuracy DNA amplification with reduced error rate. A PCR mixture contained 25 µl PrimeSTAR HS premix, 2 µl template (15 ng/µl), 2 µl of gene specific forward and reverse primer mix (1 pmol each/reaction) and 21 µl of nuclease-free water. DNA was amplified through 30 cycles of amplification by using the initial denaturation: 98°C for 10 seconds, amplification (30 cycles): denaturation 98°C for 10 seconds, annealing 55°C for 5 seconds, extension 72°C for 1 min/1000 bp, and hold at 4°C.

2.10. Colony PCR and PCR product analysis

The DNA of a single well-isolated colony was prepared by suspension in 100 µl of nuclease free water. After this the suspension was heated at 95°C for 10 min. Then centrifugation at 13000 rpm/min (Microfuge, Sigma) was used to obtain the template that was used in the PCR. Routinely, the PCR products were analyzed using gel electrophoresis.

2.11. Restriction digests of DNA

Restriction of DNA fragments was done according to manufactures' protocol (New England Biolab). The reactions were prepared by mixing 1 µl (5 U) of each restriction enzyme (*NcoI-BamHI* or *SphI-BamHI* (NEB, UK)), 1 µg of plasmid or insert DNA, 5 µl 10X CutSmart™ buffer, and nuclease- free water up to 50 µl. The mixture was incubated in a water bath at 37 ºC for 4 hours until complete digestion. Then the digested plasmids and inserts were purified using the DNA purification kit (Section 2.8).

2.12. Ligation of DNA fragments

Ligation reactions were performed in 20 µl using 2 µl (400 U/µl) of T4 DNA Ligase (New England Biolabs, UK), 2 µl of 10X T4 DNA ligase reaction buffer, and 1:3 molar ratio

of plasmid to insert. The ligation mixture was incubated at 16°C for 16 hours. After this the reaction was inactivated at 65°C for 10 min.

2.13. Transformation into *E. coli*

The ligation mixture was transferred to chemically competent *E. coli* One Shot® TOP10 (Invitrogen, UK) for plasmid propagation, or to BL21 (DE3) pLysS (Agilent Technologies, USA) for protein expression (Sambrook and Russell, 2001). Routinely, 45 µl competent cells was thawed, mixed with 5 µl ligation mixture, and placed in a prechilled 14 ml BD Falcon polypropylene round bottom tube for 30 minutes on ice. Next, the mixture was put in the water bath at 42°C for 45 seconds, and was promptly transferred back on ice for 2 min. Finally, 450 µl of LB broth was added into the sample and incubated for 1:30 hour in an Innova 44 shaking incubator set at 225 rpm at 37°C (New Brunswick Scientific, USA). Eventually, 250 µl of transformation mixture was plated on LB agar containing appropriate antibiotic, and was incubated overnight at 37°C in a static incubator.

2.14. Transformation into *S. pneumoniae*

Transformation of DNA into *S. pneumoniae* was done according to the method described by Havarstein *et al.* (1995). *S. pneumoniae* D39 wild type was grown in 10 ml BHI overnight at 37 ºC. Then the culture was diluted (1:100) in 10 ml fresh BHI and incubated at 37 ºC until OD600 had reached between 0.05-0.08. At this stage, an 860 µl bacterial culture was added with 100 µl NaOH (100 mM), 10 µl BSA (20% w/v), 10 µl $CaCl_2$ (10 mM), 2 µl (50 ng/µl) competence stimulating peptide (CSP) (Alloing *et al.*, 1996), and ~1 µg of DNA in an eppendorf tube. Finally, the mixture was incubated at 37 ºC for 3 hours, and 330 µl sample was plated out every hour over three hours on BAB containing horse blood with appropriate antibiotic at 37°C.

2.15. Construction of genetically modified strain

Targeted mutation was done by splicing overlap extension as previously described (Horton, 1995; Song *et al.*, 2005). Insertion deletion mutant was constructed by transforming the *in vitro* mutagenized SOEing construct in *S. pneumoniae*. Briefly,

spectinomycin ($Spec^R$) resistance gene cassette as well as the left - and right flanking regions of gene to be deleted were amplified. Then the DNA fragments were purified by using DNA purification kit (Promega) as described in section 2.8. After this, the purified DNA fragments were mixed at equal molar ratios. In the second step, the external primers (Upstream-SOE1746-F and Downstream-SOE1746-R) were used to fuse the three PCR segments (Table 2.4). The fused amplicons were gel-purified (Qiagen) and transformed into *S. pneumoniae*. The transformants were selected on BAB plates containing the appropriate antibiotic, and the mutations were confirmed by PCR and sequencing.

2.15.1. Amplifying SOEing fragment

The right and left flanking regions of the target gene were amplified with PrimeSTAR HS premix (Section 2.9) using the primers UF-SOE1746-F/UF-SOE1746-R and DF-SOE1746-F/DF-SOE1746-R (Table 2.4) to produce approximately 600 bp PCR products. In addition, *aadA* (1158 bp) antibiotic resistance gene was amplified from the plasmid pDL278 (Yesilkaya, 1999), using the Spec-F/Spec-R primes (Table 2.4). The primers UF-SOE1746-R and DF-SOE1746-F contain a sequence homologous to the target gene and the antibiotic resistance gene *aadA* (Table 2.4). Subsequently, the amplified PCR products were purified using the QIAquick PCR purification kit (Section 2.8).

Table 2.4. The list of primers used for insertion deletion mutations. Bold typeface indicates the homologous regions with antibiotic resistance genes.

Primers	**Sequence (5'- 3')**
UF-SOE1746-F	TTTCCAATGACGAAATTGCCT
UF-SOE1746-R-Spec	**ATTCACGAACGAAAATCGAT**AACTAATTTTTTAATTCCACG
DF-SOE1746-F-Spec	**ACAATAAACCCTTGCATAAC**AATGGTCTTGATGTTGGGAAG
DF-SOE1746-R	CCTCTCTCAAAATTATTAGTT
Spec-F	ATCGATTTTCGTTCGTGAAT
Spec-R	GTTATGCAAGGGTTTATTGT
1746-SeqM-F1	GTGAGCGTAGTTGTAACGAAC
1746-SeqM-R1	ATTTCAACATTTCTTCAAAAG
1746-SeqM-F2	CCTTCCTATTTGCTTAATTTC
1746-SeqM-R2	TCATTTCATCAGCAGACAATG

2.15.2. Fusion of SOEing fragments and transformation

The up and down stream flanking regions of the target gene as well as the amplicons for *aadA* were fused using the primers UF-SOE1746-F and DF-SOE1746-R to produce a SOEing product. For this a PCR mixture was prepared, which contained 2 µl from each of the up and down stream flanking PCR products (~20 ng/µl each) and the *aadA* gene amplicons (~20 ng/µl each), 25 µl of 2X PrimeSTAR HS premix, 1 µl of each UF-SOE1746-F and DF-SOE1746-R primer (1 pmol/µl) and up to 50 µl of nuclease-free water. The PCR conditions were described in section 2.9. The SOEing products were visualised by agarose gel electrophoresis and purified from the gel by using DNA purification kit (Promega, UK) as described in section 2.8. Then, the SOEing products were transformed into *S. pneumoniae* D39 (Section 2.14) and the transformants were selected on BAB plates supplemented with 100 µg/ml spectinomycin.

2.15.3. Confirmation of gene replacement

PCR was utilised for confirmation of insertion deletion mutations by using the genomic DNA of resistant colonies as a template. HotStarTaq *Plus* Master Mix (Section 2.9) was used for PCR confirmation, with the primers UF-SOE1746-F and DF-SOE1746-R of target gene to amplify the entire mutated region, UF-SOE1746-F and Spec-R and Spec-F and DF-SOE1746-R primers to amplify the upstream and downstream flanking regions of target gene with the antibiotic gene, respectively, and Spec-F and Spec-R to amplify the antibiotic resistance gene (*aadA*).

The gene replacement was also confirmed by DNA sequencing using two new set of primers, the primers F1 and R1 to amplify approximately 200 nucleotides before the upstream flanking region and approximately 200 nucleotides additional to the downstream flanking region of the target gene, F2 and R2 to amplify approximately 150 nucleotides before the start of antibiotic cassette and approximately 150 nucleotides away from the antibiotic cassette, respectively. Genomic DNA of the mutant strain was extracted and used as a template in PCR to amplify the entire fused constructs plus 200 nucleotides chromosomal region up and down stream of both flanks using their specific F1 and R1 sequencing primers (Table 2.4). HotStarTaq *Plus* Master Mix was used in PCR (Section 2.9) and the amplified region was purified using DNA purification kit (Section 2.8). The purified regions were sent to the Protein Nucleic Acid Chemistry Laboratory (PNACL), University of Leicester with the gene sequencing primers (SeqM-F1, SeqM-F2, SeqM-R1 and SeqM-R2).

2.16. Genetic complementation of mutants

Genetic complementation of the mutant strains was done to rule out the polar effect of the mutation using the method described by Guiral *et al.* (2006). Plasmid pCEP was used to complement the mutant strains. This plasmid has homologous sequence to *S. pneumoniae* D39 genome, and this is believed to be located on a silent transcriptional site. Therefore, reintroduction of the intact copy of gene will have no influence on the transcription of other genes after complementaion (Alloing *et al.*, 1990). Plasmid pCEP was extracted using the QIAprep spin Miniprep kit as described in section 2.6. The entire coding region with its putative promoter region was amplified using the primers XComp-

F and XComp-R (X refers to gene number), which incorporate *NcoI* and *BamHI* restriction sites, respectively (Table 2.5). PrimeSTAR HS premix was used in the PCR (Section 2.9). The amplified PCR products were purified using the DNA purification kit (Section 2.8), and visualised by agarose gel electrophoresis. The insert and the plasmid pCEP were double digested using *NcoI-BamHI* (Section 2.11) and then purified using DNA purification kit (Section 2.8). The ligation reaction was prepared as described in section 2.12.

Table 2.5. Primers used for genetic complementation. Bold typeface indicates incorporated restriction sites.

Primers	**Sequence (5′–3′)**
1745Comp-F	CG**CCATGG**GTAATTTTTAACTTTTTTTT
1745Comp-R	CG**GGATCC**CTAAACAGTATCTAGTTCCT
1746Comp-F	CG**CCATGG**TAAAGATAATAAACCTTCC
1746Comp-R	CG**GGATCC**TTAATCCGCCTTCCCAACA
MalF	GCTTGAAAAGGAGTATACTT
PCEPR	AGGAGACATTCCTTCCGTATC

2.16.1. Transformation of recombinant pCEP plasmid into *E. coli,* and confirmation by PCR and DNA sequencing

The ligation reaction was transferred into *E. coli* One Shot® TOP10 chemically competent cells. The kanamycin resistant colonies were grown on LB agar medium supplemented with 50 µg/ml of kanamycin (Section 2.13) for extracting the cloned plasmid DNA using the QIAprep spin Miniprep protocol (Section 2.6). The recombinant plasmid was used as a template in PCR with HotStarTaq *Plus* Master Mix (Section 2.9) using the primers malF and pCEPR (Table 2.5). These primers have recognition sequence on the plasmid and are designed to amplify the region surrounding the cloning site and cloned gene with its promoter region. In the absence of insert, these primers amplify approximately 263 bp product from empty pCEP, in the presence of cloned insert the primers XComp-F and XComp-R (X refer to the gene number) amplifies the cloned gene and its promoter region

plus 263 bp. The successful cloning was further confirmed by DNA sequencing using the primers malF and pCEPR.

2.16.2. Transformation of recombinant pCEP into the mutant strains and confirmation by PCR

The recombinant pCEP was transformed into the relevant mutant strains of *S. pneumoniae* (Section 2.14). The transformation resulted in several kanamycin and spectinomycin resistant colonies, while the mutant strains could not grow in the presence of kanamycin. PCR was used to confirm the successful complementation genetically using HotStarTaq *Plus* Master Mix (Section 2.9), the primers malF and pCEPR, and the primers XComp-F and XComp-R (X refer to the gene number) (Table 2.5).

2.17. Pneumococcal RNA extraction

The bacterial RNA was extracted according to Stewart *et al.* (2002). The bacterial pellet obtained from 10 ml mid-exponential phase culture was resuspended with 500 µl Trizol reagent (Invitrogen, UK), and was mixed by pipetting. Then 100 µl of chloroform was added and vortexed for 15 seconds. The samples were transferred into Lysing Matrix B tubes containing 0.1 mm silica spheres (MP Biomedicals, UK), and sonicated for 45 seconds using a PowerLyzer^{TM} 24 homogeniser (MO BIO, USA). The tubes were centrifuged at 12000 x g for 15 min at 4°C. The upper aqueous phase was transferred into a fresh tube, 250 µl of isopropanol was added to the solution, and the sample was centrifuged at 12000 x g for 10 min at 4°C to precipitate RNA. The supernatant was removed, and the pellet was added with 75% v/v Ethanol and centrifuged at 12000 x g for 5 min at 4°C. The supernatant was removed, and the pellet was dried at room temperature for 15 min. The pellet was resuspended with 100 µl of nuclease-free water, and was stored at -80°C until needed.

2.17.1. DNase Treatment

To remove any contaminating DNA, the RNA samples were treated with DNase using Ambion TURBO DNA-free^{TM} kit (Invitrogen). The extracted RNA 20 µl (~ 1.5-2 µg) was mixed with 5 µl of 10X TURBO DNase Buffer, 2 µl of TURBO DNase (2 U/µl) and

23 µl of nuclease-free water. Then, the samples were incubated in a water bath at 37°C for 30 min. The reaction was inactivated by adding 5 µl of DNase Inactivation Reagent and incubated at room temperature for 5 min. The samples were then centrifuged at 10000 x g for 1.5 min, and the aqueous phase containing RNA was transferred to a fresh tube, and stored at -80°C until further use.

2.17.2. Complementary DNA (cDNA) synthesis

Synthesis of cDNA was performed according to the manufacturer's instructions using SuperScript III reverse transcriptase (Invitrogen). ~1 µg DNase-treated RNA was mixed with 1 µl of 300 ng random primers (Invitrogen) and 1 µl of 10 mM of dNTP (Promega, UK). The mixture was heated to 65°C for 5 min. Then 4 µl of 5X First-Strand buffer, 1 µl of 0.1 M dithiothreitol (DTT) and 1 µl (200 U/µl) of SuperScrip III reverse transcriptase were added to the sample, and the total volume was brought up to 20 µl with nuclease-free water. The reaction mixture was incubated at 25°C for 5 min, and then at 50°C for 45 min. The reaction was inactivated by heating to 70°C for 15 min.

2.17.3. Microarray analysis

Microarray analysis of pneumococcal samples were done in collaboration with Prof Oscar Kuipers, University of Groningen, the Netherlands. *Streptococcus pneumoniae* D39 and its isogenic mutant strain Δ*tprA* were grown microaerobically in CDM supplemented either with 55 mM galactose or mannose as the main carbon source. At mid exponential phase of growth culture, the pellet was harvested by centrifugation, and RNA was extracted as described above (Section 2.17). The experiments were repeated with four biological replicates. RNA samples were sent to the Netherlands for microarray analysis. The influence of *tprA* on the transcriptome of *S. pneumoniae* D39 was analysed and kindly provided by Prof Oscar Kuipers.

2.17.4. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR)

qRT-PCR was performed by using the SensiMix™ SYBR® Hi-ROX kit (Bioline, UK). The reaction tubes contained 2 µl of cDNA (~15 ng), 10 µl 2X SensiMix™ SYBR Hi-ROX, 2 µl of gene specific primers (Table 2.6) and 6 µl of nuclease-free water. The

thermo cycler was used to run 40 cycles of amplification using the following conditions: activation/initial denaturation: 95°C for 10 min, amplification (40 cycles): denaturation 95°C for 20 seconds, annealing 55°C for 30 seconds, and 72°C for 20 seconds extension, when the fluorescence signal was collected. The transcription of *gyrB* was used to normalise the transcription levels of the target genes. The RT-PCR data were analysed by the comparative threshold method ($2^{-\Delta\Delta CT}$). The differences over 1.5-fold were considered significant (Livak and Schmittgen, 2001).

Table 2.6. Gene specific primers used for real-time qRT-PCR.

Primer ID	**Sequence (5'- 3')**
gyrB-RT-F	GGCTGATCCACCAGCTGAGTC
gyrB-RT-R	TCGTGTGGCTGCCAAGCGTG
SPD0558-RT-F	GCTGCAGTTATTTTAGCAGCCTA
SPD0558-RT-R	TGTTTCTCTTTATTTTCTGTTTCCTT
SPD1167-RT-F	GGAAAGAGGGAAAGTTTTAGGG
SPD1167-RT-R	TCGGCTTATTATCTTCATCGAAA
SPD1495-RT-F	GCAATTCTGGCGGAAGTAAA
SPD1495-RT-R	TTCAAACGCTTCGATGATTG
SPD1944-RT-F	TTTAGGCGCTTCACCCTATG
SPD1944-RT-R	CAAGGCAGGAAGCCAAATAA
SPD1989-RT-F	CCCAGACAAAGATTCAGCTTC
SPD1989-RT-R	GCTGCACCTTCATTTCCTTC
SPD1994-RT-F	GTGGGAATCTCAGCGTTTTC
SPD1994-RT-R	CTAGATGGCAAGCGTTCTCC
SPD0447-RT-F	TGTTTTTCCTATCGGCAGTG
SPD0447-RT-R	TTCAAGCAGACGATCCATGT
SPD1745-RT-F	GACTCTCCCAACAAACTCTTGC
SPD1745-RT-R	TTGAAAAAGTAATCCAAAGGGACT
SPD1513-RT-F	CTTTCTTCCTTGGGGCAGTC
SPD1513-RT-R	CAGCAGATCAATCCAAAAACAA

SPD1517-RT-F	TGTTTTAATGATAAGTGCTGTTGGA
SPD1517-RT-R	TTCCGCACCTTCATTTTCTT
SPD1746-RT-F	GTTACATTTGCATTGCTAGGTGTT
SPD1746-RT-R	TTAATCCGCCTTCCCAACAT
SPD1504-RT-F	GTGAAAATGGGATGGTCCAC
SPD1504-RT-R	CACTTTCGCTTCGGTAGGAG
SPD1499-RT-F	TCCTATTTTTCAAGGAGGTTCA
SPD1499-RT-R	GGCCAAACAAAGCTTGAAGA
SPD0093-RT-F	AACAAGCCTGAAGAGCAAGC
SPD0093-RT-R	TCTTGCTGGCTTTCCAATTC
SPD0936-RT-F	TTTCCTAGCCATACAGAAATGG
SPD0936-RT-R	TCAGGAGAAAATGATTGGATGA

2.18. Construction of *lacZ* fusions

The plasmid pPP1 was used for transcriptional *lacZ*-fusions (Halfmann *et al.*, 2007). The putative promoter regions of selected genes were amplified using the primers modified to incorporate the *SphI* and *BamHI* restriction sites (indicated with Fusion-F/R in Table 2.7), and was ligated into similarly digested pPP1 as described in section 2.11. The ligation reaction was then transformed into *E. coli* One Shot® TOP10 chemically competent cells (Agilent Technologies) and the transformants were selected on LA plates supplemented with 100 µg/ml of ampicillin as described in section 2.13. Fusion-Seq-F and Fusion-Seq-R primers (Table 2.7) were used to confirm the successful cloning. These primers are designated to amplify the cloned insert. HotStarTaq *Plus* Master Mix was used in colony PCR as described in section 2.10. In addition, the recombinant pPP1 was extracted from transformants (Section 2.6), and was sent for DNA sequencing using the primers Fusion-Seq-F and Fusion-Seq-R.

Table 2.7. Primers used for transcriptional *lacZ*-fusion amplification. Bold typeface indicates incorporated restriction sites.

Primers	**Sequence (5'- 3')**
Fusion-Seq-F	CTACTTGGAGCCACTATCGA
Fusion-Seq-R	AGGCGATTAAGTTGGGTAAC
P*tprA*-Fusion-F	CG**GCATGC**CTTTCTTTTTCCTTGTTTTT
P*tprA*-Fusion-R	CG**GGATCC**ATCTGAATTTCTCTGCGAGT
P*phrA*-Fusion-F	CG**GCATGC**TAAAGATAATAAACCTTCCT
P*phrA*-Fusion-R	CG**GGATCC**ATTTTTTAATTCCACGTTTT
P*nanA*-Fusion-F	CG**GCATGC**GCAGGAAGTATGGTGTAAAT
P*nanA*-Fusion-R	CG**GGATCC**CGTTCCAAATACCACTGCTC

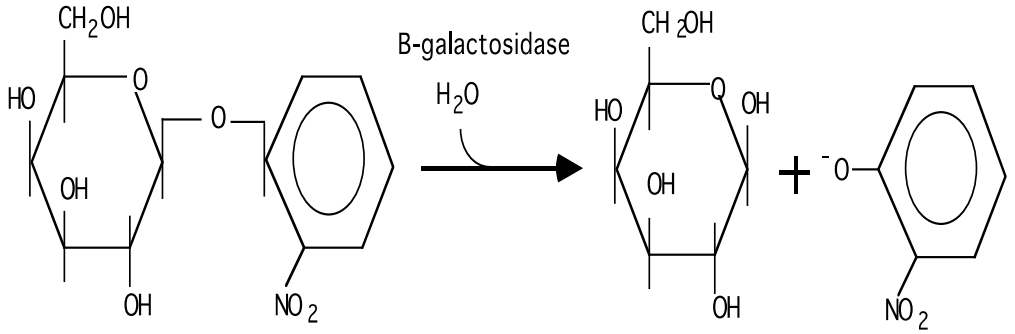
2.18.1. Transformation into *S. pneumoniae* and confirmation by PCR

The recombinant pPP1 constructs were transformed into *S. pneumoniae* (Section 2.14). The transformants were grown on BAB plates supplemented with 15 µg/ml tetracycline. PCR was used to confirm the integration of cloned plasmid into the genome of *S. pneumoniae* (the wild type D39 or mutant strains) using the primers Fusion-Seq-F and Fusion-Seq-R. HotStarTaq *Plus* Master Mix was used in PCR (Section 2.9).

2.18.2. Determination of β-galactosidase activity

β-galactosidase activity in reporter strains containing *lacZ* fusions was determined according to the protocol described by Miller (Miller, 1972; Zhang and Bremer, 1995). The level of β-galactosidase activity was determined in the cell extracts using chromogenic substrate O-Nitrophenyl β-D-galactopyranoside (ONPG). This method relies the cleavage of 2-Nitrophenyl from ONPG, which turns the colourless ONPG to

yellow colour. The amount of released 2-Nitrophenyl is proportional to the activity in the sample (Figure 2.1).



ONPG (Colourless) | Galactose (Colourless) | 2-Nitrophenyl (Yellow colour)

Figure 2.1. The mode of action for β-galactosidase in hydrolysing the chromogenic substrate 2-Nitrophenyl β-D-galactopyranoside (ONPG) to galactose and 2-Nitrophenyl.

Pneumococcal reporter strains were grown to mid-exponential phase in 10 ml CDM supplemented with 55 mM of the selected sugar microaerobically, or grown in CDM supplemented with 55 mM galactose and 100 nM LMIP with or without 10 nM synthetic peptide (PhrA10). Then the pellet was taken from 2 ml of the culture by centrifuging at 3500 rpm (Hettich MIKRO 22R, Germany) for 15 min. The supernatant was discarded, and the pellet was resuspended with 2 ml of chilled Z Buffer (0.80 g $Na_2HPO_47H_2O$, 0.28 g $NaH_2PO_4H_2O$, 0.5 ml 1 M KCl, 0.05 ml 1 M $MgSO_4$, 0.175 ml β-mercaptoethanol (BME), 40 ml dH_2O, pH 7.0). Subsequently, the absorbance of 1 ml resuspended cells was measured in OD600. Then 500 µl of the cells were diluted within 500 µl Z Buffer in a ratio of 1:1. In the next step, 100 µl chloroform and 50 µl 0.1% freshly prepared SDS were added to the samples and incubated in water bath set at 28°C for 5 min. After this, 200 µl of ONPG (4 mg/ml) solution was added to the samples and incubated at 30°C. When sufficient yellow colour had appeared, the reaction was stopped by adding 500 µl of 1 M Na_2CO_3 and the reaction time was recorded. Finally, 1.5 ml of the sample was transferred to an eppendorf tube and centrifuged for 5 min at 13000 rpm (Microfuge, Sigma), and the absorbance of the supernatant at 420 nm was measured. The β-galactosidase activity unit was determined as nanomoles of *p*-nitrophenol released per unit of time per unit of volume of cell suspensions by the equation:

$$\text{Miller Units} = 1000 \times (OD420)/(T \times V \times OD600)$$

Where: OD600 reflects cell density in the cell suspension, T= time of the reaction in minutes, V= volume of culture used in the assay in ml.

2.19. Over expression and purification of recombinant TprA

2.19.1. Transformation of recombinant plasmid into *E. coli* BL21 (DE3) pLysS and confirmation by PCR

The target gene that was cloned into pLEICS-01 plasmid was extracted from *E. coli* One Shot® TOP10 competent cells using the QIAprep spin Miniprep kit (section 2.6). Then the recombinant plasmid was transformed into *E. coli* BL21 (DE3) pLysS for protein expression (Section 2.13). PCR was performed to confirm the successful transformation using HotStarTaq *Plus* Master Mix (Section 2.9), using the recombinant plasmid as a template and the primers SPD1745-C-F/ SPD1745-C-R (Table 2.8) which incorporate 15 nucleotides homologous region to the cloning site in pLEICES-01.

Table 2.8. Primers used for protein expression. Bold typeface nucleotides are complementary to the cloning site in pLEICES-01.

Primers	**Sequence (5′–3′)**
SPD1745-C-F	**TACTTCCAATCCATG**AATACACTCGCAGAGAAATT
SPD1745-C-R	**TATCCACCTTTACTGTCA**AACAGTATCTAGTTCCTT

2.19.2. Small-scale protein expression

The *E. coli* BL21 (DE3) pLysS containing the recombinant plasmid was cultured in 10 ml LB supplemented with 100 µg/ml ampicillin overnight in a shaking incubator at 220 rpm at 37°C. Then, fresh LB supplemented with 100 µg/ml ampicillin was used to make dilution of the overnight culture (1 in 10) and the tube was incubated in a shaking incubator at 37°C with constant shaking at 220 rpm for approximately 1 h. When the OD600 reached 0.5-0.6, 1 ml culture was taken and centrifuged. The pellet was kept at -20°C and labelled as ‘before induction’. Different concentrations (0.1 to 1 M) of IPTG

(Isopropyl β-D-1-thiogalactopyranoside) was added for induction of expression and the cultures were incubated at different temperatures (18, 24, 30 and 37°C) until the OD600 reached 1.5-1.6. At this stage, 1 ml of culture was taken, and the pellet was obtained by centrifugation, which was designated as 'after induction'. A precooled AllegraTm X-22R centrifuge (Beckman Coulter, USA) was used to centrifuge the rest of the culture at 4000 x g for 15 min to take the pellet which was resuspended in 300 µl of PBS, pH 7.0, and the pellet was sonicated (Sanyo soniprep 150, Japan) at amplitude of 6 microns for 15 seconds on and 45 seconds off on ice to avoid protein denaturation This process was repeated 10 times. Then the cell lysate was centrifuged using a microfuge at maximum speed for 15 min at 4°C. The supernatant and the pellet were taken and labelled as 'crude extract' and 'after sonication', respectively. Both of these samples were stored at -20°C until further use.

2.19.3. Large-scale protein expression

The recombinant *E. coli* BL21 (DE3) pLysS was cultured in 10 ml of LB supplemented with 100 µg/ml ampicillin overnight in a shaking incubator at 220 rpm at 37°C, which was then used to inoculate 1000 ml LB supplemented with 100 µg/ml ampicillin. When the OD600 reached 0.5-0.6, 1 ml of culture was taken and centrifuged to take the pellet which was labelled as 'before induction', and stored at -20°C until use. The optimal IPTG concentration was used to induce the remaining culture, and the incubation was continued in a shaking incubator at 220 rpm, and at the optimal temperature which was determined in small-scale expression (Section 2.19.2). The culture was then grown until the OD600 of 1.5-1.6, and 1 ml of the culture was centrifuged, and the pellet was designated as 'after induction'. The pellet was then taken from the remaining culture by centrifuging at 10000 g for 30 min at 4°C, and was kept at -80°C for 24 hours. Subsequently, the pellet was resuspended in 20 ml binding buffer (20 mM Tris, 150 mM NaCl, dH_2O to a volume of 1000 ml, pH 7.45), 10 µl of 10X TURBO DNase Buffer, 2.5 µl of Ambion® TURBO™ DNase (Invitrogen, UK) and 5 µl of ProteoGuard™ EDTA-Free protease inhibitor cocktail (Takara Bio Europe, France). The mixture was sonicated at amplitude of 8 microns as described in section 2.17.1, and the lysate was centrifuged at 25000 x g for 30 min at 4°C. Then the supernatant was filtered through a 0.45 µm acrodisc filter (Fisher Scientific, UK) and labelled as 'crude extraction'. The pellet was also taken, and labelled as 'after sonication'.

2.19.4. Purification of TprA

His GraviTrap columns (GE Healthcare Life Sciences, Sweden) were used for protein purification. Firstly, the column was calibrated with 50 ml of the binding buffer. Then the crude extract was passed through the column, and 1 ml of flow through was taken and labelled as 'after column'. The column was washed with 100 ml of binding buffer, and 1 ml of flow through was taken and labelled as 'after column wash'. The protein sample was eluted in elution buffer (20 mM Tris, 150 mM NaCl, pH 7.45) containing different concentrations of imidazole (20, 40, 100, 200, 300, 500 mM). The eluted samples were collected from each concentration of imidazole and analysed as described in section 2.19.5.

2.19.5. Analysis of protein samples using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE)

SDS PAGE gels were prepared according to Sambrook *et al.* (1989). This gel was used for determination of protein expression. SDS PAGE is consisted of two gels: the resolving and stacking gel. The resolving gel contained 3.8 ml of 1.5 M Tris-HCl, pH 8.8, 150 µl of ammonium persulphate (APS) (Bio-Rad, UK), 150 µl of 10% w/v SDS, 6 µl of (0.775 g/ml) Tetramethylethylenediamine (TEMED), 7.5 ml of 30% v/v acrylamide bisacrylamide (National diagnostics, UK) and 3.4 ml dH_2O. The stacking gel was prepared with 630 µl of 1 M Tris-HCl, pH 8.8, 50 µl of APS, 50 µl of 10% w/v SDS, 5 µl of TEMED, 830 µl of 30% v/v acrylamide bisacrilamide, and 3.4 ml of dH_2O. The resolving gel was poured firstly between two glass plates and the stacking gel was poured later on top of glass plates when the resolving gel was polymerised. A comb was placed upon resolving gel to form the wells. The gel was then left to polymerise.

The pellet samples were loaded by mixing with 20 µl of PBS and 10 µl of 10X SDS protein loading buffer (6.25 ml 0.5 M Tris-HCl pH 6.8, 1 g SDS, 10 mM DTT, 50 mg bromophenol blue, 10 ml glycerol and 6.25 ml dH_2O). The protein solutions were loaded by taking 20 µl of the sample and mixed with 10 µl of 10X SDS loading buffer. Before loading, the samples were boiled for 1 min. The mini protein tetra system tank (Bio-Rad, UK) was used for separation of protein samples in the presence of 1X SDS running buffer (Tris base 15.1 g, glycine 94 g, 50 ml 10% w/v SDS and dH_2O up to 1000 ml). The comb

was removed, and the samples were loaded by placing 10 µl of each sample and protein marker Precision Plus (Bio-Rad, UK) into the wells. Electrophoresis (150 v) was performed to separate the protein samples using the negative charge of proteins towards the positive electrode across the gel. When the protein marker precision plus reached the bottom of the gel, the electrophoresis was stopped. Gels were stained using Coomassie Brilliant Blue staining solution, which was prepared by mixing: 0.4 g of 0.1% w/v coomassie brilliant blue, 40% v/v methanol, 10% v/v acetic acid, and volume was made upto 400 ml using dH_2O. The staining was done for 45 min with gentle shaking. After this, the staining solution was removed, and the gels were placed in the destaining solution (25% v/v isopropanol, 10% v/v acetic acid, and dH_2O up to 400 ml) for 1-2 hours with gentle shaking and then gels were washed with dH_2O and photographed.

2.19.6. Dialysis and quantification of TprA

Amicon Ultra-15 Centrifugal Filter Units (Millipore, UK) with a filter size of 10 kDa was used for dialysis of the fractions. The binding buffer (four column sizes) was added to the filter units and centrifuged at 4000 x g for 20 min at 4°C until only 1000 µl of protein remained. The dialyzed protein was divided into 50 µl aliquots in a PCR tubes. These tubes were exposed to liquid nitrogen for snap freezing, and then were stored at -80°C. The concentrations of the purified proteins were determined using the Bradford protein assay (Bradford, 1976) as described below in section 2.21.2.

2.19.7. MALDI-TOF analysis for protein identification

Matrix-assisted laser desorption ionization – time of flight (MALDI-TOF) mass spectrometry (PNACL, University of Leicester) was performed for analysing the purified protein to confirm its identity.

2.20. Electrophoretic mobility shifts assay (EMSA)

DNA-protein interaction was studied by EMSA. The regulatory elements in the putative promoter region of the target genes were determined using the bacterial promoter prediction tool (BPROM) (Solovyev and Salamov, 2011) and the Motif-based sequence analysis tools (MEME) (Bailey and Elkan, 1994). PCR was performed to amplify the

DNA fragments (100-195 bp), which contain the putative binding sites and the regulatory -35 and -10 elements in the upstream of each gene using the selected primers containing 5'- FAM fluorescent tags (Table 2.9) and PrimeSTAR HS premix (Section 2.9). DNA purification kit was used to purify the PCR products as described in section 2.8, and agarose gel electrophoresis was performed to visualise the amplified PCR products.

EMSAs were performed in 5X binding buffer (20 mM Tris-HCl pH 7.5, 30 mM KCl, 1 mM DTT, 1 mM EDTA pH 8.0, and 10% glycerol), which was used to incubate the protein with the promoter probes. The binding reactions in 20 µl total volume contained a constant amount of the promoter probes (~30 ng), and different concentration of purified protein (0.1-0.5 µM). The binding reaction was incubated at room temperature for 20 min.

A non-denaturing polyacrylamide gel (8%) without SDS was used to analyse the binding reaction which was prepared by mixing 4.1 ml dH_2O, 0.3 ml of 10X TB buffer, 1.6 ml acrylamide, 75 µl of 10% ammonium persulfate (APS), and 5 µl TEMED. Chilled 10X TB buffer (Tris base 89 mM + Boric acid 89 mM) was used for electrophoresis. The gel was run firstly for 20 min at 200 V without the samples to remove all traces of ammonium persulfate. After this, the samples were loaded and run for approximately 40 min at 200 V. Finally, the gel was removed from the mould and put it in the H_2O container and visualised using a Typhoon Trio$^+$ scanner (GE Healthcare Life Sciences, UK) at 488 nm blue laser.

Table 2.9. Primers used for amplifying the putative promoter regions of target genes for EMSA.

Primers	**Sequence**
gyrB-EMSA-F	ATGACAGAAGAAATCAAAAATCTGC
gyrB-EMSA-R	CCTGGACGCATACGAACAG
P*phrA* (SPD_1746)-EMSA-F	GGAATAAATAATTCTTTATGG
P*phrA* (SPD_1746)-EMSA-R	CCTTGTTTTTATTCTAACAG
P*nanA* (SPD_1504)-EMSA-F	GTATGGTGTAAATAGCATAAGC
P*nanA* (SPD_1504)-EMSA-R	TCTTTTACTACAGTTCATTTAAA
P*nanB* (SPD_1503)-EMSA-F	TATAAATTCAAGTCCCCAAAT
P*nanB* (SPD_1503)-EMSA-R	GCGACAAACAGAATAAATAAG
P(SPD_1495)-EMSA-F	GTACTTACTGACTTAATAAAA
P(SPD_1495)-EMSA-R	TTGTATTCTCCTATGTAATAA
P(SPD_1994)-EMSA-F	GATTGAGAGTTTGTAAATTTGC
P(SPD_1994)-EMSA-R	CCTTGTCCTCCTCATATCAAA
P(SPD_1517)-EMSA-F	GTAAAGGAGGAATTTGTATGA
P(SPD_1517)-EMSA-R	TTTTTCTGTTTCCTTTCATTT

2.21. Enzyme assays

2.21.1. Preparation of cell lysate

A crude extract of the bacterial cells was prepared as reported previously (Manco *et al.*, 2006; Terra *et al.*, 2010). The bacterial cultures were grown either in BHI, CDM supplemented with 55 mM of selected sugar with or without 100 nM LMIP, or in Sicard's defined medium supplemented with mucin. Then the bacterial pellet was obtained from these cultures by centrifugation, and was resuspended in 250 µl of 10 mM PBS, pH 7.0. After this, the bacterial suspension was sonicated by using a Sanyo Soniprep model 150 sonicator (7 amplitude microns) for 15 seconds followed by a 45 second off on ice. This process was repeated 7 times. Finally, the bacterial lysate was centrifuged at 13,000 rpm for 15 min at 4 ºC to take the clear lysate and was kept on -20℃ until needed.

2.21.2. Determination of protein concentration

The protein concentration in the cell extract was measured according to the method of Bradford (1976) using the Bio-Rad protein reagent (Bio-Rad Laboratories Inc., Hercules, CA) by measuring the absorbance at 595 nm using a microplate reader model infinite F50 (TECAN). Protein concentration in unknown samples was calculated from the standard curve prepared with the known concentration of bovine serum albumin (BSA).

2.21.3. Haemolytic assay

Haemolytic activity was assayed as previously described by Owen *et al.* (1994). Serial 2-fold dilutions of 50 µl of pneumococcal cell lysate were done using PBS, pH 7.0 in a round bottom microtitre plate. After this, 50 µl of 4% v/v defibrinated sheep blood (Oxoid) prepared in PBS was added to each well. The plate was incubated at 37℃ for 30 min. The haemolytic unit (HU) was expressed as the highest dilution of lysate inducing at least 50% lysis of a 4% v/v sheep blood suspension in 30 min at 37℃.

2.21.4. Neuraminidase assay

Neuraminidase activity was tested according to the Manco *et al.* (2006). 2-O-(p-nitrophenyl)-α-D-*N*-acetylneuraminic acid (pNP- NANA) (Sigma, United Kingdom) was used to assay the neuraminidase activity. Firstly, 25 µl of cell lysate (sample) was added in triplicate to each well in a flat bottom 96-well plate. After this, 25 µl of 0.3 mM pNP-NANA was added to the wells. Then the microplate was incubated at 37℃ for 2 hours. After this time, the reaction was stopped by adding 100 µl ice cold 0.5 M $NaCO_3$. Finally, the absorbance was measured at 405 nm by ELISA plate reader (Tecan Infinitive F50). The blank reaction contained 25 µl of PBS instead of the cell lysate of the sample. p-nitrophenyl (Sigma) was used to prepare a standard curve to determine the activity of neuraminidase in samples in unit that was expressed as nmol pNP released per minute per microgram of protein under standard assay conditions.

2.22. *In vivo* virulence studies

2.22.1. Preparation of standard inoculums

A sweep of colonies was inoculated in to 10 ml BHI broth for 16-18 hours at 37℃. After incubation, the cell pellet was collected by centrifugation at 3000 rpm for 15 min, and was re-suspended in 1 ml BHI serum broth (80% v/v BHI and 20% v/v filtered foetal calf serum). After this, 700 µl of the re-suspended pellet was added to 10 ml fresh pre-warmed BHI serum broth to bring the initial OD600 to approximately 0.70, and pneumococci were incubated at 37℃ for 3 to 5 hours until the OD600 was approximately 1.6. Then, the bacterial culture was divided into 1 ml aliquots in the sterile cryo tubes, and was kept at -80℃. After 24 hours, the viability of pneumococci was determined by using the method as described in section 2.3.

2.22.2. Virulence testing

Female, 10 weeks old CD-1 mice obtained from Charles River (Chesterford, UK) were used in the study. Intranasal infection was used to determine the virulence of pneumococcal strains. Ten mice were used for each strain. Mice were lightly anaesthetized with 2.5% v/v isoflurone mixed with 1.6-1.8 L O_2/minute in an anesthetic box. A 50 µl of the pneumococcal suspension in PBS containing approximately 1×10^6 CFU was placed drop wise in to the nostril of each mouse. After this, the mice were placed on their back until recovery. The actual viability of the dose was determined by plating its serial dilutions. Mice were monitored for 7 days, and the signs of disease (hunched, starry, and lethargic) were recorded. Mice that did not die for 7 days were considered as survivors and those that become lethargic were culled by dislocation of the neck. Time taken to reach lethargic state was considered as 'survival time'.

2.22.3. Bacterial count in the animal blood

Approximately 20-30 µl of blood samples were taken at predetermined time intervals post-infection from the tail vein. The blood samples were put in the tubes that had 2 µl of 2% w/v heparin to prevent clotting. Viable bacterial counts in the blood samples were determined as described previously in section 2.3.

2.22.4. Intravenous administration of *S. pneumoniae*

Mice were injected with approximately 1×10^6 CFU in 100 µl PBS through dorsal tail vein and the survival time was determined as described previously in section 2.22.2. At predetermined time points, an aliquot of blood was taken, and CFU/ml was determined as described in section 2.22.3.

2.22.5. Intraperitoneal administration of *S. pneumoniae*

Mice were injected with approximately 1×10^6 CFU/mouse intraperitoneally. Then the survival time and bacteremia were determined as previously described in sections 2.22.2 and 2.22.3.

2.22.6. Assessment of growth of pneumococcal strains in tissues

Growths of pneumococcal strains in the mice tissues were determined after intranasal infection as described previously (Terra *et al.*, 2010). For this, at 0, 3, 6, 24, 48 hours, set groups of mice were killed by cervical dislocation. Nasopharyngeal and lung tissues were transferred into 5 ml of sterile PBS. Then the samples were weighed and homogenised. Viable counts in homogenised tissues were determined by serial dilution using sterile PBS and plating onto the blood agar plates as described in section 2.3.

2.23. Statistical analysis

Graphpad Prism software (Graphpad, California, USA) was used for statistical analysis**.** When appropriate one-way or two-way analysis of variance (ANOVA) was used to compare the groups for studies, and post-test was employed to determine statistical significance. In addition, Mann-Whitney test was used to analyze the survival time. The results were expressed as means ± standard error of the mean (SEM), significance was defined as (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$

Chapter 3. Results

Section A: Construction of isogenic mutants and genetically complemented strains

To study the role of TprA/PhrA QS system in pneumococcal physiology and virulence, the allelic replacement mutagenesis was used for inactivation of the *phrA* (SPD_1746) which code for putative PhrA peptide in *S. pneumoniae* serotype 2 strain D39 and designated as Δ*phrA*. The mutant of *tprA* (SPD_1745) which code for transcriptional regulator putative TprA regulatory protein had been constructed previously by Dr Hasan Yesilkaya's group and had been designated as Δ*tprA*. Δ*tprA* was constructed using gene splicing by overlap extension PCR as described in section 3.2. Figure 3.1 shows genomic organisation of SPD_1745 and SPD_1746 in *S. pneumoniae* D39 strain. In addition, the destribution of SPD_1745 (*tprA*) and SPD_1746 (*phrA*) amongst *S. pneumoniae* and their identity with *tprA* and *phrA* in *S. pneumoniae* D39 strain were demonstrated in Table 3.1. Genetic complementations of the mutant strains were done to eliminate the possibility of polar effect due to mutation, and then the mutant strains and their genetically complemented versions were used to identify the role of these genes by growth studies and enzymatic assays. In addition, the impact of Δ*tprA* on the transcriptome of *S. pneumoniae* strain D39 was analysed by using DNA microarrays. Transcriptional reporter strains were constructed in the *S. pneumoniae* wild type D39 and the mutant strains to study the regulatory role of TprA and to determine the inducibility of TprA/PhrA in the presence of different sugars. The transcriptional reporter strains carrying putative promoter region of *nanA* linked to promoterless *lacZ* was also constructed to determine the role of TprA to regulate *nanA* gene expression. Electro Mobility Shift Assay (EMSA) was performed to detect the regulatory role of TprA for *phrA*, *nanA* and *nanB* (codes for neuraminidase B), and for three genes, SPD_1495, SPD_1517, and SPD_1994, identified to be in TprA regulon based on microarray analysis. Furthermore, *in vivo* experiments were done to determine the role of TprA/PhrA system in pneumococcal colonisation and virulence. Finally, I used linear molecularly imprinted polymers (LMIP) to interfere with TprA/PhrA quorum sensing signals selectively and modulate the phenotypic manifestation of this system in *in vitro* and *in vivo* assays in collaboration with Prof Sergey Piletsky's research group, Chemistry Department, University of Leicester.

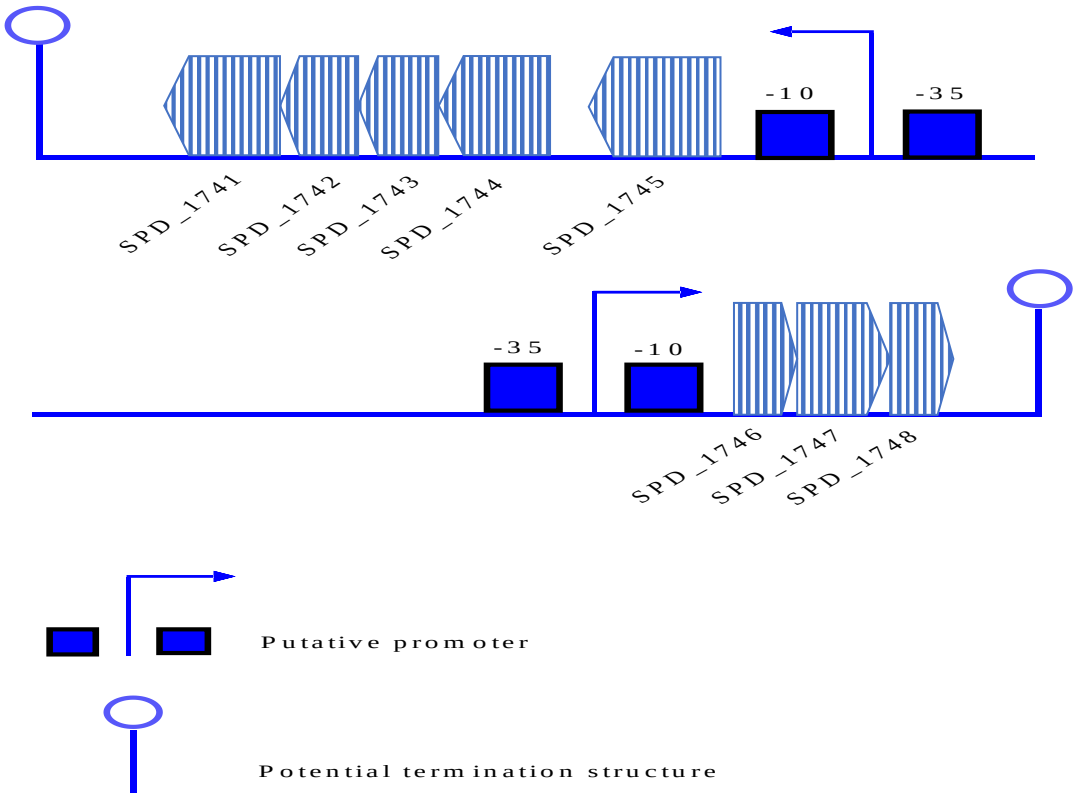


Figure 3.1. Genomic organisation of SPD_1745 (*tprA*) and SPD_1746 (*phrA*) in *S. pneumoniae* D39 strain showing the putative promoter regions and potential termination structure. The core promoter elements -10 and -35 are indicated.

Table 3.1. Destribution of SPD_1745 (*tprA*) and SPD_1746 (*phrA*) quorum sensing system in *S. pneumoniae* strains and their identity with *tprA* and *phrA* in *S. pneumoniae* D39 strain.

Strains	Identity with *tprA*	Identity with *phrA*
Streptococcus pneumoniae R6, complete genome	100%	100%
Streptococcus pneumoniae ATCC 700669 complete genome	100%	100%
Streptococcus pneumoniae Xen35, complete genome	99%	100%
Streptococcus pneumoniae TIGR4, complete genome	99%	100%
Streptococcus pneumoniae JJA, complete genome	99%	100%
Streptococcus pneumoniae strain D141 genome	99%	100%
Streptococcus pneumoniae strain D122 genome	99%	100%
Streptococcus pneumoniae strain D219 genome	99%	95%
Streptococcus pneumoniae strain 19F chromosome, complete genome	98%	100%
Streptococcus pneumoniae DNA, nearly complete genome, strain: KK1157	96%	93%
Streptococcus pneumoniae DNA, nearly complete genome, strain: KK0381	96%	92%
Streptococcus pneumoniae DNA, complete genome, strain: KK0981	96%	93%
Streptococcus pneumoniae genome assembly S_pneumo_A66_v1, chromosome: I	96%	93%
Streptococcus pneumoniae genome assembly NCTC7465, chromosome :1	96%	93%
Streptococcus pneumoniae SPN994038 draft genome	96%	92%
Streptococcus pneumoniae SPN994039 draft genome	96%	92%
Streptococcus pneumoniae gamPNI0373, complete genome	96%	100%
Streptococcus pneumoniae OXC141 complete genome	96%	92%
Streptococcus pneumoniae SPN034156 draft genome	96%	92%
Streptococcus pneumoniae SPN034183 draft genome	96%	92%
Streptococcus pneumoniae P1031, complete genome	96%	91%
Streptococcus pneumoniae SPNA45, complete genome	96%	93%
Streptococcus pneumoniae 670-6B, complete genome	96%	92%
Streptococcus pneumoniae INV200genome	96%	92%
Streptococcus pneumoniae CGSP14, complete genome	96%	92%
Streptococcus pneumoniae strain Hu15genome	96%	92%
Streptococcus pneumoniae strain Hu17genome	96%	92%
Streptococcus pneumoniae strain SP49, complete genome	96%	92%
Streptococcus pneumoniae 70585, complete genome	96%	93%
Streptococcus pneumoniae Hungary19A-6, complete genome	96%	92%
Streptococcus pneumoniae AP200, complete genome	95%	100%
Streptococcus pneumoniae MDRSPN001 DNA, complete genome	94%	96%
Streptococcus pneumoniae G54, complete genome	94%	97%

Streptococcus pneumoniae strain SWU02, complete genome	94%	96%
Streptococcus pneumoniae strain SP64, complete genome	94%	96%
Streptococcus pneumoniae strain SP61, complete genome	94%	96%
Streptococcus pneumoniae ST556, complete genome	94%	96%
Streptococcus pneumoniae A026 genome	94%	96%
Streptococcus pneumoniae TCH8431/19A, complete genome	94%	96%
Streptococcus pneumoniae Taiwan19F-14, complete genome	94%	96%
Streptococcus pneumoniae partial integrative and conjugative element ICE6094, strain Pn19	94%	98%
Streptococcus pneumoniae SPN033038 draft genome	94%	92%
Streptococcus pneumoniae SPN032672 draft genome	94%	93%
Streptococcus pneumoniae INV104 genome	94%	92%

3.1. Gene splicing by overlap extension PCR (SOEing PCR)

The allelic replacement mutagenesis was done using gene splicing by overlap extension PCR (SOEing) (Song *et al.*, 2005; Horton, 1995). Figure 3.2 shows the overall process for this method. Gene splicing by overlap extension PCR (SOEing) has several advantages. Unlike plasmid based mutagenesis systems, this method does not rely on cloning, and is a simple and quick strategy to generate site-directed mutagenesis *in vitro* using PCR without any requirement for restriction sites or ligation (Horton, 1995). This method was used to delete the genes of interest and replace them with *aadA* antibiotic casette, which provides resistance to spectinomycin. As shown in Figure 3.1, the process for allelic replacement mutagenesis and gene knockout depends on two steps of PCR. Firstly, the upstream and downstream flanking regions of target genes, and the *aadA* gene are amplified. Secondly, the UF (upstream-Forward) and DR (downstream-Reverse) primers are used to fuse the upstream and downstream flanks to the pre-amplified *aadA* gene. Then, the SOEing products are transformed into *S. pneumoniae* to replace the target gene with antibiotic casette *aadA* by homologous recombination.

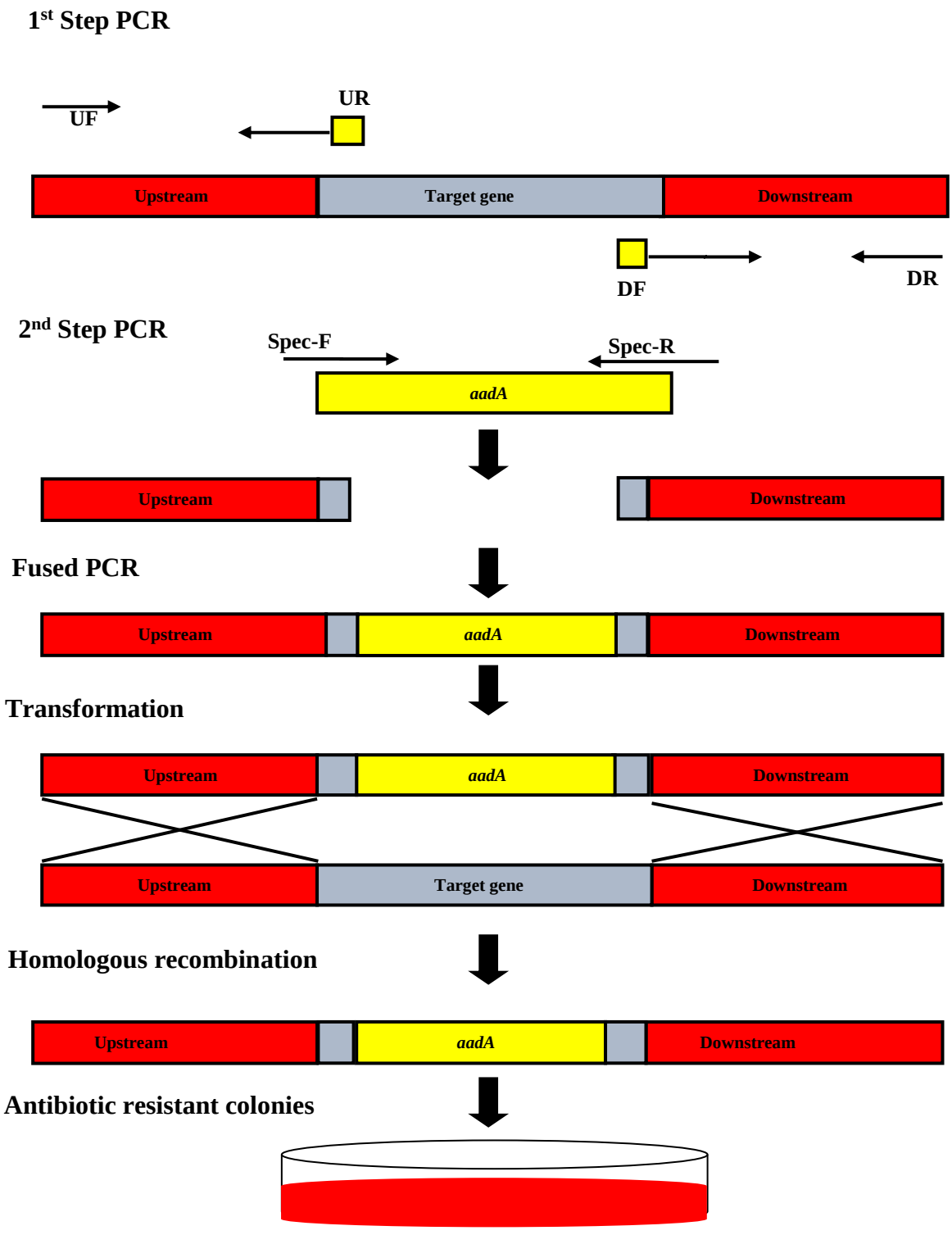


Figure 3.2. The process for allelic replacement mutagenesis and gene knockout by homologous recombination. The gene coding for *phrA* was deleted and replaced by *aadA* antibiotic resistance cassette. Primer designations: UF: forward primer of upstream flank; UR: reverse primer of upstream flank; DF: forward primer of downstream flank; DR: reverse primer of downstream flank; Spec/F and Spec/R: forward and reverse primers of spectinomycin resistance cassette. Figure constructed based on (Song *et al.*, 2005; Horton, 1995).

3.1.1. Construction of Δ*phrA*: Amplification of SOEing fragment

The upstream (UF) and downstream (DF) flanking regions (600 bp each) of target gene as well as the *aadA* (1158 bp) gene were amplified as described previously in section 2.15 and the amplified products were analysed using agarose gel electrophoresis. Figure 3.3 shows the amplification of flanking regions of target genes and the antibiotic resistance gene. The lanes 1 and 2 contain approximately 600 bp amplification products for the upstream and downstream flanking regions of *phrA* whereas the Lane 3 show the amplification product for *aadA* (1158 bp) from pDL278 (Yesilkaya, 1999). The upstream and downstream flanking regions in lanes 1 and 2 contain compatible ends with spectinomycin cassette *aadA* to generate the mutant *phrA*.

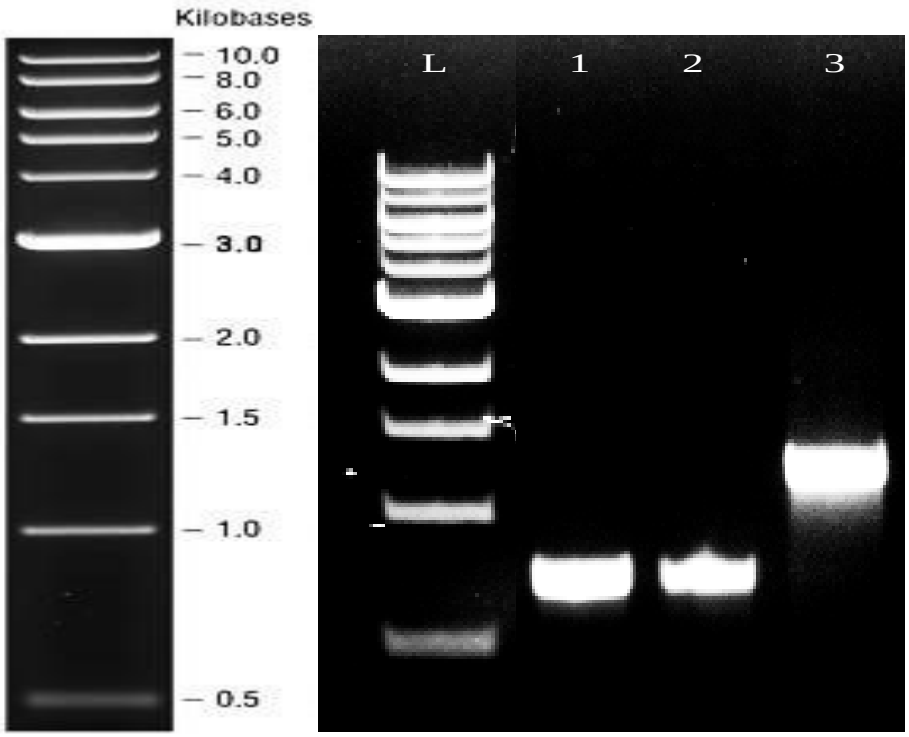


Figure 3.3. Agarose gel electrophoresis showing the flanking regions of target gene and the amplified antibiotic cassette. L- 500 ng of 1 kb DNA ladder (NEB); Lane 1 shows amplified upstream flanking region (600 bp), and lane 2 shows amplified downstream flanking region (600 bp) of *phrA*, which contain compatible terminal ends to the *aadA* spectinomycin resistance gene; Lane 3 shows amplified spectinomycin cassette *aadA* (1158 bp) from the plasmid pDL278.

3.1.2. Fusion of SOEing fragment and transformation

The upstream (UF) and downstream (DF) flanking regions of target gene were fused to the amplified antibiotic resistance gene *aadA* using the primers UF-SOE1746-F and DF-SOE1746-R (Section 2.15) (Table 2.4). The amplified PCR products were gel purified using DNA purification kit (Section 2.8), the purified products then visualised on agarose gel after electrophoresis as described in section 2.7 (Figure 3.4). As can be seen in Figure 3.4, the size of amplified PCR products in lane 1, approximately 2358 bp, were equal to the total size of upstream (600 bp) and downstream (600 bp) flanks plus the spectinomycin resistance gene *aadA* (1158 bp). The SOEing products were transformed into *S. pneumoniae* D39 (Section 2.14) and the transformants were selected on BAB plates supplemented with spectinomycin to produce mutation in *phrA* by homologous recombination.

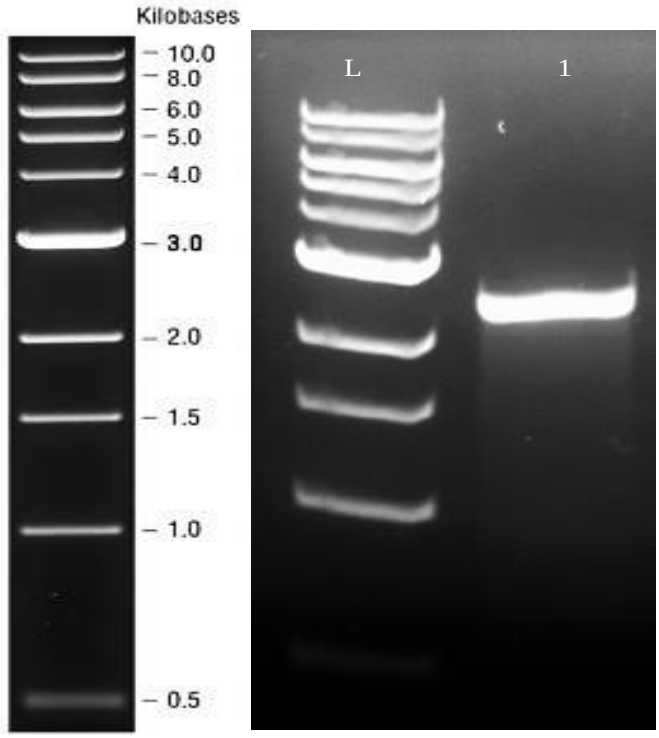


Figure 3.4. Agarose gel electrophoresis analysis showing the fused SOEing fragments. L- 500 ng of 1 kb DNA ladder (NEB); Lane 1 shows fused SOEing product approximately 2358 bp in size representing the up and downstream flanking regions of the gene *phrA* with the *aadA*, which confers resistance to spectinomycin.

3.1.3. Confirmation of mutation by PCR

Genomic DNA was extracted from the overnight culture of the putative mutant and the wild type D39 as a control (Section 2.5). PCR was performed to confirm the successful construction of mutant strain as described previously in section 2.15.3. Figure 3.5 shows the PCR strategy used to confirm the mutation. Firstly, the primers UF-SOE1746-F and DF-SOE1746-R were used to amplify the PCR product from the wild type D39 and the putative mutant. These primers designed to amplify all the SOEing fragment from the putative mutant. The amplified mutated region is expected to be larger than from the wild type since the size of spectinomycin cassette (1158 bp) is larger than the size of deleted SPD_1746 (*phrA*) gene. Secondly, the primers UF-SOE1746-F and Spec-R were used to amplify the upstream flank of target gene with the spectinomycin cassette. Thirdly, the Spec-F and DF-SOE1746-R primers were used to amplify the *aadA* cassette and the downstream flanking region of target gene. The second and third primer sets generate a product approximately 1758 bp in size from the mutant and no amplification is expected from the wild type due to absence of antibiotic resistance cassette. In addition, Spec-F and Spec-R primers were used to amplify *aadA* gene (1158 bp) from the DNA of putative mutant.

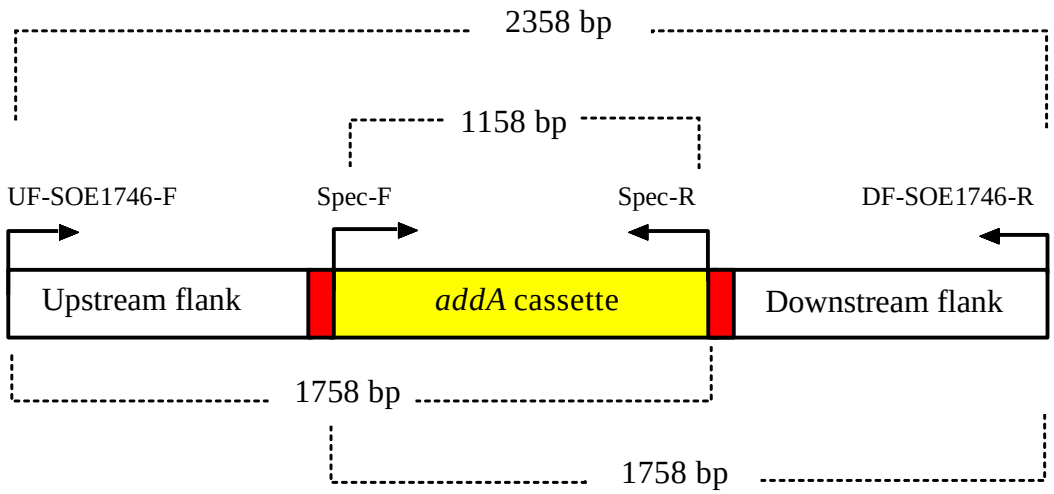


Figure 3.5. The PCR strategy was used for confirmation of gene replacement in mutant strain. Confirmation of mutation for strain is containing spectinomycin resistance, *aadA*. UF refers to upstream flank, DF indicates downstream flank. Arrows indicate the direction of primers. The expected product sizes for different primer sets have been shown.

The genomic DNA from the putative mutant strain was used as a template in the PCR to confirm the successful insertion-deletion mutation. The genomic DNA of the wild type D39 was used in the PCR as a control. Agarose gel electrophoresis was used to analyse the amplified PCR products (Section 2.7). Figure 3.6 shows successful replacement of *phrA* with antibiotic resistance cassette. Lanes 1, and 2 show PCR products amplified by the UF-SOE1746-F and Spec-R and Spec-F and DF-SOE1746-R primers, respectively. Both of the fragments had the size of 1758 bp, which is equal to the size of upstream or downstream flanking region, which is approximately (600 bp) with spectiomycin *aadA* cassette (1158 bp). Lane 3 illustrates the fragment amplified using the Spec-F and Spec-R primers from the DNA of putative mutant, while Lane 4 shows the product amplified using the primers UF-SOE1746-F and DF-SOE1746-R from the DNA of each putative mutant, approximately 2358 bp which is equal to the size of upstream and downstream flanking regions (1200 bp) with spectinomycin cassette (1158 bp). In addition, lane 5 contains the product representing the target region using the UF-SOE1746-F and DF-SOE1746-R primers from the genomic DNA of the wild type D39 as a control. The results show successful construction of Δ*phrA*.

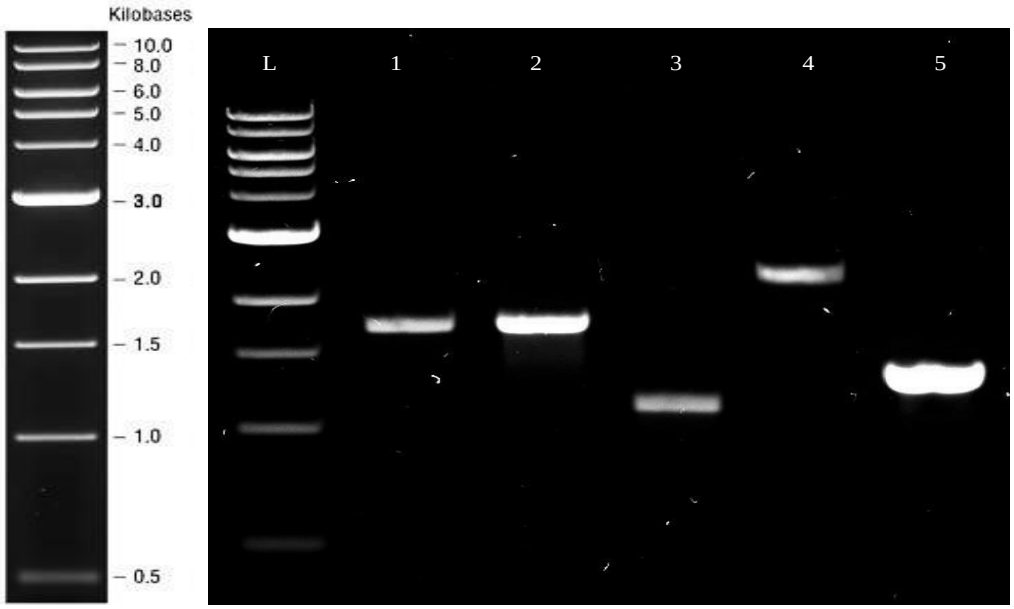


Figure 3.6. Confirmation of gene replacement. L- 500 ng of 1 kb DNA ladder (NEB). The lane 1-4 shows the amplicons obtained using the DNA of putative mutant, while the lane 5 is for products amplified using wild type DNA as a template in the PCR. Lane 1: amplified UF+ *aadA*; Lane 2: amplified *aadA* + DF; Lane 3: amplified *aadA* cassette; Lane 4: amplified UF + *aadA* + DF; Lane 5: amplified UF+ wild type gene + DF. 'UF' indicates upstream flank while 'DF' is for downstream flank.

3.1.4. Confirmation of mutation by DNA sequencing

DNA sequencing was used for further confirmation of gene replacement to ensure no unwanted mutations had occurred during homologous recombination. The primers 1746-SeqM-F1 and 1746-SeqM-R1 were used to amplify the mutated regions plus 200 bp upstream and downstream of the target region (Figure 3.7). As can be seen from this Figure, the 1746-SeqM-F1 and 1746-SeqM-R1 primers amplified the target region from the genomic DNA of mutant strain. Agarose gel electrophoresis was used to analyse the amplified fragment (Figure 3.8), which illustrates the successful amplification of the mutated region in Δ*phrA*. Lane 1 shows the entire mutated region with a size of 2758 bp, which is equal to the total size of spectinomycin cassette (1158 bp) plus 1600 bp upstream and downstream flanks of mutated gene. After amplification, the PCR product was purified using the DNA purification kit (Section 2.8) and sequenced using the 1746-SeqM-F1, 1746-SeqM-F2, 1746-SeqM-R1 and 1746-SeqM-R2 primers (Table 2.4). The 1746-SeqM-F1 and 1746-SeqM-R1 were performed to sequence the left and right flanking regions of mutated gene, whereas the primers 1746-SeqM-F2 and 1746-SeqM-R2 were used for sequencing approximately 150 bp upstream and downstream of the antibiotic resistance *aadA* gene. The results obtained from sequencing confirmed that the successful gene replacement with antibiotic resistance *aadA* gene had taken place (Appendix 1).

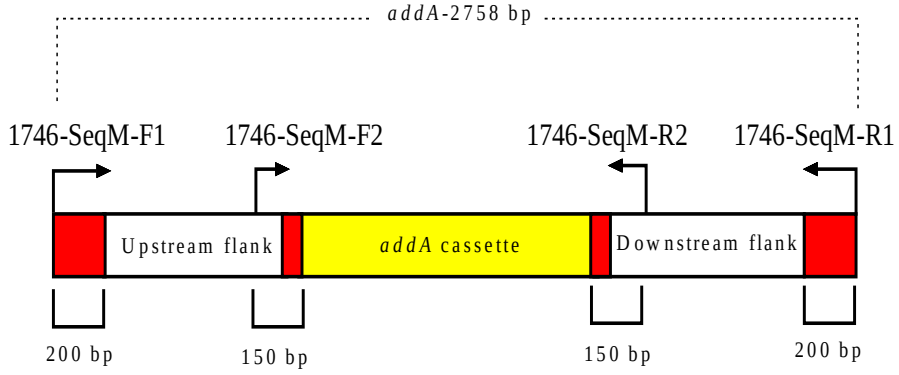


Figure 3.7. The sequencing strategy for mutation confirmation. The sequence of the primers is found in Table 2.4.

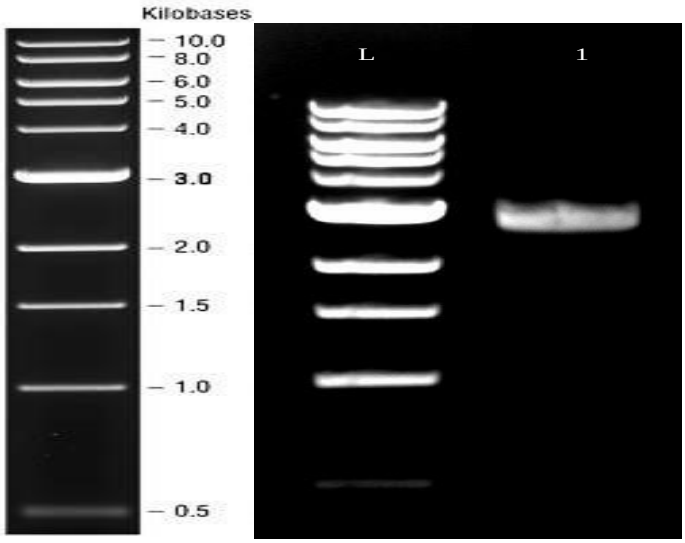


Figure 3.8. Agarose gel electrophoresis showing the amplified mutated region from the mutant strain for DNA sequencing using 1746-SeqM-F1 and 1746-SeqM-R1 primers. L- 500 ng of 1 kb DNA ladder (NEB); Lane 1 shows the amplified mutated region (2758 bp) from the genomic DNA of spectinomycin resistant mutant strain Δ*phrA*.

3.2. Genetic complementation of mutants

Genetic complementation to the mutant strain was done to rule out the possibility of polar effect of the mutation on downstream genes (Zipser, 1969). In this study, the plasmid pCEP (9540 bp) was used to complement the mutant strains Δ*tprA* and Δ*phrA* (Figure 3.9) (Guiral *et al.*, 2006). This plasmid is non- replicative in *S. pneumoniae* and replicative in *E. coli*. In addition, pCEP has 2 kb homologous sequence to *S. pneumoniae* D39 genome, and these are located on a silent transcriptional site. Therefore, reintroduction of the intact copy of gene will have no influence on the transcription of other genes.

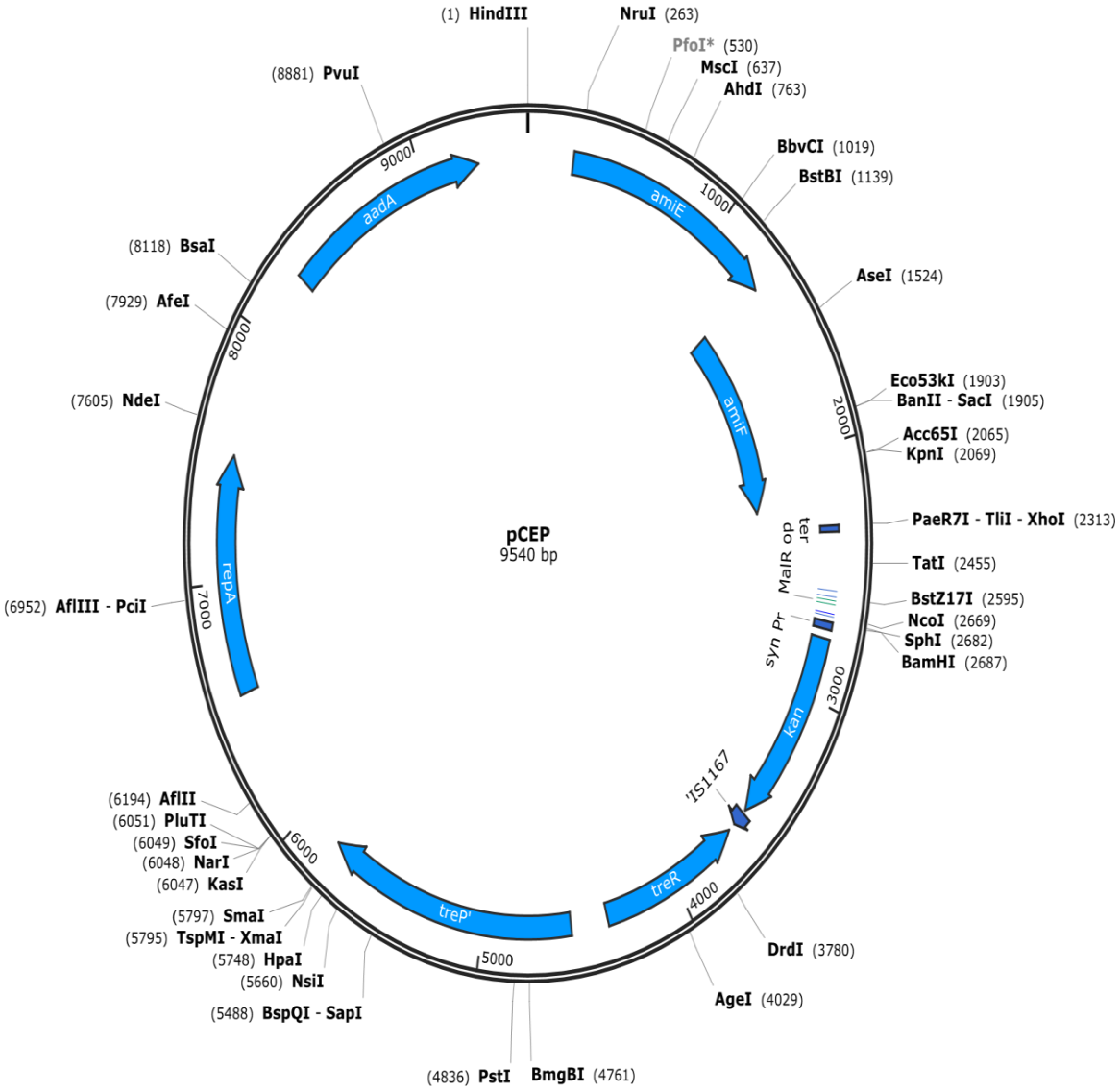


Figure 3.9. Genetic map of pCEP (Guiral *et al.*, 2006). *malR*: maltosaccharide-inducible promoter. The multiple cloning site (MCS) consists of *BstZ17I*, *NcoI*, *SphI* and *BamHI* restriction sites. *amiE*: oligopeptide ABC transporter, ATP-binding protein AmiE, *treP*: PTS system, trehalosespecific IIABC components, *kan*: kanamycin resistance cassette, *treR*: Trehalose operon repressor, *amiF*: oligopeptide ABC transporter, ATP-binding protein AmiF.

3.2.1. Construction of recombinant pCEP for complementation

The QIAprep spin Miniprep kit was used to extract pCEP and then digested using both *NcoI* and *BamHI* since these sites are not present in the cloned genes (Section 2.11). DNA purification kit was used to purify the digested pCEP to terminate the digestion process. Agarose gel electrophoresis was used to visualise the successful digestion of pCEP

(Figure 3.10). In the figure Lane 1 shows pCEP, approximately 9.5 kb, digested using *NcoI* and *BamHI*; Lane 2 shows undigested pCEP.

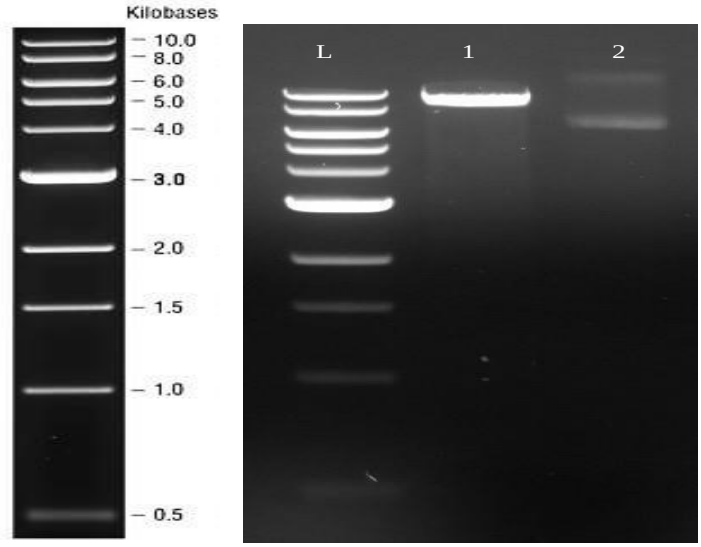


Figure 3.10. Agarose gel electrophoresis demonstrating the digested and undigested pCEP. L: 500 ng of 1 kb DNA ladder (NEB). Lane 1 shows digested pCEP using *NcoI* and *BamHI*; Lane 2 shows undigested pCEP.

The putative promoter regions of *tprA* and *phrA* along with their coding regions were amplified using XComp-F and XComp-R primers (where X indicates gene code) (Table 2.5), which are modified to include *NcoI-BamHI* restriction sites as previously described in section 2.11. The amplified PCR fragments were purified using DNA purification kit, and digested using *NcoI-BamHI*. Then, these fragments were ligated in to the same sites of pCEP (Section 2.12). An aliquot of ligation reaction was transformed in to *E. coli* TOP10 chemically competent cells for propagation. As a result, several kanamycin resistant colonies were appeared on the LB agar medium. Colony PCR was used to identify a successful transformation using MalF and PCEPR primers, which have annealing sites on pCEP and in the absence of insert, these primers amplify approximately 263 bp product from empty pCEP. Agarose gel electrophoresis was used to analyse the amplified PCR fragments (Figure 3.11). This figure shows successful cloning of the *tprA* and *phrA* inserts into pCEP since the size of each fragment amplified using MalF and PCEPR primers were equal to the size of insert plus upstream and downstream of multiple cloning site of pCEP. These recombinant plasmids were designated as pCEP*tprA* and pCEP*phrA*.

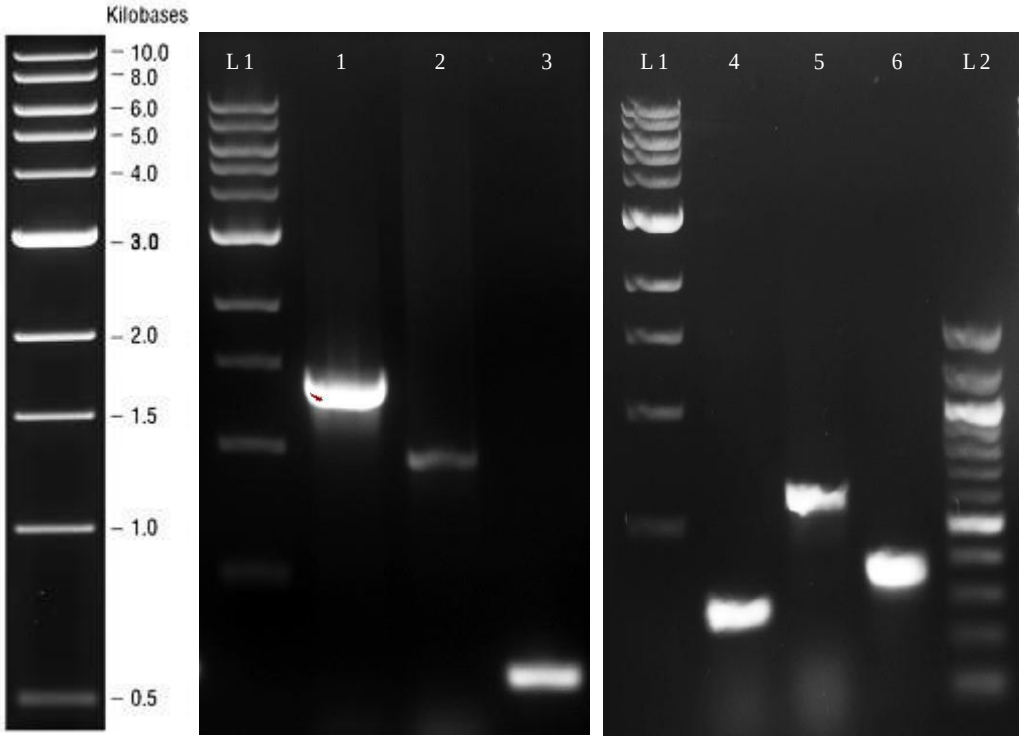


Figure 3.11. Agarose gel electrophoresis analysis showing the successful integration of target genes with their putative promoter regions into pCEP. L1: 500 ng of 1 kb DNA ladder (NEB); L2: 500 ng of 100 bp DNA ladder (NEB); Lanes 2 and 6 show amplified target genes and their putative promoter regions for *tprA* (984 bp) and *phrA* (362 bp), respectively; Lanes 1 and 5 show the amplification of *tprA* (1247 bp) and *phrA* (625 bp), respectively with their putative promoter regions plus upstream and downstream of cloning site; Lanes 3 and 4 show the amplified products for the empty plasmid, 263 bp.

3.2.2. Transformation of cloned pCEP constructs into the mutant strains, and confirmation by PCR and sequencing

Mini Prep kit was used to extract the recombinant plasmid from the *E. coli*, then the recombinant pCEP was transformed into the Δ*tprA* and Δ*phrA* strains as described in section 2.14. The transformation resulted in several kanamycin and spectinomycin resistant colonies. Colony PCR was used to confirm the successful complementation genetically using MalF/ PCEPR primers, which amplify the up and downstream of cloning site. Agarose gel electrophoresis was used to analyse the amplified PCR fragments (Figure 3.12). As can be seen from this figure, amplification of complemented genes with their putative promoter regions appear in lanes 1 and 2 by using XComp-F and XComp-R (X represents gene name) primers, which have the expected sizes of 984 bp and 362 bp for *tprA* and *phrA*, respectively, indicating the successful

complementation. In addition, DNA sequencing was done, and this also confirmed successful complementation of the mutants (Appendix 2). Subsequently, the complemented strains were designated as Δ*tprA*Comp and Δ*phrA*Comp.

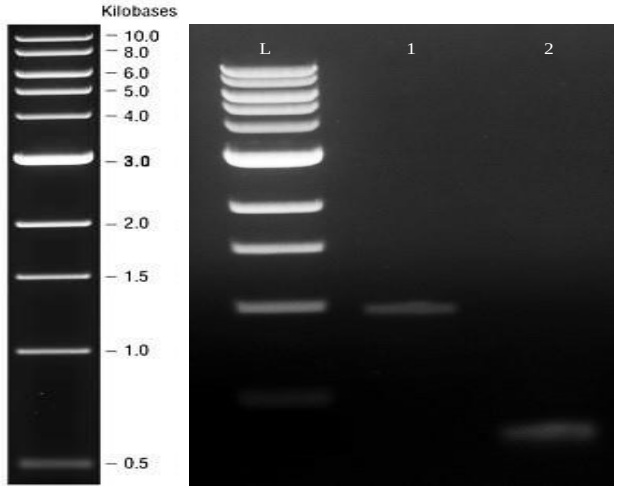


Figure 3.12. Agarose gel electrophoresis analysis to confirm the successful integration of target genes with their putative promoter regions into the chromosome of the mutant strains. L: 500 ng of 1 kb DNA ladder (NEB); Lanes 1 and 2 show the amplified target genes and their putative promoter regions for *tprA* (984 bp) and *phrA* (362 bp) from Δ*tprA* and Δ*phrA*, respectively.

Section B: Phenotypic characterisation of the mutant strains

Streptococcus pneumoniae causes several invasive infectious diseases in humans. In the first stage of infection the bacterium colonises the nasopharynx and then spread to the lower respiratory tract to cause pneumonia. It can also cause bacteraemia, otitis media, and meningitis. The human respiratory mucosa is covered with mucus, which contains mucin, a heavily glycosylated glycoprotein (King, 2010). Glycosylation plays an important biological role in the binding and interaction between the cells, stability and protection for the tissue from infection (Varki, 1993). Therefore, the bacterial modification of glycosylation and glycan structures could lead the cells to loss their function and increase the bacterial ability to colonise in the host tissue in various ways. For example, this modification can increase the bacterial adherence to the receptors, modify the host immune molecules, and finally it allows bacteria to deglycosylate host glycoproteins, leading to the release of galactose, mannose, sialic acid and *N*-acetylglucosamine for bacterial growth (King *et al.*, 2006; King, 2010; Varki, 1993). *S. pneumoniae* can utilise the mucin as a carbon source and this utilisation is linked to the expression of one of the most important virulence gene, *nanA* (codes neuraminidase A) (Yesilkaya *et al.*, 2007). Thus, to study the role of TprA genes in sugar metabolism and virulence, the TprA genes were tested for growth in the presence of galactose, mannose, *N*-acetylglucosamine, and mucin.

To explore any possible differences in the growth profiles of pneumococcal strains, they were grown in BHI and CDM supplemented with 55 mM, galactose, mannose, or *N*-acetyl glucosamine, and glucose. Moreover, Sicard's defined medium supplemented with mucin as the sole carbon source was also used to grow the pneumococcal strains at 37°C as described in section 2.4 to determine the role of *tprA* and *phrA* in sugar metabolism. In addition, the role of mutations on the production of virulence factors was also determined by measuring some important virulence factors in *S. pneumoniae*, including neuraminidase and pneumolysin. Finally, the expression of neuraminidase gene, *nanA*, was determined in the mutant *tprA* and wild type to determine the regulatory impact of TprA on *nanA*.

3.3. Growth of pneumococcal strains in BHI

The pneumococcal strains grew well in BHI micro aerobically at 37°C (Figure 3.13). The growth rate and yield were calculated as described in section 2.4 (Table 3.2). The result showed that there was no significant difference in the growth profile among the strains ($p > 0.05$).

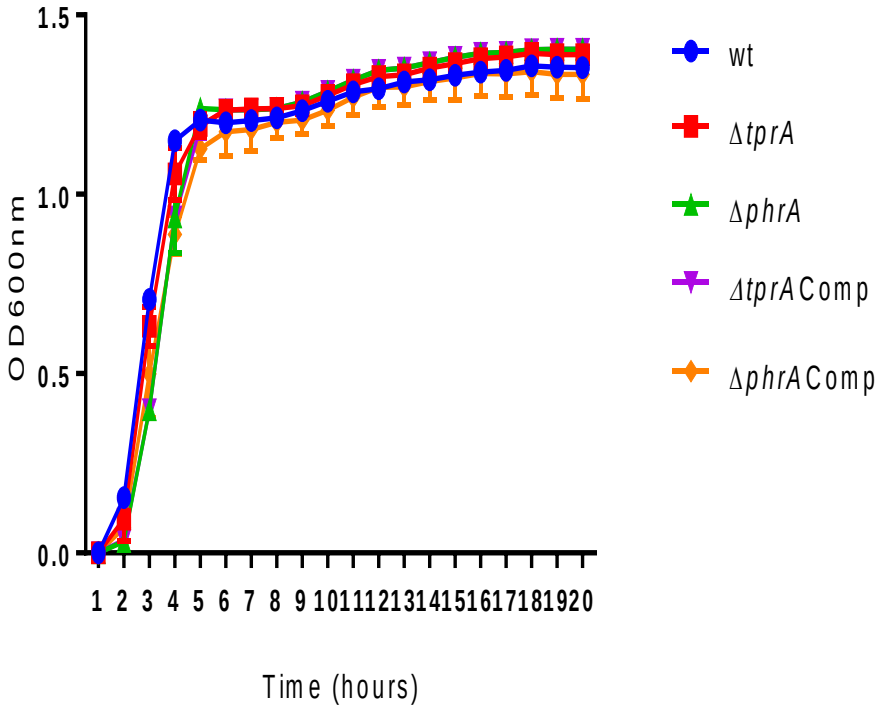


Figure 3.13. The growth of TprA, PhrA mutants, complemented strains, and wild type D39 in BHI under micro aerobic condition at 37ºC. There was no significant difference between the strains in the growth rate using one-way ANOVA and Dunnett's multiple comparisons test ($p > 0.05$). Experiment was repeated using 3 replicates of 6 independent biological samples, vertical bars indicates SEM.

Table 3.2. Growth rate (μ) and yield (maximal OD600) of pneumococcal strains grown microaerobically in BHI. Experiment was repeated using 3 replicates of 6 independent biological samples, '±' indicates standard error of means (SEM).

Strains	Growth rate (μ) h^{-1}	Growth yield (MaxOD600)
Streptococcus pneumoniae D39	0.35 ± 0.02	1.36 ± 0.022
Δ*tprA*	0.35 ± 0.015	1.38 ± 0.014
Δ*phrA*	0.31 ± 0.01	1.4 ± 0.002
Δ*tprA*Comp	0.34 ± 0.005	1.4 ± 0.002
Δ*phrA*Comp	0.30 ± 0.03	1.34 ± 0.066

3.4. Growth of pneumococcal strains in CDM supplemented with 55 mM glucose, galactose, mannose, or *N*-acetyl glucosamine

To determine the functional importance of TprA/PhrA system, the pneumococcal strains were grown in CDM supplemented either with glucose, galactose, mannose or *N*-acetyl glucosamine (Figure 3.14). There was no difference in the growth rate among strains in glucose and *N*-acetyl-glucosamine (Table 3.3 B and D) ($p>0.05$). However, on galactose the growth rates of Δ*tprA* and Δ*phrA* (0.049 ± 0.0855 h^{-1} and 0.045 ± 0.0905 h^{-1}, n=6, respectively) were significantly lower than that of wild-type strain (0.135 ± 0.0091 h^{-1}, n=6) ($p<0.0001$) (Table 3.3 A). A significant reduction in growth rates was also observed on mannose for these mutants (Table 3.3 C). The genetically complemented strains rescued the wild type growth rates ($p>0.05$), indicating that the observed phenotypic effect was not due to polarity of mutation. These results show the importance of TprA/PhrA for growth in an environment where galactose and mannose are the main source of sugars.

Table 3.3. Growth rate (μ) and yield (maximal OD600) of pneumococcal strains grown microaerobically in CDM supplemented with 55 mM (**A**) galactose, (**B**) glucose, (**C**) Mannose, or (**D**) *N*- acetyl glucosamine. Experiment was repeated using 3 replicates of 6 independent biological samples; '±' indicates standard error of means (SEM).

A

Strains	**Growth rate (μ) h^{-1}**	**Growth yield (MaxOD600)**
Streptococcus pneumoniae D39	0.135 ± 0.0091	0.857 ± 0.038
Δ*tprA*	0.049 ± 0.0855	0.437 ± 0.03
Δ*phrA*	0.045 ± 0.0905	0.344 ± 0.018
Δ*tprA*Comp	0.13 ± 0.0325	0.772 ± 0.019
Δ*phrA*Comp	0.13 ± 0.005	0.814 ± 0.031

B

Strains	**Growth rate (μ) h^{-1}**	**Growth yield (MaxOD600)**
Streptococcus pneumoniae D39	0.271 ± 0.042	1.715 ± 0.036
Δ*tprA*	0.268 ± 0.0723	1.711 ± 0.066
Δ*phrA*	0.268 ± 0.005	1.724 ± 0.017
Δ*tprA*Comp	0.2682 ± 0.0643	1.714 ± 0.057
Δ*phrA*Comp	0.268 ± 0.056	1.719 ± 0.012

C

Strains	**Growth rate (μ) h^{-1}**	**Growth yield (MaxOD600)**
Streptococcus pneumoniae D39	0.225 ± 0.06	1.554 ± 0.044
Δ*tprA*	0.099 ± 0.03	0.768 ± 0.002
Δ*phrA*	0.17 ± 0.017	1.568 ± 0.088
Δ*tprA*Comp	0.215 ± 0.009	1.544 ± 0.004
Δ*phrA*Comp	0.217 ± 0.02	1.55 ± 0.013

D

Strains	**Growth rate (μ) h^{-1}**	**Growth yield (MaxOD600)**
Streptococcus pneumoniae D39	0.216 ± 0.015	1.343 ± 0.012
Δ*tprA*	0.209 ± 0.041	1.315 ± 0.011
Δ*phrA*	0.213 ± 0.032	1.338 ± 0.007
Δ*tprA*Comp	0.217 ± 0.011	1.343 ± 0.019
Δ*phrA*Comp	0.216 ± 0.05	1.347 ± 0.051

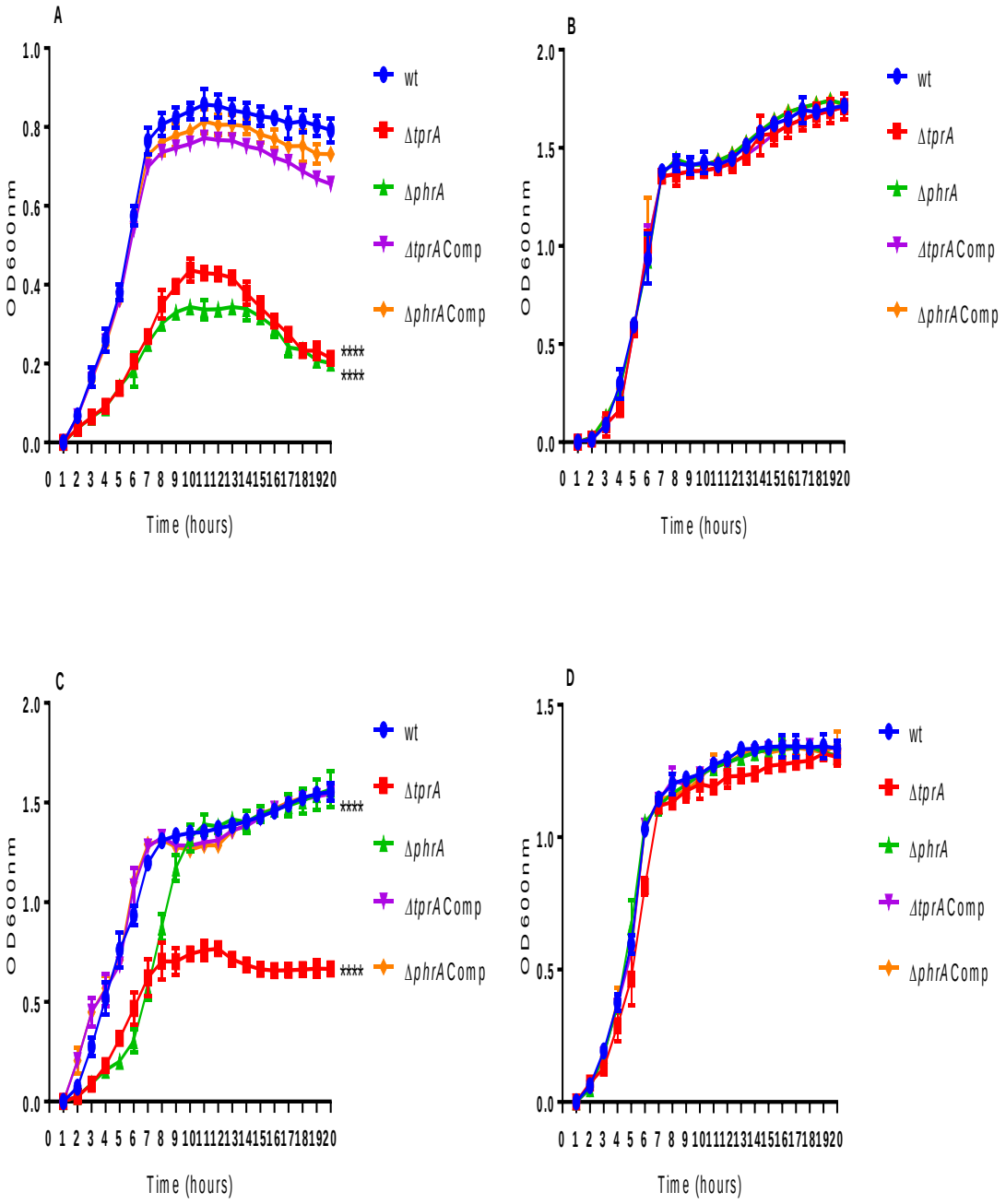


Figure 3.14. Growth curves of pneumococcal strains grown microaerobically in CDM supplemented with 55 mM (**A**) galactose, (**B**) glucose, (**C**) Mannose, or (**D**) *N*- acetyl glucosamine. Experiment was repeated using 3 replicates of 6 independent biological samples; vertical bars indicate standard error of means (SEM). Significant differences were seen by comparing the growth rates of mutant strains to the wild type D39 using one-way ANOVA and Dunnett's multiple comparisons test. (**p<0.01, *** p<0.001 and **** p<0.000 relative to the wild type).

3.5. Growth of pneumococcal strains in Sicard's defined medium with mucin

Having established the importance of TprA/PhrA system in host derived sugar metabolism, next, I evaluated whether this system is also important for utilisation of mucin. It is known that mucin contains galactose and mannose (King *et al.*, 2006). For this, the pneumococcal strains were grown in Sicard's defined medium supplemented with pork gastric mucin (Figure 3.15). PGM is commonly used for microbial interaction with mucin due to its commercial availability as a model mucin (Terra *et al.*, 2010). It was found that, *ΔtprA* had a significantly lower growth rate (0.18 ± 0.09 h^{-1}, n=6) and yield (Log_{10} 5.8 ± 0.145 CFU/ml, n=6) than the wild type D39 (growth rate: 0.34 ± 0.12 h^{-1}, n=6 and growth yield: Log_{10} 8.7 ± 0.153 CFU/ml, n=6) ($p<0.0001$). On the other hand, while Δ*phrA* had a similar growth rate, the total yield was significantly lower than the wild type D39 ($p<0.05$) (Table 3.4). The genetically complemented strains Δ*tprA*Comp and Δ*phrA*Comp grew (growth rate: 0.316 ± 0.01 h^{-1}, 0.34 ± 0.143 h^{-1}, n=6, respectively and yield: Log_{10} 8.1 ± 0.098 CFU/ml and Log_{10} 8.2 ± 0.084 CFU/ml, n=6, respectively) as well as the wild type D39 ($p>0.05$).

Table 3.4. Growth rate (μ) and yield (maximal Log_{10} CFU/ml) of pneumococcal strains grown microaerobically in Sicard's defined medium supplemented with mucin. Experiment was repeated using 3 replicates of 6 independent biological samples; '±' indicates standard error of means (SEM).

Strains	**Growth rate (μ) h^{-1}**	**Growth yield (Max Log_{10} CFU/ml)**
Streptococcus pneumoniae D39	0.34 ± 0.12	8.7 ± 0.153
Δ*tprA*	0.18 ± 0.09	5.8 ± 0.145
Δ*phrA*	0.34 ± 0.05	7.9 ± 0.033
Δ*tprA*Comp	0.316 ± 0.01	8.1 ± 0.098
Δ*phrA*Comp	0.34 ± 0.143	8.2 ± 0.084

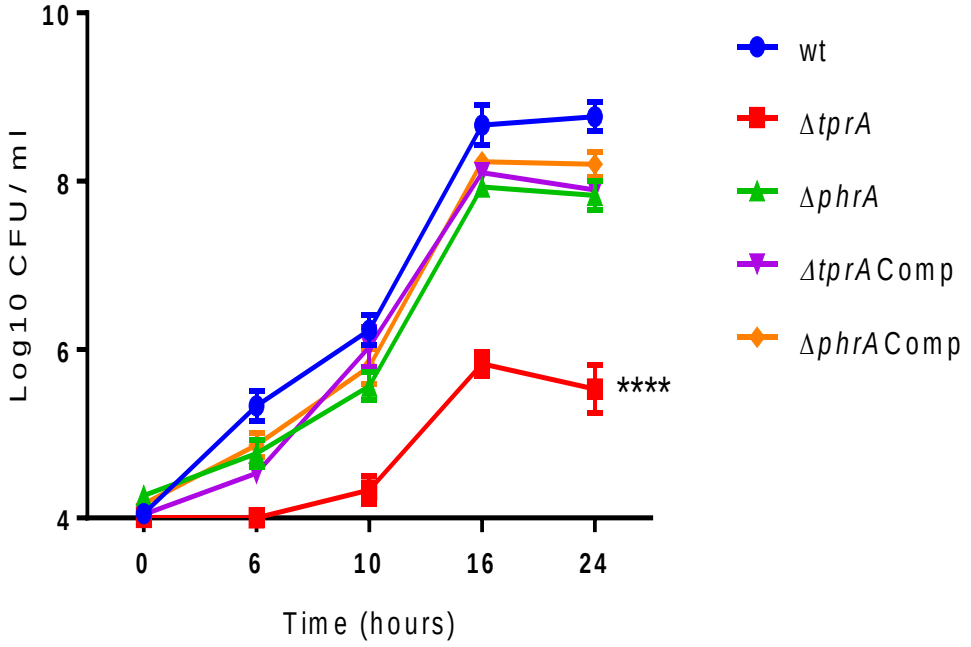


Figure 3.15. Growth profiles of pneumococcal strains grown microaerobically in Sicard's defined medium supplemented with mucin. Experiment was repeated using 3 replicates of 6 independent biological samples; vertical bars indicate standard error of means (SEM). Significant differences were seen by comparing the growth rates of mutant strains to the wild type D39 using one-way ANOVA and Dunnett's multiple comparisons test. (**p<0.01, *** p<0.001 and **** p<0.0001 relative to the wild type).

3.6. Assessing TprA/PhrA's role in production of proteins involved in virulence

TprA-PhrA was found to be important in regulation of sugar metabolism in *S. pneumoniae* especially for galactose and mannose metabolism. To further investigate this QS system's role in pneumococcal biology, I tested the contribution of this system in neuraminidase and pneumolysin production. These two proteins are known to be very important for pneumococcal virulence (Kadioglu *et al.*, 2008).

3.6.1. *tprA* plays a role to produce neuraminidase enzyme

Neuraminidase activity is required for cleavage of terminal sialic acid found in complex host glycans (Yesilkaya *et al.*, 2007). The removal of this sialic acid is critical for the activity of other glycosidases. If the terminal sialic acid is not cleaved, the other glycosidases cannot 'see' their cleavage sites (Yesilkaya *et al.*, 2007; Terra *et al.*, 2010).

Neuraminidase activity has a wide-ranging effect on pneumococcal biology, from nutrient generation, colonisation, virulence to biofilm formation (Yesilkaya *et al.*, 2007; Manco *et al.*, 2006; Parker *et al.*, 2009). The major pneumococcal neuraminidase activity is coded by *nanA* gene. I hypothesised that one reason for growth attenuation of Δ*tprA* and Δ*phrA* on mucin is due to TprA/PhrA system's impact on neuraminidase production. Neuraminidase activity was determined using pneumococcal cell extracts prepared after growing in Sicard's defined medium supplemented with mucin using a chromogenic substrate (Figure 3.16 A) or in BHI (Figure 3.16 B). When using the cell lysate from Sicard's defined medium supplemented with mucin, the result showed that Δ*tprA* had significantly less neuraminidase activity (18.9 ± 2.2 U, n= 5) than the wild type D39 (49.4 ± 2.4, n=5,) ($p<0.0001$). However, the genetically complemented strains did not show any significant difference compared with the wild type D39. In addition, when grown in BHI, there was no significant difference in neuraminidase activity among the pneumococcal strains compared with the wild type D39 (53.5 U ± 1.8, n=5) ($p> 0.05$). This was expected because in BHI is a rich medium and any difference in activity would be masked. Furthermore, quantitative reverse transcriptase PCR (qRT-PCR) was used to detect the expression of *nanA* in Δ*tprA* compared to the wild type D39. The result showed that decrease in expression of *nanA* in Δ*tprA* (2.6 ± 0.3-fold, n=3) relative to its expression in the wild type D39.

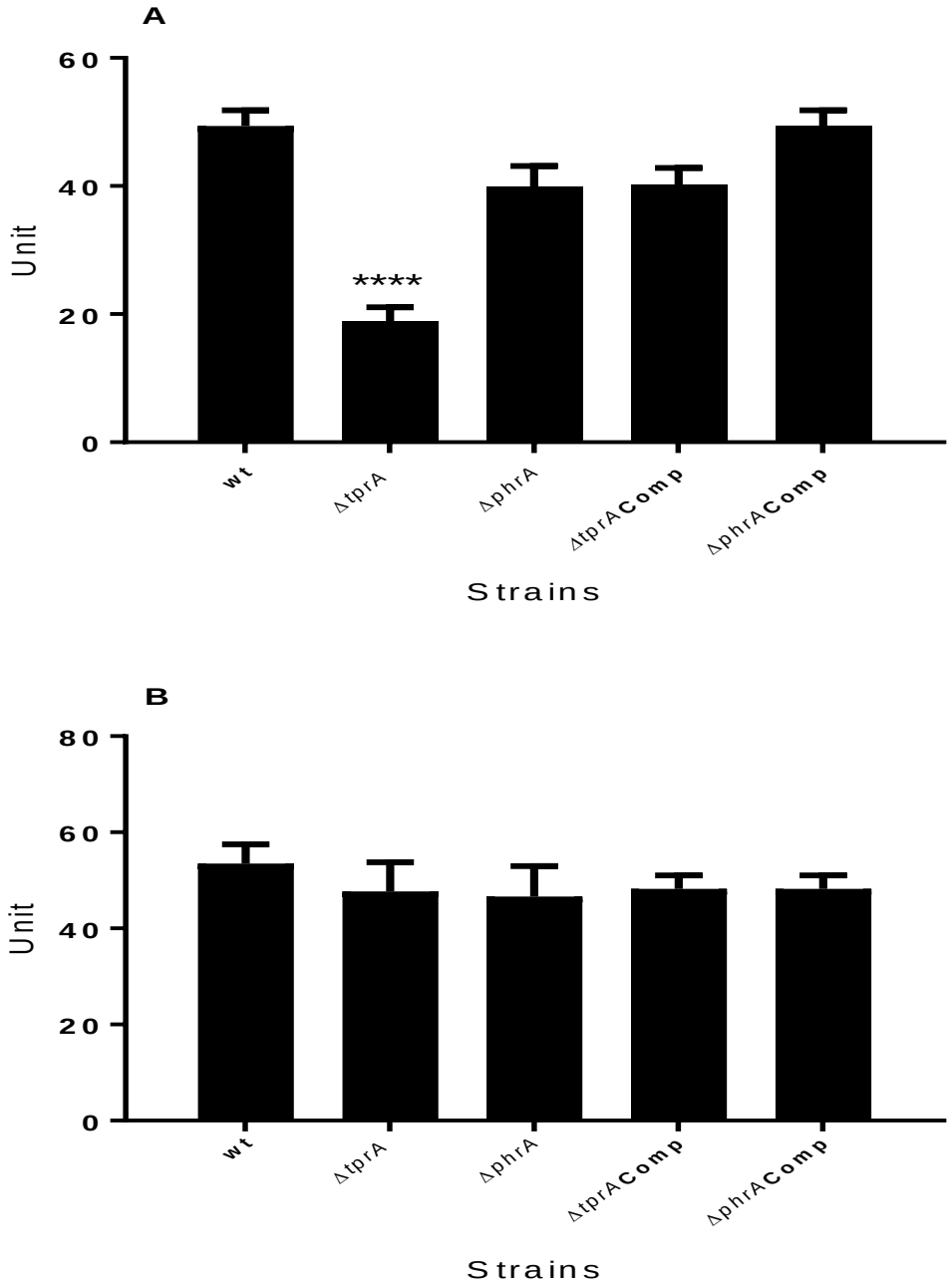


Figure 3.16. Determination of neuraminidase activity of pneumococcal strains using cell lysate of bacterial strains grown in Sicard's defined medium supplemented with mucin as sole carbon source (**A**) or in BHI (**B**). One neuraminidase unit was defined as nmol pNP released/min/μg of protein. Experiment was repeated using 3 replicates of 5 independent biological samples, error bars indicate SEM. (****$p<0.0001$).

3.6.2. Assessing haemolytic activity

The pneumococcal haemolytic activity is believed to be due to production of cytotoxin pneumolysin (Tilley *et al.*, 2005; Hirst *et al.*, 2004; Mitchell and Mitchell, 2010). The influence of *tprA* on the haemolytic activity was tested in the cell lysates of pneumococcal strains grown in BHI (Figure 3.17 B) or in Sicard's defined medium supplemented with mucin (Figure 3.17 A) by using sheep erythrocytes. There was no difference in the haemolytic activity of the strains ($p > 0.05$) when using the cell lysate of bacteria grown in BHI. The result showed that the pneumococcal strains Δ*tprA* and Δ*phrA* had similar haemolytic activity (12.6 ± 0.7 and 14.2 ± 0.1 HU/ μ g of protein, n=5, respectively) as the wild type D39, 15.03±1.17 HU/µg when using the cell lysate prepared from BHI grown cultures. However, Δ*tprA* had significantly less haemolytic activity (11.3 ± 0.17 HU/µg of protein, n= 5) than the wild type D39 (14.6 ± 0.29 HU/ μ g of protein, n=5) ($p<0.001$) when the pneumococcal strains were grown in Sicard's defined medium supplemented with mucin. In addition, the Δ*phrA* had also significantly less haemolytic activity (12.1±0.77 HU/µg of protein, n= 5) ($p<0.05$) compared to the wild type D39 when it was grown in Sicard's defined medium supplemented with mucin. The complemented strains Δ*tprA*Comp and Δ*phrA*Comp rescued the wild type haemolytic activity ($p>0.05$).

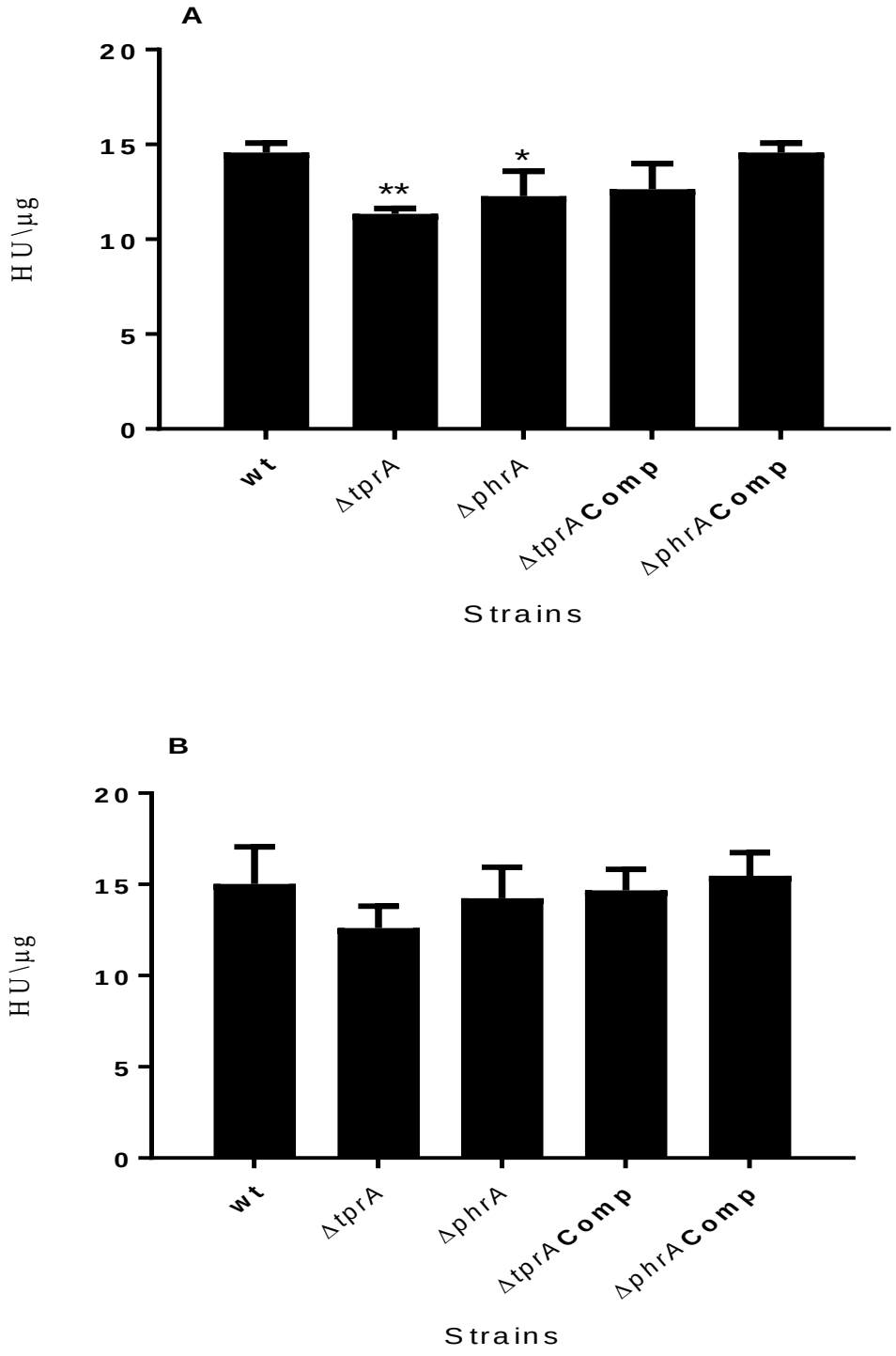


Figure 3.17. Determination of haemolytic assay of pneumococcal strains using cell lysate of bacterial strains grown in Sicard's defined medium (**A**) or in BHI (**B**). Haemolytic assay was done using 4% v/v defibrinated sheep blood. Experiments were repeated using 3 replicates of 5 independent biological samples. Error bars indicate SEM. (*$p<0.05$, *** $p<0.001$ relative to the wild type).

Section C: Construction of *lacZ* reporter system and β-galactosidase assays

3.7. Construction of *lacZ*-fusions

Transcriptional *lacZ*-fusions were constructed to investigate the response of TprA to different sugars. There are many reporter assays for studying transcriptional activity and the most common methods, include the *E. coli* β-galactosidase gene *lacZ* (Shapira *et al.*, 1983), the chloramphenicol acetyl transferase gene, *cat*, from *E. coli* (Gorman *et al.*, 1982), and the green fluorescent protein gene *gfp* from the jelly fish *Aequorea victoria* (Prasher *et al.*, 1992). The *E. coli* β-galactosidase gene *lacZ* was used in this study because it is the most popular system to determine gene regulation by a simple and inexpensive β-galactosidase assay (Miller, 1972). The plasmid pPP1was used as a promoter probe vector (Figure 3.18 A), in which the putative promoter region of target gene (the insert) drives the promoterless *lacZ* gene expression (Halfmann *et al.*, 2007). The constructs carrying promoter-*lacZ* fusions were transformed into *S. pneumoniae.* pPP1 incorporates the *lacZ* fusions into the *bgaA* locus in pneumococcal genome, which encodes for endogenous β-galactosidase. This incorporation is possible because plasmid pPP1 contains homologous regions to SPD_0562 (bgaA, encoding for endogenous β-galactosidase) and SPD_0564 of *S. pneumoniae* which helps the integration of this plasmid into the genome of pneumococci and disrupts the *bgaA* gene (Figure 3.18 B) (Zahner and Hakenbeck, 2000). This integration leads to reduce endogenous β-galactosidase activity. However, the inactivation of *bgaA* gene does not affect the growth of *S. pneumoniae* and regulatory studies (Halfmann *et al.*, 2007). Furthermore, the plasmid pPP1 contains *tetM* gene which confers tetracycline resistance in *S. pneumoniae,* and *bla* gene (beta-lactamase gene), which elicits ampicillin resistance in *E. coli*. These features of pPP1 plasmid made it more appropriate for genetic manipulation and regulatory studies.

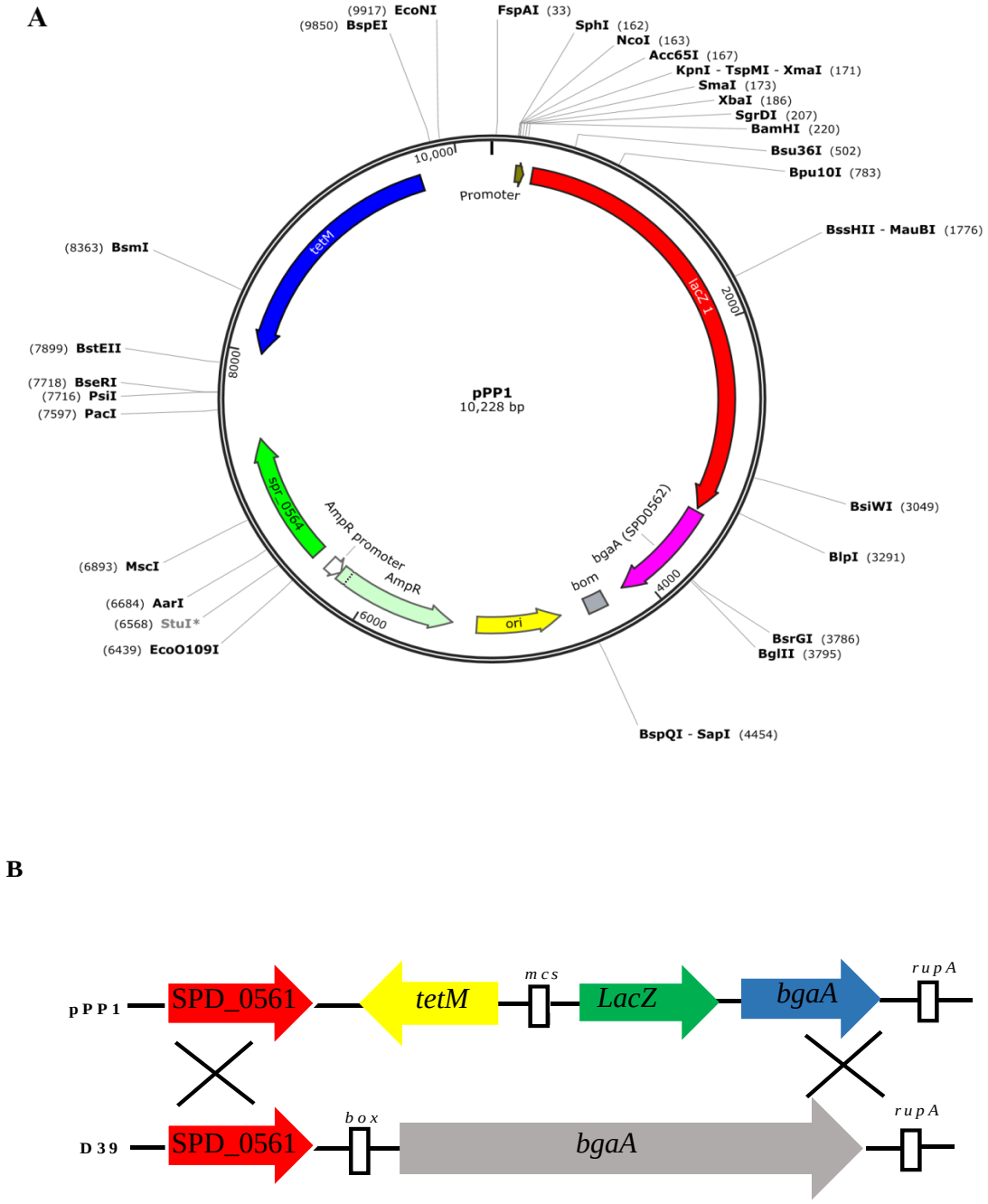


Figure 3.18. A: Genetic map of the integrative promoter probe plasmid pPP1. Several recognition regions for different restriction endonucleases are indicated. **B**: Illustration of the integration between recombinant pPP1 and *bgaA* region in D39. The endogenous *bgaA* gene and the flanking repetitive element (*box*, *rupA*), are deleted during the integration. Figure is constructed baced on (Halfmann *et al.*, 2007).

3.7.1 Overview: Construction of transcriptional reporter

The plasmid pPP1 was extracted using the QIAprep spin Miniprep kit as previously described in section 2.6. The restriction enzymes *SphI* and *BamHI* were used for pPP1 digestion as described in section 2.11. The putative promoter regions of *phrA* and *nanA* with 28 bp of the coding sequence of the genes of interest were amplified by the primers which incorporate *SphI* and *BamHI* recognition sites (Table 2.7). The PCR was done using PrimeSTAR HS premix (Section 2.9). The amplified PCR products were purified using the DNA purification kit (Section 2.8). The restriction enzymes *SphI* and *BamHI* were used to digest the inserts, which were then ligated to the similarly digested pPP1 (Section 2.12). The ligation reaction was transformed into chemically competent *E. coli* TOP10 cells. The successful ligation was confirmed using colony PCR by the Fusion-Seq-F, Fusion-Seq-R and X-Fusion-F and X-Fusion-R primers (X referred to insert name) (Section 2.10). Sequencing was done to confirm the successful cloning of the recombinant plasmids using Fusion-Seq-F and Fusion-Seq-R primers. The sequencing results demonstrated that all the recombinant plasmids contained the correct putative promoter regions of the target genes and the *lacZ* (Appendix 5).

3.7.2. Transformation of transcriptional reporter constructs into *S. pneumoniae* and confirmation by PCR

The recombinant pPP1 plasmids containing the correct inserts were transformed into *S. pneumoniae* wild type D39 and the mutant strains under study as described previously in section 2.14. The transformants were grown on blood agar in the presence of tetracycline. PCR was used to confirm the successful integration of the transcriptional constructs within the genome of *S. pneumoniae* using the Fusion-SeqF and Fusion-Seq-R primers and X-Fusion-F and X-Fusion-R (X referred to insert name) (Section 2.10). Agarose gel electrophoresis was used to analyse the PCR products (Figure 3.19). As can be seen in this figure, lanes 2, 4, and 8 show successful amplification of promoter regions (inserts) of each target gene and 138 bp upstream and downstream of the cloning site in pPP1 using the Fusion-Seq-F and Fusion-Seq-R primers. The amplified PCR products in lanes 3, 5, and 7 had the expected fragment sizes for *tprA* (219 bp), *phrA* (231 bp), and *nanA* (398 bp), respectively, as generated by X-Fusion-F and X-Fusion-R primers (X referred to insert name). Moreover, Lanes 1, 6, and 9 show the amplification of a promoterless

product using the pPP1 (253 bp) as the template using the same primers (Fusion-Seq-F and Fusion-Seq-R).

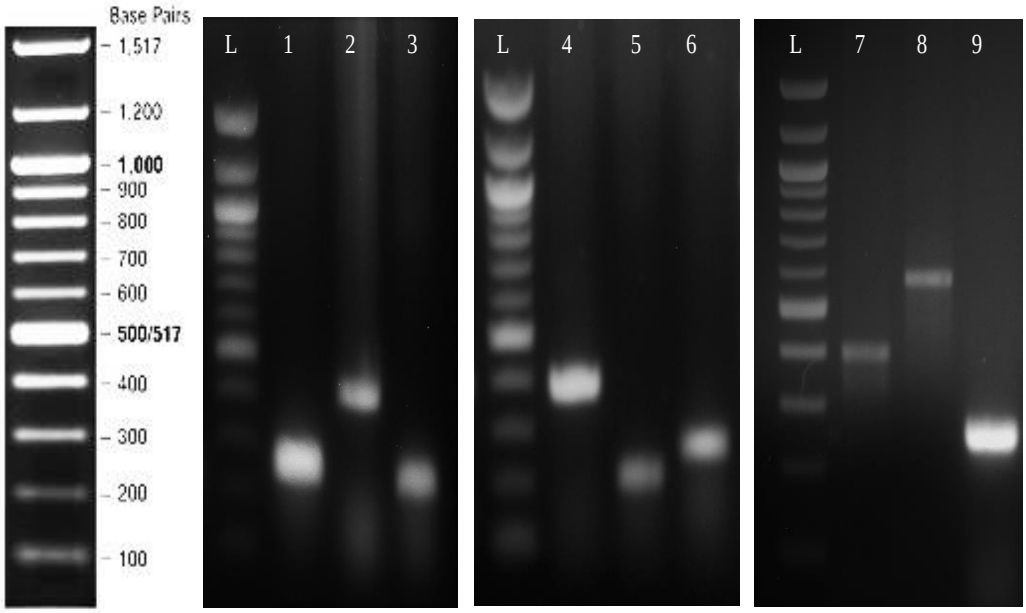


Figure 3.19. Agarose gel electrophoresis analysis to confirm the successful integration of *lacZ* reporter constructs into *S. pneumoniae*. L: 500 ng of 100 bp DNA ladder (NEB); Lanes 3, 5, and 7 show amplicons for the putative promoter regions (inserts) of *tprA* (219 bp), *phrA* (231 bp), and *nanA* (398 bp), respectively; lanes 2, 4, and 8 show the successful amplification of, 357 bp, 369 bp, and 536 bp, promoter regions (inserts) of *tprA*, *phrA*, *nanA*, respectively plus 138 bp upstream and downstream of the cloning site in pPP1; Lanes 1, 6, and 9 show the amplification of the empty plasmid, 253 bp.

3.8. Expression of transcriptional *lacZ*-fusions in the presence of different sugars

Signal transduction systems play a key role in environmental sensing. Pneumococcal strains encounter highly variable sugar availability within different host niches, for example galactose is predominant in the nasopharynx (King, 2010; Paixão *et al.*, 2015), while the blood is richer in glucose (Philips *et al.*, 2003). The inducibility of *tprA* and *phrA* were investigated using transcriptional reporter assays in microaerobically grown cultures on CDM supplemented with glucose, galactose, mannose or *N*-acetyl glucosamine. The results showed that there was no induction in β-galactosidase activity in Miller Unit (MU) in the wild type D39 containing pPP1 in the absence of any promoter regardless of presence of glucose, galactose, *N*-acetylglucosamine, or mannose (1.4 ± 0.3; 2.1 ± 0.5; 2.8 ± 0.6; and 1.5 ± 0.4 MU, n=4, respectively) compared to its expression on CDM alone (1.1± 0.3 MU, n=4, P>0.05). The transcriptional reporter strains P*tprA*-*lacZ*-wt and P*phrA*-*lacZ*-wt generated the highest β-galactosidase activity both on mannose (630.2 ± 29.8 and 793.9 ± 41.8 MU, n=4, respectively) and galactose (693.3 ± 46.8 and 881.1 ± 30.3 MU n=4, respectively) ($p<0.05$) (Table 3.5), whereas on glucose (175.6 ± 17.7 and 262 ± 50.3 MU, n=4) and *N*-acetylglucosamine (180.1 ± 14.8 and 224.4 ± 32.3 MU, n=4, respectively) no significant induction of P*tprA* and P*phrA* could be seen relative to the no sugar control (176.2 ± 14.9 and 263.6 ± 32, n=4, respectively) ($p>0.05$). These data show that the TprA/PhrA system is active when pneumococcus encounters host glycans containing galactose and mannose, such as mucin covering apical epithelial surfaces of the upper respiratory tract.

Table 3.5. Expression levels (in Miller units) of pneumococcal transcriptional *lacZ*-fusions to the promoter of *tprA* and *phrA* in wild type grown microaerobically in CDM supplemented with 55 mM of glucose, *N*-acetyl glucosamine, mannose or galactose. The activity is expressed in nmol *p*-nitrophenol/min/ml. Values are average of four independent experiments each with three replicates. ± indicates standard error of means (SEM).

Strains Genotype	Glucose	Galactose	*N*-acetylglucosamine	Mannose	CDM alone
pPP1-wt	1.4±0.3	2.1±0.5	2.8±0.6	1.5±0.4	1.1±0.3
P*tprA-lacZ*-wt	175.6±17.7	693.3±46.8	180.1±14.8	630.2±29.8	176.2±14.9
P*phrA-lacZ*-wt	262±50	881.1±30.3	224.4±32.2	793.9±41.8	263.6±32.1

3.9. Role of TprA and PhrA in *tprA* regulation

The TprA/PhrA system is induced by host-derived sugars, and both of P*tprA* and P*phrA* showed the same inducibility in CDM supplemented with different sugars (Section 3.8). Hence, the involvement of TprA and PhrA in the regulation of *tprA* was investigated using transcriptional *lacZ*-fusions to assess the regulatory interaction among the components of this putative quorum sensing system. The P*tprA-lacZ* construct was introduced into Δ*tprA*, Δ*phrA* and the wild type D39, generating the reporter strains P*tprA-lacZ*-Δ*tprA*, P*tprA-lacZ*-Δ*phrA* and P*tprA-lacZ*-D39, respectively, and β- galactosidase activities in the reporter strains were measured when the strains were grown microaerobically in CDM supplemented with 55 mM galactose, mannose, glucose or *N*-acetyl glucosamine.

The results showed that in the condition where the TprA promoter activity is high, that is presence of mannose and galactose, TprA reduces P*phrA* driven β-galactosidase activity relative to its expression in wild type D39 strain, indicating that TprA is a repressor of *phrA* ($p<0.001$ and $p<0.0001$ for mannose and galactose, respectively) (Figure 3.20). In glucose or *N*-acetylglucosamine, where the P*tprA* activity is low, TprA does not have any

effect on *phrA* expression (Figure 3.20). Moreover, it was also found that *phrA* activates its own expression on mannose and galactose, when its promoter activity is up, but this regulatory effect could not be observed on glucose and *N*-acetylglucosamine (Figure 3.20).

TprA also may influence its own expression. In glucose and *N*-acetylglucosamine, where TprA levels are lower, the absence of *tprA* or *phrA* do not influence *tprA* gene expression levels. In contrast, in galactose and mannose where TprA levels are higher, deletion of *tprA* leads to a decrease in *tprA* promoter expression, consistent with autoinduction of TprA and suggesting that expression of its regulon must depend on the balance between PhrA bound and unbound forms (Figure 3.21).

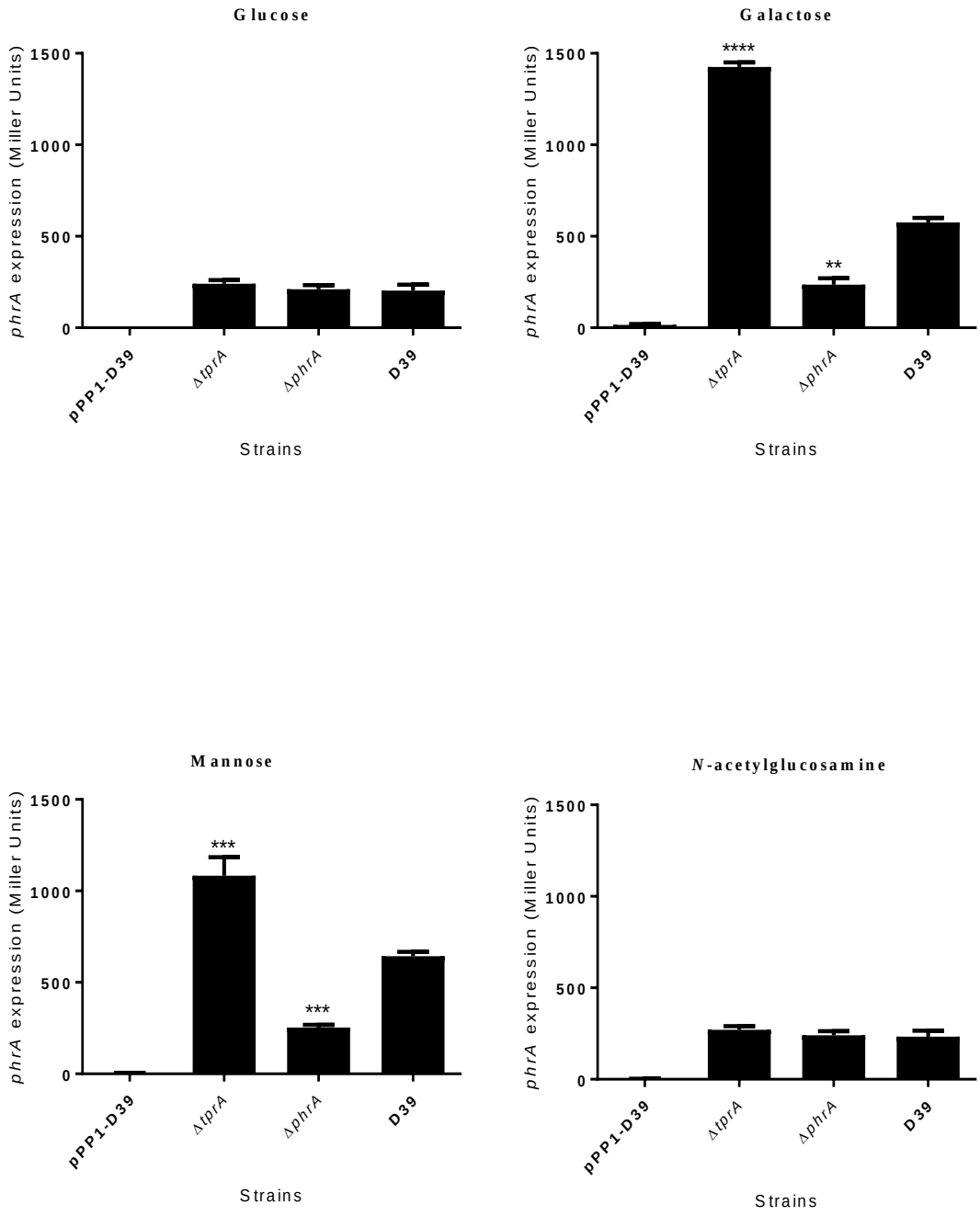


Figure 3.20. Analysis of *phrA* regulation using *LacZ* reporter assay. The activity of β-glactosidase under regulation of P*phrA* is expressed in nmol *p*-nitrophenol/min/ml. Ppp1-D39 strain referred to the wild type D39 containing pPP1 in the absence of any promoter. Values are average of three independent experiments each with three replicates. '±' indicates standard error of means (SEM), (** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$ compared to its expression in the wild type D39).

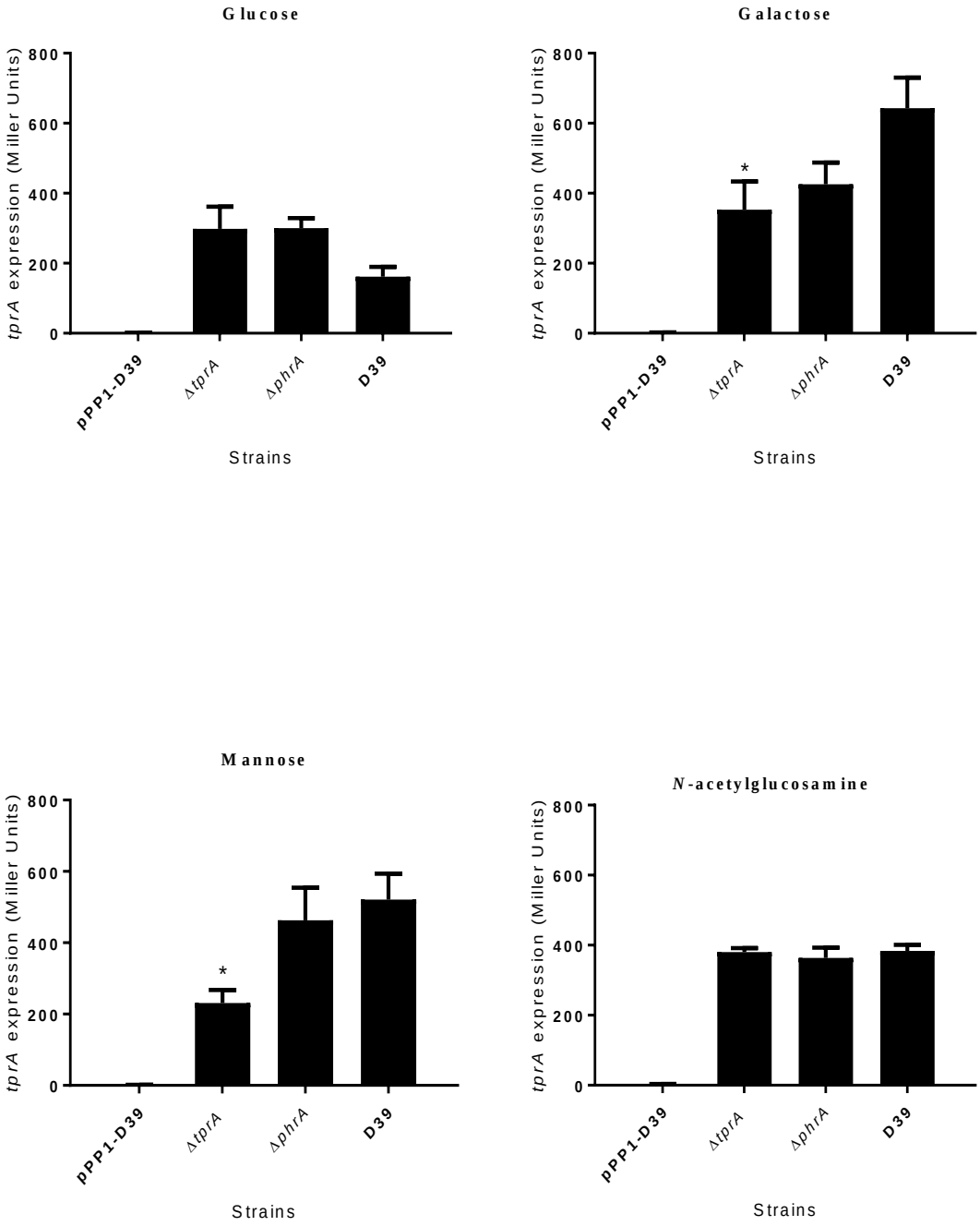


Figure 3.21. Analysis of *tprA* regulation using *LacZ* reporter assay**.** The activity of β-glactosidase under regulation of P*tprA* is expressed in nmol *p*-nitrophenol/min/ml. Ppp1-D39 strain referred to the wild type D39 containing pPP1 in the absence of any promoter. Values are average of three independent experiments each with three replicates. '±' indicates standard error of means (SEM), (*$p<0.05$ compared to its expression in the wild type D39).

3.10. TprA is an activator of *nanA* on galactose

It was shown in section B that TprA/PhrA system plays an important role in pneumococcal growth in the presence of mucin and in neuraminidase activity. Therefore, it was hypothesised that this system could play a role in regulation of *nanA*. Hence, the role of TprA/PhrA quorum sensing system in synthesis and transcription of *nanA* was investigated. This gene codes for the main pneumococcal neuraminidase activity. The putative promoter region of *nanA* (P*nanA*) was fused to promoterless *lacZ* in pPP1, and the resulting strain was transformed into the wild type D39, Δ*tprA*, and Δ*phrA*. Initially, the condition that would stimulate *nanA* expression was determined by growing the reporter strain in CDM supplemented either with glucose or galactose. The results showed that P*nanA* driven β-galactosidase activity was significantly higher when D39 was grown in CDM containing galactose (21.5 ± 0.524, n=4) than glucose (0.83 ± 0.088, n=4) compared to the promoterless pPP1 in D39 (0.91 ± 0.3; and 0.89 ± 0.1, n=4, respectively, $p<0.0001$). It was also shown that no induction of P*nanA* in all the transcriptional fusion strains under study on glucose, the wild type D39 (0.83 ± 0.088, n=4), Δ*tprA* (0.83 ± 0.12, n=4), and Δ*phrA* (0.82 ± 0.05, n=4) compared to the promoterless pPP1 in D39 (0.89 ± 0.1, n=4, $p>0.05$) (Figure 3.22). Moreover, on galactose the absence of TprA significantly reduced P*nanA* driven β-galactosidase activity (3.3 ± 0.9, n=4, $p<0.0001$) compared to its expression in the wild type D39 (21.5 ± 0.524, n=4), indicating that TprA induces *nanA* expression. Moreover, deletion of *phrA* had no impact in the expression of P*nanA* on galactose (19.13 ± 1.8, n=4, $p>0.05$) relative to its expression in the wild type D39 on galactose (Figure 3.22). This result further confirms the involvement of TprA/PhrA system in *nanA* regulation.

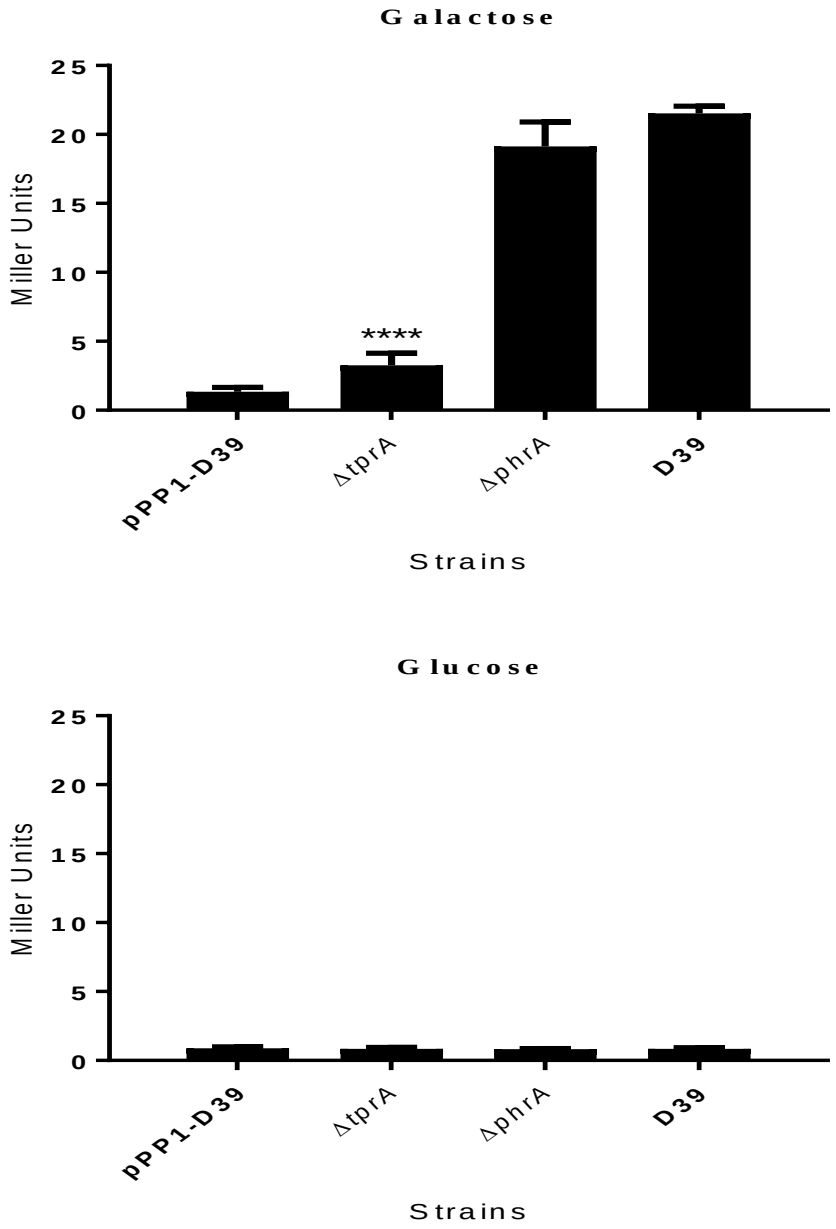


Figure 3.22. Induction of P*nanA* by TprA. P*nanA* fused to promoterless *lacZ* was transformed into either wild type D39 or Δ*tprA* and Δ*phrA*. The reporter strains were grown in CDM with 55 mM glucose or galactose. The activity of β-galactosidase under regulation of P*nanA* is expressed in nmol *p*-nitrophenol/min/ml. Ppp1-D39 strain referred to the wild type D39 containing pPP1 in the absence of any promoter. Values are average of four independent experiments each with three replicates. ± indicates standard error of means (SEM). (**** $p<0.0001$ compared to the expression in the wild type D39).

Section D: Microarray analysis and gene expression

3.11. Regulon determination for TprA

The TprA regulon in *S. pneumoniae* was identified using microarray analysis in collaboration with Prof Oscar Kuipers and Irfan Manzoor, University of Groningen, the Netherlands. To determine the regulon of TprA, galactose and mannose growth conditions were selected, where the TprA is predicted to be playing an inhibitory role, and where both the TprA promoter and PhrA promoter activities are high. The wild type and **Δ***tprA* were grown in CDM supplemented with 55 mM of galactose or mannose as the main carbon source microaerobically. When the growth reached mid- exponential phase, the total RNA was extracted and treated with DNase as described in section 2.17 and 2.17.1 respectively. Agarose gel electrophoresis was performed to determine the total RNA extraction (Figure 3.23). The experiment was repeated with three biological replicates. Lanes 1, 2, and 3 show the total RNA extracted from wild type D39 and lanes 4, 5, and 6 show the total RNA extracted from Δ*tprA* strain on CDM supplemented with galactose, and lanes 7, 8, and 9 show the total RNA extracted from wild type D39 and lanes 10, 11, and 12 show the total RNA extracted from Δ*tprA* strain on CDM supplemented with mannose. As can been in this gel image, the integrity of RNA used for gene expression study was good, and RNA preparation was free from any visible DNA contamination. Despite this, RNA preparations were treated with DNase to remove any contaminating genomic DNA as described in section 2.17.

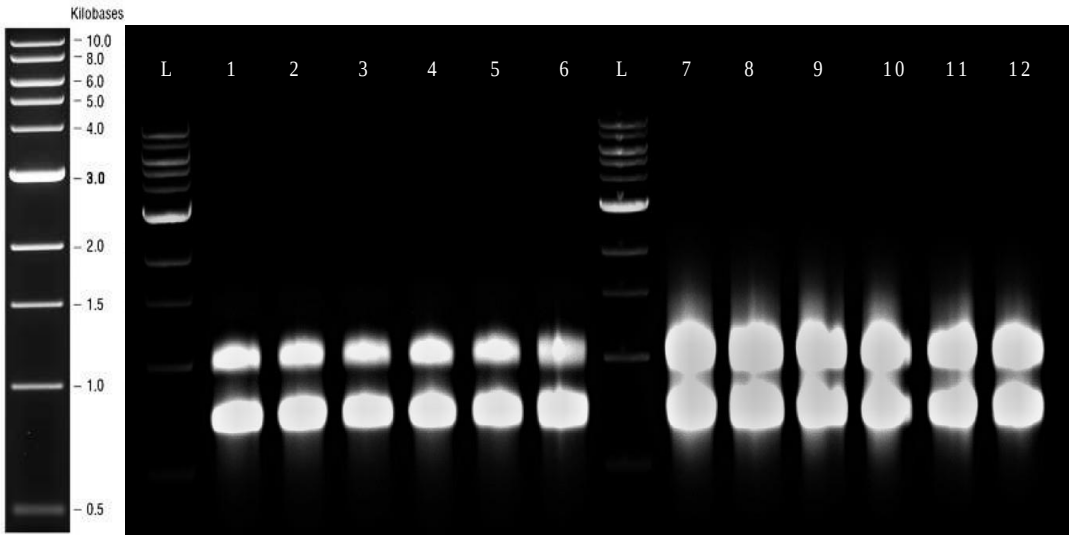


Figure 3.23. Agarose gel electrophoresis showing the total RNA extracted from the wild type (lane 1, 2, 3) and Δ*tprA* strain (lane 4, 5, 6) on CDM supplemented with galactose, and lanes 7, 8, and 9 show the total RNA extracted from wild type D39 and lanes 10, 11, and 12 show the total RNA extracted from Δ*tprA* strain on CDM supplemented with mannose. L- 500 ng of 1kb DNA ladder (NEB, UK).

The gene expression between the wild type and Δ*tprA* was compared using a microarray chips, and the results showed that on galactose, 61 genes were differentially expressed. Of these 46 were up-regulated and 15 were down regulated (Appendix 3). The up-regulated genes could be divided into 8 putative operons, of 2 or more genes. The most up-regulated is the up-regulation of 11 genes downstream of PhrA, including a bacteriocin synthesis cluster that showed between 5- and 73-fold changes. PTS and ABC transporters involved in galactose and mannose uptake, and mannose metabolism were also up-regulated by 2- to 4.5-fold. Finally, SPD_1944-SPD_1948 that contains a gene for putative CAAX amino terminal protease family protein synthesis was up-regulated by 3- to 8-fold. The notable downregulated genes included 8 tRNA genes and a GlnR family transcriptional regulator.

On mannose, 29 genes were differentially regulated. Off these 21 were up-regulated and 8 were down-regulated. The upregulated genes included 3 operons: a locus containing putative genes possibly involved in membrane protein synthesis (SPD_1514-SPD_1519), the downstream bacteriocin loci, and the hypothetical set with a CAAX protease. The two latter sets were also observed in the galactose, but the fold changes observed between

the presence and absence of TprA, are less accentuated in mannose than galactose (Appendix 4).

3.12. Confirmation of microarray results by quantitative reverse transcriptase PCR (qRTPCR)

Quantitative reverse transcriptase PCR (qRT-PCR) was used to confirm the microarray results for selected genes. cDNA was synthesised as described in section 2.17.2, DNA gyrase B gene (*gyrB*) was performed to normalize the transcription levels of target genes in parallel because the transcription of this gene is stable under different environmental stimuli (Goerke *et al.*, 2001). The primers for selected differentially expressed genes (Table 2.6 in section 2.17.4) were used to analyse the transcription levels of each gene. Comparative CT Method ($2^{-\Delta\Delta CT}$) was performed to analyse the data where the expression of each gene was normalised with *gyrB* expression. The qRT-PCR analysis showed the same trend of expression as the microarray data (Table 3.6 and Table 3.7).

Table 3.6. Fold difference in expression of genes in Δ*tprA* relative to wild type D39 strain grown micro aerobically in CDM supplemented with galactose.

Gene taq	Function	Fold difference	
		Microarray	**qRT-PCR**
SPD_0558	Cell wall-associated serine protease PrtA	2.53	2.33 ± 0.06
SPD_1167	ABC transporter, ATP-binding protein	2.86	2.58 ± 0.31
SPD_1495	Sugar ABC transporter, sugar-binding protein	3.94	3.64 ± 0.04
SPD_1746	Hypothetical protein	73.25	63.12 ± 0.04
SPD_1944	CAAX amino terminal protease family protein	4.24	4.05 ± 0.09
SPD_1989	PTS system, IID component	2.84	2.6 ± 0.23
SPD_1994	L-fuculose phosphate aldolase	2.4	2.37 ± 0.7
SPD_0447	Transcriptional regulator, MerR family protein	-2.02	-2.3 ± 0.02
SPD_1745	Transcriptional regulator PlcR, putative	-3.08	0.00 ± 0.00

‘- ‘indicates down regulation of genes, ± represents the standard deviation for three individual measurements.

Table 3.7. Fold difference in expression of genes in Δ*tprA* relative to wild type D39 strain grown micro aerobically in CDM supplemented with mannose.

Gene taq	Function	Fold difference	
		Microarray	**qRT-PCR**
SPD_0558	Cell wall-associated serine protease PrtA	2.4	2.3 ± 0.02
SPD_1513	pseudogene	3.72	3.75 ± 0.09
SPD_1517	Hypothetical protein	2.93	2.7 ± 0.1
SPD_1746	Hypothetical protein	8.7	7.9 ± 0.32
SPD_1944	CAAX amino terminal protease family protein	3.72	3.8 ± 0.15
SPD_0093	Hypothetical protein	-2.07	-1.9 ± 0.04
SPD_0936	Tn5252, relaxase	-4.81	-4.01 ± 0.12
SPD_1745	Transcriptional regulator PlcR, putative	-3.1	0.00 ± 0.00

'-'indicates down regulation of genes, ± represents the standard deviation for three individual measurements.

Section E: Electrophoretic mobility shift assay (EMSA)

It was shown that TprA plays a role in *phrA* regulation (Section C), and the results in section B showed that TprA/PhrA system involves in pneumococcal neuraminidase production. In addition, the regulon of *tprA* was investigated in the presence of galactose and mannose using microarray analysis in section D. Thus, electrophoretic mobility shift assay (EMSA) was used to detect any potential interaction between TprA and the putative promoter region of *phrA.* The interaction between TprA with neuraminidase genes in *S. pneumoniae* strain D39, *nanA* and *nanB* as well as the selected genes identified to be differentially expressed in Δ*tprA* by mircroarray analysis was also investigated (Appendix 3 and 4).

There are many techniques to detect the protein-nucleic acid interaction such as nitrocellulose filter binding, foot printing, and electrophoretic mobility shift assay (EMSA). Among these methods, EMSA is a sensitive way to identify the interaction between the protein and nucleic acid rapidly and it is considered as an essential technology for qualitative and quantitative analyses for the interaction between DNA and protein. This assay relies on the observation that protein and nucleic acid complexes move more slowly than the free nucleic acid (Garner and Revzin, 1986; Hellman and Fried, 2007). There are many techniques that can visualise the interaction between a protein and a nucleic acid in EMSA, include, ^{32}P- radiolabelled oligonucleotide probe (Hellman and Fried, 2007). The use of radio-labled oligonucleotide in EMSA has the advantage to make the protein- nucleic acid interaction hypersensitive but this method is expensive and has issues relating to the safety and short half-life of the label (Pagano *et al.*, 2011). Other labelling methods such as chemiluminescence detection, can detect the DNA band shifts but this technique requires prelabeling of the DNA, and this assay is time consuming and it can only detect the DNA band shifts without any information about the protein in the complex (Jing *et al.*, 2003; Kessler, 1992; Rodgers *et al.*, 2000). Another method is a two-colour fluorescence EMSA. This method is a simple and it detects protein and nucleic acid separately in a gel shift assay (Jing *et al.*, 2003). However, it is time consuming and more expensive compared with the use of fluorescent probe. A fluorescent probe is a rapid, simple, highly stable, sensitive method, and it provides reliable information for detection of the protein-DNA interaction (Onizuka *et al.*, 2002). In order to perform EMSA, the TprA was firstly produced and purified, then the putative promoter regions of

target genes were predicted and amplified using fluorescently labelled primers. The target DNA containing the putative promoter regions (probe) and the recombinant TprA were incubated before their analysis on non-denaturing gel by electrophoresis as covered in detail in section 3.13.

3.13. Overexpression and purification of recombinant proteins

3.13.1. Construction of recombinant strain

The cloned plasmid (SPD1745-pLEICS-01) was extracted from *E. coli* SPD1745-pLEICS-01-One Shot® TOP10 as described in section 2.6 and transformed into *E. coli* BL21 (DE3) pLysS competent cells as described in section 2.13 for protein expression. PCR was performed to confirm the successful transformation using the primers in Table 2.8 (Section 2.19). Agarose gel electrophoresis was used to visualise the amplified product (Figure 3.24).

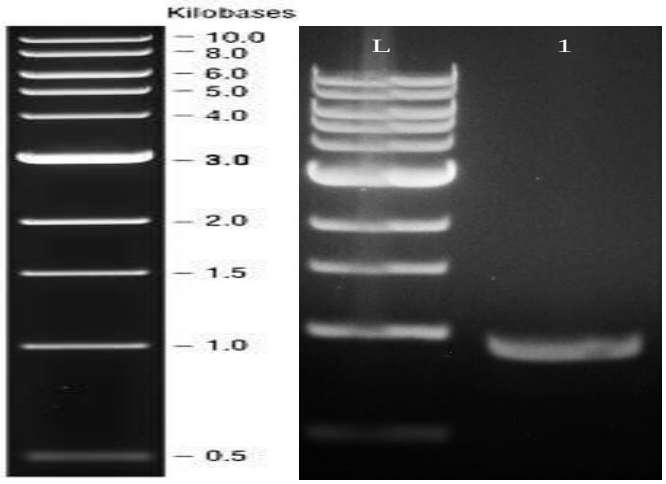


Figure 3.24. Agarose gel electrophoresis showing the amplified product for the *tprA* from the recombinant plasmid. L- 500 ng of 1 kb DNA ladder (NEB); Lane 1- *tprA* (864 bp), the lane contained between 40-100 ng DNA.

3.13.2. Small and large-scale protein expression

The *E. coli* BL21 (DE3) pLysS strain containing the recombinant plasmid (SPD1745-pLEICS-01) was grown until the OD600 reached either to 0.5-0.6 or 0.8-0.9. Then

different concentrations of IPTG (0.1, 0.2, 0.5, and 1 M) were used to induce the expression and incubated at different temperatures (18, 24, 30, 37°C) as described in section 2.19.2. The optimal conditions for TprA expression were determined to be the induction of bacteria at OD600 of 0.5-0.6 with 1 M IPTG and then further incubation for 9 hours at 18 °C or overnight at 30°C.

Large-scale TprA expression was done using the optimal conditions for protein expression. Different concentrations of imidazole buffer (25, 50, 100, 200, 300, and 500 mM) were used to elute the TprA. SDS-PAGE was used to analyse the fractions as described in section 2.19.3. The results demonstrated that the TprA was expressed in a soluble state and purified with 500 mM of imidazole elution buffer (Figure 3.25 A). The molecular weight of TprA with histidine tag (840 Da) and TEV (Tobacco Etch Virus) cleavage site (957 Da) was calculated to be 35.8 kDa for TprA. The SDS-PAGE analysis showed the expected size for TprA protein (Figure 3.25 A). The recombinant protein was dialysed against the binding buffer (Section 2.21.6) to remove any remaining imidazole, which can affect protein structure. Subsequently, the TprA was concentrated and visualised using SDS-PAGE (Figure 3.25 B).

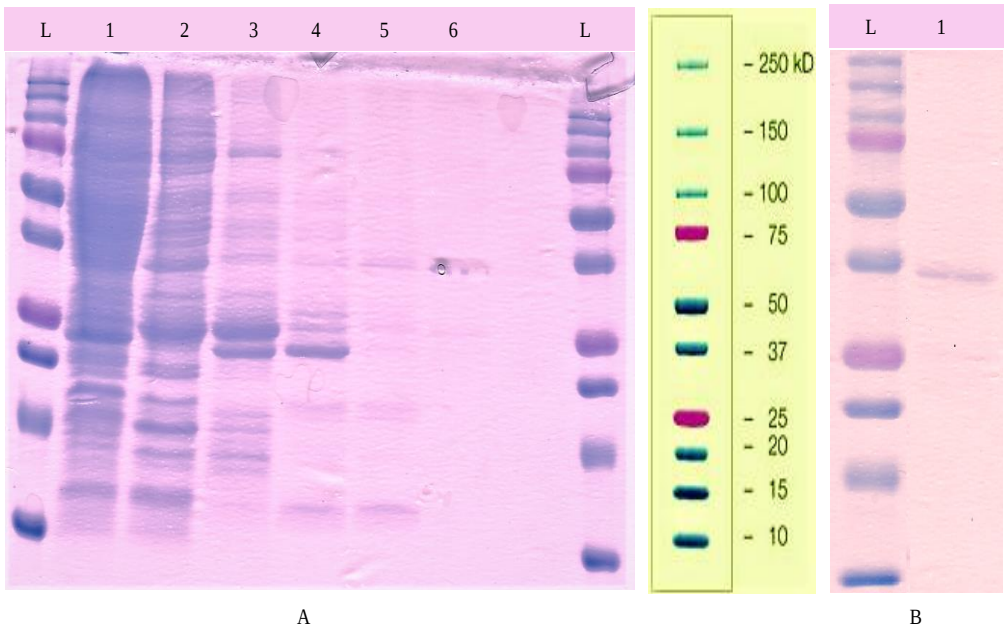


Figure 3.25. (**A**) SDS-PAGE analysis showing the purified fractions of the transcriptional regulator TprA (35.8 kDa) eluted with 500 mM imidazole elution buffer (Lane 6); L: protein molecular weight marker (Bio-Rad, UK); Lanes 1-6: protein fractions eluted with different concentrations of imidazole buffer 25, 50, 100, 200, 300, and 500 mM, respectively. (**B**) SDS-PAGE analysis showing the dialyzed and concentrated purified transcriptional regulator TprA (35.8 kDa) (Lane 1). Gel is stained with Coomassie brilliant blue.

3.13.3. MALDI-TOF analysis

Matrix-assisted laser desorption ionization- time of flight (MALDI- TOF) was used to determine the sequence and identity purified protein sample at PNACL, University of Leicester (Appendix 6). The result confirmed the identity of the purified protein sample as TprA.

3.14. Prediction of promoter region

BPROM (the bacterial promoter prediction tool) and MEME (the motif-based sequence analysis tools) were used to determine the existence of regulatory elements in the upstream of the target genes. BPROM is a bacterial promoter recognition program to find out the putative promoter region within the upstream of the genes of interest (Solovyev and Salamov, 2011). MEME is algorithm motive discovery program which is used to determine the binding sequence of transcription factor proteins on a small dataset (Bailey and Elkan, 1994)**.**

The bacterial promoter prediction tool and motif binding tool were used to detect the putative promoter regions (referred to as 'probe' from this point onward) for *phrA* (SPD_1746), *nanA* (SPD_1504), *nanB* (SPD_1503 locus), and selected genes that appeared in the *tprA* regulon as determined by the microarray analysis in the presence of galactose and mannose. These genes included SPD_1517, SPD_1994, and SPD_1495. In addition, *gyrB* (SPD_0709 locus) was used as a control in EMSA to demonstrate the specificity of binding. BPROM determined the core promoter elements (-10 and -35) within the upstream of target genes (3.26 A) and MEME detected the putative motif binding site in the upstream of the screened genes (Figure 3.26 B). In addition, the homolog of PlcR binding site known as PlcR box (TATGNAN$_4$TNCATA, where N is any base) in *Bacillus cereus* was also detected in the promoter regions of selected genes (Agaisse *et al.*, 1999) by visual inspection (Figure 3.26 C).

A

P(SPD_1504)

F

GTATGGTGTAAATAGCATAAGCTGATGTCCATCATTTGCTTATAAAGAGATATTTTAGTTTAATTGCAGC
GGTGTCCTGGTAGATAAACTAGATTGGCAGGAGTCTGATTGGAGAAAGGAGAGGGGAAATTTGGCAC
AATTTGAGATAGTTTGTTTAGTTCATTTTTGTCATTTAAATGAACTGTAGTAAAAGA
-35 -10 R

P(SPD_1503)

F

TATAAATTCAAGTCCCCAAATAGATTCATACTAGTATCTTTTGCAAAAAATAAAGGGCGACTTCCTTCAT
GAATATCAATTTCATCTATAAGGAAGGTAGCTAATTGAACTAACTTATTTATTCTGTTTGTCGC
-35 -10 R

P(SPD_1746)

F

GGAATAAATAATTCTTTATGGTAATAAACAAAAAAAAAAGTTAAAAATTACATAAAAATACTTGACTAA
ATAAAATATATCTGTTAGAATAAAAACAAGG -35
-10 R

P(SPD_1495)

F

-35

GTACTTACTGACTTAATAAAAAACAGAGGAGAGTGATGGATGAGTAGGATGAAATGAAATCGCATACA
AGAAATAAAGAACTCATTATCCAAGTTGGATACGCTTATTACATAGGAGAATACAA
-10 R

P(SPD_1994)

F

-35

AAAGATTGAGAGTTTGTAAATTTGCCTCCCTCCCCCTTCTTAGCTTTTGTGCAGGAAGGGGGGATAATT
GGTGAATTGAAAAATATTTAGTGTTTTGATATGAGGAGGACAAGG -10
R

P(SPD_1517)

F

-35 -10

GTAAAGGAGGAATTTGTATGATATACCATCGTTTAGAATAAGTCTATATTTAAAATAGAAGTTATAAGG
AATCTTCTTGAATAAAAAATGAAAGGAAACAGAAAAA
R

gyrB

F

ATGACAGAAGAAATCAAAAATCTGCAGGCACAGGATTATGATGCCAGTCAAATTCAAGTTTTAGAGGG
CTTAGAGGCTGTTCGTATGCGTCCAGG
R

B

SPD_1994	=TGTAAATTTG	CCTCCCTC	CCCCTTCTTA
SPD_1503	=AGGGCGACTT	CCTTCATG	AATATCAATT
SPD_1517	=TTATAAGGAA	TCTTCTTG	AATAAAAAAT
SPD_1504	=CATAAGCTGA	TGTCCATC	ATTTGCTTAT
SPD_1746	=AATAAATAAT	TCTTTATG	GTAATAAACA
SPD_1495	= GT	ACTTACTG	ACTTAATAAA

C

SPD_1504=		TATGGTGT	AAATAGCATA
SPD_1517=		TATGATAT	ACCATCGTTT
SPD_1994=		TATTTAGT	GTTTTGATAT
SPD_1746=		TATGGTAA	TAAACAAA
SPD_1495=	ATACGCTTAT	TACATAGG	AGAATACAA
SPD_1503=		TATAAATT	CAAGTCCCCA

Figure 3.26. A- The sequences of putative promoter regions of SPD_1504, SPD_1503, SPD_1746, SPD_1495, SPD_1994, and SPD_1517, respectively, which were used in EMSA, and the coding sequence of *gyrB* which was used as a control probe. The core promoter elements -10 and -35 are indicated. The putative binding sites according to MEME program are shown in red, and to (Agaisse *et al.*, 1999) are indicated in blue. F: indicates forward fluorescence primer whereas R: refers to the reverse primer used for amplifying the probes (Table 2.9). **B**- The putative consensus sequence using MEME program. **C**- The putative PlcR box homologs using the consensus sequence TATGNAN$_4$TNCATA (where N is any base) in *Bacillus cereus* group.

3.15. Amplification of putative promoter regions

PCR was used to amplify the putative promoter regions of SPD_1746, SPD_1504, SPD_1503, SPD_1517, SPD_1994, and SPD_1495 (P*phrA*, P*nanA*, P*nanB*, P(SPD_1517), P(SPD_1994), and P(SPD_1495), respectively, P indicates promoter), and *gyrB* used as a control probe in EMSA, using the fluorescence primers in Table 2.9 and Prime STAR HS premix (Section 2.9). The DNA purification kit was used to purify the amplified PCR products and agarose gel electrophoresis was used to analyse the amplified products (Figure 3.27).

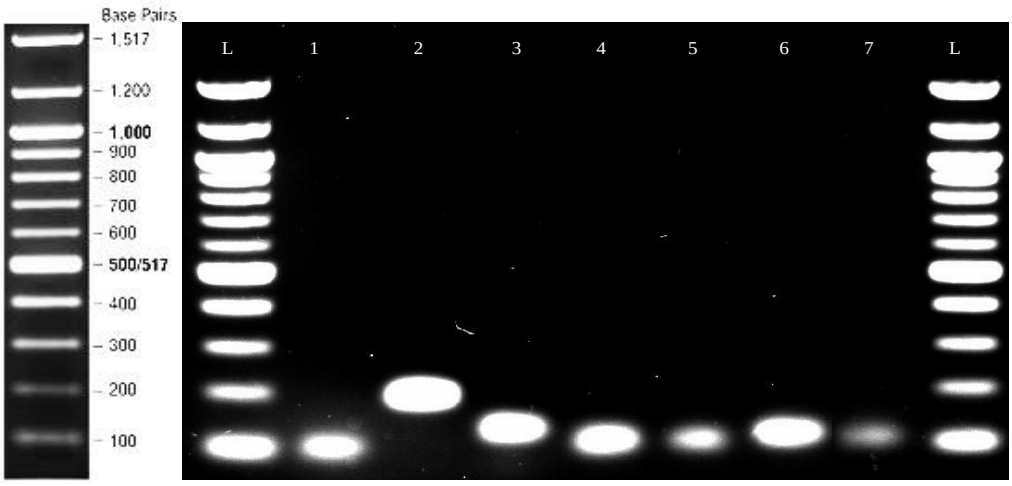


Figure 3.27. Agarose gel electrophoresis showing the promoter probe amplification of the target genes. L- 500 ng of 100 bp DNA ladder (NEB); Lane 1-7 show amplified products for P*phrA*, P*nanA*, P*nanB*, P(SPD_1517), P(SPD_1994), P(SPD_1495), and *gyrB*, respectively. Each lane contained between 40-90 ng DNA.

3.16. Electrophoretic mobility shift assay (EMSA)

3.16.1. TprA interacts directly with P(SPD_1746), P(SPD_1495), P(SPD_1517), and P(SPD_1994)

Direct interaction of TprA with the differentially expressed genes was analysed by EMSA. These genes showed the highest fold change in expression and their promoter is expected to control several genes in an operon organisation in the *tprA* regulon as determined by microarray analysis in the presence of galactose and mannose (Section D; Appendix 3 and 4). Fluorescently labelled DNA probes representing the putative

promoters of selected genes, including *phrA* (SPD_1746), a hypothetical protein upregulated in mannose (SPD_1517), sugar ABC transporter, sugar binding protein (SPD_1495), and a L-fucose phosphate aldolase upregulated in galactose (SPD_1994), were amplified, and a constant amount of each probe was mixed with increasing concentration of purified recombinant His-tagged TprA (0.1-0.5 μM). The binding reaction was analysed by non-denaturing PAGE. The results showed that in the presence of TprA, the mobility of labelled probes has changed compared to that of DNA alone, indicative of TprA interaction with these DNA probes (Figure 3.28), and the band shift increased with increasing TprA concentration. In lanes 1 and 2 in B and D of Figure 3.28 show the analysis of binding reaction in the absence or presence of up to 0.5 μM TprA *gyrB* was included as a negative control probe. The results showed that no change in mobility of *gyrB* could be detected. Thus, TprA binding was specific, as the recombinant protein could not bind to *gyrB*, which is not within TprA regulon.

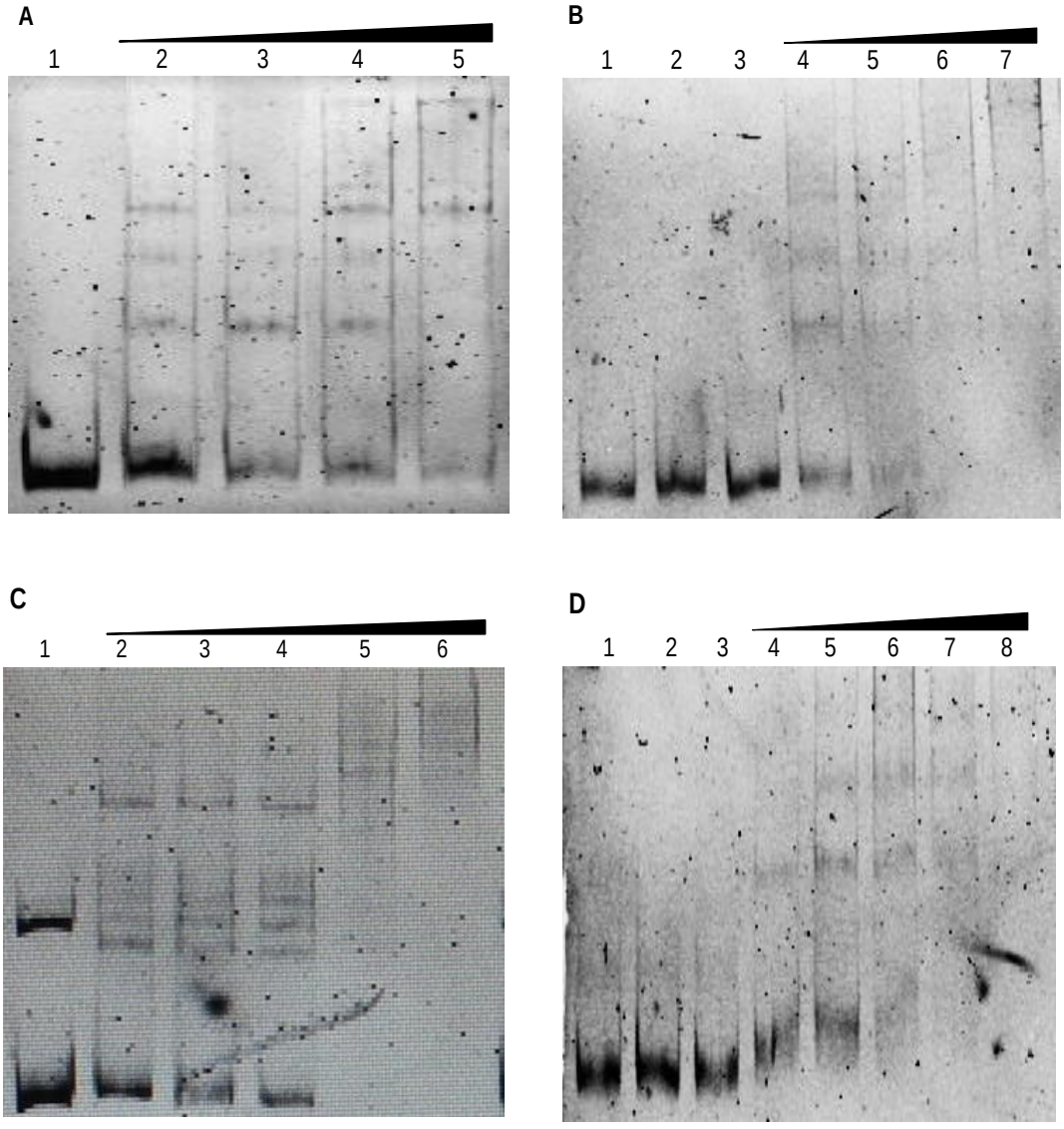


Figure 3.28. EMSA analysis showing the direct interaction of His-TprA with P*phrA* (**A**), PSPD_1495 (**B**), PSPD_1517 (**C**), and PSPD_1994 (**D**). **A**- In lane 1- P*phrA* probe alone (~50 ng); Lanes 2 to 5 P*phrA* probe with 0.1 to 0.4 μM His-TprA protein, respectively. Positive protein-DNA interactions are seen in lanes 2-5. **B**- Lane 1- P*gyrB* probe alone (~50 ng); Lane 2- P*gyrB* probe + 0.5 μM His-TprA; Lane 3- PSPD_1495 probe alone (~50 ng); Lanes 4 to 7 PSPD_1495 probe with 0.2 to 0.5 μM His- TprA protein, respectively. Positive protein-DNA interactions are seen in lanes 4-7. **C**- Lane 1- PSPD_1517 probe alone (~50 ng); Lanes 2 and 6 show PSPD_1517 probe with 0.1 to 0.5 μM His-TprA protein, respectively. Positive protein-DNA interactions are seen in lanes 2-6. **D**- Lane 1- P*gyrB* probe alone (~50 ng); Lane 2- P*gyrB* probe + 0.5 μM His-TprA; Lane 3- PSPD_1994 probe alone (~50 ng); Lanes 4 to 8 PSPD_1994 probe with 0.1 to 0.5 μM His- TprA protein, respectively. Positive protein-DNA interactions are seen in lanes 4-8.

3.16.2. TprA shows direct interaction with P*nanA* and P*nanB*

Neuraminidase has an important role in mucin utilisation, pneumococcal virulence and colonisation (Yesilkaya *et al.*, 2007; Manco *et al.*, 2006). In this study, it was shown that TprA plays an essential role in the production of neuraminidase activity in *S. pneumoniae* (Section B). TprA also plays a role in pneumococcal growth in the presence of mucin (Section B). In addition, the transcriptional *lacZ* fusions showed that TprA activates the expression of *nanA* in the presence of galactose (Section C). Therefore, TprA-P*nanA* interaction was further investigated by EMSA. I also included *nanB*, which is the second gene that codes for neuraminidase activity, in this analysis. Thus, increasing amount of purified His-tagged TprA (0.1-0.5 μM) were mixed with a constant amount of P*nanA* or P*nanB*, and the binding reactions were analysed. The results showed that TprA binds to both *nanA* and *nanB* promoters in a dose dependent manner and the band shift increased with increasing protein concentration (Figure 3.29).

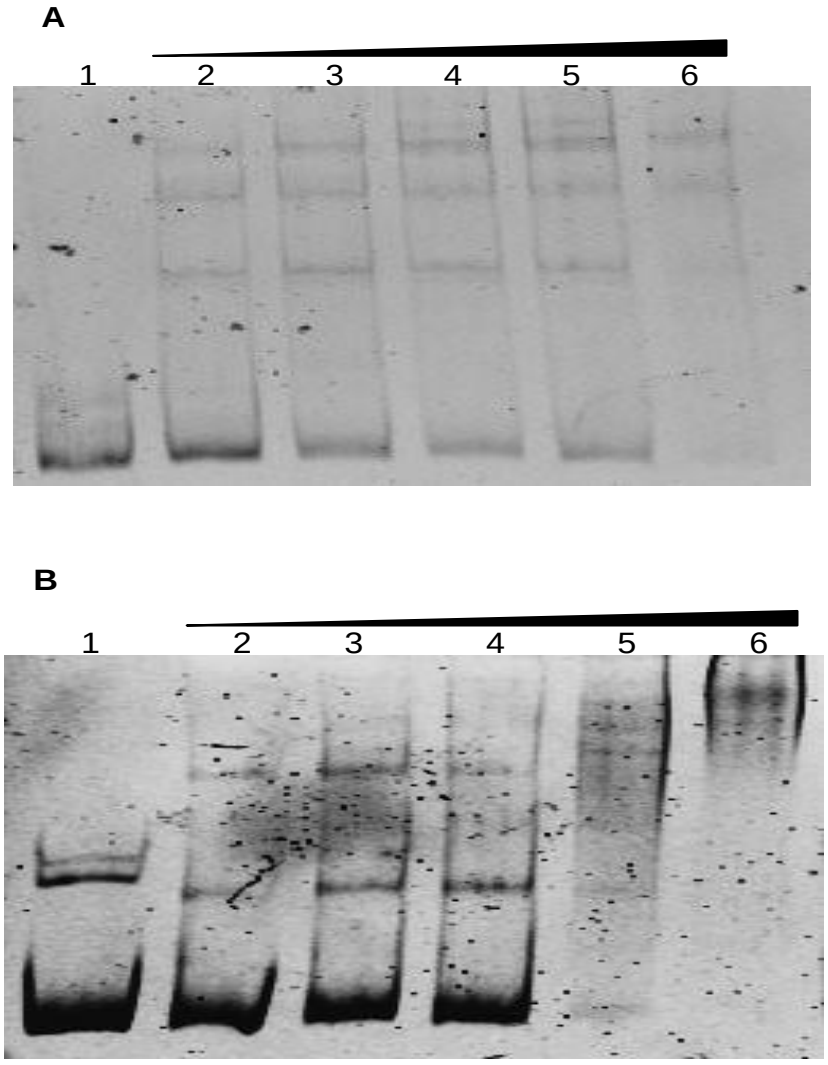


Figure 3.29. EMSA analysis showing the direct interaction of His-TprA with P*nanA* (**A**) and P*nanB* (**B**). **A**- Lane 1 shows P*nanA* probe alone (~30 ng); Lanes 2 to 6 P*nanA* probe with 0.1 to 0.5 µM His- TprA protein, respectively. Positive protein-DNA interactions are seen in lanes 2-6. **B**- Lane 1 P*nanB* probe alone (~50 ng); Lanes 2 to 6 P*nanB* probe with 0.1 to 0.5 µM His- TprA protein, respectively. Positive protein-DNA interactions are seen in lanes 2-6.

Section F: *In vivo* virulence studies

3.17. Virulence studies

The involvement of TprA/PhrA system in host derived sugar metabolism and neuraminidase activity led me to hypothesis that this system also plays a role in virulence. To test this hypothesis, I used a mouse model of pneumonia that develops after intranasal infection, systemic infection model initiated by intravenous injection, systemic infection model initiated by intraperitoneal injection. In addition, I tested TprA/PhrA's role in the chinchilla otitis media model in collaboration with Dr Luisa Hiller, Carnegie Mellon University.

3.17.1. Virulence test of pneumococcal strains by assessment of median survival time after intranasal infection

The mouse cohort infected with approximately 5×10^5 CFU/mouse wild type D39 strain survived (50 hr ± 5.1, n=10), whereas all the mice infected either with Δ*tprA* or Δ*phrA* survived the challenge ($p<0.0001$) (Figure 3.30 A). In addition, the complemented strains were as virulent as the wild type D39 strain as there was no difference in median survival time ($p>0.05$) (Figure 3.30 A). Consistent with the survival data, the numbers of D39 strain progressively increased at 24 h (Log_{10} 1 ± 0.7 CFU/ml, n=10) and 48 h (Log_{10} 3.1 ± 1.1 CFU/ml, n=10) post-infection, while no mutant pneumococci could be detected in the blood of mice that were infected by either with *ΔtprA* or Δ*phrA* at 24 and 48 h post infection (Figure 3.30 B).

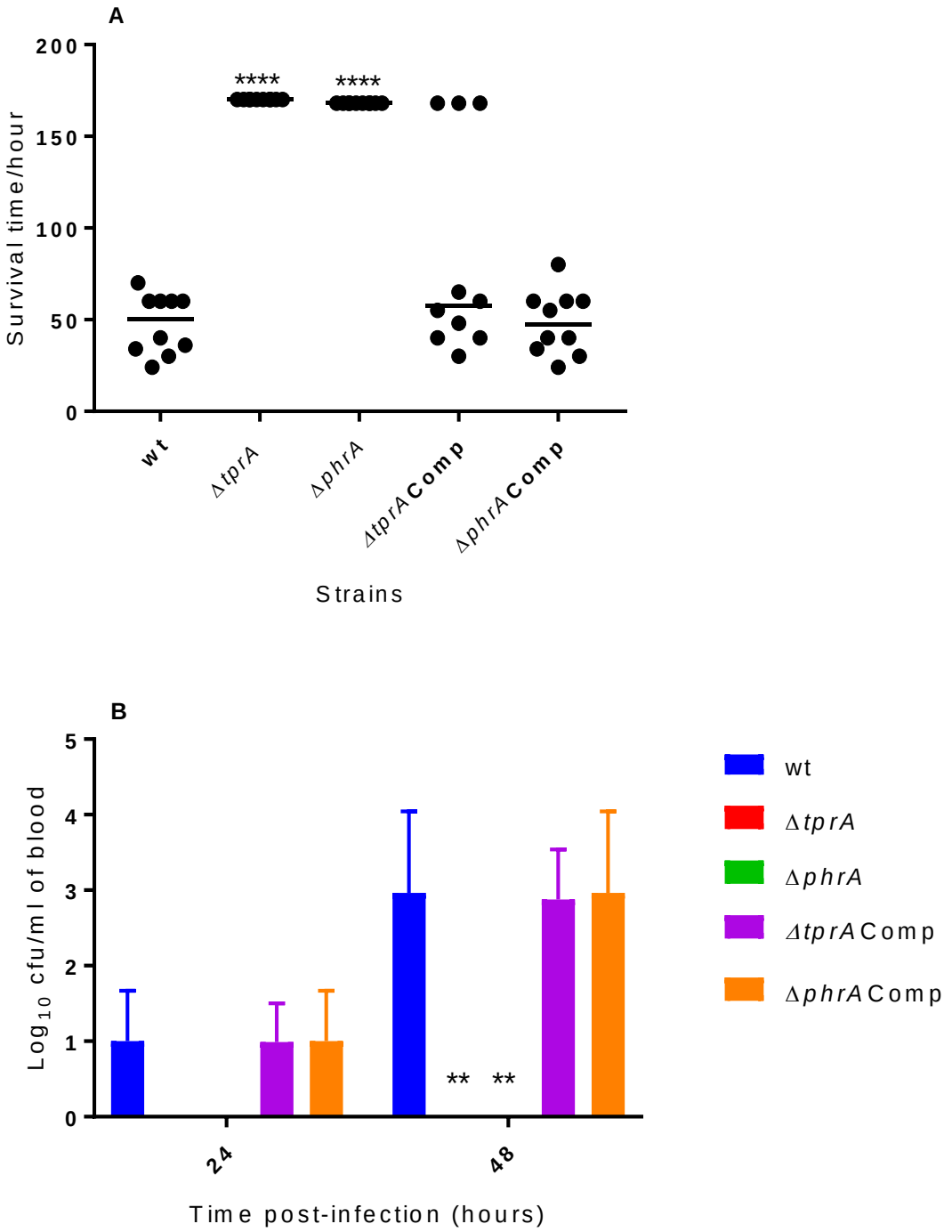


Figure 3.30. Virulence test of pneumococcal strains after intranasal infection using 5×10^5 CFU/mouse dose. (**A**) Survival time of infected mice. Each dot represents survival time of individual mouse, horizontal lines represent median survival time) (**B**) Progression of bacteraemia after intranasal infection with pneumococcal strains. Error bars indicate SEM. (**p<0.01, **** p<0.0001 relative to the wild type).

Next, the experiment was repeated with a high challenge dose, approximately 1.6×10^6 CFU/mouse. The wild type was still observed to be the most virulent, the Δ*phrA* infected cohort survived significantly longer (63 h ± 9.7, n=10) than the wild type infected cohort (48 h ± 4.5, n=10), and all the mice infected with Δ*tprA* survived the course of infection (168 hr ± 0.00, n=10) ($p<0.01$ and $p<0.0001$ for Δ*phrA* and Δ*tprA*, respectively) (Figure 3.31 A). Reintroduction of an intact copy of either *phrA* or *tprA* back to the respective mutants reconstituted the virulence as the cohorts infected either with Δ*phrA*Comp (55 h ± 2.7, n=10) or Δ*tprA*Comp (57.5 h ± 17.7, n=10) had similar median survival time as the wild type D39 infected cohort ($p>0.05$). These finding demonstrate that the TprA/PhrA system constitutes a novel pneumococcal virulence determinant.

As before, the blood samples were taken from the tail vein to determine the extent of dissemination across the cohorts challenged with the high dose (Figure 3.31 B). As expected, the numbers of D39 wild-type strain increased progressively at 24 h (Log_{10} 1.98 ± 0.57 CFU/ml, n=10) and 48 h (Log_{10} 4.2 ± 0.8 CFU/ml, n=10) post-infection. In contrast, no pneumococci could be recovered from the blood of Δ*tprA* infected cohort at these time points. Interestingly, despite being partially virulent, no Δ*phrA* could be detected from the blood. Complementation provided substantial rescue, in that at 24 hr and 48 hr post infection, the numbers of complemented mutants, Δ*tprA*Comp (Log_{10} 0.99 ± 0.5 CFU/ml and Log_{10} 2.88 ± 0.67 CFU/ml, respectively, n=10) and Δ*phrA*Comp (Log_{10} 1.1 ± 0.52 CFU/ml and Log_{10} 3.2 ± 0.6 CFU/ml, respectively, n=10), increased progressively (Figure 3.31 B). These findings suggest that dissemination and/or survival in the blood stream requires the TprA/PhrA system.

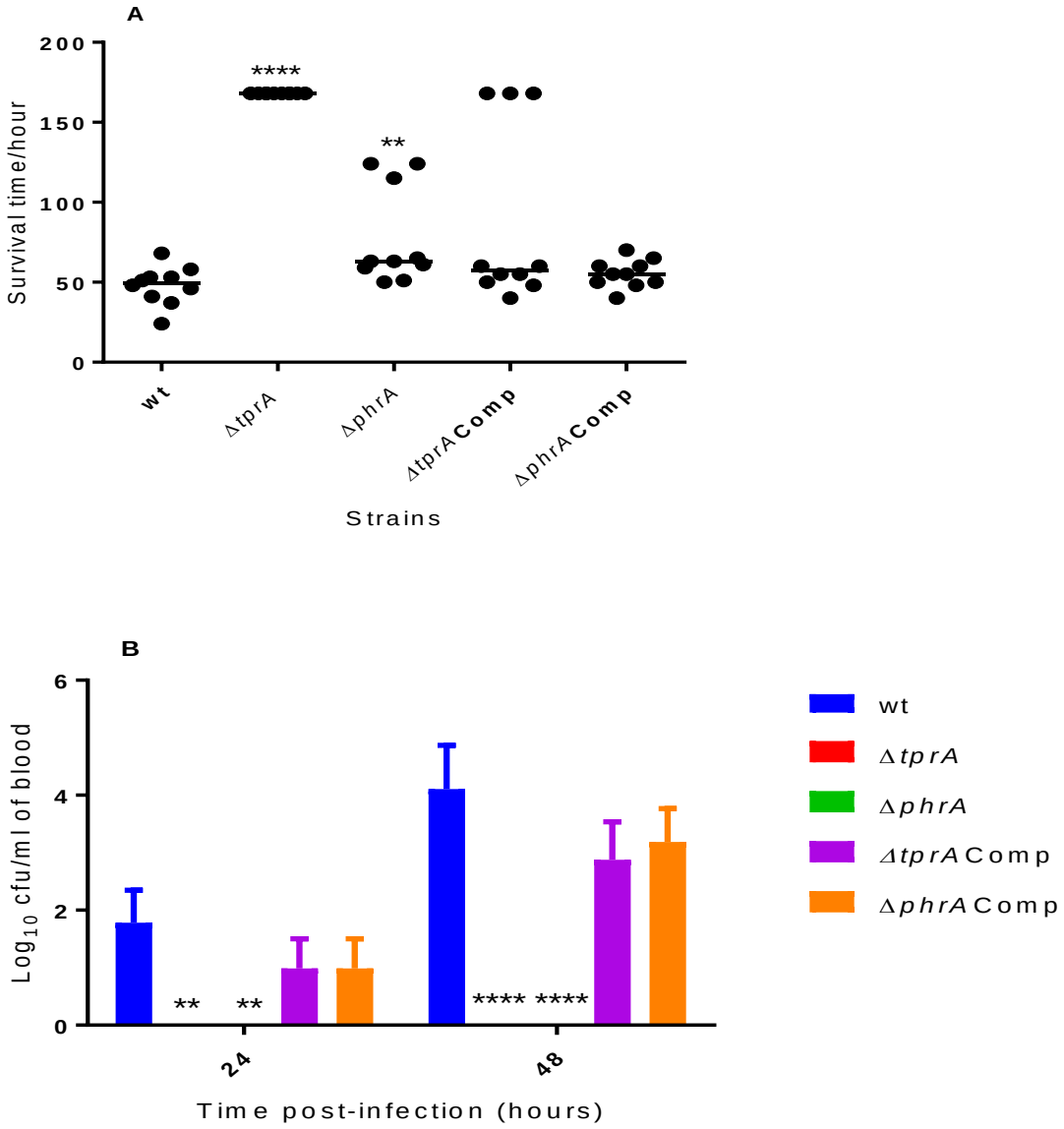


Figure 3.31. Virulence test of pneumococcal strains after intranasal infection using 1.6×10^6 CFU/mouse dose. (**A**) Survival time of mice infected with the pneumococcal strains. Each dot represents survival time of individual mouse and horizontal line represents median survival time (**B**) Progression of bacteraemia after infection. Error bars indicate SEM. (**p<0.01, **** p<0.0001 relative to the wild type).

The growth profiles of pneumococcal strains in respiratory tissues, nasopharynx and lung, were also determined after intranasal infection using 1.6×10^6 inoculum dose. Wild-type and mutants were detected in both tissues immediately post-inoculation. The numbers of wild-type D39 strain increased gradually at 24 h (Log_{10} 3.52 ± 0.12 CFU/mg, n=5) and

48 h (Log_{10} 4.02 ± 0.08 CFU/mg, n=5) post-infection in the nasopharynx (Figure 3.32 A). In contrast, no increase was observed in the numbers of either mutant. The difference in bacterial load between the wild type and the mutants at 48 h post infection was statistically significant in both tissues ($p<0.0001$). Moreover, in the lungs the Δ*tprA* was cleared after 24 hr post-infection, the numbers of Δ*phrA* did not change, and the number of wild type increased (Figure 3.32 B). These data suggest that TprA/PhrA aid growth in the nasopharynx and the lungs, and further that TprA is critical for survival in the lung.

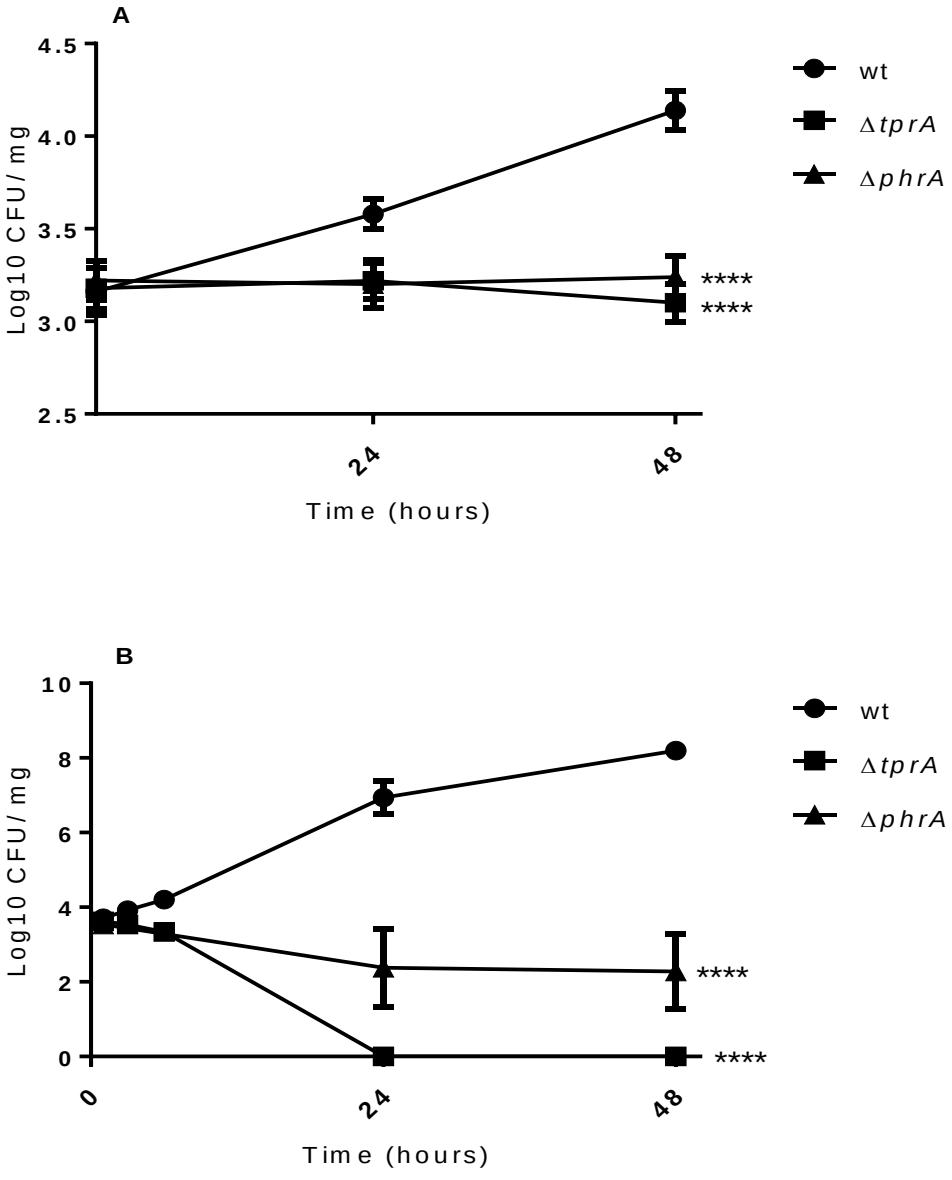


Figure 3.32. Survival of pneumococcal strains in nasopharynx (**A**) and lungs (**B**). Mice were infected intranasally with approximately 1.6×10^6 CFU/mouse. Each datum point was collected from 5 animals. Error bars indicate SEM, (**** $p<0.0001$ significantly different compared to the wild type D39).

3.17.2. Systemic infection model initiated by intravenous injection

The *in vitro* data suggest that TprA/PhrA should not play a key role in an intravenous model of inoculation, given higher levels of glucose in the blood. To test this, I compared the wild type, Δ*tprA* and Δ*phrA* in a systemic infection model by administering the strains through dorsal tail vein to determine if the strains are attenuated in their growth in blood. The results showed that all the mice infected either with Δ*tprA* or Δ*phrA* survived the challenge, whereas 5 out of 6 mice infected with D39 died (median survival: 52 hr ± 20.2, n=6) (Figure 3.33 A). Moreover, the CFU/ml of D39 at 24- and 48 hr post infection was Log_{10} 3.25 ± 0.56 (n=6) and Log_{10} 5.95 ± 1.36 (n=6), respectively, and Δ*tprA* and Δ*phrA* could not be detected in the blood at these time points (Figure 3.33 B). These data suggest that TprA/PhrA do influence pneumococci in the blood stream, suggesting that these genes are expressed in the blood.

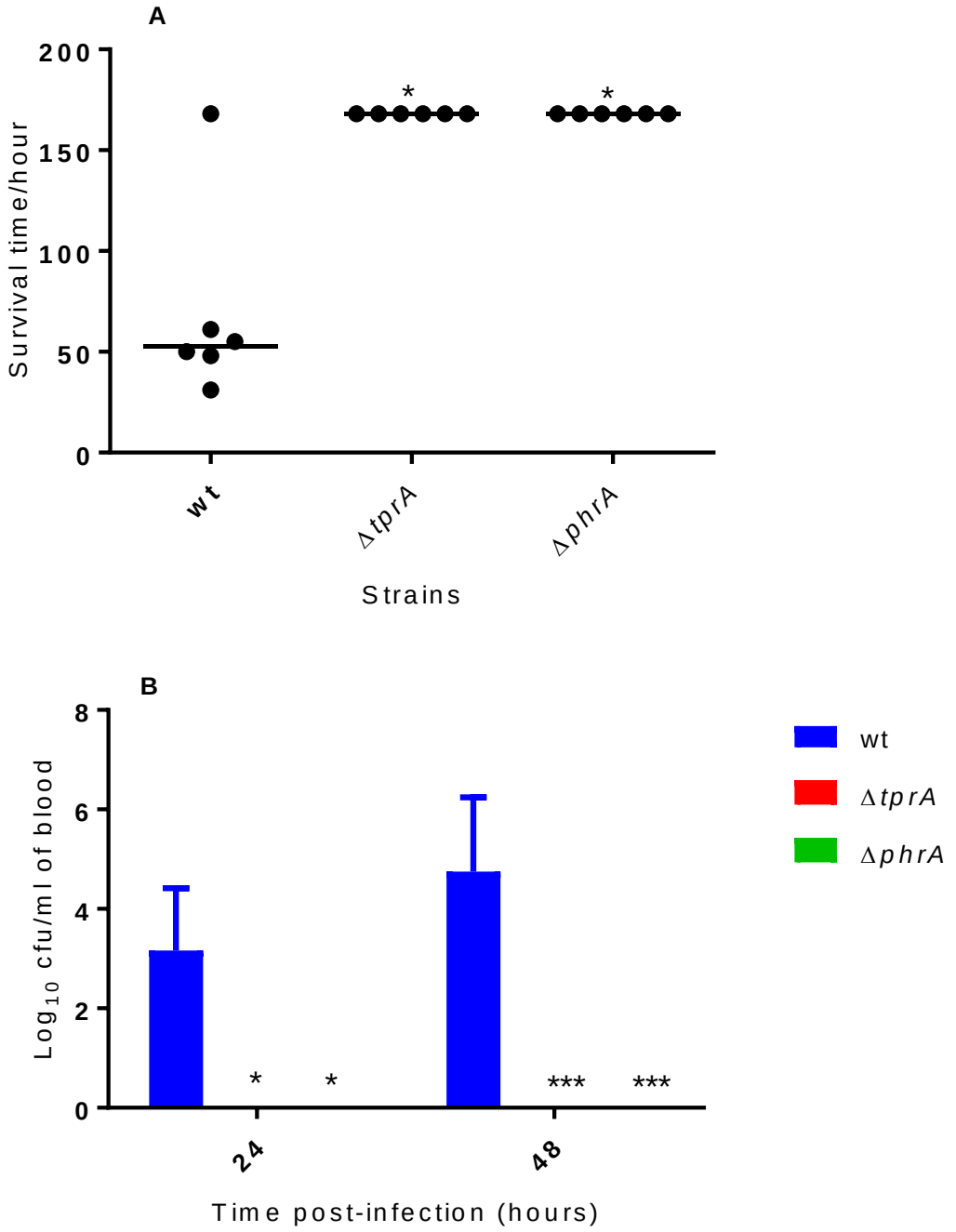


Figure 3.33. A= Median survival time of mice injected intravenously through dorsal tail vein with 1×10^6 pneumococci, the median survival times were recorded after intravenous injection of 6 mice for each strain with *tprA* and *phrA* mutants and wild type D39 for 7 days. There was significant difference in the median survived time of mice injected with the mutants of *tprA* and *phrA* compared to the wild type D39 (*p<0.05). **B**= The growth of wild type D39, *tprA*, and *phrA* mutants in the blood of mice. There was significant difference in blood counts of mutants compared with the wild type D39 at 24 hr (*p<0.05) and 48 hr post infection (***p<0.001).

3.17.3. Systemic infection model initiated by intraperitoneal injection

In addition to direct administration into the blood, I tested *ΔtprA* and *ΔphrA* in intraperitoneal model. In intraperitoneal injection, the median survival time of the wild type infected mice was 20 h ± 0.6 (n=3), while the mice that received Δ*tprA* or Δ*phrA* survived the course of infection (120 hr ± 0.00, n=3, p<0.0001) (Figure 3.34). This data provides further support that TprA/PhrA system is required for pneumococcal survival not only in respiratory tract but also in blood and peritoneum.

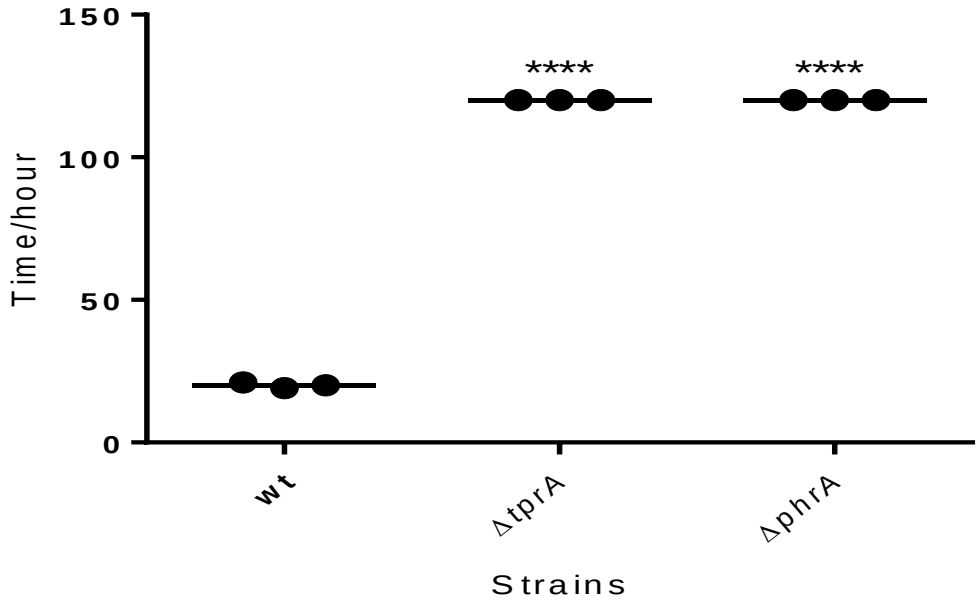


Figure 3.34. Median survival time of mice injected intraperitonealy with the pneumococcal strains. The median survival times were recorded after intraperitoneal injection of 3 mice for each strain for 5 days. There was significant difference in the median survived time of mice injected with Δ*tprA* and Δ*phrA* compared to the wild type D39 (****p<0.0001).

3.17.4. Chinchilla otitis media model

Streptococcus pneumoniae is one of the biggest causes of otitis media (Melegaro *et al.*, 2006). To test the role of TprA/PhrA in the middle ear, composed of a complex sugar environment, the chinchilla otitis media model was employed in collaboration with Dr Luisa Hiller, Department of Biological Sciences, Carnegie Mellon University, USA. To

this end, wild-type D39 and Δ*tprA* strains were transbullarly inoculated (100 CFUs/ear) in cohorts of 10 animals each. Animals were monitored daily for duration of 10 days during which time they either succumbed to disease or were euthanized upon reaching extreme morbidity. When compared to wild type, the Δ*tprA* mutant caused significantly less mortality ($p<0.001$). Only one out of ten animals inoculated with wild type survived, while nine out of ten animals inoculated with the mutant survived. Further, virulence was partially restored in animals inoculated with *tprA* complement strain Δ*tprA*Comp, where five animals survived (Figure 3.35). The combined animal studies suggest that TprA/PhrA play an important role in the growth of pneumococcus in the nasopharynx, the middle ear, the lungs, and the blood.

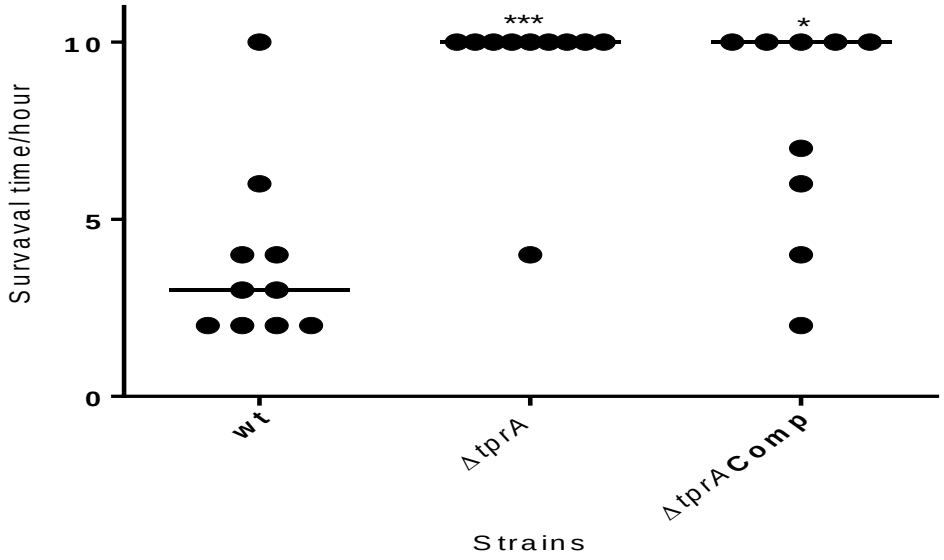


Figure 3.35. Analysis of pneumococcal strains in chinchilla otitis media model. Chinchillas were injected through ear canal either with D39 (40 CFU/ear), Δ*tprA* (85 CFU/ear), or Δ*tprA*Comp (24 CFU/ear). Each dot represents survival time of individual mouse. Horizontal line represents median survival time. (*$p<0.05$, and ***$P<0.001$).

Section G: Modulation of TprA/PhrA quorum sensing system by linear imprinted copolymers with anti-infective properties

In the current study, it was shown that the TprA/PhrA system is a major virulence determinant. It is important for pneumococcal growth on galactose and mannose as well as on mucin (Section B), the primary carbon sources for pneumococcus *in vivo* (Robb *et al.*, 2017; Kahya *et al.*, 2017).The PhrA peptide expression is induced both by galactose and mannose (Section C). Independent deletion mutation of TprA and PhrA both abrogates pneumococcal virulence in a mouse model of pneumonia and septicaemia, as well as in a chinchilla model of otitis media (Section F). Thus, TprA/PhrA system is a potential drug target. Therefore, it was suggested that interference with the binding of PhrA with TprA would block the phenotypic manifestations of the TprA/PhrA QS system. The inducer peptide (PhrA10) was used to create the LMIP as described in section 2.2. The C-terminal end of this peptide with SNGLDVGKAD sequence was used as template for LMIP preparation. The LMIP was prepared by solid-phase imprinting by Dr. Antonio Guerreiro, Dr. Elena Piletska, and Proffessior Sergey Piletsky in the Chemistry Department, University of Leicester (Motib *et al.*, 2017). In this study, it was shown that the first example of QS control in Gram-positive bacteria by using low molecular weight linear molecularly imprinted polymers, which can both bind to PhrA peptide and also block the TprA target.

3.18. Evaluation of LMIP in cell free system

This work has been done in collaboration with Dr Antonio Guerreiro. Affinity of LMIP to PhrA10 peptide was studied by surface plasmon resonance (SPR) (Figure 3.36**)**. Results indicate PhrA10 binds LIMP in a dose-dependent and sequence-specific manner. Dissociation constant (K_D) was estimated to be 8.16 µM (Figure 3.36 A). Conversely, the K_D of the interaction between LMIP and a scrambled peptide with the same amino acids (DAKGVDLGNS) and in similar concentration range was much higher >0.13 mM though practically no binding was observed (Figure 3.36 B). Effect of LMIP/PhrA10 binding on to TprA receptor was assessed by SPR. For this, TprA was immobilized on a SPR chip then PhrA10 (ranging from 0.2 to 50 µM) injected in order to establish the minimal concentration of PhrA10 necessary for binding to occur to TprA, which was 25 µM. PhrA10 at 25 µM (Figure 3.36 C) was then co-injected on the TprA receptor with LMIP

at a concentration of 100 nM as used in the microbiological inhibition assays. LMIP significantly altered the binding behavior of PhrA10 to TprA with 84% reduction in binding response and reducing the dissociation rate of the complex, resulting in irreversible binding. No measurable interaction was observed upon injection of 100 nM LMIP onto immobilised TprA in absence of PhrA10.

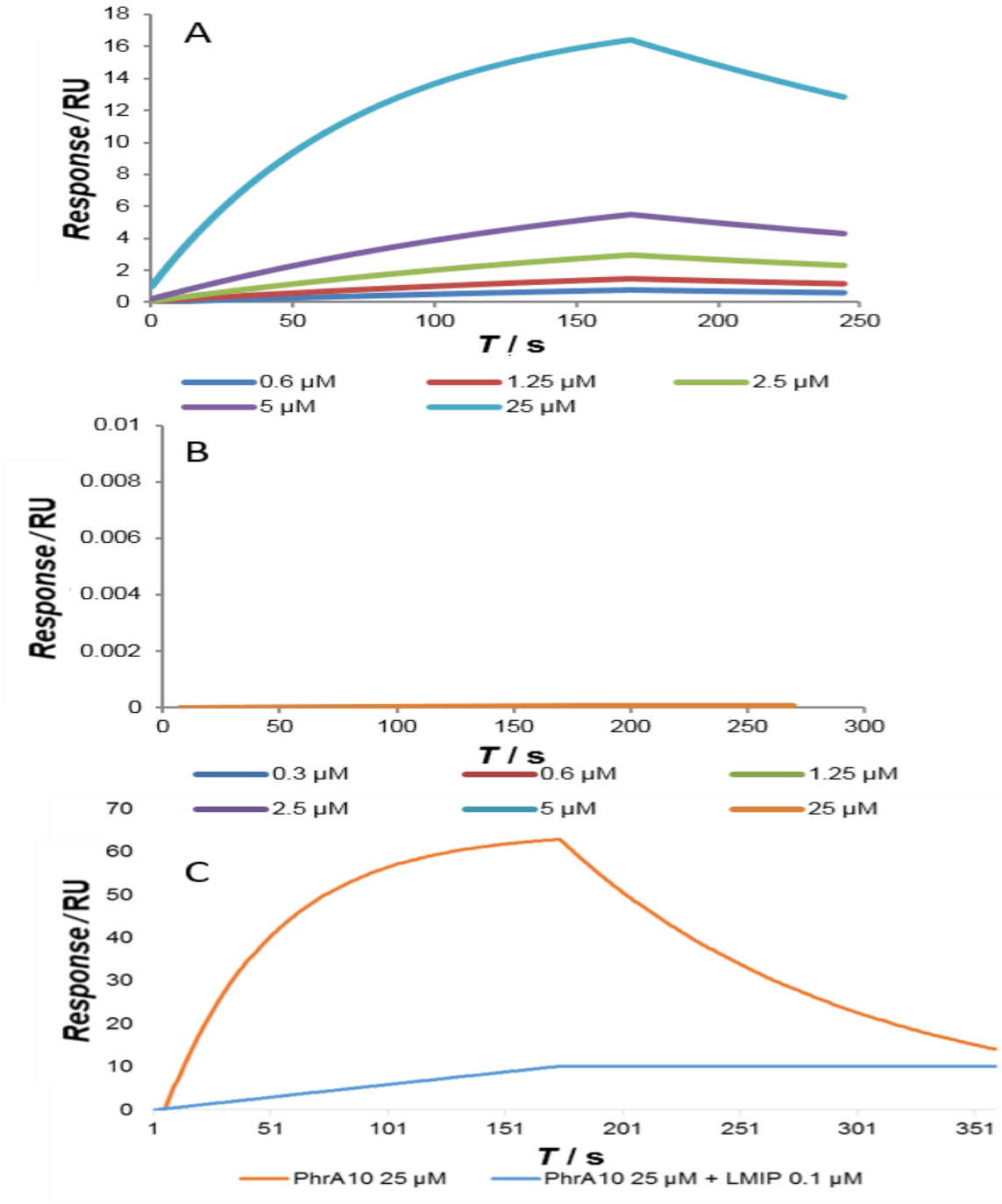


Figure 3.36. SPR sensorgrams. (**A**) Injections of PhrA10 onto immobilized LMIP, (**B**) injections of scrambled PhrA10 onto LMIP. (**C**) PhrA10 at 25 µM injected onto immobilized TprA (curve PhrA10) and PhrA10 (at 25 µM) injected alongside LMIP onto immobilized TprA. This analysis has been done in collaboration with Dr Antonio Guerreiro (Motib *et al.*, 2017).

3.19. LMIP inhibits PhrA10 induced β-galactosidase activity

In order to demonstrate the specific binding and consequent blocking of TprA receptor by the LMIP, LMIP was firstly tested to determine if it would attenuate the induction of β-galactosidase activity driven by the *phrA* promoter (P*phrA*) using P*phrA-lacZ-*Δ*phrA* strain, a strain deficient in endogenous production of PhrA in the presence of galactose, for which the TprA/PhrA system is required (Section C). This was done to eliminate interference by endogenously produced PhrA and increase the chances of any discernible effect of LMIP on transcription. In this study, it was determined that 10 nM was the lowest concentration of PhrA10, which significantly induce the β-galactosidase activity of P*phrA-lacZ-*Δ*phrA* strain on galactose. Addition of 10 nM PhrA10 resulted in approximately 5.7-fold increase in the activity relative to a control that was not induced with the peptide (Figure 3.37**)**. In contrast, in the presence of 100 nM LMIP the β-galactosidase activity was still higher than the control (no PhrA10) but was significantly lower, by 1.8-fold, compared to induction by PhrA10 alone ($p<0.001$), demonstrating the efficacy of LIMP in inhibiting promoter activation by PhrA10.

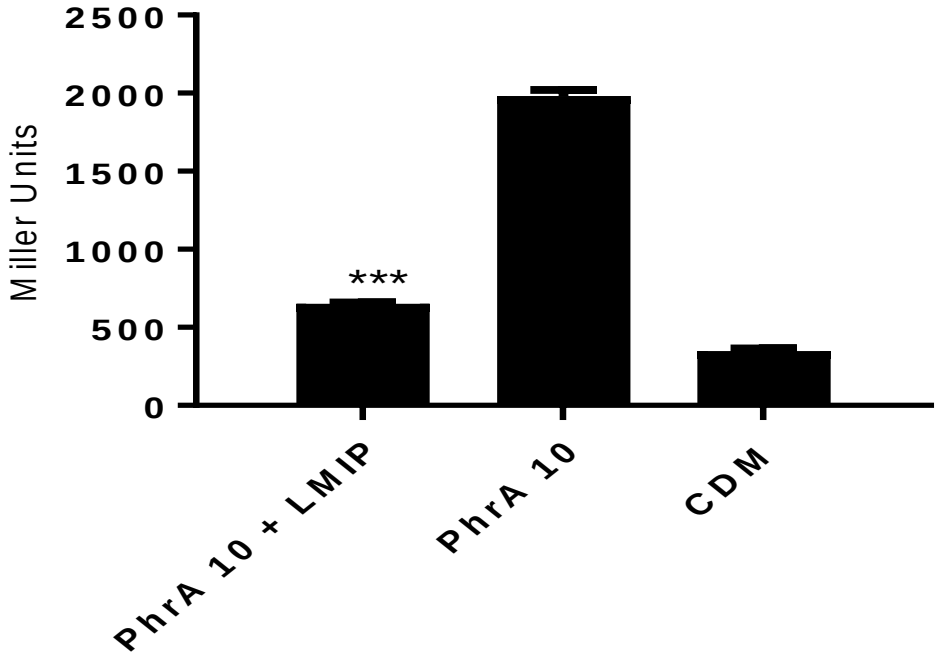


Figure 3.37. Analysis of *phrA* expression (in Miller units) using *LacZ* reporter assay in the presence of LMIP and PhrA10. LMIP inhibits β-galactosidase activity driven from the promoter of *phrA* (P*phrA*) in Δ*phrA*, a strain deficient in endogenous production of PhrA. P*phrA* was induced with 10 nM synthetic PhrA10 peptide or with PhrA10 plus 100 nM LMIP, and galactosidase activity without PhrA in CDM supplemented with 55 mM galactose was also included. The activity of β-galactosidase under regulation of P*phrA* is expressed in nmol *p*-nitrophenol/min/ml. Values are average of four independent experiments each with three replicates. ± indicates standard error of means (SEM), (*** $p<0.001$).

3.20. Growth of *S. pneumoniae* in the presence of LMIP

To demonstrate the growth inhibitory effect of LMIP, wild-type strain was grown in CDM supplemented with galactose, in presence of varying concentration of LMIP (25-100 nM) (Figure 3.38 A). Growth yield was significantly lower at all the concentrations tested and growth rate was significantly decreased at 100 nM LMIP compared to the control in absence of LMIP. However, LMIP had no influence on growth on glucose (Figure 3.38 B).

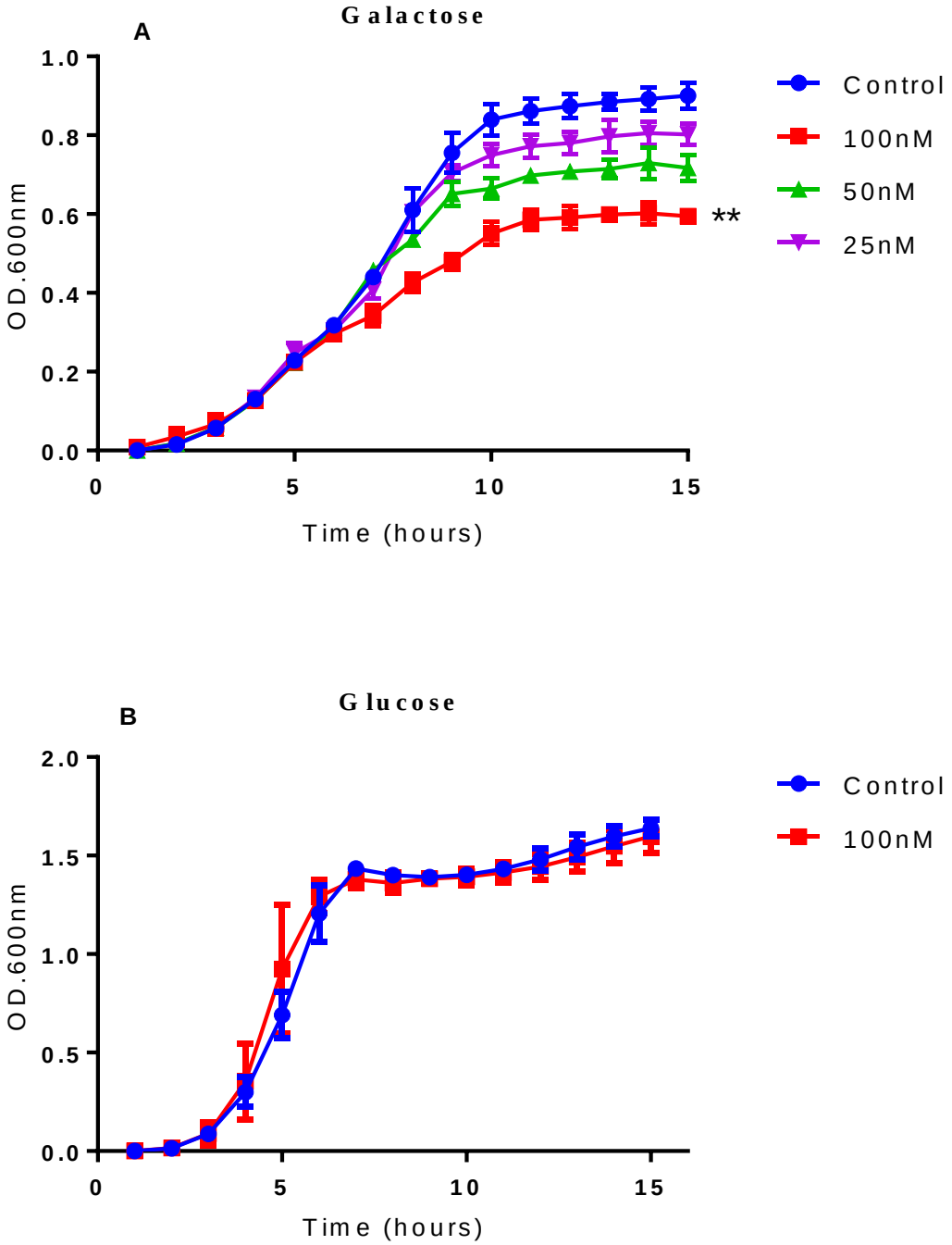


Figure 3.38. Growth curves of *Streptococcus pneumoniae* D39 grown microaerobically in CDM supplemented with 55mM galactose or glucose in the presence of 25-100 nm LIMP. 25-100 nM LMIP attenuates *S. pneumoniae* growth in medium supplemented with galactose (**A**) but not with glucose (**B**). Control cultures did not receive LMIP. Experiment was repeated using 3 replicates of 3 independent biological samples; '±' indicates standard error of means (SEM). Significant differences were shown by comparing the growth rates in the presence of each concentration of LIMP to the control. (**p<0.01).

3.21. Influence of LMIP on pneumococcal virulence *in vitro* and *in vivo*

***In vitro* evaluation.** TprA was found to regulate neuraminidase (Section C), thus its activity was assessed in pneumococcal cultures grown on galactose supplemented or not with 100 nM LMIP. Neuraminidase is responsible for cleaving complex host sugars, playing an important role in pneumococcal colonisation and invasiveness (Yesilkaya *et al.*, 2007; Manco *et al.*, 2006). There was a significant decrease in neuraminidase activity (26.7 ± 1.5 U) in the presence of LMIP compared to the control that did not contain LMIP (53.3 ± 3.0 U) ($p<0.001$) (Figure 3.39), indicating that LMIP by blocking TprA abrogates neuraminidase activity, hence virulence.

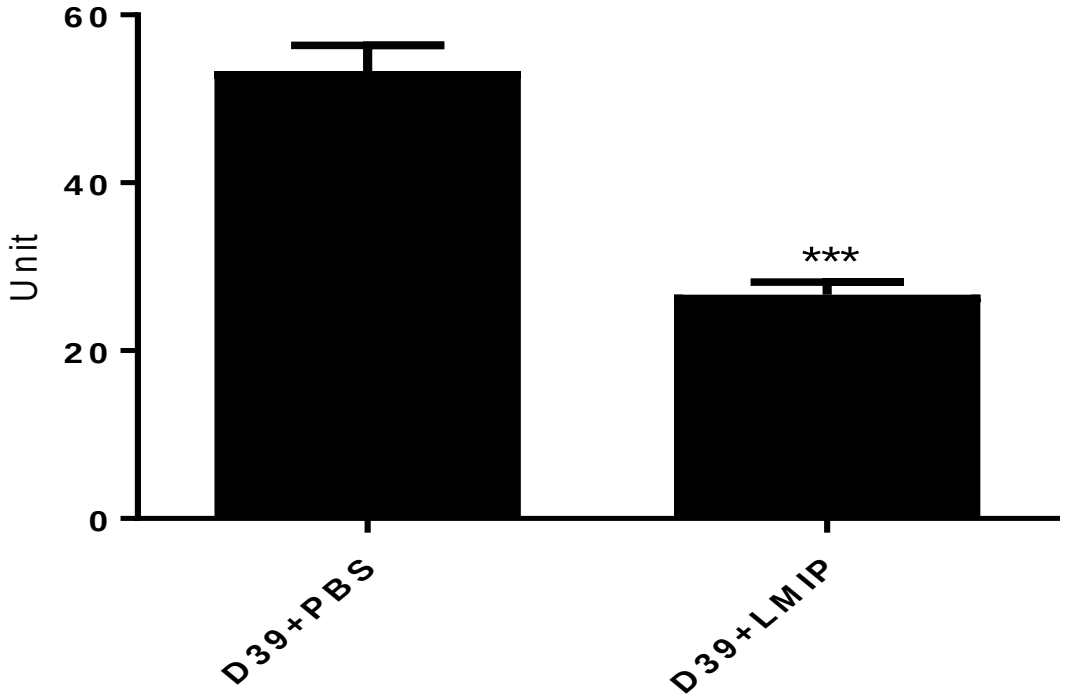


Figure 3.39. Determination of neuraminidase activity of *S. pneumoniae* strain D39 in the presence of LIMP. The cell lysate of the pneumococcus was prepared when the bacterium was grown in CDM supplemented with 55 mM galactose in the presence of 100 nm LIMP or PBS. One-unit neuraminidase activity was defined as nmol pNP released/min/µg of protein. Experiment was repeated using 3 replicates of 3 independent biological samples, error bar indicates SEM. (***$p<0.001$).

***In vivo* evaluation.** Before testing LMIP impact in pneumococcal infection, the toxicity of LMIP was tested in mouse using mucosal and intraperitoneal routes by administering

100 nM LMIP. The preliminary experiments did not show any toxicity of LMIP as measured by observation of signs of disease. Thus, LMIP impact on virulence was further confirmed by *in vivo* evaluation against lethal pneumococcal challenge in a mouse model of pneumococcal pneumonia that progresses to bacteremia (Figure 3.40). Three groups were infected intranasally either with 7.5×10^5 pneumococci/mouse in 50 µL PBS (control), or with 1.4×10^6/mouse suspended in the same volume containing 100 nM LMIP solution in PBS. The third group was infected with 1.4×10^6/mouse, in the presence of 100 nM LMIP targeting the scrambled 10 aa peptide (LMIPscramble). The results showed that despite receiving a higher infective dose the cohort that received the inoculum together with PhrA10 LMIP had significantly lower blood counts (Log10 0.61 ± 0.28 CFU mL^{-1}, n=15) than the cohort that had received the dose in PBS (Log10 2.96 ± 0.54 CFU mL^{-1}, n=13) 24 hr post-infection ($p<0.01$). There was no difference between the number of pneumococci in control and LMIPscramble groups (Log10 2.49 ± 1.04 CFU mL^{-1}, n=5) ($p>0.05$) (Figure 3.40 B). This shows that LMIP prevents the translocation of pneumococci from lungs to blood. Consistent with reduction in bacterial blood count, the cohort that received LMIP survived significantly longer (65 hr ± 15.1, n=13) than control (37 hr ± 8.4, n=15) ($p<0.0001$), whereas there was no difference between LMIPscramble group (44 hr ± 4.6, n=5) and the control ($p>0.05$) (Figure 3.40 A). In conclusion, it was found that LMIP interfere with the QS signals selectively, and curtail the phenotypic manifestation of this system in *in vitro* and *in vivo* assays. These results indicate that LMIP can be developed as an effective anti-infective against *S. pneumoniae*.

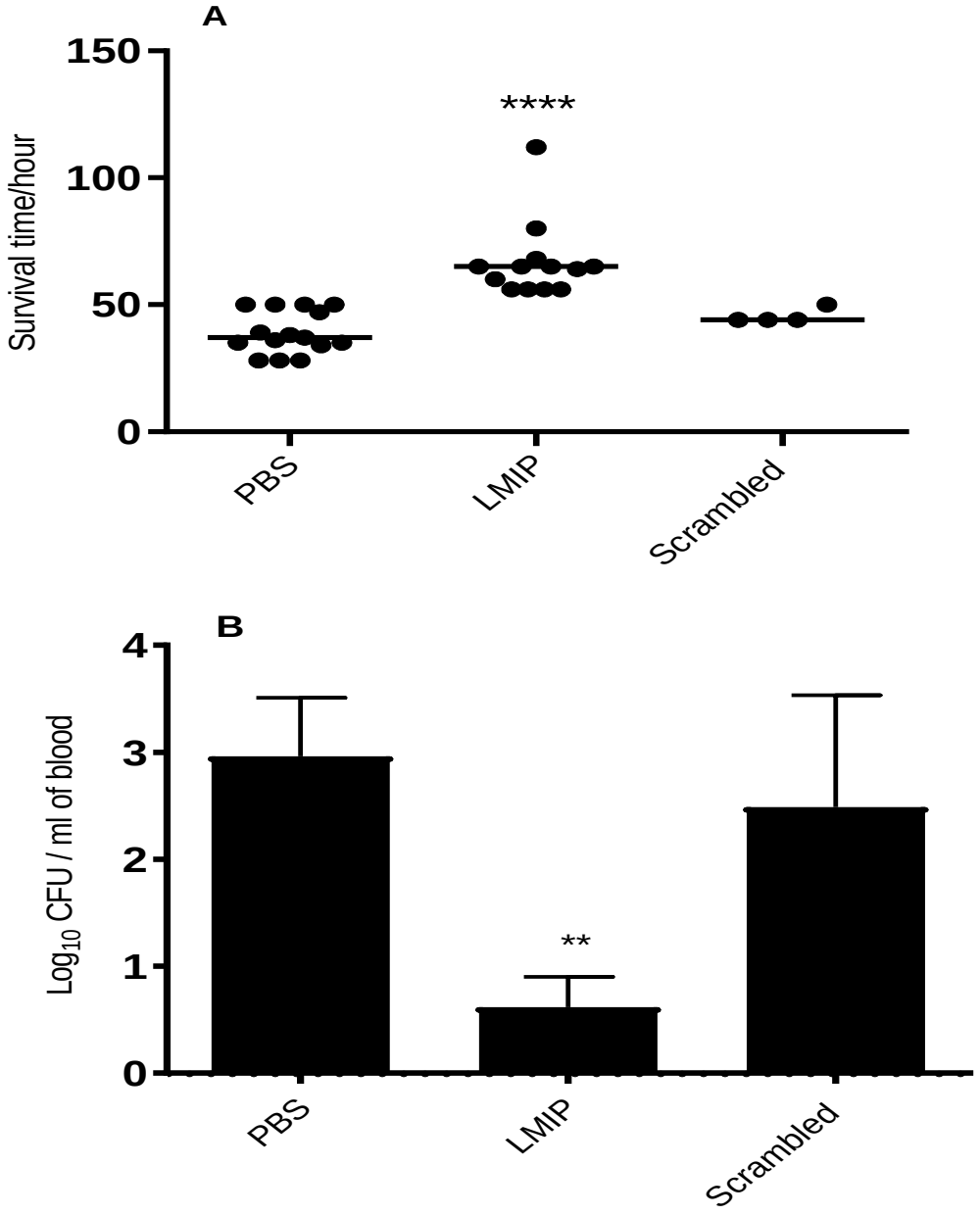


Figure 3.40. *In vivo* evaluation of LIMP against lethal pneumococcal challenge. Three groups of mice were infected intranasally either with 7.5×10^5 pneumococci/mouse in 50 µL PBS (control), or with 1.4×10^6/mouse suspended in the same volume containing 100 nM LMIP solution in PBS. The third group was infected with 1.4×10^6/mouse, in the presence of 100 nM LMIP targeting the scrambled 10 aa peptides (LMIP scramble). (**A**) Survival time of infected mice (Each dot represents survival time of individual mouse, horizontal line represents median survival time), and (**B**) Progression of bacteraemia which was shown after infection. Error bar indicate SEM, (**p<0.01, **** p<0.0001).

Chapter 4. Discussion

Streptococcus pneumoniae colonises the nasopharynx asymptomatically but also it causes important diseases such as pneumonia, bacteremia, meningitis, and otitis media (Barthelson *et al.*, 1998; Kadioglu *et al.*, 2002). It causes more deaths than other invasive bacterial species (World Health Organization, 2014). The pneumococcal infections are important public health threats and these infections are compounded by rising trend of antibiotic tolerant and resistant pneumococcal strains (Giedraitiene *et al.*, 2011).

One approach to overcome pneumococcal disease is to define critical metabolic networks which are required for virulence and target these pathways pharmaceutically. Sugar metabolism plays an essential role for pneumococcal energetics. *S. pneumoniae* is a fermentative microbe that relays on sugar metabolism as energy source (Paixão *et al.*, 2015). This is also evident from the fact that more than 30% of transporters in the pneumococcal genome are involved in the carbohydrate uptake, and the microbe has ability to ferment at least 32 different sugars (Tettelin *et al.*, 2001; Bidossi *et al.*, 2012). Carbohydrate metabolism plays an essential role in pneumococcal pathogenesis as it provides the bacterium with crucial nutrients during infection (Shelburne *et al.*, 2008b). While glucose is the preferred energy source *in vitro* (Carvalho *et al.*, 2011), the concentration of this sugar in the human respiratory tract is below 1 mM (Philips *et al.*, 2003). Therefore, in the nasopharynx, the pneumococcus depends on host glycoproteins as energy source (Paixão *et al.*, 2015; Yesilkaya *et al.*, 2007). Mucin is one of the major host glycoproteins (Yesilkaya *et al.*, 2007). It covers the surfaces of respiratory epithelial cells. Ninty percent of dry weight of mucin glycoproteins is composed of carbohydrates, including *N*-acetylglucosamine (GlcNAc), *N*-acetygalactosamine (GalNAc), *N*-acetylneuraminic acid (NeuNAc), galactose (Gal), and fucose (Fuc). The sugar residues are attached to the protein core through a *N*-acetylgalactosamine moiety (Sheehan *et al.*, 1991; Rose and Voynow, 2006; Terra *et al.*, 2010). The pneumococcus can utilise these sugars from mucin to enhance its ability for colonisation, adherence and biofilm formation, and for growth (King, 2010).

Among these host derived sugars, galactose and galactosamine are probably the most noteworthy sugars, as they are the most abundant sugars found in mucin and have a major impact on pneumococcal metabolism, colonisation and virulence (Terra *et al.*, 2010;

Paixão *et al.*, 2015). The pneumococcus is able to release and utilise mucin galactose via the activities of at least two galactosidases, BgaC and BgaA (Terra *et al.*, 2010). It has also been shown that pneumococcus catabolises galactose via the tagatose and the Leloir pathway (Von Wright and Axelsson, 2012; Paixão *et al.*, 2015), and both pathways are required for pneumococcal growth on galactose, colonisation and virulence (Paixão *et al.*, 2015). Therefore, it can be concluded that the pneumococcus is evolved to utilise galactose and the utilisation of this sugar has a major impact on pneumococcal metabolism and *in vivo* survival. The question needs addressing is that how the pneumococcus regulates its galactose metabolism. This study aimed to find an answer to this question.

The mechanism of adaptation in tissues with different sugar composition is not fully understood but is very likely that multiple regulators are involved, including quorum sensing systems (Zheng *et al.*, 2011). A wide range of bacterial phenotypes are regulated by QS systems, including growth, biofilm formation, and virulence factor expression (Rutherford and Bassler, 2012; Zhu *et al.*, 2002). In addition, it was also shown that QS could coordinate other bacterial cellular functions, include DNA replication, cell division, and carbon metabolism (Schuster *et al.*, 2003; Wagner *et al*., 2004).

These communication systems depend on the secretion and recognition of signalling molecules in a cell density dependent manner (Bouillaut *et al.*, 2008), and in Gram positive bacteria these signal molecules are made out of peptides. Examples of Gram positive QS systems include RRNPP family regulators. The RRNPP family is composed of Rgg (regulator gene of glucosyltransferases), Rap (aspartyl phosphate phosphatases), NPrR (neutral protease regulator), PlcR (Phospholipase C regulator) and PrgX (pheromone responsive gene regulator) family regulators. The genes that often encode for the RRNPP family proteins and their signaling pro-peptides are located in the bacterial chromosomes or in the plasmids. The pro-signaling peptides are exported out of bacterial cell and processed to the mature signaling peptide by proteases. Then this mature peptide is transported in to the cell via oligopeptide permease (OPP) and subsequently interacts with RRNPP receptor protein (Gohar *et al.*, 2008; Pottathil and Lazazzera, 2003).

In this study, the quorum sensing TprA/PhrA system of *S. pneumoniae* has been studied. It was found that it is involved in galactose and mannose metabolism, required for

transcriptional activation of neuraminidase A activity, and is essential for pneumococcal survival and virulence in a mouse model of pneumonia and bacteraemia, as well as in chinchilla otitis media model. It was also demonstrated that this system can be targeted by LMIP to modulate pneumococcal survival on host-derived sugars, and its neuraminidase activity. LMIP were found to interfere with the binding of PhrA with TprA to block the phenotypic manifestations of the TprA/PhrA QS system both *in vitro* and *in vivo*.

4.1. TprA/PhrA quorum sensing system is important for pneumococcal sugar metabolism, survival, and virulence

In order to identify the functional role of TprA/PhrA quorum sensing system in *S. pneumoniae,* the manipulation of *tprA* and *phrA* were required. Insertion deletion mutagenesis was used for deletion of target gene and insertion of antibiotic resistance marker by SOEing PCR mutagenesis (Horton *et al.*, 1995). This method is very effective and rapid because it does not require cloning, hence less laborious (Horton *et al.*, 1995). SOEing PCR mutagenesis is established by two steps of PCR to form the SOEing product, which is then transformed to the wild type strain D39 to allow insertion-deletion mutagenesis. *S. pneumoniae* has natural ability to take the foreign DNA, defined as competence state, which relies on cell density or growth phase (Lee *et al.*, 1998; Morrison and Baker, 1979). The competence state develops in early exponential phase of pneumococcal growth, in this time point, the pneumococcus can readily uptake and integrate the foreign DNA at a homologous site within the genomic DNA (Lacks, 1997).

In order to rule out the polar effect of mutations, genetic complementation was used for phenotypic restoration of the mutants. The plasmid pCEP (Guiral *et al.*, 2006) was used for complementation of Δ*tprA* and Δ*phrA*. The results showed the successful complementation as the genetically complemented mutants displayed phenotype similar to the wild type strain. This meant that the observed phenotypic features in the mutants were due to mutations rather than the polar effect of the mutation.

In pneumonia model that develops after intranasal infection, the results showed that both TprA and PhrA have a significant role in pneumococcal virulence since the median survival time of mice infected intranasally with Δ*tprA*, and Δ*phrA* was significantly

higher than the wild type infected cohort. In addition, no bacterial growth could be detected in the blood of mice infected by these mutants. When the inoculum dose increased, from approximately 5×10^5 CFU/mouse to approximately 1.6×10^6 CFU/mouse, it was noted that the contribution of individual components of TprA/PhrA quorum sensing system in virulence varied in pneumonia model. For example, regardless of dose used, the *tprA* mutation rendered the pneumococcus avirulent, whereas the *phrA* mutant retained some of its virulence when a high inoculum dose was used for infection. Interestingly, in the cohort infected with a high dose of Δ*phrA,* pneumococci could be detected in the lungs of sick animals, but not in blood, while at a lower dose no Δ*phrA* could be detected neither in the lungs nor in blood.

Furthermore, the difference in phenotype between Δ*tprA* and Δ*phrA* were also obvious when these strains were grown *in vitro* in the presence of mucin. These differences suggest that although PhrA activation is required for efficient functioning of TprA, for example for translocation of *S. pneumoniae* from lungs into the blood, TprA may activate virulence determinants even in the absence of PhrA, or alternatively other unknown peptide (s) may activate TprA in the absence of PhrA as the pneumococcus has other peptide activated QS systems such as short hydrophobic peptides (SHP) of Rgg/SHP transcriptional regulators (Cook and Federle, 2014).

It was demonstrated by Cook *et al.* (2013) that the synthesis and secretion of SHP1520 by GBS can control Rgg2/3-regulated gene expression in GAS in trans. Similarly, SHP2/3 production by GAS could stimulate RovS-mediated gene expression in GBS. Activation of homologous QS systems by the noncognate peptide has also been reported in *S. pneumoniae*. In collaboration with Dr Hiller's group, I demonstrated that unlike type 2 D39 strain which has only TprA/PhrA, *S. pneumoniae* PMEN1 strains contain two homologs of TprA/PhrA QS systems (TprA/PhrA and TprA2/PhrA2) (Kadam *et al.*, 2017). We showed that PhrA2 activates TprA/PhrA, both in PMEN1 cells as well as in *S. pneumoniae* type 2 D39 strain.

The growth profiles of pneumococcal strains in respiratory tissues, nasopharynx and lungs, were also determined after intranasal infection. Wild-type strain D39, Δ*tprA,* and Δ*phrA* were detected in both tissues immediately post-inoculation. The numbers of wild-type strain D39 increased gradually in the nasopharynx, in contrast, no increase was

observed in the numbers of either mutant. The difference in bacterial load between the wild type and the mutants at 48 hr post infection was statistically significant in both tissues. Moreover, in the lungs the Δ*tprA* was cleared after 24 hr post-infection, the numbers of Δ*phrA* did not change, and the number of wild type increased. These results suggest that TprA/PhrA support growth in the nasopharynx and the lungs, and further that TprA is critical for survival in the lung. Attenuation in pneumococcal growth in these tissues can be explained by the decrease in availability of galactose due to reduction in total neuraminidase activity. Given that initial removal of terminal sialic acid is needed for other glycosidases to 'see' their substrate, in the circumstances when the neuraminidase activity is low, pneumococcal galactosidases will be less efficient in release of galactose from host glycoproteins in these tissues (Blanchette *et al.*, 2016). Decrease in the availability of galactose has at least two implications for the microbe: first the microbe will be less able to generate metabolic energy, and second its virulence will attenuate. Paxio *et al.* (2015) has demonstrated previously that inability to utilise galactose either via Leloir or tagatose pathway abbroagte pneumococcal colonisation and virulence.

Furthermore, the role of TprA/PhrA system in the middle ear was tested as the middle ear composed of a complex sugar environment (Kobayashi *et al.*, 1985; Juhn and Huff, 1976). The chinchilla otitis media model was employed in collaboration with Dr Luisa Hiller and Anagha Kadam, Department of Biological Sciences, Carnegie Mellon University, USA. Wild type strain D39 and Δ*tprA* strain were transbullarly inoculated to the animals, the results showed that the Δ*tprA* caused significantly less mortality compared to the wild type strain D39. It is very likely that the abrogation in virulence in middle ear infection is also largely, but not exclusively, due to consequence of reduced ability to release and utilise galactose. Previously, it has been shown that in middle ear the pneumococcus is able to induce mucin gene expression, suggesting strongly that the microbe relies on host glycans for its nutritional requirements. It was shown that after pneumococcal inoculation of rat middle ear, Muc1-Muc5 mucin genes are expressed in the middle ear mucosa in a time-dependent manner (Tsuboi *et al.*, 2001). In the Eustachian tube, the Muc2, Muc4 and Muc5 mucin genes were expressed in both control and pneumococcal inoculation groups. Another supporting line of evidence for nutritional aspect of decrease in pneumococcal virulence originates from a study which showed that middle ear effusions

(MEE) containing *S. pneumoniae* had substantial levels of neuraminidase activity (LaMarco *et al.*, 1984).

The crucial role of TprA/PhrA QS system in sugar metabolism is supported by the observation that the inactivation of *tprA* and *phrA* resulted in significantly reduced growth yield and rate compared to the wild type strain D39 when grown in CDM supplemented with galactose or mannose. However, Δ*tprA,* Δ*phrA,* growth was not affected in BHI, or CDM supplemented with glucose or *N*-acetylglucosamine suggesting that TprA/PhrA system does not play a role in rich media, nor in glucose or *N*-acetylglucosamine metabolism. These results are in line with previous reports that demonstrated the TprA/PhrA quorum system in *S. pneumoniae* is active at high density in the presence of galactose, one of the main sugars in the human nasopharynx, while this system is not active in the media containing glucose, the main carbon source in the blood (Hoover *et al.*, 2015). Thus, galactose and mannose have a crucial role in regulation of TprA/PhrA system. However, this does not exclude TprA/PhrA system's involvement in other metabolic functions. Indeed, both Δ*tprA* and Δ*phrA* were virulent in septicaemia model, which develops after direct administration of the inoculum into blood. Given that glucose concentration is higher than galactose and mannose in blood, abrogation of virulence is unlikely due solely to galactose and mannose metabolism, supporting the view that TprA has functions other than its role in sugar metabolism. Indeed, further evidence for this postulation comes from TprA regulon analysis, which showed that in addition to its involvement in sugar metabolism, TprA controls the expression of genes involved in other pathways, including SPD_1753 (serine protease, subtilase family protein), SPD_1744 (CAAX amino terminal protease family protein), and SPD_1947 (transcriptional regulator, putative), which appeared to be up regulated in the Δ*tprA* compared to their expression in the wild type strain D39 in the presence of galactose and mannose. In addition, SPD_1517 locus (hypothetical protein) appeared up regulated in the presence of mannose, and SPD_0936 (Tn5252, relaxase) down regulated in the presence of galactose and mannose. These results indicate that TprA might be controlling the expression of some other essential pathways required for infection.

Among the notable gene classes that are regulated by TprA include tRNA genes (n=7) on galactose, which are important for protein synthesis. These genes are up regulated by TprA. In the mutant strain, protein synthesis, therefore, is expected to be less efficient

than the wild type, however, this has to be confirmed by additional techniques such as polysome analysis, which is used to determine translation activity of mRNAs in the cells (Poria and Ray, 2017). In addition, both on galactose (n=16) and mannose (n=10), TprA controls the expression of 26 hypothetical genes. Currently, the function of these genes is not known. However, it is likely that some of these genes are also important for pneumococcal virulence. Future studies need to be done to determine their functional roles.

Another notable gene that was controlled by TprA and may be responsible for reduced virulence was *merR,* also known as GlnR. This gene is up regulated by TprA, and was demonstrated to control transcriptional repression of genes involved in glutamine synthesis and uptake (*glnA* and *glnPQ*), glutamate synthesis (*gdhA*), and the gene encoding the pentose phosphate pathway enzyme Zwf (Kloosterman *et al.*, 2006b). Recently, Al-Bayati *et al.* (2017) showed that GlnR and CcpA are involved in regulation of both *pflA* and *pflB,* which are responsible for the synthesis of active pyruvate formate lyase and contributes to pneumococcal colonisation and virulence as was demonstrated in a mouse model of pneumococcal colonisation and virulence. The regulatory role of TprA on GlnR clearly demonstrates that TprA has regulatory impact on pyruvate metabolism. Further work needs to be done to explore the full extent of this regulation.

Hoover *et al.* (2015) showed that the TprA/PhrA system controls the expression of a lantibiotic gene cluster that encodes members of the bacteriocin family of antimicrobial peptides. However, they were unable to establish this system's importance in invasive pneumococcal disease, presumably due to differences in host strain background, mutation method used, or some minor differences in inoculum preparation for infection. The results in this study showed that TprA/PhrA system plays a crucial role in the pneumococcal growth in the nasopharynx, the middle ear, the lungs, and the blood stream.

QS systems play a major role in virulence not only in *S. pneumoniae* but other Gram-positive bacteria too. It was shown that the pathogenicity of *B. thuringiensis* strain 407 and *B. cereus* strain ATCC 14579 are controlled by PlcR. The analysis of a *plcR* mutant in an insect species, *Galleria mellonella*, and in BALB/c mice, showed that the parental strain caused 70% mortality whereas the mortality rate in the mutant infected cohort was only 7 % in larvae model. In addition, the nasal instillation of the mice showed that 100

% mortality was caused by the parental strain while there was no mortality with Δ*plcR*, and the results were the same if vegetative cells or spores were used. Thus, PlcR play an essential role in the pathogenesis of these bacteria (Salamitou *et al.*, 2000). It was reported that PlcR of *Bacillus thuringiensis* regulates extracellular virulence factor genes coding for degradative enzymes, cell surface proteins and enterotoxins (Agaisse *et al.*, 1999). In addition, it was shown that PlcR is a pleiotropic virulence regulator for controlling extracellular virulence factors since 90 % of the PlcR regulon genes in *B. cereus* encode proteins for interface between the bacteria and their environment, including, protease, hemolysins, proteins involved in peptidoglycan synthesis, and antibacterial peptides, which may protect the bacterium from competition with other bacterial species and their bacteriocins (Gohar *et al.*, 2008). It was shown that the role of PlcR in virulence was linked to its contribution to biofilm formation and the production of putative virulence factors (Gohar *et al.*, 2002).

The involvement of TprA/PhrA QS sytem was studied using *LacZ* reporter assays. The transcriptional *lacZ*-fusion results showed that the promoters of *tprA* and *phrA* are not induced in the presence of glucose and *N*-acetylglucosamine. This was consistent with the growth of mutants, which grew as well as the wild type in CDM supplemented with these sugars. In the same line, it was shown that PlcR positively regulates its own expression and the expression starts at the onset of the stationary phase of *B. thuringiensis*, and the expression of system is influenced by the medium used (Lereclus *et al.*, 1996). I demonstrated regulatory interaction between TprA and PhrA. The transcriptional *lacZ*-fusion analysis showed that on mannose and galactose where the TprA promoter activity is high, TprA represses P*phrA* driven β-galactosidase activity relative to its expression in wild type D39 strain, indicating that TprA is a repressor of *phrA.* On the other hand, in the presence of glucose or *N*-acetylglucosamine, where the P*tprA* activity is low, TprA does not have any effect on *phrA* expression. Consistently, the analysis of microarray results of this study showed that the expression of *phrA* (SPD_1746) is up regulated in the *tprA* mutant relative to its expression in the wild type D39 during growth on galactose or mannose (Appendix 3 and 4), Similarly, Hoover *et al.* (2015) showed that TprA is an inhibitor of *phrA* expression and this system is not active when the pneumococcus is grown in rich media and capable of signalling between the cells only when grown in media containing galactose. Furthermore, EMSA was used to identify the direct interaction between the purified TprA and the fluorescently labelled

DNA probes representing the putative promoter sequence of *phrA* (SPD_1746), and the results showed direct interaction between TprA and *phrA.* Therefore, TprA directly bind with PhrA to form a complex which is essential for TprA regulon expression. In here, factors effecting TprA and phrA have not been determined. However, in future the interaction between TprA and *phrA* can be studied quantitatively in the presence of, for example, glucose and galactose by using EMSA.

The differential expression of *tprA* and *phrA* in response to galactose as opposed to glucose seems to be mediated through the carbon catabolite control mechanism of Gram positive bacteria. It was shown that the promoter of *phrA* contains a *cre* binding site for the CcpA of *S. pneumoniae* (Carvalho *et al.*, 2011), and they also showed that the expression of both *tprA* and *phrA* were up regulated in the *ccpA* mutant compared with their expression in the wild type strain through microarray analysis when the cells were grown in the media containing glucose. Therefore, the recent results indicate that the loss of activity of the TprA/PhrA system in cells grown in the presence of glucose may be due to the inhibitory effect of CcpA in the presence of glucose.

It was found that the predicted protein structure of transcriptional regulator TprA in *S. pneumoniae* strain D39 is similar to protein structure of PlcR of *B. thuringiensis* and *B. cereus* (Hoover *et al.*, 2015). PlcR in *Bacillus cereus* is reported to contain a helix turn helix motif in its N-terminal sequence. It is activated through binding with its cognate signalling peptide PapR, and this binding is mediated by tetratricopeptide repeat type regulatory domain (Grenha *et al.*, 2013). The C-terminal helix of PlcR regulatory domain is binding with the N-terminal residue of PapR heptapeptide determines the specificity of this system (Bouillaut *et al.*, 2008). This binding induces conformational changes via binding of PapR and its target DNA sequence which defined as a PlcR box.

Previous studies failed to identify a conserved TprA binding motif in the putative promoter regions of TprA regulated genes in *S. pneumoniae* (Hoover *et al.*, 2015). In this study, a putative binding sequence for TprA was determined using MEME in the upstream of the selected genes identified to be regulated by TprA by gene expression studies, including *phrA* (SPD_1746), *nanA* (SPD_1504), *nanB* (SPD_1503 locus), SPD_1517, SPD_1994, and SPD_1495. In addition, I identified a sequence resembling to *plcR* box, TATGNAN$_4$TNCATA (where N is any base) of *B. cereus.* This is a highly

conserved palindromic region which is the specific target sequence for PlcR binding. It is located in the upstream regions of PlcR regulated genes and is essential for transcription (Agaisse *et al.*, 1999). In future, the pneumococcal genomes should be screened with these putative binding sequences to identify the genes potentially regulated by TprA, and the significance of this binding should be verified experimentally.

Growth studies of pneumococcal strains in Sicard's medium supplemented with mucin showed that Δ*tprA* had attenuated the pneumococcal growth on mucin and this is likely due to TprA/PhrA system's impact on neuraminidase production. When using the cell lysate from Sicard's defined medium supplemented with mucin, the result showed that Δ*tprA* had significantly less neuraminidase activity compared with the wild type D39 strain. Glycosidases, including neuraminidase, are responsible for cleaving monosaccharaides from host glycans. The initial sialic acid cleavage is critical as this process exposes other internal sugar linkages to the activity of pneumococcal glycosidases, such as galactosidases and mannosidases, which cleave galactose and mannose, respectively (King, 2010). The reduction in neuraminidase activity in the Δ*tprA* is consistent with growth attenuation of this mutant on mucin, very likely due to reduced concentration of free sugars and subsequent nutrient deprivation. NanA plays a significant role in nasopharyngeal colonisation and, pneumococcal survival in the upper respiratory tract and blood (Manco *et al.*, 2006). Neuraminidase activity has been shown to play a role in formation of pneumococcal biofilm (Parker *et al.*, 2009). In fact, a galactose-specific biofilm phenotype of *nanA* was recently reported (Blanchette *et al.*, 2016), hence TprA may be providing the regulatory link between galactose, *nanA*, and biofilms.

The results of transcriptional reporter assays showed that the expression of *nanA* is up regulated by TprA on galactose but not on glucose. Similarly, RT-PCR analysis showed that the expression of *nanA* is downregulated in Δ*tprA* compared to its expression in the wild type strain D39. However, microarray analysis of Δ*tprA* relative to the wild type strain D39 did not show difference in *nanA* expression between the mutant and wild type (Appendix 3 and 4). This is very likely due to the differences in sensitivity of assay platforms. It was reported that qRT-PCR is highly sensitive and allows quantification of small changes in gene expression. qRT-PCR is known to have a greater dynamic range for detecting small changes in gene expression than microarray (Allanach *et al.*, 2008). On the other hand, the PhrA had no impact on the expression of *nanA* neither on galactose

nor on glucose. This is in line with the growth studies of Δ*phrA* in the presence of mucin, which grew as well as the wild type. This may suggest that *tprA* may have *phrA* dependent and independent regulatory functions. Furthermore, EMSA showed that TprA binds to both *nanA* and *nanB* promoters in a dose dependent manner. The ability of TprA to bind with both neuraminidase genes in *S. pneumoniae* suggests that TprA play an essential role to regulate pneumococcal neuraminidase production.

Having seen the involvement of TprA/PhrA system in pneumococcal virulence, I investigated additional mechanisms by which this quorum sensing system contributes to pneumococcal virulence. Therefore, in addition to neuraminidase, TprA/PhrA's role in production of pneumolysin was also investigated. Pneumolysin (Ply) is an important virulence factor and it is found in most clinical strains of *S. pneumoniae* (Mitchell and Mitchell, 2010). It was shown that Ply is required to cause pneumonia (Kadioglu *et al.*, 2002), and it is involved in pneumococcal bacteraemia (Orihuela *et al.*, 2004). Further, it was observed that Ply is important for pneumococcal migration from the lungs to bloodstream (Berry *et al.*, 1999; Orihuela *et al.*, 2004). Loss of pneumolysin led to significantly lower numbers of *Streptococcus pneumoniae* serotype 2 strain D39 in the nasopharynx, trachea, and lungs (Kadioglu *et al.*, 2002). Thus, the influence of *tprA* on the haemolytic activity, which originated mainly due to Ply activity, was tested in the cell lysates of pneumococcal strains that were grown in BHI or in Sicard's defined medium supplemented with mucin.

In this study, the results showed that there was no significant difference in the haemolytic activity among pneumococcal strains when using the cell lysate in BHI. However, Δ*tprA* and Δ*phrA* had significantly less haemolytic activity compared with the wild type strain D39 when using the bacterial cell lysate prepared from Sicard's defined medium supplemented with mucin. Pneumolysin is one of the cholesterol dependent cytolysin protein which belongs to the family of pore forming toxins synthesised by more than 20 species of Gram-positive bacteria. This toxin causes damage on the cholesterol containing membranes of host cells to form a transmembrane pore which leads to cell lysis and death (Tilley *et al.*, 2005; Marriott *et al.*, 2008; Mitchell and Mitchell, 2010). It was shown that CcpA mediates repression of toxin gene expression in *Clostridium difficile* (Antunes *et al.*, 2011). The presence of glucose in the bacterial growth medium strongly represses

cytotoxin synthesis of *Clostridium difficile*, linking carbon source utilisation to cytotoxin gene expression (Dupuy and Sonenshein, 1998; Antunes *et al.*, 2011).

It was observed that the pneumolysin of *S. pneumoniae* is expressed during the late logarithmic phase of growth (Benton *et al.*, 1997). Similarly, the release of cytotoxin of *C. difficile* happens during the late logarithmic phase of growth (Kazamias and Sperry, 1995; Dupuy and Sonenshein, 1998). Therefore, the expression of this toxin may be mediated by quorum sensing system which is active in late exponential phase of growth. Moreover, it was shown that PlcR activates the transcription of a two-gene operon encoding for the phosphatidylcholine specific phospholipase C (PlcB or PC-PLC) and the sphingomyelinase CerB, and the products of these genes form the haemolytic component designated as cereolysin AB (Gilmore *et al.*,1989; Lereclus *et al.*, 2000). It was observed that the level of cytolytic activity of *B. thuringiensis* and *B. cereus* were significantly reduced in the Δ*plcR* strains compared with the wild type strains using sheep, horse, and human erythrocytes and *G. mellonella* haemocytes (Salamitou *et al.*, 2000).

The results of this study showed that TprA/PhrA quorum sensing system is important for virulence due to its role in expression of pneumolysin and neuraminidase. It is clear that TprA/PhrA quorum sensing system plays a key role in pneumococcal sugar metabolism. Therefore, my data suggest that TprA/PhrA quorum sensing system can be used as a target to develop novel anti-infective against pneumococcal diseases.

4.2. Modulation of TprA/PhrA quorum sensing system by linear imprinted copolymers with anti-infective properties

There is an increase in the prevalence of multidrug resistant bacteria and this problem requires new approaches to identify effective antiinfectives. Bacteria can exploit several types of resistance mechanisms, include antibiotic inactivation, target modification, or changed permeability of cell membrane, through innate or acquire resistance. Acquired resistance can be occurred through mutation in chromosomal genes, transfer resistant plasmids, and bacteriophages (Giedraitiene *et al.*, 2011). In order to avoid known mechanisms of bacterial resistance, effective new drugs must be designed to fight the infection, avoid resistance, and protect the natural microbiome (Brooks and Brooks, 2014). Current antibiotics excert their action through their ability to kill or inhibit

bacterial growth by inhibition of the vital cellular processes, including DNA replication, protein synthesis, and cell wall biosynthesis (Murima *et al.*, 2014). The antibiotic resistance is significantly increased against every new antibiotic used. Thus, the alternative approach is to target functions essential for infection, such as virulence factors which cause host damage and disease. This approach also allows expanding the repertoire of bacterial targets as it has been reported that there is a limited number of antiinfective targets, preserve the host endogenous microbiome, and reduce the selective pressure on critical biological processes required for survival (Clatworthy *et al*., 2007).

QS systems are considered as potential targets to design antininfectives. Approaches to target QS systems relies on one of three main strategies: i. interference with autoinducer synthesis, ii. prevention of ligand/receptor interactions, and, iii. destruction of the autoinducer (Grandclément *et al.*, 2015). These methods have been used to manipulate the QS systems in different bacterial species but particularly in the Gram-negative bacteria to inhibit or control bacterial infections (Huang *et al.*, 2003; Gonzalez and Keshavan, 2006). It is believed that as QS systems control virulence but not the viability of bacterial pathogens, the likelihood of antibiotic resistance would be less than those of traditional antibiotics (Rasmussen and Givskov, 2006).

In Gram negative bacteria, acyl homoserine lactones (AHL) is a QS signal molecule which involved in increase in antimicrobial resistance (Rasmussen and Givskov, 2006). QS signals can be inactivated through enzymes which degrade or modify the AHL, including, lactonases, amidases, and reductases (Zhang *et al.*, 2002; Lin *et al.*, 2003; Bijtenhoorn *et al.*, 2011), or by chemical compounds disrupting the QS pathways which are known as QS inhibitor (Grandclément *et al.*, 2015). Other mechanisms of signal interference are that signal biosynthesis inhibition such as triclosan which inhibit the enoyl-ACP synthesis that is essential compounds in AHL biosynthesis (Hoang and Schweizer, 1999), or reduction in the amount of QS signal receptor proteins. It has been reported that some QS signals produced by a microorganism can interfere effectively with QS signaling of other bacteria (Taga and Bassler, 2003), and many of QS signals in bacteria share structural similarities with the QS signal of other bacterial species especially from the same family, suggesting that signal interference may be possible between these species (McClean *et al.*, 1997). In addition, it was reported that some bacterial species such as *P. aeruginosa*, are able to degrade their own autoinducing

substance (AHL signals) (Zhang *et al.*, 2002; Huang *et al.*, 2003). In Gram positive bacteria, interference mechanisms have been reported in two component regulatory system based QS system of *Staphylococcus aureus* which regulates its virulence factor production via autoinducing peptide (AlP), which activates virulence factors synthesis in a strain specific manner while inhibiting the expression of virulence in other strains (Lyon *et al*., 2002).

Molecular imprinting technology (MIT) is an approach to design artificial receptors with high selectivity and specificity for the target molecule which used in the imprinting procedure, and resistance to high temperature and pressure. Molecular imprinted polymer able to recognise and bind selectively only with its target template molecule. However, design a new molecular imprinting polymer required more time for optimisation, synthesis, washing, and testing (Vasapollo *et al.*, 2011). Alternatives include synthetic cross-linked adsorbents like molecularly imprinted (and non-imprinted) polymers which are not biodegradable. These have been used to remove homoserine lactones from solution and prevent QS (Hentzer *et al*., 2003; O'Loughlin *et al.*, 2013). Linear random copolymers have been tested, but less successfully due to lower affinity towards the signalling molecule (Piltska *et al.*, 2011; Piletska *et al.*, 2010). Unfortunately, crosslinked polymers have limitations as drug candidates since it is impossible to elucidate their random structure (Cavaleiro *et al.*, 2015). Affinity ligands made with linear molecularly imprinted polymers (LMIP) can overcome some of these limitations as they are smaller in size and can conceivably be sequenced (Magennis *et al.*, 2014). These are ideal attributes which should allow for their use as drug candidates or drug discovery tools. Therefore, in this study, LIMP was used to modulate TprA/PhrA QS system in *S. pneumoniae*.

TprA/PhrA system was chosen as QS target to develop new anti-infective for two reasons. First, it is widespread across bacilli and streptococci thus relevant to many human pathogens (Hoover *et al.*, 2015). In this study, it was shown that the TprA/PhrA system is a major virulence determinant. It is important for pneumococcal growth on galactose, mannose, and mucin which are the main carbon sources for pneumococcal growth in animal tissues (Robb *et al.*, 2017; Kahya *et al.*, 2017). The PhrA peptide expression is induced both by galactose and mannose. Loss of TprA and PhrA attenuated pneumococcal virulence in mice model of pneumonia and septicaemia, and in a chinchilla

model of otitis media. In addition, QS system homologs are absent in mammalian hosts and conserved among pathogenic bacteria decreasing the possibility of host-toxicity and ensuring targets in human pathogens, respectively (Clatworthy *et al.*, 2007). Moreover, it is not essential for survival but is required for virulence and its inhibition would treat disease, while the non-vital nature of the QS should limit the selective pressure that drives the spread of drug resistance (Rasmussen and Givskov, 2006).

It was envisaged that by interfering with the binding of PhrA with TprA would block the TprA/PhrA QS system. In order to prepare a modulator of this system, its inducer peptide (PhrA10) was used for LMIP preparation. PhrA10 is the active processed signal peptide with a capability to induce PhrA expression (Hoover *et al.*, 2015). The C-terminal end of this peptide with the following sequence, SNGLDVGKAD, was used as template for LMIP preparation. The LMIP was prepared in collaboration with Prof Sergey Piletsky's research group, Chemistry Department, University of Leicester, using solid-phase imprinting method. This is a versatile technique, which can be used to obtain affinity ligands in a short time period, 2-4 days, as opposed to months required for generation of traditional ligands such as immunoglobulins (Poma *et al.*, 2014; Canfarotta *et al.*, 2016). While molecularly imprinted polymers are usually cross-linked, in order to obtain water-soluble low molecular weight ligands, the cross-linking agent from the monomer mixture was ommitted (Xue *et al.*, 2011). This way, low-molecular weight ligands could be produced, which also allows for the use of higher effective (molar) concentrations, as compared to other imprinted polymer formats such as micro/nano particles. This is especially important in the case of a target such as PhrA, where the biologically effective concentration is in the micromolar range (Piletska *et al.*, 2011; Piletska *et al.*, 2010).

In this study, I showed the first example of QS modulation in Gram-positive bacteria by using low molecular weight linear molecularly imprinted polymers, which can both bind to the PhrA peptide and also block the TprA target (Motib *et al.*, 2017). An added advantage of the present approach is the fact that soluble low molecular weight ligands are more appropriate as anti-infective agents, unlike micro-sized polymers which have very limited prospects for *in vivo* applications (Venkataraman *et al.*, 2011). In addition to their therapeutic use, LMIP can be used as a study tools to manipulate QS systems without resorting into to genetically altered mutant strains, which may cause polar effect.

I demonstrated that LMIP selectively inhibited TprA/PhrA QS system. It was observed that the wild-type strain D39 was less able to utilise galactose and the transcriptional activation of *phrA* expression as well as neuraminidase activity, which is important for pneumococcal virulence (Manco *et al*., 2006), could be inhibited in the presence of LMIP. This strongly suggests that the interaction of LMIP with the PhrA peptide blocked pneumococcal growth and abrogated virulence. LMIP effect was specific to Phr10 as it did not work against nonspecific peptide. The results suggest that inhibition occurs by sequestration of active PhrA by LMIP. Additionally, it may be due to the irreversible binding of PhrA to the TprA receptor in the presence of LMIP, effectively blocking QS activity. The TprA inhibition was only observed when both PhrA10 and LMIP were co-injected, and not with free LMIP alone. This interaction can be attributed to the blocking of the receptor binding site by the LMIP-peptide complex. Currently, it is not known whether LMIP interacts with the target peptide inside or outside the pneumococcal cell. Future studies utilising fluorescently labelled LMIP should be able to answer this question.

In this study, *in vivo* results indicated the anti-infective utility of LMIP. LMIP was not toxic in the concentration that was used in mucosal and intraperitoneal routes, these experiments did not show any toxicity of LMIP as measured by observation of signs of disease. However, more detailed toxicity experiments are needed. The results showed that increased the survival time of mice compared to cohort that did not receive LMIP, and reduced pneumococcal blood count significantly 24 hr post-infection. This shows that LMIP modulates pneumococcal phenotype *in vivo*. Moreover, the action of LMIP was specific because the LMIP targeting non-specific peptide was ineffective *in vivo*. In this study, the LMIP was administered simultaneously with the bacterial inoculum in order to optimise any discernible impact of LMIP *in vivo*. Although LMIP were found to be protective against lethal pneumococcal challenge, it consisted mainly of sequences containing three acrylamide, three butyl acrylamide, and two or three acrylic acid blocks, which are toxic compounds (Friedman, 2003). Therefore, future studies should focus on generating compounds from non-toxic material.

Despite its limitations, LMIP is a good drug discovery tool. For example, the existing LMIP sequence can be used a template for fragment based drug discovery tool. Fragment based drug discovery (FBDD) is an approach identifies, firstly, very small molecules that

have low affinity ligands (fragments) which have approximately half the size of typical drugs. The fragments are then expanded or joined together to generate larger and higher affinity ligands (drug) (Erlanson *et al.*, 2004; Erlanson, 2011; Norton, 2014). These are selected according to the basis of their ability to bind to the target and inhibit it in the functional assay (Erlanson *et al.*, 2004). The most important advantage of FBDD is that this approach provides a more rapid and effective way of identifying ligands for protein target. In this study, it has been demonstrated that LMIP is similar in nature to fragment-based drug discovery with added advantage that the sequence of appropriate fragments is guided by a self-assembly process taking place during molecular imprinting (Baker, 2013). Therefore, this study provides a novel target for drug discovery.

Final Remarks

In this study it was shown that TprA/PhrA quorum sensing system plays a crucial role for sugar metabolism, survival, and virulence of *S. pneumoniae*. Through transcriptional *lacZ*-fusions, it was determined that TprA/PhrA quorum sensing is active in the presence of galactose and mannose but not in the presence of glucose and *N*-acetylglucosamine. It was shown in this study that TprA is an activator of *nanA* expression in the presence of galactose and inactivation of TprA reduced the neuraminidase activity of cells grown in the presence of mucin. It was shown that TprA regulation of *nanA* and *nanB* is due to direct interaction of TprA with the putative promoters of *nanA* and *nanB*. In addition, *tprA* or *phrA* mutation reduced the haemolytic activity of *S. pneumoniae* significantly. Loss of *tprA* or *phrA* leads to significant attenuation in pneumococcal virulence and survival in nasopharynx, lungs, blood, and middle ear.

This study demonstrated that LMIP selectively inhibited TprA/PhrA system, rendering the wild-type strain less able to utilise galactose, and reducing neuraminidase activity and the transcriptional activation of *phrA* expression, and appeared no visible toxicity after intranasal or intraperitoneal administration in the murine model. It was also shown that LMIP modulates pneumococcal phenotype in a mouse model of pneumococcal pneumonia that progresses to bacteremia. The findings provide proof of principle that LMIP can be used to block Gram-positive quorum-sensing peptides as a novel drug to target Gram positive pathogens.

Future work

Quorum sensing systems are complex organisations, which are important in coordination of important microbial processes including virulence. Therefore, a better understanding of their function and operation are required. In this study, it was shown that this system is active in the presence of galactose. While I studied TprA's role mainly in galactose metabolism, it is very likely that TprA/PhrA quorum sensing system plays additional roles other than its involvement in sugar metabolism and its regulatory impact on neuraminidase expression. This is supported by the fact that TprA regulon contains genes that are not involved in sugar metabolism and the inactivation of TprA renders pneumococcus avirulent in septicaemia model that develops after intravenous administration of pneumococci.

To test TprA/PhrA systems involvement in other biological processes, the reporter strains constructed in this study can be tested in host relevant environmental conditions, such as at different temperature and pH range, in the presence of different metals, and oxygen concentration. Once the inducing conditions are determined, regulon for TprA can be identified using RNAseq analysis of Δ*tprA* and the wild type strain. After analysis of regulon data, the key genes of relevant pathways can be mutated as described in section 2.15 and these mutants can be tested using microbiological and biochemical assays similar to those that are described in this study.

It was determined in this study that TprA/PhrA activates the expression of *nanA* in the presence of galactose, and through EMSA, the purified TprA showed direct binding with both *nanA* and *nanB*. However, TprA/PhrA regulatory function on *nanB* should be further tested using transcriptional *lacZ*-fusion as described in section 2.18. Moreover, TprA's regulatory impact on *nanA* and *nanB* should be determined at different pH. Because it has been reported that NanA (pH 5.5-6.5) and NanB (pH 5–5.5) have different pH range and optima (Hayre *et al.*, 2012).

In this study, it has been shown that TprA plays an important role in neuraminidase expression. Neuraminidase activity plays an important role in pneumococcal biofilm formation and attachment (Parker *et al.*, 2009). Therefore, it is also required to determine

the impact of TprA/PhrA system in biofilm formation using human airway epithelial cells as described previously (Parker *et al.*, 2009).

TprA/PhrA quorum sensing system play a major role in pneumococcal sugar metabolism. Capsule synthesis is linked to sugar metabolism, most of the compounds required for capsule synthesis are produces during catabolism of sugars (Bentley *et al.*, 2006). Thus, it will be interesting to determine the role of this system in cell wall and capsule synthesis by measuring the glucuronic acid concentration of Δ*tprA* and Δ*phrA*, and determine the thickness of the cell wall using fluorescently labelled vancomycin incorporation (Ng *et al.*, 2004).

In this study, it was shown that TprA/PhrA quorum sensing system is involved in pneumolysin production. In addition, it was observed that the pneumolysin is expressed during the late logarithmic phase of growth (Benton *et al.*, 1997). This may suggest the involvement of a quorum sensing system, which is also active in late exponential phase when the cell density is high. Thus, in-depth experiments are required to determine the role of TprA/PhrA system in regulation of pneumolysin expression through qRT-PCR and *lacZ*-fusion analysis. To establish the regulatory role of TprA over *ply*, *ply::lacZ* construct can be generated and its inducibility in selected conditions in the wild type and mutant strain can be determined as described in section 2.18. Further experiments can establish whether TprA-*ply* interaction is direct or indirect by using recombinant TprA and EMSA.

Pneumococcus is exposed to different environmental conditions in host. In nasopharynx, the concentration of oxygen is high, while blood is nearly anaerobic, respiratory tract is rich in galactose while blood is rich in its content of glucose. Thus, it is reasonable to assume that *tprA*/*phrA* expression differs in different tissues during infection. To determine the expression of this system during infection, mice can be infected with *S. pneumoniae* as described in section 2.22. After the onset of disease signs, blood, nasopharyngeal, and lung tissues can be collected for extraction of pneumococcal mRNA. After cDNA synthesis, the relative expression of *tprA* can be determined by quantitative reverse transcriptase real time PCR as describe in section 2.17.4.

In this study, it was detected that there are two putative binding sites for TprA. It is not known exactly which one of these putative sites TprA binds or whether TprA has higher affinity for one site over another. To determine this, DNase foot printing assay can be used, and subsequently the binding site can be determined by Maxam-Gilbert sequencing of DNase treated samples (Higuchi *et al.*,1988).

In addition to TprA/PhrA, in pneumococcus there is also another PlcR homolog coded by SPD_1786 in *S. pneumoniae* D39 strain. The role of this second putative PlcR homolog is not known. Therefore, it is interesting to study the role of the second PlcR homolog (SPD_1786) in *S. pneumoniae*, and determine the communication between these two systems, if any. To test the function of SPD_1786, a similar experimental strategy as employed in this study can be adapted.

It was showen that TprA/PhrA system is active in the presence of galactose and mannose, and inactive in the presence of glucose or *N*-acetylglucosamine, suggesting this may be due to CcpA regulation. Therefore, the interaction between *tprA* and *phrA* with CcpA can be investigated by EMSA as described in section 2.20.

LMIP was tested for modulation of pneumococcal growth on host-derived sugars, for its impact on neuraminidase activity, and for activation of P*phrA*. I was also able to determine the *in vivo* utility of LMIP by mixing it with the pneumococcal inoculum. In future, the utility of LMIP can be tested in a more challenging clinical situation, such as a treatment agent. In addition, LMIP's *in vivo* utility can be evaluated using different doses and dosing regimens.

Appendix 1

DNA sequencing showing successful gene replacement with antibiotic cassette. Highlighted sequence represents the start of antibiotic cassette.

(A) Δ*phrA* (SPD_1746) with spectinomycin cassette

Alignment with SPD1746-SeqM-F1

```
Query  13       GTAACGAACTCTGATGTAGCCAAATAAAAACTCTTGATGGTCCAAATTTTTTGTCTGATA  72
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738503  GTAACGAACTCTGATGTAGCCAAATAAAAACTCTTGATGGTCCAAATTTTTTGTCTGATA  1738562

Query  73       CAACTCTATTAAATGAGAGTAGTTTGCCTCATATTCTTGTTCACGACCCACTAAGGAATA  132
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738563  CAACTCTATTAAATGAGAGTAGTTTGCCTCATATTCTTGTTCACGACCCACTAAGGAATA  1738622

Query  133      GAAATTAGATAGAGTATTCAACGCCTTTAAATAAATCAGAGTATTTGAAGAGACTTTTAA  192
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738623  GAAATTAGATAGAGTATTCAACGCCTTTAAATAAATCAGAGTATTTGAAGAGACTTTTAA  1738682

Query  193      TAATATATTTTCCAATGACGAAATTGCCTCACACTTACTGTCATATTGATAGAAGTCAAT  252
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738683  TAATATATTTTCCAATGACGAAATTGCCTCACACTTACTGTCATATTGATAGAAGTCAAT  1738742

Query  253      TATAGATTTAATCCATTCAAGGTAAGTTCGGTCTTCTAATGTTAGAAAAGTGCTTCGTTC  312
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738743  TATAGATTTAATCCATTCAAGGTAAGTTCGGTCTTCTAATGTTAGAAAAGTGCTTCGTTC  1738802

Query  313      TACCTCTATTTTATAAAGATATTCTAAATCGTCATAATTTCTGTCATCTAATAGGCGAGC  372
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738803  TACCTCTATTTTATAAAGATATTCTAAATCGTCATAATTTCTGTCATCTAATAGGCGAGC  1738862

Query  373      AGATAGATGTTTGAAATTAGAGAGGTTAGACTTAACTTCGATTTGTTCATTGAAAAAGTA  432
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738863  AGATAGATGTTTGAAATTAGAGAGGTTAGACTTAACTTCGATTTGTTCATTGAAAAAGTA  1738922

Query  433      ATCCAAAGGGACTTCAAGTCGTTGAGAGAGTTTGAATAACAAGTCTGCGGAGGGAATAAA  492
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738923  ATCCAAAGGGACTTCAAGTCGTTGAGAGAGTTTGAATAACAAGTCTGCGGAGGGAATAAA  1738982

Query  493      ATGACCTCTTTCAATTTTACTAATCTGGCTTTGTTCACAAATTCCTTCTGCAAGAGTTTG  552
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738983  ATGACCTCTTTCAATTTTACTAATCTGGCTTTGTTCACAAATTCCTTCTGCAAGAGTTTG  1739042

Query  553      TTGGGAGAGTCTCAACTCTTTTCTTTTCAATCTGAATTTCTCTGCGAGTGTATTCATTAA  612
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1739043  TTGGGAGAGTCTCAACTCTTTTCTTTTCAATCTGAATTTCTCTGCGAGTGTATTCATTAA  1739102

Query  613      AGATAATAAACCTTCCTATTTGCTTAATTTCATTATAAAGTTTTTAGT  660
                ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1739103  AGATAATAAACCTTCCTATTTGCTTAATTTCATTATAAAGTTTTTAGT  1739150
```

Alignment with SPD1746-SeqM-F2

```
Query  165   ATCGATTTTCGTTCGTGAATACATGTTATAATAACTATAACTAATAACGTAACGTGACTG  224
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1412  ATCGATTTTCGTTCGTGAATACATGTTATAATAACTATAACTAATAACGTAACGTGACTG  1471

Query  225   GCAAGAGATATTTTTAAAACAATGAATAGGTTTACACTTACTTTAGTTTTATGGAAATGA  284
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1472  GCAAGAGATATTTTTAAAACAATGAATAGGTTTACACTTACTTTAGTTTTATGGAAATGA  1531

Query  285   AAGATCATATCATATATAATCTAGAATAAAATTAACTAAAATAATTATTATCTAGATAAA  344
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1532  AAGATCATATCATATATAATCTAGAATAAAATTAACTAAAATAATTATTATCTAGATAAA  1591

Query  345   AAATTTAGAAGCCAATGAAATCTATAAATAAACTAAATTAAGTTTATTTAATTAACAACT  404
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1592  AAATTTAGAAGCCAATGAAATCTATAAATAAACTAAATTAAGTTTATTTAATTAACAACT  1651

Query  405   ATGGATATAAAATAGGTACTAATCAAAATAGTGAGGAGGATATATTTGAATACATACGAA  464
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1652  ATGGATATAAAATAGGTACTAATCAAAATAGTGAGGAGGATATATTTGAATACATACGAA  1711

Query  465   CAAATTAATAAAGTGAAAAAAATACTTCGGAAACATTTAAAAAATAACCTTATTGGTACT  524
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1712  CAAATTAATAAAGTGAAAAAAATACTTCGGAAACATTTAAAAAATAACCTTATTGGTACT  1771

Query  525   TACATGTTTGGATCAGGAGTTGAGAGTGGACTAAAACCAAATAGTGATCTTGACTTTTTA  584
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1772  TACATGTTTGGATCAGGAGTTGAGAGTGGACTAAAACCAAATAGTGATCTTGACTTTTTA  1831

Query  585   GTCGTCGTATCTGAACCATTGACAGATCAAAGTAAAGAAATACTTATACAAAAAATTAGA  644
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1832  GTCGTCGTATCTGAACCATTGACAGATCAAAGTAAAGAAATACTTATACAAAAAATTAGA  1891

Query  645   CCTATTTCAAAAAAAATAGGAGATAAAAGCAACTTACGATATATTGAATTAACAATTATT  704
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1892  CCTATTTCAAAAAAAATAGGAGATAAAAGCAACTTACGATATATTGAATTAACAATTATT  1951

Query  705   ATTCAGCAAGAAATGGTACCGTGGAATCATCCTCCCAAACAAGAATTTATTTATGGAGAA  764
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1952  ATTCAGCAAGAAATGGTACCGTGGAATCATCCTCCCAAACAAGAATTTATTTATGGAGAA  2011

Query  765   TGGTTACAAGAGCTTTATGAACAAGGATACATTCCTCAGAAGGAATTAAATTCAGATTTA  824
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  2012  TGGTTACAAGAGCTTTATGAACAAGGATACATTCCTCAGAAGGAATTAAATTCAGATTTA  2071

Query  825   ACCATAATGCTTTACCAA  842
             ||||||||||||||||||
Sbjct  2072  ACCATAATGCTTTACCAA  2089
```

Appendix 2

DNA sequencing of pCEP constructs carrying the intact copies of target genes and their putative promoter regions for genetic complementation analysis.

(A) *tprA*, SPD_1745

Alignment with Comp-Seq-F

```
Query  80       AACTTTTTTTTTTGTTTATTACCATAAAGAATTATTTATTCCTTTAACTTGCTATTTTGA  139
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1739210  AACTTTTTTTTTTGTTTATTACCATAAAGAATTATTTATTCCTTTAACTTGCTATTTTGA  1739151

Query  140      ACTAAAAACTTTATAATGAAATTAAGCAAATAGGAAGGTTTATTATCTTTAATGAATACA  199
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1739150  ACTAAAAACTTTATAATGAAATTAAGCAAATAGGAAGGTTTATTATCTTTAATGAATACA  1739091

Query  200      CTCGCAGAGAAATTCAGATTGAAAAGAAAAGAGTTGAGACTCTCCCAACAAACTCTTGCA  259
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1739090  CTCGCAGAGAAATTCAGATTGAAAAGAAAAGAGTTGAGACTCTCCCAACAAACTCTTGCA  1739031

Query  260      GAAGGAATTTGTGAACAAAGCCAGATTAGTAAAATTGAAAGAGGTCATTTTATTCCCTCC  319
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1739030  GAAGGAATTTGTGAACAAAGCCAGATTAGTAAAATTGAAAGAGGTCATTTTATTCCCTCC  1738971

Query  320      GCAGACTTGTTATTCAAACTCTCTCAACGACTTGAAGTCCCTTTGGATTACTTTTTCAAT  379
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738970  GCAGACTTGTTATTCAAACTCTCTCAACGACTTGAAGTCCCTTTGGATTACTTTTTCAAT  1738911

Query  380      GAACAAATCGAAGTTAAGTCTAACCTCTCTAATTTCAAACATCTATCTGCTCGCCTATTA  439
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738910  GAACAAATCGAAGTTAAGTCTAACCTCTCTAATTTCAAACATCTATCTGCTCGCCTATTA  1738851

Query  440      GATGACAGAAATTATGACGATTTAGAATATCTTTATAAAATAGAGGTAGAACGAAGCACT  499
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738850  GATGACAGAAATTATGACGATTTAGAATATCTTTATAAAATAGAGGTAGAACGAAGCACT  1738791

Query  500      TTTCTAACATTAGAAGACCGAACTTACCTTGAATGGATTAAATCTATAATTGACTTCTAT  559
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738790  TTTCTAACATTAGAAGACCGAACTTACCTTGAATGGATTAAATCTATAATTGACTTCTAT  1738731

Query  560      CAATATGACAGTAAGTGTGAGGCAATTTCGTCATTGGAAAATATATTATTAAAAGTCTCT  619
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738730  CAATATGACAGTAAGTGTGAGGCAATTTCGTCATTGGAAAATATATTATTAAAAGTCTCT  1738671

Query  620      TCAAATACTCTGATTTATTTAAAGGCGTTGAATACTCTATCTAATTTCTATTCCTTAGTG  679
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738670  TCAAATACTCTGATTTATTTAAAGGCGTTGAATACTCTATCTAATTTCTATTCCTTAGTG  1738611

Query  680      GGTCGTGAACAAGAATATGAGGCAAACTACTCTCATTTAATAGAGTTGTATCAGACAAAA  739
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738610  GGTCGTGAACAAGAATATGAGGCAAACTACTCTCATTTAATAGAGTTGTATCAGACAAAA  1738551

Query  740      AATTTGGACCATCAAGAGTTTTTATTTGGCTACATCAGAGTTCGTTACAACTACGCTCAC  799
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738550  AATTTGGACCATCAAGAGTTTTTATTTGGCTACATCAGAGTTCGTTACAACTACGCTCAC  1738491

Query  800      TACCTAGTATCAAAGGAAAAATATAATGAAGCCATCCAAGAAGCTCTTGAAACGATTGAA  859
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738490  TACCTAGTATCAAAGGAAAAATATAATGAAGCCATCCAAGAAGCTCTTGAAACGATTGAA  1738431

Query  860      CTCTGTAAACAAAGACAGACAAGCTACCAATTGGCTCCTCTACTTATTCTTGTAGGAAAT  919
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738430  CTCTGTAAACAAAGACAGACAAGCTACCAATTGGCTCCTCTACTTATTCTTGTAGGAAAT  1738371

Query  920      GCTGGAGCCAAATTTCTAGACAGAGAACAAGTCAAAAATTATTATATAGAAGCAAGAGAG  979
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738370  GCTGGAGCCAAATTTCTAGACAGAGAACAAGTCAAAAATTATTATATAGAAGCAAGAGAG  1738311

Query  980      TTATGTAAGATTTATAACAATCCTTTAATGTTGATGAAGATAGAAAATTATTTGAAGGAA  1039
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1738310  TTATGTAAGATTTATAACAATCCTTTAATGTTGATGAAGATAGAAAATTATTTGAAGGAA  1738251

Query  1040     CTAGATACTGTTTAG  1054
                |||||||||||||||
Sbjct  1738250  CTAGATACTGTTTAG  1738236
```

(B) *phrA*, SPD_1746

Alignment with Comp-Seq-F

```
Query  78       TAAAGATAATAAACCTTCCTATTTGCTTAATTTCATTATAAAGTTTTTAGTTCAAAATAG  137
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1739100  TAAAGATAATAAACCTTCCTATTTGCTTAATTTCATTATAAAGTTTTTAGTTCAAAATAG  1739159

Query  138      CAAGTTAAAGGAATAAATAATTCTTTATGGTAATAAACAAAAAAAAAAGTTAAAAATTAC  197
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1739160  CAAGTTAAAGGAATAAATAATTCTTTATGGTAATAAACAAAAAAAAAAGTTAAAAATTAC  1739219

Query  198      ATAAAAATACTTGACTAAATAAAATATATCTGTTAGAATAAAAACAAGGAAAAAGAAAGG  257
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1739220  ATAAAAATACTTGACTAAATAAAATATATCTGTTAGAATAAAAACAAGGAAAAAGAAAGG  1739279

Query  258      GGTTTCATTGCATGAGAAAAAAACGTGGAATTAAAAAATTAGTTACATTTGCATTGCTAG  317
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1739280  GGTTTCATTGCATGAGAAAAAAACGTGGAATTAAAAAATTAGTTACATTTGCATTGCTAG  1739339

Query  318      GTGTTTTTATGTTTAGTAATACAATTCCTTACCAACAGTTTATTCAGAAGAATAAACAAT  377
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1739340  GTGTTTTTATGTTTAGTAATACAATTCCTTACCAACAGTTTATTCAGAAGAATAAACAAT  1739399

Query  378      TGGAGATTCGAGTGCAATCGCAAAAGAAGTCCAATGGTCTTGATGTTGGGAAGGCGGATT  437
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1739400  TGGAGATTCGAGTGCAATCGCAAAAGAAGTCCAATGGTCTTGATGTTGGGAAGGCGGATT  1739459

Query  438      AA  439
                ||
Sbjct  1739460  AA  1739461
```

Appendix 3

Table A. Summary of transcriptome comparison of *S. pneumoniae* D39 Δ*tprR* and wild-type grown in CDM plus galactose. (Downregulated genes).

Gene tag[a]	Function[b]	Ratio[c]	P-value
spd_0015		-2.07	1.05E-02
spd_0447	Transcriptional regulator, MerR family protein	-2.02	8.18E-03
spd_0756		-3.66	4.20E-04
spd_0913	Hypothetical protein	-1.99	8.31E-03
spd_0936	Tn5252, relaxase	-2.06	9.16E-02
spd_1410		-4.81	7.12E-04
spd_1683		-3.1	9.87E-03
spd_1685		-2.01	1.48E-02
spd_1687		-2.62	2.46E-03
spd_1694		-3.37	7.31E-04
spd_1695		-2.44	6.89E-03
spd_1696		-3.02	1.79E-03
spd_1697		-2.08	1.31E-02
spd_1745	Transcriptional regulator PlcR, putative	-3.08	7.85E-04
spd_1882		-3.16	1.67E-03

[a]Gene numbers refer to D39 locus tags. [b]D39 annotation/TIGR4 annotation. (Hoskins *et al*., 2001; Lanie *et al*., 2007), [c]Ratios >2.0 or <2.0 (wild-type + 55 mM galactose/wild-type + 0 mM galactose).

Table B. Summary of transcriptome comparison of *S. pneumoniae* D39 Δ*tprR* and wild-type grown in CDM plus galactose. (Upregulated genes).

Gene tag[a]	Function[b]	Ratio[c]	P-value
SPD_0008	Septum formation initiator, putative	2.96	7.27E-03
SPD_0010	Hypothetical protein	2.66	1.12E-02
SPD_0120	Hypothetical protein	2.14	2.46E-02
SPD_0323	Polysaccharide polymerase	2.33	1.57E-02
SPD_0325	Hypothetical protein	2.18	2.09E-02
SPD_0453	Type I restriction-modification system, S subunit	3.29	2.36E-03
SPD_0558	Cell wall-associated serine protease PrtA	2.53	4.03E-02
SPD_0559	PTS system IIA component, putative	2.34	5.86E-03
SPD_0560	PTS system, IIB component, putative	2.91	7.13E-04
SPD_0750	Hypothetical protein	2.16	1.64E-02
SPD_0915	Iron-compound ABC transporter, iron compound-binding protein	2.56	3.87E-03
SPD_0920	Hypothetical protein	2.12	7.90E-03
SPD_0932	Hypothetical protein	2.16	6.32E-03
SPD_1057	PTS system, IIB component, putative	2.76	1.28E-03
SPD_1058		2.52	2.69E-03
SPD_1167	ABC transporter, ATP-binding protein	2.86	5.02E-03
SPD_1169	Oligopeptide ABC transporter, permease protein	2.24	6.89E-03
SPD_1172	N-acetylmannosamine-6-phosphate 2-epimerase 2, putative	3.39	6.87E-04
SPD_1495	Sugar ABC transporter, sugar-binding protein	3.94	3.87E-04
SPD_1496	PTS system, IIBC components	4.55	1.98E-04
SPD_1497	N-acetylmannosamine-6-phosphate 2-epimerase 2, putative	3.61	4.39E-04
SPD_1595	Hypothetical protein	2.37	4.52E-03
SPD_1746	Hypothetical protein	73.25	2.15E-07
SPD_1747	Hypothetical protein	7.95	9.39E-05
SPD_1748	Hypothetical protein	15.1	1.20E-05
SPD_1749	Bacteriocin formation protein, putative	13.08	1.75E-05
SPD_1750	Multimeric flavodoxin WrbA (general function prediction only)	14.38	8.33E-06
Spd_1751	Hypothetical protein	49.43	7.00E-07

[a]Gene numbers refer to D39 locus tags. [b]D39 annotation/TIGR4 annotation. (Hoskins *et al*., 2001; Lanie *et al*., 2007), [c]Ratios >2.0 or <2.0 galactose.

Appendix 4

Table A. Summary of transcriptome comparison of *S. pneumoniae* D39 Δ*tprA* and wild-type grown in CDM plus mannose. (Downregulated genes)

Gene tag[a]	Function[b]	Ratio[c]	P-value
Spd_0093	Hypothetical protein	-2.07	1.05E-02
Spd_0094	Hypothetical protein	-2.02	8.18E-03
Spd_0095	Hypothetical protein	-3.66	4.20E-04
Spd_0308	ATP-dependent Clp protease, ATP-binding subunit	-1.99	8.31E-03
Spd_0703	Hypothetical protein	-2.06	9.16E-02
Spd_0936	Tn5252, relaxase	-4.81	7.12E-04
Spd_1745	Transcriptional regulator PlcR, putative	-3.1	9.87E-03
Spd_1874	LysM domain protein	-2.01	1.48E-02

[a]Gene numbers refer to D39 locus tags. [b]D39 annotation/TIGR4 annotation. (Hoskins *et al*., 2001; Lanie *et al*., 2007), [c]Ratios >2.0 or <2.0 (wild-type + 55 mM mannose/wild-type + 0 mM mannose).

Table B. Summary of transcriptome comparison of *S. pneumoniae* D39 Δ*tprR* and wild-type grown in CDM plus mannose. (Upregulated genes)

Gene tag[a]	Function[b]	Ratio[c]	P-value
Spd_0558	Cell wall-associated serine protease PrtA	2.4	5.60E-03
Spd_1513		3.72	5.35E-05
Spd_1514	ABC transporter, ATP-binding protein	4.93	5.69E-04
Spd_1515	Hypothetical protein	2.62	1.55E-02
Spd_1516	Hypothetical protein	4.04	2.90E-03
Spd_1517	Hypothetical protein	2.93	2.75E-02
Spd_1746	Hypothetical protein	8.7	1.65E-07
Spd_1747	Hypothetical protein	7.37	7.77E-06
Spd_1748	Hypothetical protein	9.49	4.35E-07
Spd_1749	Bacteriocin formation protein, putative	4.39	1.55E-05
Spd_1750	Bultimeric flavodoxin WrbA (general function prediction only)	3.4	5.61E-05
Spd_1751	Hypothetical protein	9.49	5.96E-08
Spd_1753	Serine protease, subtilase family protein	7.5	7.58E-08
Spd_1754	Hypothetical protein	4.52	2.21E-06
Spd_1755	ABC transporter, ATP-binding protein	3.08	8.26E-05
Spd_1756	Hypothetical protein	3.95	6.47E-06
Spd_1944	CAAX amino terminal protease family protein	3.72	5.70E-05
Spd_1945	Hypothetical protein	2.04	2.96E-02
Spd_1946	Hypothetical protein	4.71	9.12E-06
Spd_1947	Transcriptional regulator, putative	3.92	8.85E-05
Spd_1948	Hypothetical protein	4.21	9.46E-06

[a]Gene numbers refer to D39 locus tags. [b]D39 annotation/TIGR4 annotation. (Hoskins *et al*., 2001; Lanie *et al*., 2007), [c]Ratios >2.0 or <2.0 (wild-type + 55 mM mannose/wild-type + 0 mM mannose).

Appendix 5

DNA sequencing of pPP1 constructs carrying the putative promoters of target genes for transcriptional *lacZ*-fusion analysis.

(A) P*tprA* (SPD_1745)

Alignment with Fusion-Seq-F

```
Query  43       CTTTCTTTTTCCTTGTTTTTATTCTAACAGATATATTTTATTTAGTCAAGTATTTTTATG  102
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1739278  CTTTCTTTTTCCTTGTTTTTATTCTAACAGATATATTTTATTTAGTCAAGTATTTTTATG  1739219

Query  103      TAATTTTTAACTTTTTTTTTTGTTTATTACCATAAAGAATTATTTATTCCTTTAACTTGC  162
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1739218  TAATTTTTAACTTTTTTTTTTGTTTATTACCATAAAGAATTATTTATTCCTTTAACTTGC  1739159

Query  163      TATTTTGAACTAAAAACTTTATAATGAAATTAAGCAAATAGGAAGGTTTATTATCTTTAA  222
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1739158  TATTTTGAACTAAAAACTTTATAATGAAATTAAGCAAATAGGAAGGTTTATTATCTTTAA  1739099

Query  223      TGAATACACTCGCAGAGAAATTCAGAT  249
                |||||||||||||||||||||||||||
Sbjct  1739098  TGAATACACTCGCAGAGAAATTCAGAT  1739072
```

(B) P*phrA* (SPD_1746)

Alignment with Fusion-Seq-F

```
Query  44       TAAAGATAATAAACCTTCCTATTTGCTTAATTTCATTATAAAGTTTTTAGTTCAAAATAG  103
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1739100  TAAAGATAATAAACCTTCCTATTTGCTTAATTTCATTATAAAGTTTTTAGTTCAAAATAG  1739159

Query  104      CAAGTTAAAGGAATAAATAATTCTTTATGGTAATAAACAAAAAAAAAAGTTAAAAATTAC  163
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1739160  CAAGTTAAAGGAATAAATAATTCTTTATGGTAATAAACAAAAAAAAAAGTTAAAAATTAC  1739219

Query  164      ATAAAAATACTTGACTAAATAAAATATATCTGTTAGAATAAAAACAAGGAAAAAGAAAGG  223
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1739220  ATAAAAATACTTGACTAAATAAAATATATCTGTTAGAATAAAAACAAGGAAAAAGAAAGG  1739279

Query  224      GGTTTCATTGCATGAGAAAAAAACGTGGAATTAAAAAAT  262
                ||||||||||||||||||||||||||||||||||||||||
Sbjct  1739280  GGTTTCATTGCATGAGAAAAAAACGTGGAATTAAAAAAT  1739318
```

(C) P*nanA* (SPD_1504)

Alignment with Fusion-Seq-F

```
Query  43       GCAGGAAGTATGGTGTAAATAGCATAAGCTGATGTCCATCATTTGCTTATAAAGAGATAT  102
                |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1525824  GCAGGAAGTATGGTGTAAATAGCATAAGCTGATGTCCATCATTTGCTTATAAAGAGATAT  1525765

Query  103      TTTAGTTTAATTGCAGCGGTGTCCTGGTAGATAAACTAGATTGGCAGGAGTCTGATTGGA  162
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1525764  TTTAGTTTAATTGCAGCGGTGTCCTGGTAGATAAACTAGATTGGCAGGAGTCTGATTGGA  1525705

Query  163      GAAAGGAGAGGGGAAATTTGGCACCAATTTGAGATAGTTTGTTTAGTTCATTTTTGTCAT  222
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1525704  GAAAGGAGAGGGGAAATTTGGCACCAATTTGAGATAGTTTGTTTAGTTCATTTTTGTCAT  1525645

Query  223      TTAAATGAACTGTAGTAAAAGAAAGTTAATAAAAGACAAACTAAGTGCATTTTCTGGAAT  282
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Sbjct  1525644  TTAAATGAACTGTAGTAAAAGAAAGTTAATAAAAGACAAACTAAGTGCATTTTCTGGAAT  1525585

Query  283      AAATGTCTTATTTCAGAAATCGGGATATAGATATAGAGAGGAACAGTATGAATCGGAGTG  342
                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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Query  343      TTCAAGAACGTAAGTGTCGTTATAGCATTAGGAAACTATCGGTAGGAGCGGTTTCTATGA  402
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Sbjct  1525524  TTCAAGAACGTAAGTGTCGTTATAGCATTAGGAAACTATCGGTAGGAGCGGTTTCTATGA  1525465

Query  403      TTGTAGGAGCAGTGGTATTTGGAACG  428
                |||||||||||||||||||||||||
Sbjct  1525464  TTGTAGGAGCAGTGGTATTTGGAACG  1525439
```
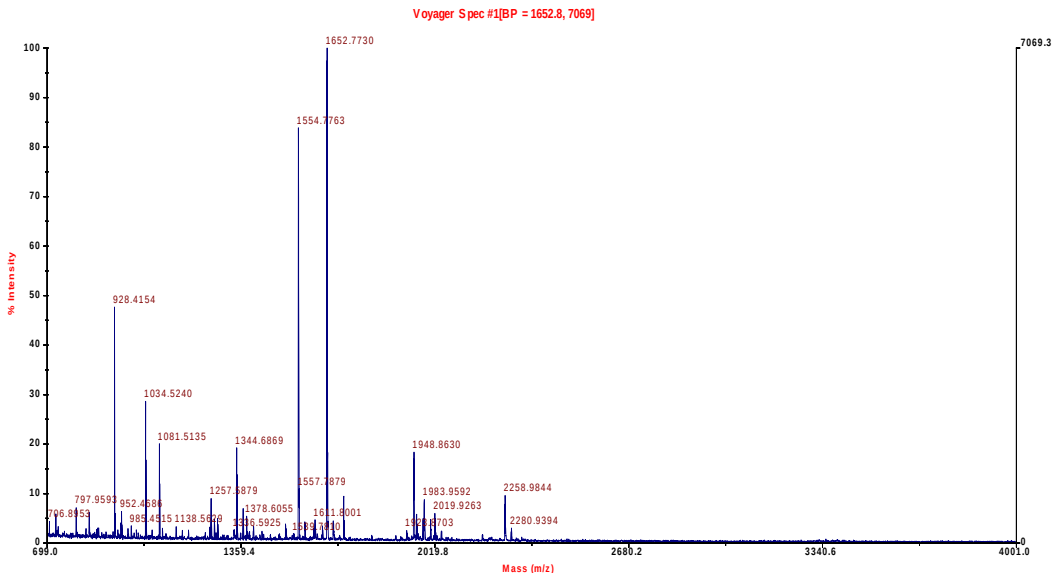

Appendix 6

MALDI-TOF mass spectrometry confirming the identity of the recombinant protein.

TprA



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