Streptococcus pneumoniae - stress hormone interactions

Thesis submitted for the degree of
Doctor of Philosophy
at the University of Leicester

by

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September 2017

Statement of originality

This accompanying thesis submitted for the degree of PhD entitled "*Streptococcus pneumoniae*-stress hormone interactions" 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 October 2013 and September 2017.

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

Streptococcus pneumoniae is one of the most important bacterial pathogens of humans causing a wide range of mild to life-threating diseases. It is also a commensal microorganism in the nasopharynx of up to 60% of people. Fundamental aspects of its ability for transition from colonisation to an infectious state as well as how bacterial-host interactions influence this process are largely unknown. In the field of microbial endocrinology, it has been well established in mainly Gram-negative bacteria that stress hormones such as norepinephrine epinephrine and dopamine play an essential role in determining the outcome of bacterial infections. This study successfully established the conditions to investigate *S. pneumoniae*-stress hormone interactions using modified serum-SAPI media. 13 mutants lacking two-component regulatory system and 4 two-component system fusion reporter strains were created, and examined for their role in *S. pneumoniae*-stress hormone interactions. This study demonstrated that *S. pneumoniae* is stress hormone responsive and has mechanisms to recognise and process host stress hormones by a transferrin-iron delivery mechanism, which evidence suggests might be mediated via the TCS09 system since hormone-induced growth and radiolabelled norepinephrine and Fe uptake were reduced in a ΔTCS09 mutant. In addition, the pneumococcal response to stress hormone exposure resulted in a change in cell-cell association from chains into diplococci and cell morphology by reducing cell size and the capsule. Furthermore, the pneumococcal exposure to norepinephrine also increased biofilm formation and significantly altered metabolism. The analysis of *in vivo* experiments indicated that a stress hormone encounter might trigger translocation from the nasopharynx into the lungs, which may enhance *S. pneumoniae* in its transition from commensal to pathogen. Therefore, the pneumococcal ability to respond to host stress signals may be key to its capacity to cause life-threatening pneumonia, septicaemia and meningitis.

Acknowledgments

I would like to thank my supervisors Dr. Hasan Yesilkaya and Dr. Primrose Freestone for their valuable and continues support during my study. I cannot express in words how thankful I am for their intellectual and scientific assistance during lab work as well as encouragement, patience and critical feedback during the writing of this thesis.

I would also like to express my gratitude to my colleagues in lab 125 for their informative meetings and friendship.

In addition, I am very thankful to the progress review panel members, Prof. Mike Barer and Dr. Ed Galyov for their advice and feedback, which was greatly appreciated.

Additionally, I would like to thank the staff of Protein Nucleic Acid Chemistry Laboratory (PNACL) for their great work in sequencing DNA fragments with the data collection and analysis.

I would like to thank Dr. Morten Danielsen and staff of MS-Omics (Frederiksberg, Denmark) for their assistance during Metabolomics analysis.

I am very grateful to my sponsors, Majmaah University in Saudi Arabia and the Saudi Cultural Bureau in London, for providing the funds of my PhD study.

I dedicate this thesis to my father, who would be very proud, and my beloved mother for her endless wishes and prayers. I also dedicate this thesis to my wife for her support and patience throughout my entire study.

Abbreviations

OD	Optical density	v/v	Volume per volume
SDS	Sodium dodecyl sulphate	w/v	Weight per volume
BSA	Bovine serum albumin	x g	Gravity force
CFU	Colony forming unit	g	Gram
EDTA	Ethylene diamine tetra acetic acid	mg	Milligram
PBS	Phosphate buffered saline	µg	Microgram
RNA	Ribonucleic acid	ml	Millilitre
DNA	Deoxyribonucleic acid	µl	Microliter
PCR	Polymerase chain reaction	µM	Micromolar
CAP	Community-acquired pneumonia	bp	Base pair
CSP	Competence stimulating peptide	Dop	Dopamine
TCS	Two-component system	Tf	Transferrin
IPD	Invasive pneumococcal disease	Epi	Epinephrine
CDM	Chemically defined medium	BAB	Blood agar base
ICU	Intensive care unit	NE	Norepinephrine
VAP	Ventilator-associated pneumonia	LB	Luria-Bertani broth
TCA	Tricarboxylic acid cycle	BHI	Brain heart infusion

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Chapter 1. Introduction

1.1. Project background

Streptococcus pneumoniae is one of the most common causes of community and hospital acquired pneumonia as well as otitis media, meningitis and septicaemia (Bogaert et al., 2004). In addition, pneumococci harmlessly colonise the upper respiratory tract in approximately 10% of healthy adults and 60% of children. This situation is known as carriage (Bogaert et al., 2004;Dudley et al., 2001). Despite the pneumococcal carriage and range of diseases, fundamental aspects of its ability for transition from colonisation to invasive mode are not fully understood. What are the host signals involved in these events and how do pneumococci recognise, process and respond to such signals?

Microorganisms communicate with their hosts by the assistance of several host molecules, such as hormones that are released from eukaryotic cells and sensed by bacteria, events known as inter-kingdom signalling (Freestone et al., 2008a). Such hormonal communication is part of a relatively recent research discipline that has been named Microbial Endocrinology. This field of study aims to understand and examine the microorganism-host interaction in both health and disease. The influence of stress-associated hormones on bacterial growth and the outcome of infection have been demonstrated in several studies (Freestone et al., 2008a;Lyte, 2004;Sharaff and Freestone, 2011). However, how the pneumococcus responds to the stress hormones is not known in detail. Stress is defined as "consequences of the failure of a human or animal to respond appropriately to emotional or physical threats to the organism, whether actual or imagined" (Reiche et al., 2004). Stressors can be physical, psychological or environmental factors, and when stress is sensed by central nervous system (CNS), various neurochemicals such as hormones are released. These hormones include a wide range of neuropeptides, glucocorticoids (e.g. cortisol), and catecholamines including dopamine, adrenaline (epinephrine) and noradrenaline

(norepinephrine) (Lyte and Freestone, 2010). It has been repeatedly suggested in an increasing number of reports that stress-associated chemicals may play a critical role in the determination of the outcome of bacterial infections (Freestone et al., 2008a). The recognition of stress hormone involvement in increasing the risk of infection has arisen from the evidence that stress-related molecules negatively affect immune function and have impact on bacterial growth and virulence (Freestone et al., 2008a;Reiche et al., 2005). Previous studies have shown that catecholamines increase growth of a number of, mainly Gram-negative, pathogenic bacteria such as *Pseudomonas aeruginosa*, *Salmonella typhi*, *Escherichia coli* and *Campylobacter jejuni* (Freestone et al., 2008a). The catechol-containing moiety within catecholamine plays a major role of such growth stimulating effect by forming an iron complex inside transferrin (Tf) or lactoferrin (Lf). This weakens Fe binding and subsequently facilitates bacteria to access the otherwise inaccessible Fe-complex (Sandrini et al., 2010). Catecholamines have also had a direct impact on bacterial virulence. For example, augmented *E. coli* O157:H7 attachments to intestinal epithelial cells as well as secretory and inflammatory responses were induced by norepinephrine (NE) (Vlisidou et al., 2004). In infection caused by *Vibrio parahaemolyticus*, for instance, NE also has shown an increased level of cellular cytotoxicity and enterotoxicity (Nakano et al., 2007). More importantly, treatment of critically ill patients by catecholamine inotropes (epinephrine and dopamine) has shown increased levels of pseudomonad and staphylococcal biofilm formation as well as supporting microbial recovery after antibiotic exposure (Freestone et al., 2008a;Freestone et al., 2012;Lyte et al., 2003).

Most of the data about these interactions between bacteria and stress hormones has been obtained from the findings of studies on Gram-negative intestinal pathogens. Knowledge of Gram-positive bacteria-catecholamine interactions are relatively little, particularly, the infection significance of *S. pneumoniae*. This is despite an older finding that patients with pneumococcal pneumonia had a remarkable increase of plasma stress hormone levels in comparison with healthy people (Feldman et al., 1989). Moreover, mice exposed to stress have been shown to be more sensitive to pneumococcal infection (Kim et al., 2011). A recent study using a tissue culture and

animal biofilm model of infection has shown that the pneumococci recognise host factors such as glucose, cell lysates, ATP and norepinephrine as signals to disperse from biofilms and to subsequently cause invasive disease (Marks et al., 2013). In spite of these significant findings, *S. pneumoniae* response to catecholamines *in vitro* and *in vivo* as well as the molecular mechanisms by which catecholamine signals are recognised and processed by pneumococci are largely unknown.

1.2. The pneumococcus

Streptococcus pneumoniae (also known as the pneumococcus), was first independently isolated by Louis Pasteur and George M. Sternberg, when Pasteur inoculated rabbits with saliva of pneumococci-asymptomatic carriers, and observed quick progression to septicaemia (Sternberg, 1881;Pasteur, 1881). Initially, Pasteur described the pneumococcus surrounded by "an aureole which corresponds perhaps to a material substance", which was later recognised as the pneumococcal capsule (Pasteur, 1881). It is worth recognising the roles played by pneumococci since its discovery in the establishment of several scientific principles. The sera of people who had recovered from pneumonia protected rabbits against the disease leading to the discovery of passive immunity by Felix and Georg Klemperer, who made the first descriptions of humoral immunity (Klemperer and Klemperer, 1891). Pneumococcal disease was also involved in the development of the most influential drugs of the 20th century, Penicillin, whereas pneumococcal polysaccharide contributed towards vaccine production (Grabenstein and Klugman, 2012). It is also worth mentioning the role of this microorganism that led to a transforming principle, which later verified to be the DNA (Griffith, 1928) through observing the ability of non-encapsulated pneumococcal strains to acquire the capsular type of the heat-killed encapsulated strain after co-administration to mice (Watson et al., 1993;Kazanjian, 2004).

Streptococcus pneumoniae is a Gram-positive bacterium belonging to the *Streptococcaceae* family. *S. pneumoniae* appears as lancet shaped diplococci in pairs and short chains, and forms α-haemolytic mucoid colonies surrounded by green zones on

the surface of blood agar. The pneumococci are susceptible to ethylhydrocupreine HCl (optochin) and autolyse quickly when treated with detergents (Garrity, 2006). The pneumococcal optimum pH range is between pH 6.5-8, but during pneumococcal growth, lactic acid is produced, bringing the pH of the culture to approximately pH 5.0. Therefore, the medium is often buffered with serum to minimise the effect of the potentially lethal drop of the pH to protect the bacterium (Rotta, 1986). Regarding metabolism, pneumococci are fermentative facultative anaerobic bacteria and grow well with 5% CO_2. The pneumococci are catalase negative since they are not able to synthesise haem compounds. Hence, red blood cells are usually added to the culture media as a source of catalase (Rotta, 1986).

1.2.1. Pneumococcal virulence factors

A microbial virulence factor can be simply defined as a component of microbes which contributes to virulence and subsequently damages the host (Casadevall and Pirofski, 1999). The pneumococci have an arsenal of virulence factors mainly surface-associated proteins that have direct interaction with the host (Jedrzejas, 2001). The most studied pneumococcal virulence factors include: the polysaccharide capsule (PSC), pneumolysin (Ply), choline-binding proteins (CbpA) also called pneumococcal surface protein C (PspC), neuraminidases (NanA and NanB), hyaluronate lyase (Hyl), pneumococcal surface protein A (PspA), autolysin (LytA), pneumococcal surface antigen A (PsaA), enolase (Eno) and pneumococcal adhesion and virulence A protein (PavA) (Jedrzejas, 2001;Kadioglu et al., 2008). The spatiotemporal importance of these virulence elements during pneumococcal infection can vary, although the maximum virulence of the pneumococcus involves the collective action of all these factors (Kadioglu et al., 2008).

Clinical and experimental studies have shown that the PSC is the most important virulence determinant for pneumococci. It is essential throughout colonisation, invasion, and dissemination from the respiratory tract. The capsule also protects against mechanical clearance by mucous secretion and aids the bacteria in the

transition to epithelial surface (Nelson et al., 2007). The highly negatively charged PSC inhibits phagocytic CR3-iC3b interaction as well as interaction with IgG component Fcγ- Fc (Nelson et al., 2007;Hammerschmidt et al., 2005). The capsule minimises exposure to numerous antibiotics and limits autolysis (Bogaert et al., 2004;Kadioglu et al., 2008). Different pneumococcal serotypes can naturally colonise the nasopharynx in otherwise healthy people. However, when they manage to translocate from the mucosal barrier to disseminate inside the host, they can cause severe damage and diseases (Gillespie, 1989). Approximately 20 out of over 90 serotypes are able to cause serious invasive diseases (Mulholland, 1999;Musher, 1992). This is due to pneumococcal ability to adhere to and invade various epithelial and endothelial cells via cell-associated mechanisms for internalisation (Adamou et al., 1998;Cundell et al., 1995;Elm et al., 2004;Ring et al., 1998;Talbot et al., 1996;Zhang et al., 2000). It has been demonstrated that PSC modulation is a vital process throughout the transition from carriage to invasive disease in another pathogen inhabiting the nasopharynx. For example, phase-off capsule production in *Neisseria meningitidis* improves tissue invasion whereas phase-on is essentially required in order to survive under systemic infection conditions (Hammerschmidt et al., 1996). In *S. pneumoniae*, several studies have shown that the PSC plays a significant role in the pneumococcal pathogenesis and systemic dissemination rendering the organism resistant to complement-related opsonophagocytosis (Avery and Dubos, 1931;Brown, 1985;Hardy et al., 2001;Kelly et al., 1994;van Dam et al., 1990;Watson and Musher, 1990;Winkelstein, 1984). It is also reported that encapsulated pneumococci support colonisation of the nasopharynx compared with unencapsulated strains, though strains with markedly decreased capsule levels were adequate for murine carriage compared with the wild type (Magee and Yother, 2001).

1.2.2. Pneumococcal disease

In spite of the two current vaccines for the prevention of invasive pneumococcal disease, a polysaccharide 23-valent and a pneumococcal conjugate 13-valent vaccine, pneumococci remain the most frequent cause of bacterial meningitis and community-

acquired pneumonia (CAP) worldwide. It is reported by the World Health Organisation (WHO) that an estimated annual 1.6 million deaths can be attributed to invasive pneumococcal disease (IPD) (Schuchat et al., 1997;Ortqvist et al., 2005;Ludwig et al., 2012). IPD generally refers to the pneumococcal presence inside the otherwise sterile host sites including blood, cerebrospinal, pleural and cerebrospinal fluids, while non-invasive state of pneumonia and other conditions like sinusitis and otitis media do not usually accompany positive blood cultures (Ludwig et al., 2012) (Figure 1.1).

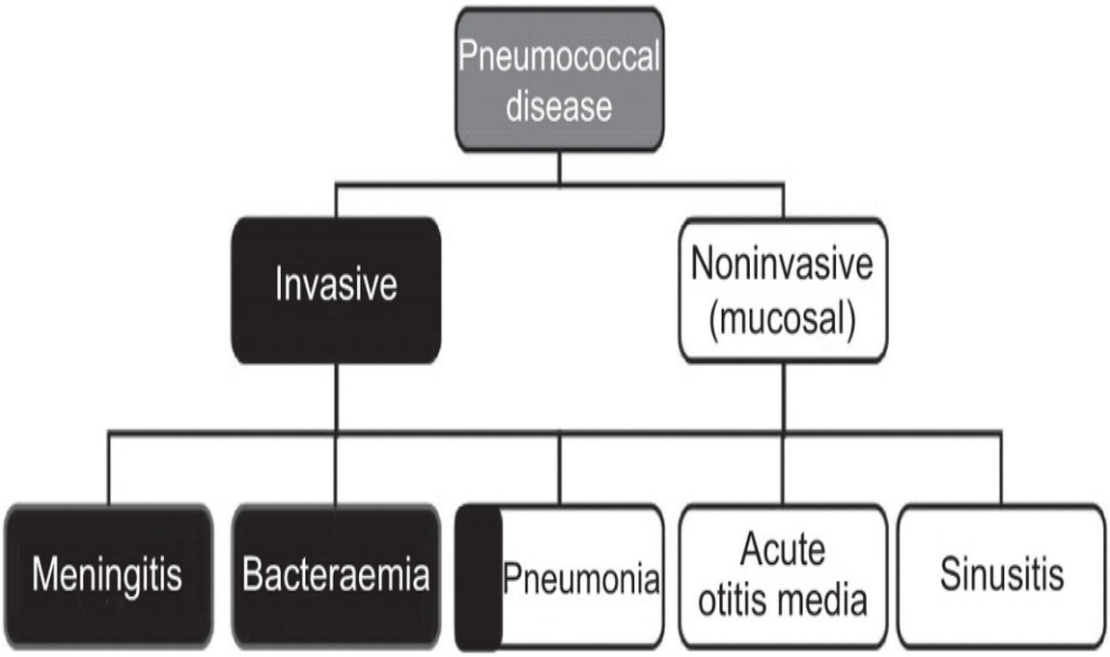


Figure 1.1. Pneumococcal invasive and non-invasive disease. Invasive disease is recognised when *S. pneumoniae* is isolated from naturally sterile body fluids while pneumonia can be non-invasive or invasive if bacteraemic (Ludwig et al., 2012).

Pneumococcal pneumonia has caught medical attention due to the high incidence and mortality rate of CAP particularly in old population. The estimated annual incidence rate of CAP in the US is 6 cases per 1000 adults, and although mortality rate is 2-3%, it can reach up to 25% and 12% in hospitalised patients with and without bacteraemia, respectively (Mandell, 2004;Marrie et al., 2003;Fine et al., 1996;Marrie and Huang,

2005). In the UK, the pneumococci are the most isolated pneumonia-causing pathogen (Lim et al., 2001). However, the largest incidence of IPD is found in Africa where there are about 4 million cases every year and death rates caused by pneumococcal bacteraemia can exceed 30% (Berkley et al., 2005;Scott, 2007). Depending on age and geography, serotypes accountable for IPD can vary significantly, however, serotypes 1, 2 (used in this study), 4, 5, 7F, 9V, 14, and 16 are predominately isolated from invasive diseases (Brueggemann et al., 2003;Kronenberg et al., 2006;Hausdorff et al., 2000). Acute Otitis Media (AOM) is considerably less severe than invasive disease, though much more prevalent accounting for annual 30 million visits to American health care settings mainly by children, 25% of which are *S. pneumoniae*-associated cases (Klein, 2000;Kilpi et al., 2001). AOM occurs when the pneumococci manage to translocate from nasopharynx cavity to the middle ear through Eustachian tube. Regardless of their abilities to cause invasive disease, most pneumococcal serotypes were found to have an equal tendency of causing AOM, which is linked to their prevalence in nasopharyngeal carriage (Hanage et al., 2004).

Colonising strains of *S. pneumoniae* can readily be detected in approximately 10%, 40% and more than 60% of healthy adults, children and infants in day-care settings, respectively (Bogaert et al., 2004;Dudley et al., 2001). Many clinical and laboratory studies have identified a number of risk factors for pneumococcal pneumonia and invasive pneumococcal disease. The high-risk factors for IPD include age; children under 2 years and adults older than 65 of age, alcoholism, asplenia, HIV and influenza infection as well as diabetes mellitus and defect in humoral immunity (immunoglobulin and complement deficiencies)(van der Poll and Opal, 2009). The defects of host defences, particularly, can lead to massive changes in the host–pathogen interaction, which is also an advantage even to low virulence strains in invading the immunocompromised host (Kadioglu et al., 2008;Orihuela et al., 2004;Zhang et al., 2007). Another definite risk factor is the recent acquisition of an invasive strain, which is considered far more important than the duration of colonisation as a determinant of subsequent risk of IPD (Brueggemann et al., 2003). Other studied IPD factors of moderate risk include crowding, poverty, isolated population, cigarette smoking,

chronic lung diseases and low pneumococcal vaccine use (Bogaert et al., 2004;Smith et al., 1993). It is suggested by studies on the conjugate seven-valent pneumococcal vaccine in children that the response of humoral immunity can lead to a massive reduction in the likelihood risk of pneumococcal transmission in vaccine recipients (Bogaert et al., 2004;Millar et al., 2008). However, the total decline of incidence was associated with a minor increase in IPD from the non-vaccine serotypes (Hicks et al., 2007), a pattern that appears to be ongoing, and has caught the attention for the development of other vaccine formulations (van der Poll and Opal, 2009).

1.2.3. Pneumococcal carriage

Although pneumococci can be isolated from the nasopharynx of chimpanzees and equine hosts, pneumococcal infections are almost exclusive to the human population (Whatmore et al., 1999;Chi et al., 2007). The carriage state of pneumococci within the mucosal surfaces of the upper respiratory tract facilitates an entrance to all disease manifestations (Figure 1.2). Pneumococcal carriage is believed to be the precursor for the horizontal transmission (person-to-person direct contact) of pneumococcus in the population (Bogaert et al., 2004), whereas the closeness of animal species to humans may result in carriage in specific circumstances (Chi et al., 2007).

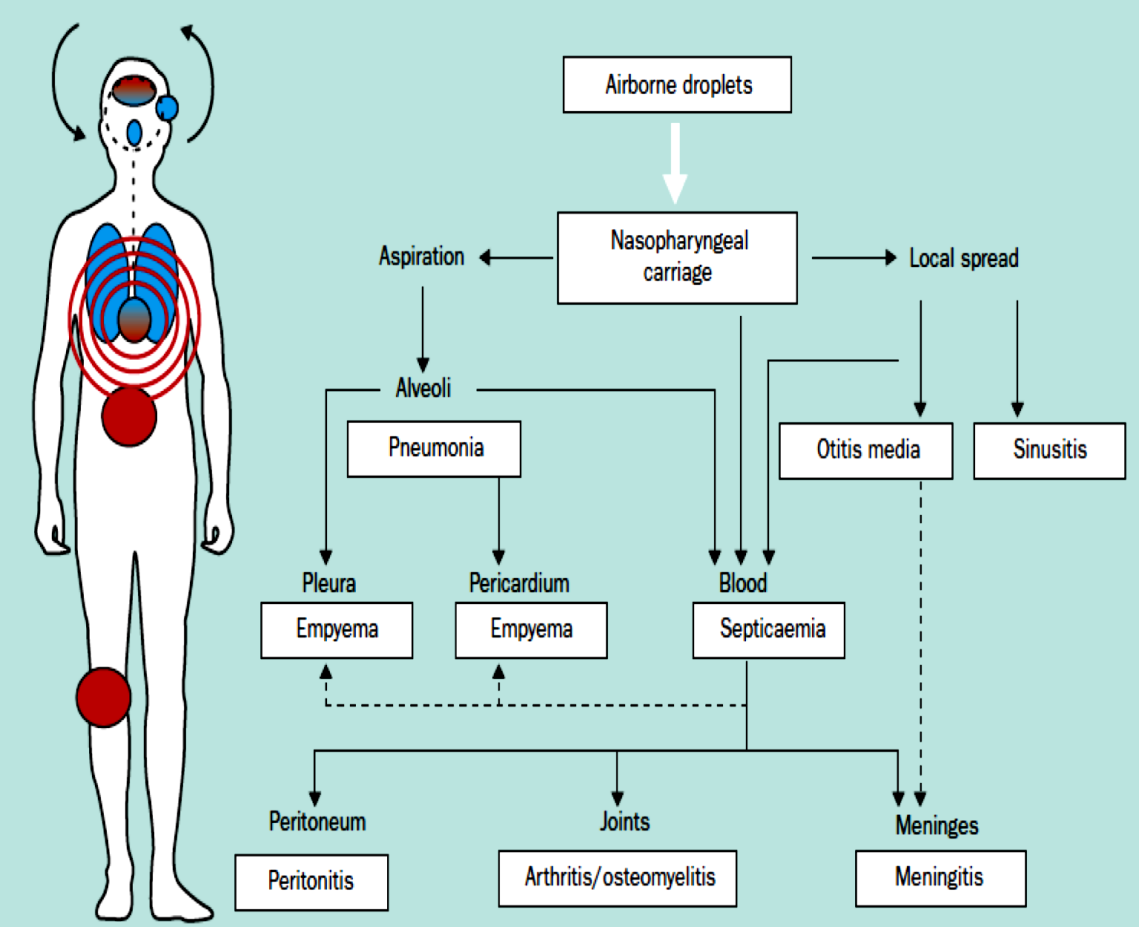


Figure 1.2. Pneumococcal routes of infection. The diagram illustrates the dissemination of *S. pneumoniae* within the host. All subsequent routes initiates from upper respiratory bacterial intake and nasopharyngeal carriage, which can then progress to otitis media, pneumonia, meningitis and septicaemia (Bogaert et al., 2004).

Pneumococcal carriage in the developing and developed world occurs within the first few years of life with 50% of children less than 5 years old are being colonised at any given time (Hansman et al., 1985;Hill et al., 2008;Syrjanen et al., 2001). The prevalence of colonisation declines significantly from over 50% down to less than 20% after 5 years of age (Abdullahi et al., 2008;Hussain et al., 2005). This dramatic change in the rate of carriage is believed to be associated with the development of the immune system, since children less than 2 years old produce only low levels of serum antibodies against antigens such as pneumococcal capsular polysaccharide (Douglas et al., 1983). Furthermore, they also produce markedly low anti-capsular IgG compared to their mothers when colonised by a particular serotype (Soininen et al., 2001).

Nasopharyngeal colonisation is an intermittent state, and persistence of carriage therefore can vary from a few days to a few months of any colonising strain before being cleared or rather replaced by a subsequent colonisation episode by an independent strain (Smith et al., 1993;Erasto et al., 2010). This turnover occurs faster in adults, and is probably due to constant exposure to pneumococcal strains through nasopharyngeal colonisation (Goldblatt et al., 2005;Rapola et al., 2000). Approximately, one third of carriage cases are accounted by more than one pneumococcal serotype, and up to six serotypes can be isolated from the nasopharynx at any given time (Meats et al., 2003;Hill et al., 2008;Hansman et al., 1985). The most commonly isolated colonising serotypes in children are 6, 19, 14 and 23 regardless of geographical restriction while colonising serotypes for adults have a much broader distribution (Meats et al., 2003;Soininen et al., 2001;Goldblatt et al., 2005). Pneumococcal transition from asymptomatic nasopharyngeal carriage to invasiveness is poorly understood. In addition, the association between carriage and disease is rather complex as epidemiological evidence suggests the involvement of factors such as age, serotype, vaccination status and previous encounter to pneumococci. For instance, 6, 19, 14 and 23 serotypes related to carriage are also able to cause invasive disease (Pineda et al., 2002). Moreover, other serotypes known to cause invasive disease have shorter colonisations period than the less invasive ones that persist longer in the nasopharynx (Sleeman et al., 2006). Nevertheless, it is probably incorrect to consider that variation in pneumococcal carriage is merely due capsular serotype. For example, a study demonstrated an increased prevalence of carriage in association with *in vitro* shorter lag phase growth in minimal medium, which suggests that under specific condition, the genetic background of the pneumococcal strains may also affect the outcome during carriage (Battig et al., 2006).

1.2.4. Pneumococcal biofilm formation in carriage and disease

Microbial biofilm can be generally described as communities of sessile cells attached to each other or to a substratum while embedded in a mixture of polymeric substances (Donlan and Costerton, 2002). Bacterial ability to attach to different surfaces and form

biofilms is an important aspect of virulence, particularly when developing a respiratory infection. Naturally biofilm-forming bacteria are widely distributed in the environment and are able to colonise specific sites. These environments include, for instance, the endocarditic decay caused by *Streptococcus viridans,* and the respiratory mucous membrane of cystic fibrosis (CF) patients by *P. aeruginosa* (Jiménez-gómez et al., 2007). The main characteristic of biofilms is their antibacterial resistance and the evasion of host immune responses due to the protective biofilm matrix, enabling bacterial persistence and dissemination (Costerton et al., 1999;Chole and Faddis, 2003;Lewis, 2008;Sanchez et al., 2011). Biofilm formation seems to be also a very important aspect of pneumococcal colonisation as well as virulence during invasive diseases since it has been demonstrated that the pneumococcal biofilm is highly structured during nasopharyngeal colonisation (Marks et al., 2012b). The clinical significance of *S. pneumoniae* biofilm is poorly understood despite the evidence of pneumococcal biofilm detection *in vivo* during infection courses of chronic rhinosinusitis, otitis media and pneumonia (Hall-Stoodley et al., 2006;Hoa et al., 2009;Reid et al., 2009;Sanchez et al., 2010). Essentially, hormone-derived inotropes therapeutically used in intensive care units (ICU) are reported to increase the growth and biofilm formation within indwelling medical devices (e.g. intravenous lines) such as the case of the skin-dwelling *Staphylococcus epidermidis*, which leads to subsequent infections in hospitalized patients (Lyte et al., 2003). Moreover, inotrope agents such as dobutamine and isoprenaline are given to approximately half of patients in the ICU and about 56% of pneumococcal infected patients are admitted to the ICU (Smythe et al., 1993). More importantly, hospital-acquired pneumonia associated with ventilator known as ventilator-associated pneumonia (VAP) represents a major challenge in controlling bacterial pneumonia. VAP is defined as pneumonia occurring within 48-72 hours of endotracheal intubation and accompanied by signs of systemic infection, distinctive sputum characteristics and detection of an etiologic agent (Kalil et al., 2016). It accounts for approximately 50 % of all hospital-associated pneumonia cases and occurs in about 9-27 % of all mechanically ventilated patients (Kalil et al., 2016;Vincent et al., 1995;Chastre and Fagon, 2002). In the ICU, VAP is the second most common nosocomial infection mainly affecting mechanically ventilated patients (Hunter,

2012;Afshari et al., 2012). Typically, *S. pneumoniae* is one of the causative agents of early-onset VAP along with *Haemophilus influenzae*, *Staphylococcus aureus*, and certain other antibiotic-sensitive enteric Gram-negative bacilli, while late VAP are frequently caused by drug-resistant bacteria including *Pseudomonas aeruginosa, Acinetobacter* and methicillin-resistant *S. aureus* (MRSA) (Hunter, 2012). Recently, a study found that the pneumococcal treatment with norepinephrine or with other host signals (such as cell lysate, ATP and glucose) on biofilms formed *in vitro,* and *in vivo* using biotic surfaces led to dispersion of the pneumococcus from the biofilm, and those dispersed cells were different in phenotype from those of both biofilm and broth-grown planktonic bacteria (Marks et al., 2013).

1.2.5. Competition in the nasopharynx

It is estimated that the human naso/oro pharynx can be inhabited by several hundred species of diverse microflora (Aas et al., 2005). Thus, any subsequent pneumococcal carriage may be influenced by existing normal flora either with pneumococcal strains itself and/or other species of commensal bacteria or viruses. For instance, when a pneumococcal strain encounters a similar pre-existing strain within the nasopharynx, both strain populations may persist if not increase in numbers (Margolis et al., 2010). However, when the pneumococcus encounters another pneumococcal strain, it can then secrete serotype-specific antimicrobial peptides, which can disrupt the rival pneumococcal growth during colonisation (Dawid et al., 2007). This may explain why one particular serotype may dominate over others in density as seen in multi-serotype carriage in humans (Gray et al., 1980). Other factors influencing interspecies colonisation might be the production of hydrogen peroxide (H_2O_2) by pneumococci under aerobic conditions, which can kill *H. influenzae* and *Neisseria meningitidis* (Pericone et al., 2000) contrary to the evidence that such physiological levels of H_2O_2 are not sufficient to antagonise co-colonisation (Margolis et al., 2010). Furthermore, when pneumococci co-exist with a species such as *H. influenzae*, the pneumococcus must then avoid the immunity created by its companion coloniser. The pneumococcus can itself evade the innate immunity stimulated by one or more of its elements such as

capsule, IgA, protease and PspA. However, the intranasal co-inoculation of both *S. pneumoniae* and *H. influenzae* results in the recruitment of a substantial increase in neutrophils compared to those seen with each organism alone, which leads to the preferential opsonophagocytosis of pneumococci over *H. influenzae* (Lysenko et al., 2005). This kind of discriminating pressure shows that pneumococcal strains with large polysaccharide capsules are more capable of surviving in the nasopharyngeal niche (Lysenko et al., 2010).

1.3. Bacterial interaction with host signals

During infection, pathogenic bacteria will inevitably encounter various host signals and conditions including temperature, pH, nutrient limitation, etc. Such environmental signals stimulate adaptive responses in bacteria to these specific situations, and also affect virulence genes expression at the right place and time (Freestone et al., 2008a). In fact, pathogenic bacteria encounter stressful conditions as soon as they contact their host, and for most, this involves a change in temperature (Fang et al., 2016). For example, pathogens of the respiratory tract must withstand a range of host antimicrobial peptides like those produced via epithelial cells (Grubor et al., 2006), and adapt to hyperosmolarity (Henderson et al., 2014), oxygen limitation (Worlitzsch et al., 2002) and nitrosative stress (Lundberg, 2008). Enteric pathogens, on the other hand, must cope with the extremely low pH levels of stomach (Foster, 1999) as well as dietary-generated reactive nitrogen species (Duncan et al., 1995). In addition, these pathogens experience membrane-active antibacterial peptides (Ouellette, 2011), free fatty acids, bile salts, fluctuating oxygen levels and osmolarity (Guiney, 1997). Hence, bacteria have developed powerful systems enabling not only the detection of these stresses and host signals, but also trigger adequate responses, which aid in their survival and even proliferation under these changing environments (Fang et al., 2016).

1.3.1. Temperature

A temperature shift from ambient to the host body environment is a key signal for many pathogens leading to the expression of several thermo-regulated virulence genes. The temperature-dependent regulation in Gram-negative bacteria is controlled by the nucleoid-like protein Hha and the histone-like protein H-NS. For instance, when *S. enterica* and *E. coli* are exposed to a shift from 25 to 37°C, about 200 and 126 genes, respectively, exhibit H-NS-dependent upregulation (Ono et al., 2005;White-Ziegler and Davis, 2009). The H-NS regulates the expression of virulence genes such as the *Salmonella* pathogenicity islands, the temperature-responsive gene *virF* and the *E. coli* hemolysin operon (Falconi et al., 1998;Madrid et al., 2002;Navarre et al., 2006). Another example of temperature pathogen interaction is the case of *Bordetella pertussis*, which senses changing temperature through the regulation of the BvgA/BvgS TCS. A high temperature shift results in an accumulation of the response regulator BvgA, which then stimulates the expression of virulence genes (Prugnola et al., 1995). The pneumococcal response regulator TCS05 (*ciaR*) controls high-temperature requirement A gene (*htrA*), which renders pneumococci tolerant to temperature shifts that they often encounter in the environment (Ibrahim et al., 2004).

1.3.2. pH

Infective bacteria experience acidic pHs in several places in the host including skin and endocytic vesicles of the intracellular degradative system in addition to the gastrointestinal and genital tracts. Several pathogenic bacteria adapt to pH change by using their two-component systems (TCSs), for instance, EnvZ/OmpR, PmrB/PmrA and PhoQ/PhoP systems through sensing low environmental pH levels before initiating a series of signalling cascades leading to bacterial adaptation to pH changes (Fang et al., 2016). It has already been established that bacteria alter genes expression in response to pH changes via transcriptional regulators such as Fur, sE and sS alternative sigma factors, which were found controlling gene expression when interacting with bacterial RNA polymerase. Furthermore, many regulators contribute to bacterial virulence in the

case of salmonella for example, and those include OmpR and PhoP (Fang et al., 1992;Belden and Miller, 1994;Lee et al., 2000). Dependent and independent-phosphorylation signaling via ArsR/ArsS TCS assist *Helicobacter pylori* to cope with the stomach's acidic environment. This TCS system regulates the release and handling of urease, which facilitates buffering of gastric acid in urea-dependent matter (Marcus et al., 2016). Moreover, acid-triggered activation of the TCS response can promote virulence in *Salmonella enterica* as OmpR regulates expression of the pathogenicity island-2 type III secretion system (Chakraborty et al., 2015), which supplies proteins that initiate infection and further stimulate acid sensation via other TCS, PhoP/PhoQ system (Alpuche Aranda et al., 1992;Martin-Orozco et al., 2006). The rapid production of lactic acid during sugar fermentation by all lactic acid-producing bacteria including all streptococcal species requires them to respond appropriately to the predictable environmental acidification resulting from their metabolism (Reqeuena, 2012). In addition, virulent streptococci must also cope with the acidic pH levels they might experience in the events of abscesses and necrotic lesions formation (Simmen and Blaser, 1993). In *S. pneumoniae*, *fabM* was found to be essential to control acidification and withstand low pH conditions, since mutation within the transcriptional regulator of *fab* operon, caused higher values of saturated fatty acids and increased acid sensitivity (Lu and Rock, 2006).

1.3.3. Oxygen

An additional adaptation challenge encountered by many pathogens is shifting from external aerobic environments to the host oxygen-limited condition leading to the consequent alteration of their metabolic pathways. Again, TCS systems such as SrrA/SrrB in *S. aureus* and ResD/ResE in *Bacillus subtilis*, for example, assist these microbes to survive by regulating genes essential for hypoxic environments (Kinkel et al., 2013;Durand et al., 2015). Similarly, ArcB/ArcA in *E. coli* and DosR/DosS in *M. tuberculosis* control the adaptive response to this hypoxia stress (Alvarez et al., 2013;Sardiwal et al., 2005). For facultative anaerobes such as *E. coli*, a protein called FNR is the typical oxygen-sensing transcription factor involved in response to the shift

from aerobic to anaerobic metabolism. This regulator senses the availability of oxygen using [4Fe-4S] and [2Fe-2S] clusters (Crack et al., 2004;Reinhart et al., 2008). The aerobic to anaerobic shift has also an impact on the cellular NADH/NAD+ balance, which is sensed by a repressor Rex that is found in some Gram-positive bacteria including *S. aureus*. The Rex protein represses genes involved in adaptation to anaerobiosis, nitrate and nitrite respiration and lactate fermentation when NADH levels are elevated (Pagels et al., 2010;Ravcheev et al., 2012). The upper respiratory tract environment is rich in oxygen; *S. pneumoniae* has mechanisms to withstand oxygen and its reactive derivatives. This involves the transcriptional repressor Rgg, which is found to be vital during higher oxygen levels and pneumococcal virulence in mice model of infection (Bortoni et al., 2009). Other gene products are also found to be important for pneumococcal virulence and survival under oxidative stress conditions such as MnSOD, *psaA* and *psaD* (Yesilkaya et al., 2000;Tseng et al., 2002).

1.3.4. Nutrients

Bacterial access to essential nutrients such as amino acids and carbohydrates greatly varies within the host environment. Intracellular environments can be extremely restricted whereas places such as the host intestinal tract is rich with various nutrients. Throughout the gut, for instance, some breakdown products of carbohydrates such as glucose and fatty acids serve as signals to induce virulence gene expression of pathogenic bacteria (Fang et al., 2016). Bacterial regulators like FruR/Cra, which are widely found in enteric pathogens, and can sense the availability of carbohydrate sources that promote either glycolysis or gluconeogenesis through fructose-1-phosphate and fructose-1,6-bisphosphate interactions (Ramseier et al., 1993). In enterohemorrhagic *E. coli* (EHEC), *S. enterica* and *Shigella flexneri*, this system regulates virulence gene expression in addition to the appropriately directed carbon flow function (Yoon et al., 2009;Gore and Payne, 2010;Njoroge et al., 2012). A small molecule called guanosine tetra/pentaphosphate also mediates the response to amino acid starvation following interactions with ribosomes that are stalled because of amino acid malnutrition (Haseltine and Block, 1973;Agirrezabala et al., 2013). This molecule also

interacts with RNA polymerase and various proteins to stimulate virulence gene expression in bacteria like *E. coli* EHEC, *S. aureus*, *Vibrio cholerae*, *S. enterica*, *Yersinia pestis*, *Francisella tularensis* and *Burkholderia pseudomallei* (Charity et al., 2009;Pal et al., 2012;Müller et al., 2012;Geiger et al., 2010;Sun et al., 2009;Nakanishi et al., 2006). CodY plays a role in regulating virulence gene expression of Gram-positive bacteria in response to amino acid accessibility (Geiger et al., 2010;Lobel et al., 2015). When amino acid concentrations are critically low, *Listeria's* CodY stimulates expression of the virulence regulator PrfA, whereas CodY's role in regulating virulence gene of *S. aureus* is more complicated (Fang et al., 2016). Pneumococcal nutrient regulators such as CodY, CcpA and (p)ppGpp play a major role in metabolism coordination to ensure that environmental signals like nutrient sources are utilised to maintain energy production needed for growth and virulence gene expression within the host changing environment (Reqeuena, 2012).

1.3.5. Metals

Metal restriction is probably one of the most essential kinds of nutrient limitation experienced by bacteria in the host. In fact, the term ‘‘nutritional immunity’’ can be used for sequestration of metals including iron, zinc and manganese, which play a major role in bacterial ability to replicate (Becker and Skaar, 2014). Such metals, along with enzymes and proteins are collectively involved in a wide range of vital processes such as metabolism and DNA replication. Pathogens sense metal presence by metal-binding transcription elements that control the expression of genes associated with metal uptake (Fang et al., 2016). Regarding zinc and manganese acquisition, Zur regulates ZnuABC (high-affinity zinc acquisition system) while MntR (manganese-binding transcriptional regulator) and OxyR (oxidative stress regulator) control manganese acquisition, which subsequently prevents oxidative damage (Patzer and Hantke, 2000;Anjem et al., 2009). MntR is found in both Gram-negative and Gram-positive bacteria and work by repressing the expression of manganese acquisition systems (Waters et al., 2011;Lieser et al., 2003). There are at least five pneumococcal proteins that are shown to be directly involved in the acquisition of host zinc: transcription

regulator AdcR, AdcC, AdcB, AdcA and AdcAII (Dintilhac and Claverys, 1997;Dintilhac et al., 1997;Bayle et al., 2011). Manganese is another key metal and cofactor for many pneumococcal proteins that sustain colonisation and invasiveness, and it is present in different locations and concentrations inside the human host. The importer of manganese in the pneumococcus is the PsaBCA ATP-binding cassette (ABC) transporter, which pumps the ions metal from extracellular environment (i.e., human host) to pneumococcal cells (Honsa et al., 2013). For most pathogenic bacteria, iron plays an essential role in their growth within their host and for cellular processes such as energy production, oxygen transport and DNA replication (Skaar, 2010). Inside the mammalian host, iron is highly restricted representing an important innate immune defence against pathogenic bacterial infection. In the human body, around 70 % of iron is secured in red blood cells and much of remaining is tightly bound by high affinity iron-binding proteins; transferrin (Tf) in serum and lactoferrin (Lf) in extra-cellular fluids. The normal existence of partially iron-saturated status of Tf and Lf ensures that the concentration of free iron in host tissues is lower than 10^{-20} M. This level of Fe is too low for most infectious bacteria to grow which require around 10^{-6} M (Lyte and Freestone, 2010). Unsurprisingly, pathogenic bacteria are extremely adaptive microorganisms and have various mechanisms for liberating this essential nutrient. One of bacterial strategies to overcome the extremely limited iron availability is by using Tf-binding proteins (Tbps), which are highly conserved bacterial proteins (Ratledge and Dover, 2000) such as; two-protein TbpA and TbpB system in *N. meningitidis* and *N. gonorrhoeae*. Collaboratively, these Tbps bind specifically to the Tf of their particular host species and subsequently internalise the iron into the bacterial cells (Powell et al., 1998). However, enteropathogenic *E. coli* not known to possess Tbps was able to bind to Tf by its surface porins proteins OmpA and OmpC (Sandrini et al., 2013).

Another strategy of iron acquisition used by infectious bacteria is the secretion of low molecular weight siderophores. These catecholate or hydroxymate molecules have very high affinity for ferric iron (Ratledge and Dover, 2000) and are crucial elements in the mechanism by which catecholamine hormones induce bacterial growth (Lyte and

Freestone, 2010). Siderophores are low molecular substances (usually less than 1 kDa) and produced by some fungi and many bacteria in situations of iron deficiency (Andrews et al., 2003). Siderophore production by bacteria, however, does not necessarily render them as being pathogenic (Wandersman and Delepelaire, 2004). Over 500 various types of siderophores have been identified and many have shown to be secreted by some Gram-negative as well as Gram-positive bacteria. For siderophore non-producing bacteria, an alternative strategy of iron acquisition is achieved by direct binding of bacterial specific receptors to host Tf and Lf (Krewulak and Vogel, 2008). These receptors have been found in Gram-negative bacteria such as *N. gonorrhoeae*, *N. meningitis* and *H. influenzae* (Price et al., 2004;Perkins-Balding et al., 2004;Khan et al., 2007). Another example includes Fur (ferric uptake regulator), which is a transcriptional repressor released under iron limitation conditions and which regulates genes supporting the growth and survival of bacteria such as *Salmonella* when inside its host (Troxell et al., 2011). Fur also activates iron acquisition systems including the iron-chelating siderophores; enterobactin and salmochelin (Ernst et al., 1978;Bagg and Neilands, 1987;Hantke et al., 2003). Iron-responsive regulators such as DtxR and IdeR are also important for iron homeostasis in *Corynebacterium diptheriae* and *Mycobacterium tuberculosis*, respectively (Boyd et al., 1990;Yellaboina et al., 2004;Pandey and Rodriguez, 2014). Since the pneumococci occupy different niches, it is of great importance to sense extra- and intracellular nutrient levels and this is achieved by the two-component systems (TCSs) which regulate such events (Lange et al., 1999;Throup et al., 2000). Iron is an essential metal for pneumococcal viability and pathogenicity, hence a regulator like the RitR (repressor of iron transport) of the TCS plays an important role in iron acquisition and found to be required during murine lung infection (Throup et al., 2000;Ulijasz et al., 2004). Pneumococcal Surface Protein A (PspA) has also been identified as lactoferrin-binding protein (Hammerschmidt et al., 1999).

1.3.6. Stress hormones

In human and animal systems, stress hormones such as catecholamines and glucocorticoids are released into the blood by the sympathetic nervous system in response to stress which is mainly associated with "fight or flight" response (Selye, 1976). Although stress hormone release in response to threat can increase heart rate, blood pressure and awareness, all of which can subsequently lead to a quick reaction to any dangerous situation, the stress state can also be negative and the outcome could lead to disease or rarely death (McEwen, 2005). The catecholamines are produced by the adrenal medulla, while the glucocorticoids are produced by adrenal cortex, and they both assist in restoring homeostasis. In addition to these two main classes of stress hormones, others are also involved during stress responses such as; opioids, arginine vasopressin and somatosin (Lyte and Freestone, 2010).

More than 50 of the bacterial species that occupy the large niches of the mammalian body have been found to be able to sense and respond to host stress hormones (Lyte and Freestone, 2010). It has been shown that there is up to a 5 log higher magnitude of bacteria growing in blood or serum-containing media when catecholamines are present compared to controls (Freestone et al., 2008a). The ubiquitous distribution of catecholamines in the human body where bacteria are located might have led to the evolution of the normal flora developing mechanisms to perceive any changes of stress hormones levels (Lyte and Freestone, 2010). For instance, when humans are exposed to long lasting emotional stress, this may significantly result in the development of bacterial-associated periodontal disease. In addition, when using a serum-based medium, catecholamine hormones such as norepinephrine (noradrenaline) and epinephrine (adrenaline) were found to have stimulatory effects on oral cavity colonising bacteria that are involved in such periodontal infections (Roberts et al., 2002;Roberts et al., 2005). With regards to respiratory pathogenic bacteria such as *Klebsiella pneumoniae*, *Bordetella pertussis* and *B. bronchiseptica*, for example, the catecholamine hormones have also been shown to be effective growth simulators (Freestone et al., 2008a). Moreover, the *B. bronchiseptica* stimulated growth by

norepinephrine resulted in inducing the expression of *bfeA* gene, which is in control of a siderophore receptor shown to be vital for growth *in vivo* studies (Freestone et al., 1999;Anderson and Armstrong, 2006).

The catecholamines stress hormones are of a particular interest in this study, which include norepinephrine (NE), epinephrine (Epi) and dopamine (Dop). They are derived from the amino acid tyrosine and possess a catechol group composed of a benzene ring with two hydroxyl side groups as well as primary or secondary side-chain amine (Figure 1.3) (Lyte and Freestone, 2010). The enzyme involved in the synthesis of catecholamines is tyrosine hydroxylase, which is regulated by a cAMP-associated protein kinase and norepinephrine complex feedback system. The tyrosine is catabolized in the autonomic sympathetic nervous system where the hydroxylation stage begins inside the cytoplasm of postganglionic nerve termini. Next step occurs in the same division of nervous system, requires catalysis effect of L-aromatic amino acid decarboxylase in order to produce the first synthesized catecholamine, Dop. After the production of dopamine, it is transported and stored in the sympathetic nerve terminal vesicles. It can, also, be processed by the activation of β-hydroxylase to produce norepinephrine, which is stored in vesicles by adenosine triphosphate (ATP) and secreted by a triggering action. Upon receiving a stress related stimuli, electrical impulses are originated through the central nervous system and this eventually leads to the release of NE, which will interact with adrenergic receptors and the final product is Epi (Lyte and Freestone, 2010).

1.4. How do stress hormones enhance bacterial growth?

It has been identified that the iron-associated catechol moiety present in siderophores such as ferrichrome and enterobactin, is also found in catecholamine stress hormones as well as their related inotropes such as dobutamine and isoprenaline (Figure 1.3). This understanding has shed some light on the underlying mechanism by which stress hormones stimulate bacterial growth (Freestone et al., 2002). Several studies have reported that the catecholamines, inotrope agents and even plant-extracted

compounds containing catechol molecules (tannic, chlorogenic and caffeic acids) all are capable of stimulating bacterial growth (Anderson and Armstrong, 2006;Anderson and Armstrong, 2008;Coulanges et al., 1998;Freestone et al., 2000;Freestone et al., 2002;Freestone et al., 2003;Freestone et al., 2007b;Freestone et al., 2007c;Freestone et al., 2008a;Freestone et al., 2008b;Roberts et al., 2002;Roberts et al., 2005;Williams et al., 2006). The growth enhancing mechanism has indicated the involvement of the catechol part of the catecholamine, which like siderophores enables bacteria to acquire the otherwise inaccessible Tf or Lf-bound iron. (Lyte et al., 1996;Freestone et al., 1999).

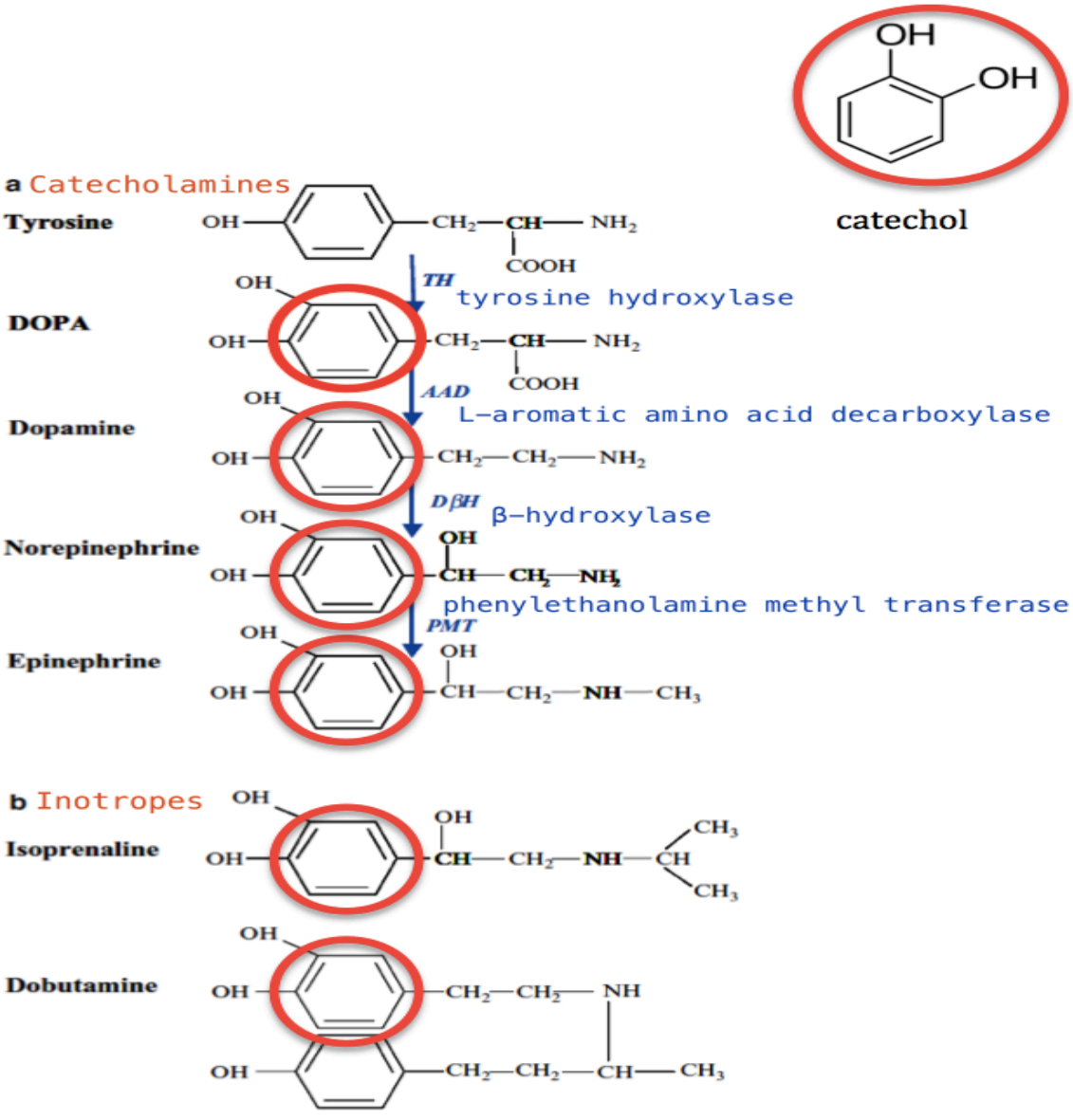


Figure 1.3. Chemical structure of catechol containing catecholamines (a) and inotropes (b). Cited and modified from (Lyte and Freestone, 2010).

1.5. Bacterial Two-component system (TCS)

Bacterial adaptation, by which bacteria perceive and respond to environmental changes including host stress hormones levels, is often regulated by key mechanisms called two-component regulatory system or two-component signal transduction system (TCS) (Hoch, 2000;Stock et al., 2000). As its name suggests, the basic bacterial TCSs normally consist of two proteins (Figure 1.4): a membrane-bound sensory histidine kinase (HK) and its cognate, a cytoplasmic response regulator (RR). Once a stimulus is received by the sensory domain of HK, this results in its auto phosphorylation. The signal is further transmitted by phosphorylation of the cognate RR, which then binds to a promoter region and eventually regulates transcription of genes or functioning proteins under its control (Paterson et al., 2006). The TCSs systems are widely spread among many bacteria some of which are rather essential for viability and others found to be responsible for modulating a variety of bacterial cellular responses such as; sporulation, chemotaxis, pathogenicity and photosynthesis (Fabret and Hoch, 1998;Lange et al., 1999;Martin et al., 1999;Throup et al., 2000;Hoch, 2000;Stock et al., 2000). TCS has caught scientific attention due to the fact that it is absent in vertebrates and thus making this field a potential target for antimicrobial development (Barrett and Hoch, 1998).

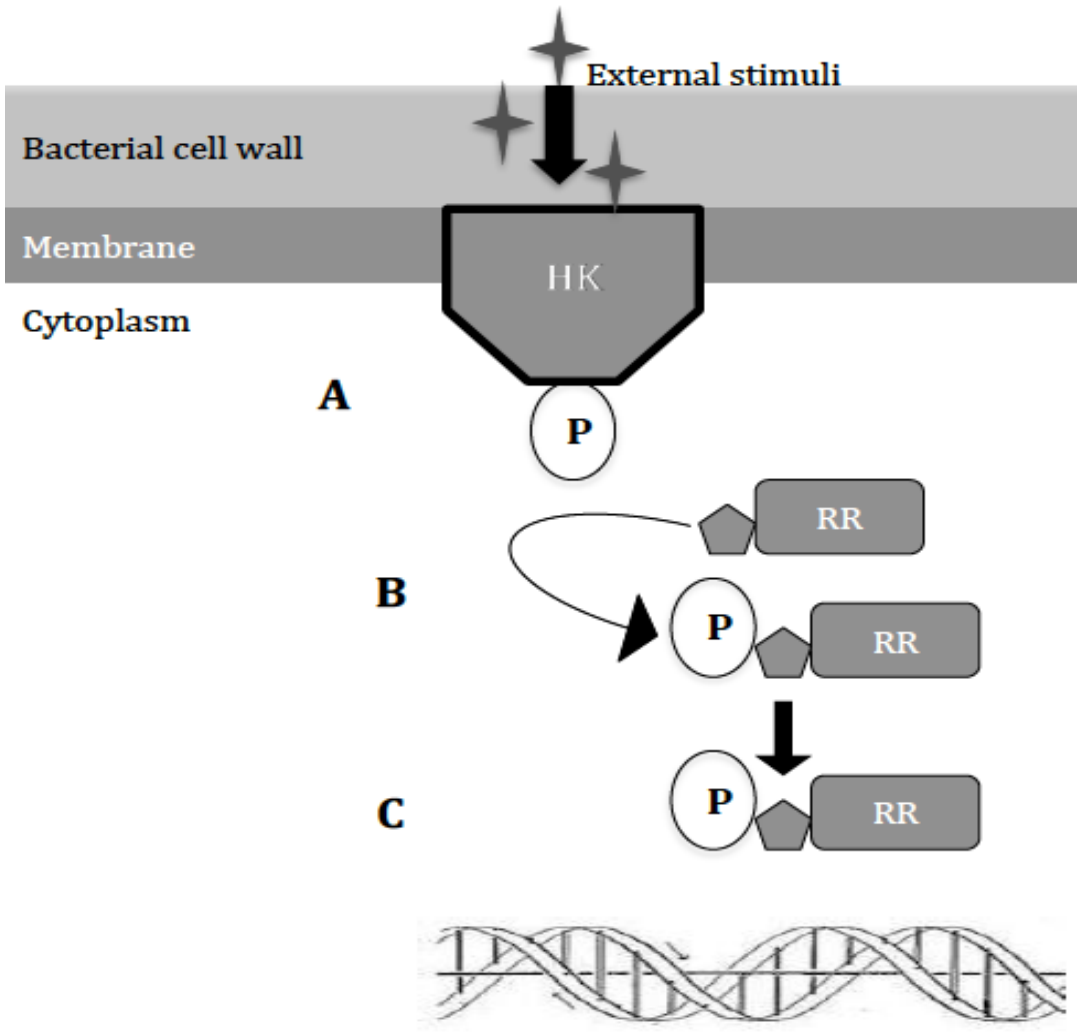


Figure 1.4. Bacterial two-component system (TCS). Membrane-bound histidine kinase (HK) receives external stimuli resulting in its auto-phosphorylation **(A)**. Further transfer of the stimuli **(B)** is achieved by phosphorylation of its cognate cytoplasmic-associated response regulator (RR), which regulates genes under its control **(C)** (Paterson et al., 2006).

1.6. Pneumococcal TCS

Initially, only four pneumococcal TCSs had been discovered (Guenzi et al., 1994;Novak et al., 1999;Pestova et al., 1996) but after genome sequencing of pneumococcal DNA, the full repertoire has become more apparent (figure 1.5), with 13 HK: RR pairs and an unpaired 'orphan' RR (RitR) have been identified (Lange et al., 1999;Throup et al., 2000;Gomez-Mejia et al., 2017).

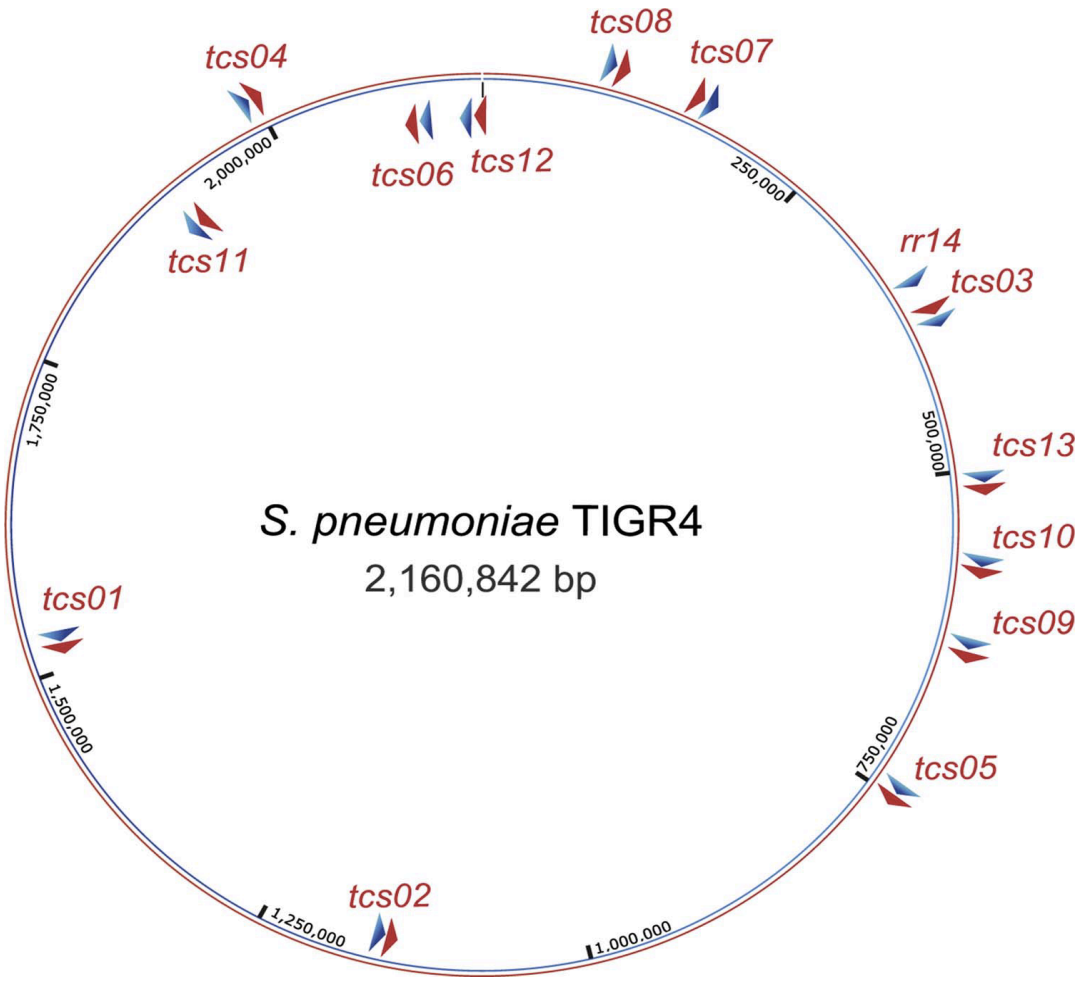


Figure 1.5. Genomic illustration of TCS in *S. pneumoniae* TIGR4. A circular genomic map represents the direction and location of the pneumococcal two-component regulatory systems. The arrangement of various TCS is indicated by the red and blue arrows histidine kinase (HK) and response regulator (RR), respectively (Gomez-Mejia et al., 2017).

The transcription regulators as output domains of TCSs usually switch gene expression off and on in response to certain signals (figures 1.6 and 1.7), (Gao and Stock, 2009;Krell et al., 2010). For example, the response regulator of TCS05 system is in direct control of 16 promotors that drive transcription of 29 genes including small non-coding RNAs or so called cia-controlled small RNAs (csRNAs). The csRNAs are involved in ß-lactam resistance, autolysis, virulence and competence (Halfmann et al., 2007b;Tsui et al., 2010;Schnorpfeil et al., 2013;Mann et al., 2012). Furthermore, the serine protease HtrA which is produced by another member of the CiaR regulon aids in the regulation of many of these phenotypes (Ibrahim et al., 2004;Cassone et al., 2012;Kochan and Dawid, 2013). TCS05 was found to be significantly active under various *in vitro* laboratory conditions and *in vivo* animal models of virulence and colonization and was active in different *S. pneumoniae* clinical isolates (Halfmann et al., 2011;Throup et al., 2000;Ibrahim et al., 2004;Tsui et al., 2010;Marra et al., 2002;Kumar et al., 2010;Sebert et al., 2002;Lanie et al., 2007).

The roles of pneumococcal TCSs in virulence have been investigated, which provided the foundation for pneumococcal TCSs biological analysis. Up to 2006, all known pneumococcal TCSs have demonstrated a role in virulence with the exception of TSC03, TSC10 and TCS11 (Paterson et al., 2006). Therefore, studying their virulence profile of the pneumococci is beyond the scope of this project. The pneumococcus is naturally competent microorganism due to TCS12, which is the most studied TCS in *S. pneumoniae.* The competence state of the pneumococci facilitates the uptake of external DNA from the environment and then incorporate it into its genome (Seitz and Blokesch, 2014). This TCS contains HK encoded by *comD* and its cognate the RR encoded by *comE,* both of which can sense and process the competence stimulating peptide (CSP). Their functions and role in virulence are well-studied, for example, TCS12 (*comE*) regulates other components of the competence machinery, which ultimately activates many other genes involved in stress response, DNA uptake and processing as well as bacteriocin production (Peterson et al., 2004;Paterson et al., 2006). The genes involved in the competence were also found to contribute to pneumococcal virulence as seen in TCS12 (*comD*) mutant, which was attenuated in

models of bacteraemia and pneumonia in serotype 2 strain D39, serotype 3 and serotype 4 strain TIGR4 (Bartilson et al., 2001;Lau et al., 2001;Hava and Camilli, 2002). The response regulator RR02 of the pneumococcal TCS02 (*vicR*) is the only TCS found to be essential for pneumococcal viability (Lange *et al.*, 1999; Paterson *et al.*, 2006), which has homology to a TCS in *S. aureus* and *B. subtilis* (Paterson et al., 2006). TCS02 was also shown to be involved in regulating the cell wall, the expression of the virulence factor *pspA* and fatty acid metabolism (Wagner et al., 2002). Another important TCS is TCS05 (CiaH/R), which is shown to be a key regulator by influencing competence, virulence and antibiotic resistance in *S. pneumoniae* (Guenzi et al., 1994;Guenzi and Hakenbeck, 1995;Throup et al., 2000;Zahner et al., 2002). Its role in virulence is thought to occur via the activation of *htrA*, which is a major pneumococcal virulence factor since both HtrA and CiaR knockout mutants showed similar attenuation in murine intranasal infection model (Ibrahim et al., 2004). Additionally, TCS05 protects the cells from the stress caused by competence development (Dagkessamanskaia et al., 2004). TCS13 and TCS04 also contribute to the pneumococcal virulence as *rr13* mutant significantly reduced respiratory infection on an animal model by 10000-fold in comparison with the wild type while *rr04* mutant was attenuated in murine pneumonia (Throup et al., 2000). TCS09 is yet another vital component of the pneumococcal TCSs system as mutation in *rr09* displayed nonvirulent behaviour in mouse models of pneumonia and bacteraemia resulting in the survival of all infected mice accompanied with rapid clearance of the bacteria (Blue and Mitchell, 2003). Homology data of amino acid sequence suggest that the HK09 is similar to that of extracellular domain of McpA/B proteins of Bacillus subtilis. Although they are involved in controlling chemotaxis via sensing environmental nutrient concentrations (Lange et al., 1999), the extracellular stimuli of TCS09 is unknown. All other TCSs have been found to be non-essential to the pneumococcus virulence such as TCS03, TCS10 and TCS11 (Paterson et al., 2006). The orphan response regulator RitR is a unique TCS as it is not located next to a cognate *hk* in the pneumococcal genome. This regulator also contributes to the virulence of the pneumococcus since mutants lacking this TCS showed a substantial reduction in bacterial counts of the lungs of a murine pneumonia model compared to the counts of wild type strain (Throup et al., 2000). It also plays a role in iron regulation

and when deleted, the iron uptake increased, which made pneumococci more vulnerable to iron-dependent killing (Ulijasz et al., 2004).

Microarrays analysis revealed that the response regulator of TCS03 system (LiaR) is in control of stress response genes such as *hrcA* and *grpE*. The same study concluded that TCS03 system (LiaRS) is stimulated via the activity of the PGN hydrolases CbpD, LytA and LytC and regulates the stress relief proteins in *S. pneumoniae*. This stimulation has been shown to be TCS03 response regulator-dependent, which controls 6 transcriptional units with a total of 18 genes (Eldholm et al., 2010). Interestingly, the authors used promotor deletion analysis to detect the binding site of the response regulator of TCS03 as expected for the *B. subtillis* homolog (Jordan et al., 2006). Microarray studies on the upstream regions of the genes showing an effect in TCS02 (WalR also called VicR) mutant showed that 6 of these genes exhibited a variant of direct repeat consensus sequence found for the response regulator of TCS02 in *S. aureus* and *B. subtilis*. Using DNA-footprinting, WalR was found to bind to *pspA* and *pcsB* in *S. pneumoniae*. In addition, the binding of this essential response regulator to an upstream region of *fabT* was also observed (Ng et al., 2005). Microarray analysis of *S. pneumoniae* revealed that the expression of the cellobiose operon is under the control of TCS08 (SaeRS) (McKessar and Hakenbeck, 2007). Another microarray study reported that mutant lacking the response regulator of TCS09 resulted in 102 genes differentially expressed compared to the wild-type strain D39. The set of genes down-regulated in the ΔTCS09 mutant were found to be encoding proteins involved in carbohydrate metabolism and facilitate the uptake of sugars such as mannose and fructose (Hendriksen et al., 2007). TCS05 system is in direct control of 16 promotors that drive transcription of 29 genes including small non-coding RNAs or so called cia-controlled small RNAs (csRNAs). The csRNAs are involved in ß-lactam resistance, autolysis, virulence and competence (Halfmann et al., 2007b;Tsui et al., 2010;Schnorpfeil et al., 2013;Mann et al., 2012). Furthermore, the serine protease HtrA which is produced by another member of the CiaR regulon aids in the regulation of many of these phenotypes (Ibrahim et al., 2004;Cassone et al., 2012;Kochan and Dawid, 2013).

There are similarities between TCS12 (ComDE) and TCS13 (BlpRH) systems. For instance, a DNA recognition site identified for BlpR shares a high similarity with that of ComE. BlbR contains two 9 base pair direct repeats, showing a strong conservation with ComE binding motif except two base pairs (Knutsen et al., 2004). TCS06 (CbpRS) system is a regulatory transcription factor for PspC (also called CbpA), which is one of the key adhesion molecules in *S. pneumoniae* (Ma and Zhang, 2007; Standish et al., 2005; Agarwal et al., 2010; Elm et al., 2004; Voss et al., 2013; Zhang et al., 2000). In fact, it is reported that the upregulation of PspC occurs after binding of the response regulator of TCS06 to a 19-bp conserved sequence upstream of a promoter region of the *pspC* (Ma and Zhang, 2007; Standish et al., 2005).

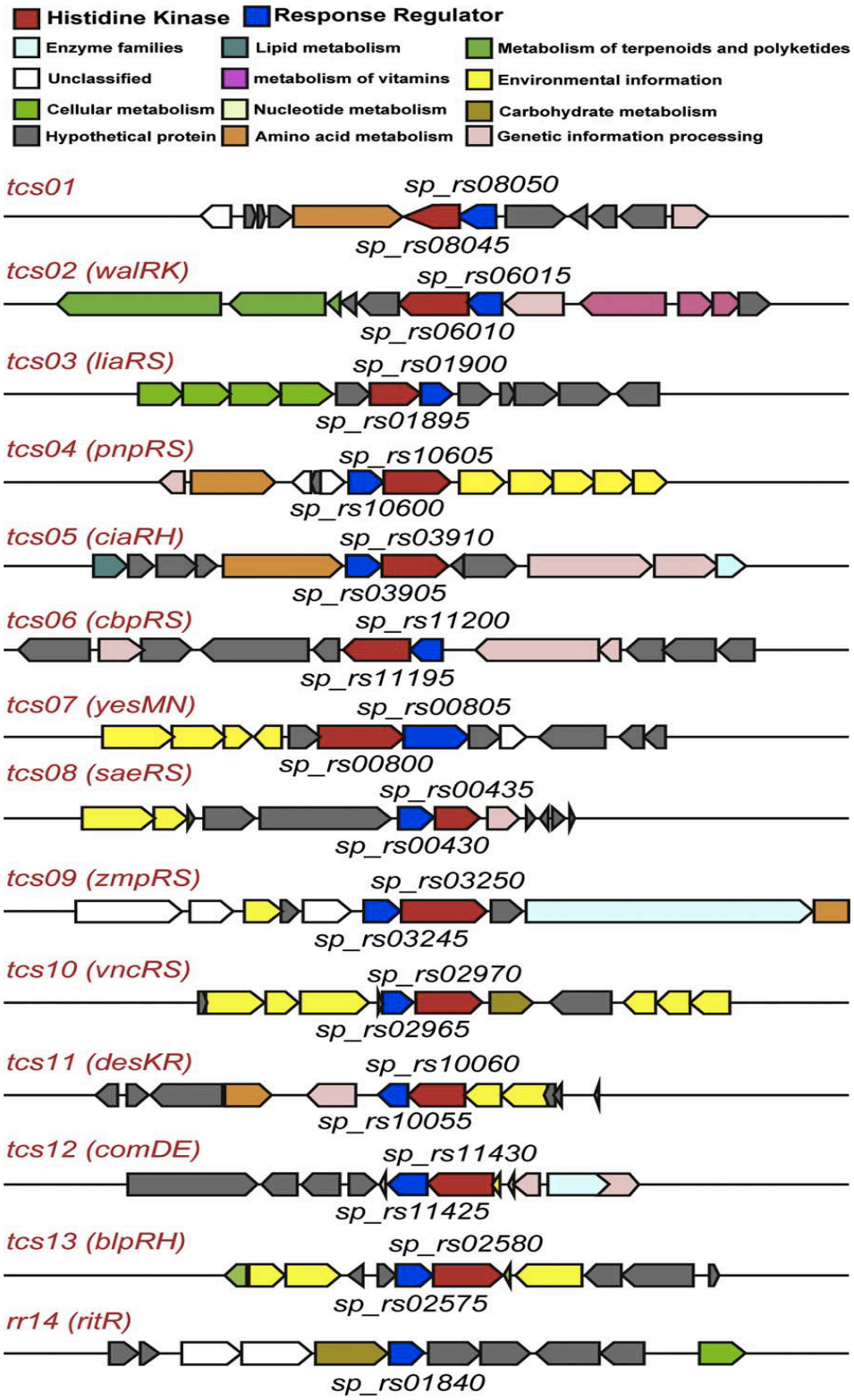


Figure 1.6. Genomic representation diverse classes of TCS flanking genes in *S. pneumoniae* TIGR4 genome. The arrangement of the TCS is indicated by the red and blue arrows histidine kinase (HK) and response regulator (RR), respectively (Gomez-Mejia et al., 2017).

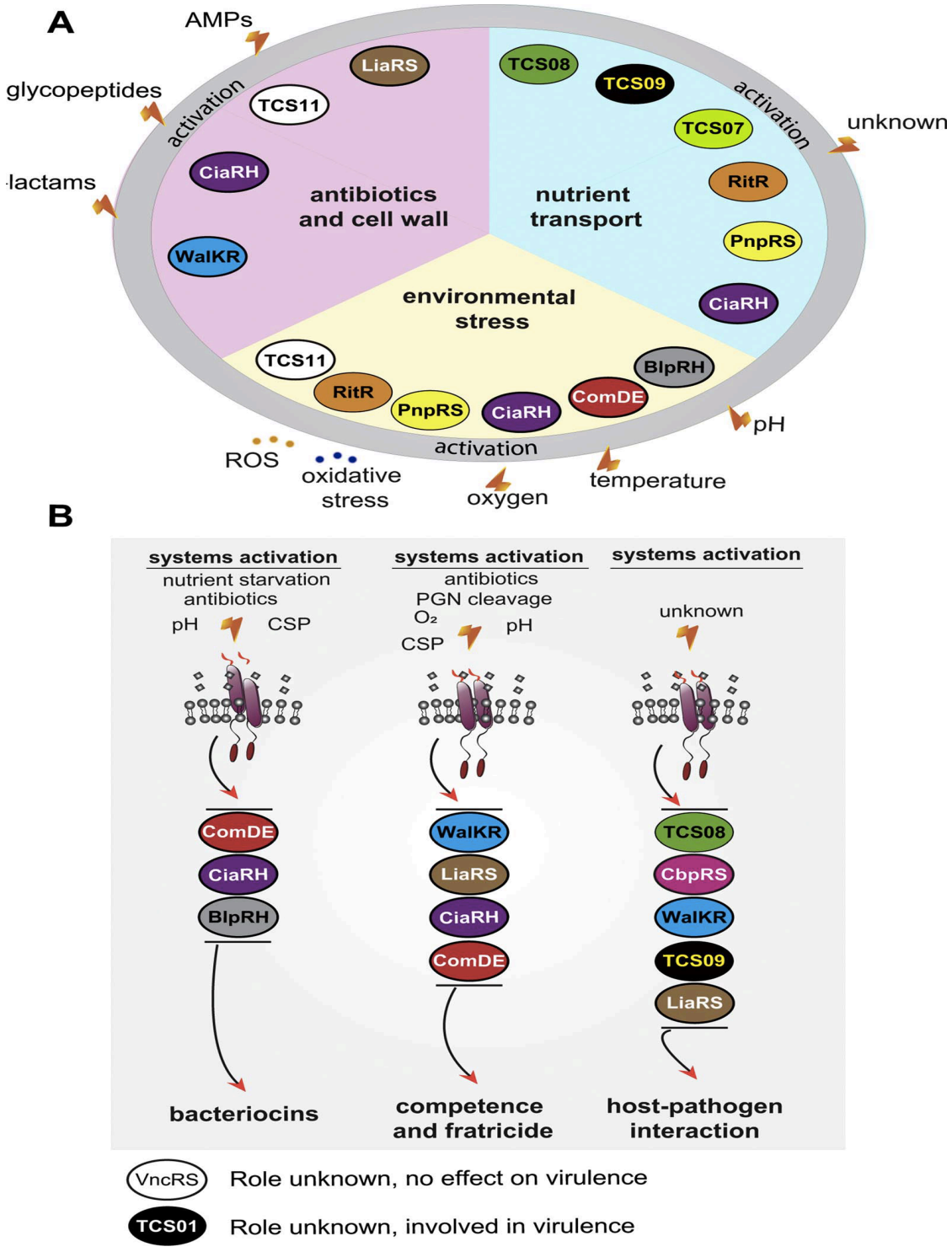


Figure 1.7. TCS regulatory network in *S. pneumoniae*. Summary of the TCS role in response to environmental stimuli **(A)** and pathophysiological processes **(B)**. The spark icon indicates the studied molecules and activation environmental factors for various TCSs in pneumococci. "unknown" refers to a specific process that has not been elucidated (Gomez-Mejia et al., 2017).

1.7. Project aims

The aims of this study are, firstly, to elucidate the mechanism(s) by which catecholamine stress hormones increase pneumococcal growth. Second, it is important to identify the genetic cascades responsible for recognition and processing of inotrope signals, including the determination of whether TCS receptors are involved. The third and final aim of the project is to evaluate the role of stress hormones in the pneumococcal transition from colonisation to invasive mode using an animal model of infection.

Chapter 2. Materials & Methods

2.1. Chemicals and biological materials

Chemicals used in this study were purchased from Sigma unless otherwise stated. The bacterial strains and plasmids constructed and used in this study are listed in Table 2.1.

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

Strains/Plasmids	Description/Use	TCS*	Source
S. pneumoniae			
D39	Serotype 2 strain		Lab stock
ΔTCS012	Spc^R; ΔSPD_2063	TCS012	This study
ΔTCS013	Spc^R; ΔSPD_0468	TCS013	This study
ΔTCS05	Spc^R; ΔSPD_0701	TCS05	This study
ΔTCS08	Spc^R; ΔSPD_0081	TCS08	This study
ΔTCS04	Spc^R; ΔSPD_1908	TCS04	This study
ΔTCS03	Spc^R; ΔSPD_0352	TCS03	This study
ΔTCS07	Spc^R; ΔSPD_0158	TCS07	This study
ΔRitR	Spc^R; ΔSPD_0344	TCS14	This study
ΔTCS06	Spc^R; ΔSPD_2020	TCS06	This study
ΔTCS10	Spc^R; ΔSPD_0524	TCS10	This study
ΔTCS01	Spc^R; ΔSPD_1446	TCS01	This study
ΔTCS09	Spc^R; ΔSPD_0574	TCS09	This study
ΔTCS11	Spc^R; ΔSPD_1798	TCS11	This study
ΔpPP2::wt	Tet^R; ΔpPP2-wt		This study
ΔPTCS03::*lacZ*-wt	Tet^R; ΔPTCS03-*lacZ*-wt	TCS03	This study
ΔPTCS05::*lacZ*-wt	Tet^R; ΔPTCS05-*lacZ*-wt	TCS05	This study
ΔPTCS09::*lacZ*-wt	Tet^R; ΔPTCS09-*lacZ*-wt	TCS09	This study

Strains/Plasmids	Description/Use	Source
Escherichia coli		
E. coli top10	plasmid propagation	Laboratory stock
E. coli top10	Competent cells	This study
E. coli top10	Amp^R; ΔpPP2-top10	This study
Plasmids		
pDL278	Amplification of spc^R (*aadA*)	Yesilkaya, 1999
pPP2	Promoterless *lacZ* for transcriptional fusions; Amp^R, Tet^R	Halfman *et al*., 2007

*TCS cited from (Paterson et al., 2006).

2.2. Culture media and reagents used

According to requirement, different culture media were used to grow bacterial strains within experiments as follows:

Brain Heart Infusion (BHI): 53286, SIGMA, UK.

Blood Agar (BAB): Blood Agar Base (CM0055, Oxoid) supplemented with 5% (v/v) horse blood (SR0050C, Thermo).

Luria Agar (LA): Luria-Bertani broth (LB) solidified with 1.5% (w/v) agar

Serum-SAPI (a serum-based minimal medium): It is composed of bovine serum 30% and (v/v) SAPI 70% (v/v).

Adult bovine serum**:** B9433, SIGMA, UK.

SAPI: 6.25 mM ammonium nitrate, 3.35 mM KCl, 2.7 mM glucose, 1.84 mM KH_2PO_4 and 1.01 mM $MgSO_4$, adjusted to pH 7.5 by 1 M KOH (Lyte and Ernst, 1992;Lyte and Ernst, 1993).

Just prior to use, 100 µl of 0.8 g/ml of sodium pyruvate and the following vitamins (0.015 mg biotin, 5 mg choline, 0.6 mg nicotinamide, 2.4 mg pantothenate, 0.6 mg

pyridoxal HCl, 0.3 mg riboflavin and 0.6 thiamine) were added to the serum-SAPI medium according to vitamins preparation of Sicard's minimal medium (Sicard 1964).

CDM medium:

Table 2.2. Composition of CDM used for growth of pneumococcal strains.

Components	**g l^{-1}**	**Components**	**g l^{-1}**
Buffers/Salts		**Amino acids**	
Na_2-β-glycerophosphate	26	Alanine	0.24
$(NH_4)_3$ citrate	0.6	Arginine	0.124
KH_2PO_4	1.0	Asparagine	0.352
Na-acetate	1.0	Aspartate	0.4
Na-pyruvate	0.1	Cysteine-HCl	0.4
Vitamins		Glutamate	0.5
Choline-HCl	10	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		Valine	0.324
Adenine	1.0	**Micronutrients**	
Uracil	1.0	$MgCl_2$	20
Xanthine	1.0	$CaCl_2$	3.8
Guanine	1.0	$ZnSO_4$	0.5

Z buffer: 0.06 M $Na_2HPO_4.7H_2O$, 0.04 M $NaH_2PO_4.H_2O$, 0.01 M KCl, 0.001 M $MgSO_4$ and 0.05 M β-mercaptoethanol (BME). The buffer was adjusted at pH 7.0 and stored at 4°C.

Phosphate buffer: 0.06 M $Na_2HPO_4.7H_2O$, 0.04 M $NaH_2PO_4.H_2O$. The buffer was adjusted at pH 7.0 and stored at room temperature.

Transformation buffer TFB I: 30 mM K-acetate (CH_3CO_2K), 50 mM $MnCl_2$, 100 mM KCl, 10 mM $CaCl_2$ and 15% (v/v) glycerol. The buffer was adjusted at pH 5.8, filter sterilised and stored at 4°C.

Transformation buffer TFB II: 10 mM Na-MOPS ($C_7H_{14}NNaO_4SNa$), 75 mM $CaCl_2$, 10 mM KCl and 15% (v/v) glycerol. The buffer was adjusted at pH 6.5, filter sterilised and stored at 4°C.

Where appropriate, all liquid-based culture media were subjected to autoclaving at 125°C for 15 minutes at 15 psi (pound per square inch), and stored at 4°C or room temperature until needed. According to requirements, various antibiotics were added to liquid or solid media.

2.3. Bacterial growth conditions

Streptococcus pneumoniae D39 type 2 and its isogenic mutants were routinely grown under microaerophilic conditions at 37°C on Brain Heart Infusion broth (BHI) or Blood Agar Base (Oxoid) containing 5% (v/v) horse blood. According to manufacturer instructions, all media were prepared and sterilised by autoclaving at 121°C for 15 min. When needed, growth media were supplemented with 100 μg/ml of spectinomycin or 15 μg/ml of tetracycline. According to requirements, pneumococcal strains were also grown microaerophilic or anaerobically in a modified serum-based minimal medium (serum-SAPI) (Lyte and Ernst, 1992) and chemically defined medium (CDM) (Kloosterman et al., 2006a) supplemented with glucose or galactose (Table 2.2).

The serum-SAPI medium was modified by adding glucose and vitamins of Sicard's medium to support pneumococcal growth, and used to mimic the *in vivo* conditions *S. pneumoniae* would experience in its host whereas the CDM medium was used to test the effects of stress hormones and sugars on pneumococcal growth. Initially, the growth profile of the pneumococcal strains in the serum-based medium were examined in micro-aerobic environment with or without 50 μM of norepinephrine and 50 μM of ferric iron Fe $(NO_3)_3$ as control. The ferric nitrate was included to prove that growth failure in serum containing medium resulted from a lack of iron uptake in the pneumococcus, and not from serum's sensitivity itself. Moreover, concentrations ranging from 1 to 50 μM of the catecholamines hormones norepinephrine, epinephrine and dopamine were tested on pneumococcal wild type and its derived mutants.

Growth experiments were then carried out using a Multiskan™ GO Microplate Spectrophotometer (Thermo Scientific, UK). For growth assays, 100 μl replicates of serum-SAPI medium or CDM supplemented with or without the stress hormones and iron, as well as bacterial suspensions of around 10^2-10^3 CFU/ml were added into wells of a flat bottom 96 well microtiter plate. The 96 well microtiter plate was then placed inside the spectrophotometer and programmed to run for 20-30 hours at 34, 37 or 39°C

with reading every hour at OD_{600} nm. For growth in anaerobic environment, the above preparation was held in an anaerobic chamber and the microtiter plate was completely sealed with a MicroAmp® Optical Adhesive Film (Invetrogen, UK) before transferring the plate into the microtiter plate reader, which was set to read every hour without shaking. For microaerophilic conditions, the growth assays preparation was carried out on a laboratory bench instead of the anaerobic chamber and the plate was then sealed with the MicroAmp® Optical Adhesive Film (Invetrogen, UK) before transferring the plate into the microtiter plate reader, which was set to read every hour without shaking whereas in aerobic conditions, the microtiter plate was not sealed with the adhesive film and the microtiter plate reader was set on constant shaking. Maximum growth yield was considered to be the highest OD value of the bacterial growth, whereas specific growth rate was calculated by linear regressions of growth plots of In (OD_{600}) against time within the logarithmic phase of bacterial growth using the equation below:

$$(\mu)\ h^{-1} = \text{In}\ OD_2 - \text{In}\ OD_1 \ /\ t_2 - t_1$$

Where In is the logarithm of a number, while OD_2 and OD_1 are the cell densities at the time t_2 and t_1 respectively (Neidhardt et al., 1990).

For transformation and propagation purposes, Luria broth (LB) (10 g/l NaCl, 10 g/l tryptone and 5 g/l yeast extract) or Luria Agar (LA) LB containing 1.5% w/v bacteriological agar) were used to grow *E. coli* by shaking at 37°C with or without 100 µg/ml Ampicillin according to requirement.

2.4. DNA extraction

Bacterial DNA was extracted according to a published protocol with modification (Saito and Miura, 1963). Overnight bacterial cultures grown in BHI at 37°C were centrifuged at 10,000 x g for 10 minutes. After discarding the supernatant, pellets were mixed with 400 µl TE buffer containing 25% (w/v) sucrose. This mixture was then transferred into

a sterile 1.5 ml tube containing 60 µl of 500 mM EDTA, 40 µl of 10% w/v freshly prepared sodium dodecyl sulphate (SDS) and 2 µl of proteinase K (12.5 mg/ml). The DNA-containing sample was incubated at 37°C for 1-2 hours before centrifuging at 10000 x g for 5 minutes. The sample supernatant was then transferred into a sterile 1.5 ml before adding an equal volume of chloroform: isoamyl alcohol (24:1) with gentle mixing until emulsion was formed. This was followed by 10 minutes centrifuging at 10000 x g to obtain the upper aqueous phase, which was then treated again with the chloroform and centrifugation. The next step involved transferring the upper aqueous phase to a 1.5 ml tube containing 500 µl of 100% ethanol and 20 µl of 3 M sodium acetate. The mixture was centrifuged at 10000 x g for 5 minutes followed by replacement of the 100% ethanol with 500 µl of 70% ethanol before spinning down at 10000 x g for 5 minutes. Finally, the pellet was left to dry prior to re-suspension in 250 µl TE buffer and then kept at 4°C until needed.

2.5. Agarose gel electrophoresis

Agarose gel electrophoresis was routinely used to assess the integrity of DNA and PCR products (Meyers et al., 1976) on a 1% (w/v) of agarose gel (Bioline, UK). The gel was prepared in 1X TAE buffer (1 mM EDTA 40, mM Tris-acetate, pH 8.0) containing 0.2 µg/ml ethidium bromide to stain the DNA samples. The samples were normally mixed with 5 µl of 6X gel loading dye (New England Biolabs (NEB), UK) prior to loading them into the previously prepared wells of the agarose gel. A DNA ladder of 1 kb or 100 bp (NEB, UK) was also separately loaded as a control for an approximate measure of the DNA size and concentration. For electrophoresis, 80-90 volts was applied to samples in the gel for about an hour before they were visualised under UV light.

2.6. Polymerase chain reaction PCR

Both HotStarTaq *Plus* Master Mix (Qiagen, UK) and PrimeSTAR HS premix (Clontech, USA) were utilised for PCR amplification. The latter is a powerful proofreading enzyme used for amplifying and cloning DNA samples as well as insertion deletion mutations.

A typical PCR reaction in a total volume of 50 µl was composed of 19 µl of nuclease-free water, 2X 25 µl of PrimeSTAR HS premix (2X dNTP mixture 0.4 mM each, 1.25 U/25 µl PrimeSTAR HS DNA Polymerase and 2X PrimeSTAR buffer containing 2 mM Mg^{2+}), 3 µl of DNA as a template (15-30 ng/µl) and 3 µl of gene-specific forward and reverse primer mix (1 pmol each primer/reaction).

A thermal cycler (Techne, UK), was set for 30 cycles of amplification under the following conditions:

Description	Temperature	Time	Cycles
Initial denaturation	98°C	10 sec	1
Amplification	98°C	10 sec	30
	55°C	15 sec	30
	72°C	1 min/1000 bp	30
Final extension	72°C	10 min	1

The HotStarTaq *Plus* Master Mix was normally used for confirmation purposes. A typical PCR confirmation reaction contained 6 µl of nuclease-free water, 2 µl DNA template (15-30 ng/µl), 10 µl of 2X HotStarTaq *Plus* Master Mix (HotStarTaq *Plus* DNA Polymerase, 400 µM of each dNTP and PCR Buffer with 3 mM $MgCl_2$) and 2 µl of gene specific primers (1 pmol each primer/reaction). A thermal cycler (Techne, UK), was set for 35 cycles of amplification under the following conditions:

Description	Temperature	Time	Cycles
Initial denaturation	95°C	5 min	1
Amplification	95°C	45 sec	35
	55°C	45 sec	35
	72°C	1 min/1000 bp	35
Final extension	72°C	10 min	1

2.7. Colony PCR

A single isolated colony was selected from an agar culture and mixed with 200 µl of nuclease free water. Next, the mixture was boiled for 15 min at 95°C, and centrifuged at 8000 x g for 1 min to obtain the supernatant, which was used as a DNA template. Alternatively, a broth-based culture was used to prepare the DNA template. This was obtained by centrifuging approximately 500 µl of broth culture at 8000 x g before re-

suspending in 100-300 µl of nuclease free water. Then, aliquots of this were used as a DNA template. Depending on the purpose of work, various sets of primers were used for a routine PCR procedure as mentioned in section 2.6.

2.8. DNA purification from PCR and agarose gel electrophoresis

All amplified DNA and PCR products were extracted and purified using Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The DNA fragments of interest were precisely cut from gels by a sterile scalpel before transferring into a clean 1.5 ml eppendorf tube. The excised gels or PCR products were dissolved and vortexed after adding 10 µl of Membrane Binding Solution (4.5 M guanidine isothiocyanate) per 10 mg of agarose gel slice or 10 µl of PCR product, respectively. The mixture was then incubated for about 10 min at 65°C to completely dissolve the gel slices or PCR products before transferring the mixture into a SV Minicolumn assembly to recover the DNA from the mixture. This was followed by centrifugation for 1 minute at 8000 x g to discard the supernatant. The SV Minicolumn was washed and centrifuged twice with 700 µl and 500 µl for 1 and 5 minutes at 8000 x g, respectively, with Membrane Wash Solution (80% ethanol, 10 mM potassium acetate pH 5.0 and 16.7 µM EDTA pH 8.0). The collection column was then transferred into a sterile 1.5 ml eppendorf tube and added with 30-50 µl of nuclease-free water. Finally, it was incubated for about 1 minute at room temperature before centrifugation for 1 minute at 8000 x g. At this step, the SV Minicolumn was discarded and the tube containing the purified DNA was kept at -20°C until required.

2.9. Plasmid Extraction

To extract the plasmids used in this study, the QIAprep spin Miniprep kit (27106, Qiagen, UK) was used. *E. coli* cultures containing plasmids of interest were transferred into a clean 1.5 ml tube before centrifuging at 6000 x g for 10 minutes. The pellets were re-suspended and gently mixed in 250 µl buffer P1, which contained RNase and 250 µl buffer P2 (blue colour developed). Then, 350 µl of buffer N3 was added to the mixture

and mixed again by inverting the tube few times (until a white colour developed) and immediately followed by centrifugation at 8000 x g for 10 minutes. Next, the lysate was transferred into a QIAprep spin column and centrifuged at 8000 x g for 1 minute. The QIAprep spin column was then washed with 500 µl Buffer PB to remove traces of nucleases, and centrifuged for 1 minute at 10000 x g. The flow-through was discarded, and the column was washed with 750 µl of PE buffer and centrifuged at 8000 x g for 1 minute. The flow-through was discarded again and the column underwent another centrifugation for drying off any remaining ethanol and buffers that could interfere with later enzymatic reactions. The column was placed inside a sterile Eppendorf tube before adding about 30-50 µl DNA-RNA free water and left to be absorbed for 1 minute. Finally, the tube was centrifuged at 10000 x g for 1 minute to recover the plasmid DNA, which was then measured by NANODROP 1000 spectrophotometer (Thermo Scientific) and stored at -20°C until needed.

2.10. Restriction digest and cloning

The plasmids pPP2 (Halfmann et al., 2007a) was utilised in this project for the construction of transcriptional *lacZ*-fusions and genetically complemented strains, respectively. The plasmid pPP2 and the inserts were both double digested by two sets of restriction enzymes, *SphI* and *BamHI* (NEB, UK). The components of a typical reaction were 1 µg of each plasmid or insert, 1 µl of restriction endonuclease (10 U/µL), 5 µL CutSmart™buffer, and DNA-free water topping up to 50 µl. This mixture was then incubated at 37°C for 3-4 hours. The final step of digestion was to purify the digested DNA molecules using the Wizard® SV Gel and PCR Clean-Up System as described in section 2.8. The ligation process was carried out in a 20 µl reaction mixture. It contained 2 µl of T4 DNA Ligase (400 U/µL, NEB, UK), 2 µl of 10 X T4 DNA ligase buffer (1 Mm ATP, 10 Mm $MgCl_2$, 10 Mm DTT, 50 Mm Tris-HCl and pH 7.5) and required amount of plasmid and insert to bring molar ratio of plasmid to insert 1 to 3. Lastly, the mixture was incubated for 16 hours at 16°C before being deactivated at 65°C for 10 minutes.

2.11. Competent *E. coli* preparation

Competent *E. coli* top10 strains were chemically prepared according to the protocol described previously (Hanahan, 1983). Firstly, a single *E. coli* colony was selected from an LA plate and inoculated into 5 ml of LB containing 20 mM $MgSO_4$ and incubated for 24 hours at 37°C. The following day, 1 ml of the overnight culture was transferred into 100 ml of LB medium and incubated for 2-3 h until the OD_{550} had reached 0.7 to 0.8. At this point, the culture was centrifuged at 2000 x g for 10 minutes and was re-suspended in 30 ml of sterile cold Tfb I buffer and incubated for approximately 30 minutes. This was followed by centrifugation at 2000 x g for 10 minutes at 4°C before re-suspending the bacterial pellets in 4 ml of Tfb II buffer. Small aliquots were then stored at -80°C until needed.

2.12. Transformation of *E. coli*

The chemically competent *E. coli* strains were transformed by the heat shock method. The first step was mixing about 7-10 µl of the ligated pPP2 plasmid (20-50 ng/µl) with 50-100 µl of the competent *E. coli* in an autoclaved 1.5 tube and immediately placed on ice for 30 minutes. After the cold incubation, the tube was quickly transferred into pre-heated water bath at 42°C for just 1 minute. Then, the tube was moved back into the ice for 2-3 minutes before transferring the tube mixture into 500 µl pre-heated LB medium and incubated for 1 hour at 37°C on a shaking incubator. This following step was plating out approximately 250 µl of the bacterial culture into LA plate containing 100 µg/ml ampicillin and incubation for 24 hours at 37°C. Next day, the positive colonies were selected and analysed by PCR, and stored at -80°C until required.

2.13. Transformation into *S. pneumoniae* D39

The constructs were genetically transformed into pneumococci according to the protocol described by (Bricker and Camilli, 1999). Firstly, the overnight grown pneumococci were diluted at 1:100 in 10 ml of fresh BHI broth, and incubated at 37°C

until the OD_{600} reached between 0.05-0.08. At this point, 860 µl of the bacterial suspension was added into an eppendorf tube containing 100 µl of 100 mM NaOH, 10 µl of 100 mM $CaCl_2$, 10 µl of 20% (w/v) BSA, 5-10 µl of a linear DNA product or plasmid (1 µg) and 2 µl of 50 ng/ml of competence stimulating peptide (CSP) (Alloing et al., 1996). The mixture was then incubated for 3 hours at 37°C. Up to 330 µl of the culture was plated every hour on BAB plates containing appropriate antibiotics, and incubated overnight in a candle jar at 37°C.

2.14. Mutagenesis by gene splicing overlap extension (SOEing) PCR

SOEing is a PCR-based method in which mutation is generated *in vitro* without reliance on restriction sites. It relies on the production of a PCR construct through addition or modification of sequences at its ends allowing the product can itself be used to prime DNA synthesis in a subsequent overlap-extension reaction to create mutant or recombinant molecules. SOEing PCR was done according to the protocol originally described previously (Horton, 1995;Song et al., 2005) as illustrated in figure 3.1 in chapter 3. Firstly, upstream and downstream (left and right flanks) regions of genes of interest as well as *aadA* gene (spc^R), which confers resistance to spectinomycin, were amplified. Second, the three amplified fragments were fused using LF/F and RF/R primers. Finally, the fused PCR end product was introduced to pneumococcal D39 by transformation. The homologous recombination between the flanks leads to the deletion of the target gene and insertion of cassette conferring resistance to spectinomycin.

2.14.1. Amplification of SOEing constructs

Spe/F and Spe/R primers were designed to amplify the spectinomycin resistance gene *aadA* (1158 bp) obtained from pDL278 vector (Yesilkaya, 1999). Additionally, two pairs of gene-specific primers LF/F-X, LF/R-X, RF/F-X and RF/R-X (X= gene code) were also designed to amplify the left and right fragments of target gene creating approximately 800 bp PCR products for each flank. Primers used for the amplification

of *spc*^{R} cassette and the left and right flanking regions of each individual gene are shown in Table 2.3. Sections homologous to the cassette are highlighted in bold. All SOEing fragments were amplified with PrimeSTAR HS premix (section 2.6) using the designed primers, and were purified via the previously described Wizard® SV Gel and PCR Clean-Up System kit in section 2.8.

Table 2.3. Primers used for allelic replacement mutagenesis. Sections homologous to the *aadA* gene are highlighted in bold typeface.

PRIMERS	5` → 3`
Spe/F	ATCGATTTTCGTTCGTGAAT
Spe/R	GTTATGCAAGGGTTTATTGT
LF/F-TSC12	TCAGATATGGTAAGTACGAT
LF/R-TSC12	**TATTCACGAACGAAAATCGAT**AAACTTTCATTCAAATTCC
RF/F-TSC12	**AACAATAAACCCTTGCATAAC**TCAAAAGTGATTGACAATTAGC
RF/R-TSC12	TTACAAGGAGGAAATATGCAAG
LF/F-TSC13	TTCTAGTTCAATCAAGAATCGA
LF/R-TSC13	**TATTCACGAACGAAAATCGAT**ATATTCTCATCTTCTTACTC
RF/F-TSC13	**AACAATAAACCCTTGCATAAC**CTTACACTGAGCTAGGAGAG
RF/R-TSC13	AGCTGGTTAAGAGGTTAGTGTAG
LF/F-TSC05	ATGAAGGAACGGATGCTGAAAC
LF/R-TSC05	**TATTCACGAACGAAAATCGAT**CTCTCTGCATTTTACATGAG
RF/F-TSC05	**AACAATAAACCCTTGCATAAC**GATGTTCAGTAAACTTAAAAAAAC
RF/R-TSC05	CGCATATTTCGGACTTCTTCCA
LF/F-TSC08	AGGTGATGGTATGTTGACTCG
LF/R-TSC08	**TATTCACGAACGAAAATCGAT**GTCTTTCCCATCTGTCTCTCC
RF/F-TSC08	**AACAATAAACCCTTGCATAAC**GACAAACATGAAACTAAAAAG
RF/R-TSC08	GTCCTTCTCCAGCTTAGCCACC
LF/F-TSC02	ACTTGCCTGAGCCAGCTGTAG
LF/R-TSC02	**TATTCACGAACGAAAATCGAT**GTATTTTTTTCATATGTTCACC
RF/F-TSC02	**AACAATAAACCCTTGCATAAC**TAATGCTTGATTTACTGAAAC
RF/R-TSC02	GTGAAAGATGGAGGAGATCCG
LF/F-TSC04	GACTTGTGACAATCGCTTGAG
LF/R-TSC04	**TATTCACGAACGAAAATCGAT**GTTTTGTCATCTATTATCTCC
RF/F-TSC04	**AACAATAAACCCTTGCATAAC**GGAGTTATAGATGAAACGCT
RF/R-TSC04	GGTTTCAGCAAAGCCCTTAA
LF/F-TSC03	CTCAAGCGTTTATTGGCAGG
LF/R-TSC03	**TATTCACGAACGAAAATCGAT**GTAAAATTTTCATCTTTACTCC
RF/F-TSC03	**AACAATAAACCCTTGCATAAC**GGAGTTTTAGATGAGTTTAGC
RF/R-TSC03	GCTAGAGGTACTTGCTTGCT
LF/F-TSC07	CGGATTGACATTTCCGAGAAAG
LF/R-TSC07	**TATTCACGAACGAAAATCGAT**ACTTTATACATTTTCTCCCT
RF/F-TSC07	**AACAATAAACCCTTGCATAAC**CTATACTATAAGATTTGTATTCC
RF/R-TSC07	CCTTGACCAAATCCTTGATATGAA
LF/F-RitR	GGCTGAAATCTTTACTGAGTGG
LF/R-RitR	**TATTCACGAACGAAAATCGAT**GTTTCCCCATGGCTGACCTA
RF/F-RitR	**AACAATAAACCCTTGCATAAC**GCAAGAATAGAAAAGCAGTT
RF/R-RitR	GAGCAGGTTTGCTTCTATCAAT
LF/F-TSC06	GATATGACCCAGAGATGGGAG
LF/R-TSC06	**TATTCACGAACGAAAATCGAT**AAATGTTCATCTCTCTCCCT
RF/F-TSC06	**AACAATAAACCCTTGCATAAC**TAGCTTATGATAAAAAATCC
RF/R-TSC06	GGATAATTCTAAGACTGGCTAAAGG

PRIMERS	5`→ 3`
LF/F-TSC10	GAGCTTCTCAGTTCTCCTCC
LF/R-TSC10	**TATTCACGAACGAAAATCGAT**AATTTTCATACTTTAACTGC
RF/F-TSC10	**AACAATAAACCCTTGCATAAC**GCGAAAATGAAACGAACAGG
RF/R-TSC10	GACCTTCATCTTCTCCAGCT
LF/F-TSC01	TATCCTTTGATAACCCGCAGTC
LF/R-TSC01	**TATTCACGAACGAAAATCGAT**TCTTGTGCATGCGCTTCTCC
RF/F-TSC01	**AACAATAAACCCTTGCATAAC**GCATGCTTGATTGGAAACAA
RF/R-TSC01	CCACGTTCAAATACTCGGAG
LF/F-TSC09	GCCTATCCTGTCATTGATGC
LF/R-TSC09	**TATTCACGAACGAAAATCGAT**TGTAGGTCATGCTCTGCTCC
RF/F-TSC09	**AACAATAAACCCTTGCATAAC**AGACCGATGAAGCGTTCTTC
RF/R-TSC09	CAATGATGACACGCCAAGTAC
LF/F-TSC11	CTTGCTCAAGTCCTTGTTGTG
LF/R-TSC11	**TATTCACGAACGAAAATCGAT**ATACTTTCATCTTAGTTTCTC
RF/F-TSC11	**AACAATAAACCCTTGCATAAC**GGTTGGTTATGATGATATAATATTTTC
RF/R-TSC11	CTTCTTTCTCGTTATCTTCATCGC

2.14.2. Fusion and transformation of SOEing fragments

A second PCR reaction was carried out using the amplified PCR products as one template mixture (the two-flanking region of a given gene and *spc*^{R} gene). The PCR amplification using LF/F-X and RF/R-X primers produced a linear fused product, which was used to transform *S. pneumoniae* type 2 D39 strain. The 50 µl PCR reaction consisted of 2 µl of the left, 2 µl of right flank PCR products (~20 ng /µl each), 4 µl of *spc*^{R} gene cassette (~20 ng /µl), 25 µl of 2X PrimeSTAR HS premix, 3 µl of mixed primer LF/F-X and RF/R-X (1 pmol/ µl) and 14 µl of molecular grade water. The PCR conditions were mentioned previously in section 2.6. Following the amplification of the linear fused product, the amplicons were subjected to agarose gel electrophoresis, and the band of interest was purified by Wizard® SV Gel and PCR Clean-Up System (Promega, USA) as earlier described in section 2.8. Eventually, the fused product was then introduced to *S. pneumoniae* type 2 D39 strain for genetic transformation, as described in section 2.13, and were grown and selected on BAB plates supplemented with 100 µg/ml spectinomycin.

2.14.3. Confirmation of mutation by PCR

Multiple PCR amplifications with different set of primers confirmed the mutagenesis of target genes in *spc*^{R} colonies. The extracted DNA (as described in section 2.4) from selected transformants was utilised as a template for the PCR reactions while the genomic wild type DNA was used as a control. The LF/F-X and RF/R-X primers were used for the amplification of wild type DNA and for integration of mutation in selected transformants. Additionally, the Spe/F and Spe/R primers were used to amplify the *spc*^{R} cassette of the transformants. Furthermore, LF/F-X and Spe/R mixed primers were used to amplify the cassette and left flanking region of each target gene while Spe/F and RF/R-X mixed primers amplified the cassette and the right flanking region of each gene. The PCR reactions and conditions using the HotStarTaq *Plus* Master Mix were as previously described in section 2.6.

2.14.4. Confirming the mutants by DNA sequencing

The successful allelic gene replacements were confirmed through DNA sequencing of the mutants. To confirm the status of mutation, two new pairs of primers were designed (Table 2.4). The F1-X-Seq primer was used to amplify about 100 nucleotides away from the left flank while R1-X-Seq primer was used to amplify about 100 nucleotides away from the right flank of the mutated gene. In addition, F2-X-Seq primer was used to amplify about 180 nucleotides upstream of the *spc*^{R} cassette insertion whereas R2-X-Seq primer was used to amplify about 180 nucleotides downstream of the cassette insertion. As a result, the entire mutated region as well as an additional 100 nucleotides chromosomal region adjacent to the flanks were amplified using F1-X-Seq and R1-X-Seq primers. Similarly, the DNA from putative mutants was also used to amplify the cassette and 180 nucleotides upstream or downstream using F2-X-Seq and R2-X-Seq, respectively. The PCR conditions and purification of the amplified products used are previously described in section 2.6 and 2.8, respectively. Finally, the samples were then sent for sequencing at The Protein Nucleic Acid Chemistry Laboratory (PNACL), University of Leicester or Eurofins (MWG, Germany), provided with gene specific

sequencing primers; F1-X-Seq, F2-X-Seq, R1-X-Seq and R2-X-Seq (X=gene code).

Table 2.4. Primers used for pneumococcal DNA sequencing.

PRIMERS	5`→ 3`
F1-TSC12-Seq	GGAACTAGCCTATTTTGACGAGG
R1-TSC12-Seq	GACATATAGTCTAATTTGTTTAC
F2-TSC12-Seq	CAGGAGATCTATTTGCCTTAGG
R2-TSC12-Seq	ACTACCAACTGAGCTATGGCGG
F1-TSC13-Seq	TCAAAGTGGAAGCGGTCATAG
R1-TSC13-Seq	CTGCTCCATATCCTCCTCTTC
F2-TSC13-Seq	ACTAAAGCCCATACCGTACAG
R2-TSC13-Seq	GATAGACCTATACCGAACAAAGG
F1-TSC05-Seq	AGGAAGAGTCTTATCTGGTGG
R1-TSC05-Seq	CTCCGGCTTAATCCCATCAT
F2-TSC05-Seq	GTTCTTTGAGCCGCAACTTTC
R2-TSC05-Seq	GCTTATCGTCCACCGAAGTAT
F1-TSC08-Seq	CAGGTGTCAACCATGTGATTC
R1-TSC08-Seq	GTCACACTGATTGAAAGCTGG
F2-TSC08-Seq	ATGATGGATAGTGGGCTGAC
R2-TSC08-Seq	GACTGGCAATAGGAGAAAGAGA
F1-TSC04-Seq	TGAGCCCTTGATAACCACTG
R1-TSC04-Seq	GGTCGTCCATTGTATAGGCA
F2-TSC04-Seq	TTTCTTGTCTTGTTGCTCCTG
R2-TSC04-Seq	GCAACTTCCCATTTCCAAAGG
F1-TSC03-Seq	AATCTCAAGCGTTTATTGGCAG
R1-TSC03-Seq	CCATTATCACATAAACAGGCAAAC
F2-TSC03-Seq	GACAATGGGATTGGTTTCCAG
R2-TSC03-Seq	GTTAGCAGCCACATATTGGTAT
F1-TSC07-Seq	GGGTTTGAGCCTTCTCTTTATTG
R1-TSC07-Seq	CTCAACATCTCCGTTATCCAC
F2-TSC07-Seq	CCAAGCCAGCTACAGTGATC
R2-TSC07-Seq	GCAACTTGTTTCTGAGCAGC
F1-RitR-Seq	TGGTATTGAGTACGGTGATATGC
R1-RitR-Seq	CCATTTGCCTCGTACATATTTCC
F2-RitR-Seq	CCAACTTTCTCAGCAGCTATTAC
R2-RitR-Seq	CTTATCAACAAATCGGCCACC
F1-TSC06-Seq	TTATGGTCAAGCCTTTAGTGG
R1-TSC06-Seq	CACTCTGTCCACTCTTGGAAT
F2-TSC06-Seq	TTGTAGATAGAGCTGACGTGG
R2-TSC06-Seq	GACTTTGAATAAGACTGGAGTAAGC
F1-TSC10-Seq	AAGAGTTGAATGGCATCAGTG
R1-TSC10-Seq	CTATCATCTCGCAATTCCTGC
F2-TSC10-Seq	ATAAATCCGAAAGCAGTGGTG
R2-TSC10-Seq	CTTGCTCGATACTCTGCCTAT

PRIMERS	5` → 3`
F1-TSC01-Seq	GAACGATGATTTGGTGAGCAG
R1-TSC01-Seq	GATAGATAAAGGCCAAGTCCAGA
F2-TSC01-Seq	TCTAACTCATTCAAGGGAAGGTC
R2-TSC01-Seq	CTGCGTTTCCACCAATATGTC
F1-TSC09-Seq	TGATGAGGTGGCTAAGAAATACG
R1-TSC09-Seq	CACACTAAGCAGAGACAGACA
F2-TSC09-Seq	CTGTGGCTATCTTCCTAGTTTAC
R2-TSC09-Seq	GAATAAAGTGGCTAGTCTGGC
F1-TSC11-Seq	GTTGGGAATACTGCCTTTGTG
R1-TSC11-Seq	GAGAACAATCGGAGTACGTGA
F2-TSC11-Seq	TAGAACGGACAGAGAAGGAAC
R2-TSC11-Seq	GAAACTAAGCCCTCCTAAAGT

2.15. Construction of *lacZ*-fusions

2.15.1. Restriction digest, cloning and *E. coli* transformation

The construction of the transcriptional *lacZ*-fusions was performed based on the previously described method (Halfmann et al., 2007a) by the use of the integrative pPP2 plasmid, which was obtained from Dr. Reinhold Bruckner (Kaiserslautern, Germany). To begin with, the pPP2 was extracted using the previously described QIAprep spin Miniprep kit in section 2.9. Then**,** the putative promoter regions of the selected genes along with 28 bp coding sequence of each target gene were amplified using primers modified to incorporate *SphI* and *BamHI* sites as shown in Table 2.5. The promoters of gene under study were amplified by PCR using HotStarTaq plus Master Mix and purified by Wizard® SV Gel and PCR Clean-Up System before analysing on agarose gel electrophoresis as previously described in sections 2.6, 2.8 and 2.5, respectively. The plasmid pPP2 and the inserts were both double digested by two sets of restriction enzymes, *SphI* and *BamHI* as explained in section 2.10. Following the digestion, the samples were purified through the Wizard® SV Gel and PCR Clean-Up System, and the digested pPP2 was compared with an undigested pPP2 (control) on agarose gel electrophoresis. Next, the digested inserts and plasmid were ligated as

already described in section 2.10, prior to transforming an aliquot of the resulting ligation mixture to the TOP10 *E. coli* (section 2.11). Ultimately, *E. coli* transformants were grown on LA plates supplemented with 100 μg/ml of ampicillin as described in section 2.12.

Table 2.5. Primers used for amplification of transcriptional *lacZ*-fusions. Underlined nucleotides refer to the incorporated restriction enzyme recognition sites.

PRIMERS	5`→ 3`
TCS05-Fusion-F	GACGCATGCGTAACATCGGTATGGGAATCAAG
TCS05-Fusion-R	GCGGGATCCGAAACTCCTCCTTATTAAA
TCS03-Fusion-F	GACGCATGCAGGAGCGGGTTGAAGATATG
TCS03-Fusion-R	GCGGGATCCCAAGCCCAAACGGACCA
TCS09-Fusion-F	GACGCATGCCTTTGAGAAACCTGTGGC
TCS09-Fusion-R	GCGGGATCCCCTTGTCTTACCAGATATTC

2.15.2. Confirmation of cloning for construction of *lacZ*-fusions by PCR

Initially, colony PCR was used (before extracting the DNA) to confirm the successful construction of *lacZ*-fusion. Fus-seq-UF: 5'-CTACTTGGAGCCACTATCGA-3'and Fus-seq-DR: 3'-AGGCGATTAAGTTGGGTAAC-5' fusion primers were designed to amplify approximately 70 bp of the upstream of any given cloned putative promoter region as well as 68 bp of *lacZ* gene. The reason was to indicate the successful ligation in the correct location of each cloned promoter within pPP2. The DNA fragments were amplified by using HotStarTaq plus Master Mix as described previously in section 2.6, while the recombinant plasmid DNA was extracted from the positive bacterial colonies and was further analysed by genomic DNA sequencing using the Fus-seq-UF and Fus-seq-DR primers.

2.15.3. Transformation of reporter constructs to *S. pneumoniae*

Transformation of the recombinant pPP2 plasmids into *S. pneumoniae* D39 wild type and selected TCS mutants was performed according to the previously described

protocol in section 2.13. Only transformants grown on BAB plates containing 15 µg/ml tetracycline were selected. Post-transformation, colony PCR (as described in section 2.6) was initially used (before extracting the DNA) to confirm the successful transformation with the recombinant pPP2 plasmids. PCR confirmed the successful integration of *lacZ*-fusions within the genome of pneumococci using HotStarTaq *Plus* Master Mix, as described in section 2.6, as well as the Fus-seq-UF and Fus-seq-DR primers, whose recognition sites are directly located up and downstream of the cloning site, respectively.

2.16. β-galactosidase activity assay

This assay was performed according to the protocols of (Zhang and Bremer, 1995), and (Miller, 1972). The promoter responsiveness to stress hormones was determined by measuring β-galactosidase activity, coded by promoterless *lacZ* gene under the transcriptional control of promoter under investigation. The quantity of β-galactosidase from the cell extracts was determined by the gradual appearance of yellow colour. This is a result of the hydrolysis of the chromogenic substrate (lactose mimic) known as ONPG (O- Nitrophenyl β-D-galactopyranoside) by β-galactosidase enzyme as shown in Figure 2.1.

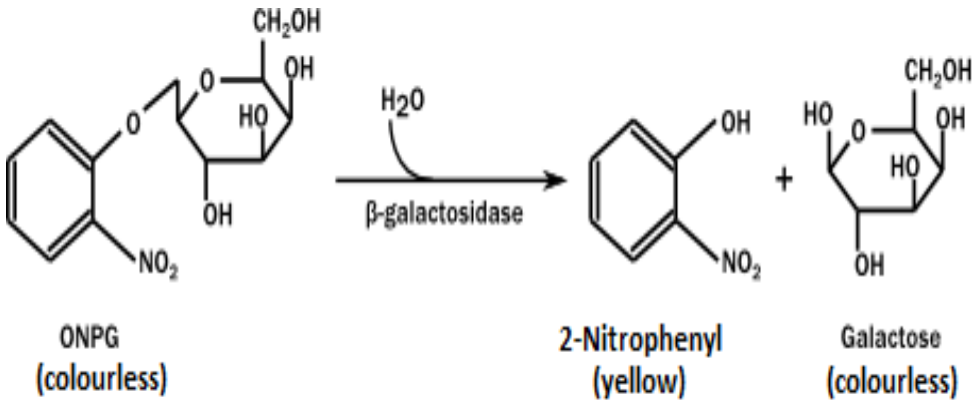


Figure 2.1. Illustration of the β-galactosidase mode of action; the β-galactosidase enzyme hydrolyses O-nitrophenyl-β-galactopyranoside (ONPG) to 2-Nitrophenyl and galactose.

Pneumococcal *lacZ*-fusion strains (the reporter strains) were grown to mid-exponential phase on serum-SAPI medium with and without 50 μM of norepinephrine. A 2-ml aliquot of the cultures was then centrifuged at 4000 x g for 10 min and re-suspended in 2 ml of chilled Z buffer (section 2.2). The optical density of 1 ml of the mixture was measured at 600 nm, and blanked against Z buffer. Next was the addition of about 100 μl of Triton™ X-100 and leaving the culture for 10-30 minutes at room temperature. This was followed by addition of 200 μl of O-nitrophenyl-β-galactopyranoside (ONPG) (4 mg/ml solution = 40 mg ONPG + 10 ml phosphate buffer, as described in section 2.2). The next step was incubating the test tube at 30°C until the yellow colour developed to a certain level (Luria broth-like colour). At this point, the reaction was stopped by adding 400 μl of 1 M Na_2CO_3, and the start and the end-point of the reaction time was recorded. The sample tubes were then centrifuged at maximum speed for 5 minutes before transferring 1 ml into cuvettes to record the optical density at 420 nm. Lastly, the specific β-galactosidase activity produced was measured by the following equation:

$$\text{Miller Units} = 1000 \times (OD_{420}) / (T \times V \times OD_{600})$$

Where OD_{420} = optical density at the end of the test, T = Reaction time in minutes, V = Culture volume in milliliters and OD_{600} = optical density of the washed culture suspension.

2.17. Radiolabelled Norepinephrine and transferrin uptake assays

Norepinephrine (NE) and transferrin (Tf) uptake assays were performed according to a previously published report (Sandrini et al., 2014). To test the ability of *S. pneumoniae* wild type and selected mutants to acquire iron from Tf, a serum-SAPI medium containing filter-sterilized ^{55}Fe-Tf (2×10^5 cpm ml^{-1}) supplemented with 50 μM NE or an equivalent volume of PBS, pH 7.0 (control), was used. Washed pneumococcal cultures containing approximately 1×10^7 CFU/ml per reaction were added and incubated at 37°C for 24 hr. For analysis of NE internalization, pneumococcal wild type

and selected mutants' cultures were similarly grown but supplemented with 1×10^5 cpm per ml of ^{3}H-norepinephrine. Cultures were harvested by centrifugation at 8000 x g for 10 min, washed in PBS and assayed for cell numbers and for radiolabel incorporation. Radioactivity was measured by mixing samples with 2 ml of emulsifier-safe scintillant (Canberra-Packard, Pangbourne, U.K.) for counting in the tritium channel of a Minaxi Tri-Carb 400 series scintillation counter (Canberra-Packard).

2.18. Biofilm formation assays

Stress hormone effect on biofilm formation was examined using the crystal violet attachment assay (Merritt et al., 2005). Pneumococcal strains of approximately 10^6 CFU/ml were statically cultured in the serum-SAPI medium for 16 hours in 200 µl volumes in triplicate using 96 well plates. After incubation, non-attached bacteria and culture supernatants were removed and the wells were washed 3 times with PBS. The wells were then dried at room temperature. After drying, crystal violet (0.5% v/v) was added for 1-2 minutes. Then, the wells were washed 3 times with PBS, tapped to remove residual liquid, and the plate was dried at room temperature. A mixture of 80% (v/v) ethanol and 20% (v/v) acetone was then added to elute the stain, and the attachment was determined by measuring the absorbance at 595 nm.

2.19. Catecholamine antagonism assays

These assays were carried out to investigate the nature of the putative pneumococcal adrenergic and/or dopaminergic receptor(s) by which catecholamines bind and exert their effects. The protocol and concentrations of antagonists used were chosen based on the previously published report (Freestone et al., 2007a). Catecholamine antagonism experiments were performed similarly to the catecholamine pneumococcal growth assays as described in section 2.3. Controls contained equivalent volumes of the solvent utilised to dissolve the antagonist or catecholamine hormones (distilled water), which were then sterilised by passage through a 0.2mm Acrodisc syringe filter. Three different types of antagonists were used: phentolamine (α-

adrenergic receptor specificity), propranolol (β-adrenergic) and chlorpromazine (dopaminergic), and concentrations ranging from 2 to 200 μM were added to the growth assays. To determine whether inhibitory to bacterial growth was directly affected by the antagonists, all antagonism of catecholamine-induced growth assays were also performed in the presence of a concentration of 50 μM Fe (ferric iron Fe $(NO_3)_3$) to overcome the iron-restriction of serum-based medium and to confirm the antagonist was not toxic *per se.*

2.20. Phenotypic characterisation of catecholamine-exposed pneumococci

To understand the nature of catecholamine effects on pneumococcal growth phenotypically, bacterial cells were examined under an optical microscope after exposure to 50 μM of norepinephrine in serum-containing media using INFINITY 1 camera and INFINITY ANALYZE imaging software. 78 pneumococcal cells were measured in size for each group of samples. In addition, cell arrangement of catecholamine-treated and untreated pneumococci was also examined under the light microscope. The growth of pneumococci wild type was also tested under different conditions such as temperature of 34, 37 and 39°C and in aerobic, micro-aerobic and anaerobic environments.

2.21. Capsule extraction and glucuronic acid assay

Pneumococcal polysaccharide capsule was extracted as previously described (Domenico et al., 1989). The first step comprised mixing 500 μl of bacterial culture with 100 μl of 1% Zwittergent 3-14 detergent in 100 mM of citric acid, pH of 2.0. This mixture was then incubated at 50°C for 20-30 min. The next step was centrifugation at 8000 x g for 5 min before transferring 300 μl of the supernatant into a new 1.5 ml tube, and absolute ethanol was added to a final concentration of 80%. Then, the solution was incubated at 4°C for 20 min followed by centrifugation at 8000 x g for 5 min. Finally, the pellet was then dissolved in 200 μl of distilled water. The glucuronic acid assay was carried out according to a previous protocol of (Bitter and Muir, 1962). After extracting

and purifying the capsular samples, 0.5 ml of each sample was used for determining the amount of glucuronic acid. Next, 3 ml of 0.025 M sodium tetraborate solution (Borax) was added to the samples, which were incubated at 100°C for 10 minutes. This was followed by cooling them on in ice bath before adding 0.1 mL of 0.125% w/v carbazole solution. The samples were then heated again at 100°C for 10 minutes and cooled to room temperature. Finally, absorbance was measured using a spectrophotometer at a wavelength of 530 nm. A standard curve of known glucuronic acid concentrations, 0, 10, 20, 40, 60, 80, and 100 µg ml^{-1}, was prepared for calculating the concentration of glucuronic acid in the samples (Figure 2.2). Ultimately, the concentration of capsule in samples was expressed in µg of glucuronic acid per 100 CFU (Favre-Bonte et al., 1999;Lai et al., 2003).

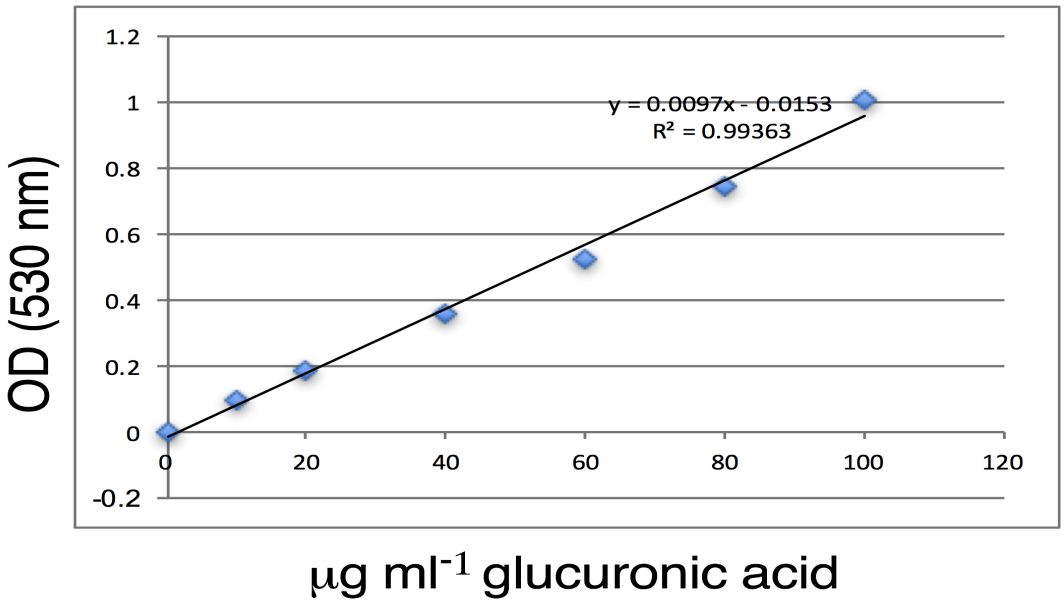


Figure 2.2. Glucuronic acid calibration plot.

2.22. Metabolomics preparation

Pneumococcal samples were prepared for extracellular and intracellular metabolite analysis according to the previously described protocol (Hartel et al., 2012). Following growing under the required conditions, pneumococci were treated with 10 mm sodium

azide and sedimented at 8000 x g at 5°C for 15 min. They were washed with 40 mm Tris/HCl, pH 7.4 before extraction with cold methanol containing 0.2 mM norvaline as internal standard. The samples including a medium control, extracellular and intracellular metabolites were sent to MS-Omics (Frederiksberg, Denmark) for metabolic analysis. The pneumococcal metabolites were analysed using Gas Chromatography – Mass Spectrometry (GC-MS), which is one of the most applied analytical tools in metabolomics. GC-MS is capable of identifying hundreds of metabolites due to its high separation power. Most of raw GC-MS data are processed by MS Omics developed software, which uses the powerful PARAFAC2 model that facilities the extraction of more compounds and cleaner MS spectra compared with other GC-MS software.

2.23. *in vivo* studies

2.23.1. Preparation of bacterial inoculum

Prior to *in vivo* experiments, bacterial inoculum was prepared in one of two ways for infection. The first one was exposing the bacterial inoculum to NE or PBS for short time prior to the administration of infection dose. This was started by growing pneumococcal strain at 37°C on BHI until the OD_{500} reached approximately 1.4-1.6. Then, the bacterial culture was centrifuged at 3000 rpm (Sorvall legend T, Thermo Scientific) for 15 min followed by discarding the supernatant. The next step was re-suspending the pellet by adding 1 ml of fresh serum broth (20% (v/v) filter-sterilised fetal calf serum and 80% (v/v) BHI). Only 700 µl of this dense bacterial cells was inoculated into a 10-ml fresh BHI serum broth tube in order to bring the OD_{500} to about 0.7. This was followed by incubation until the growth reached 1.6 of OD_{500} when the pneumococcal culture was divided into 500 µl aliquots and kept at -80°C until required. Subsequently, the inoculum was prepared for administration as below (section 2.23.2), and incubated either with 50 µM NE or PBS at room temperature before administration to mice. The second bacterial inoculum, on the other hand, was prepared by growing the pneumococci with or without 50 µM NE for a much longer period to investigate the

effects of the hormone on pneumococcal behaviour inside the host. Hence, the inoculum was prepared by growing the bacteria overnight with or without the hormone on serum-SAPI as previously described in section 2.3.

2.23.2. Preparation of infectious dose

Aliquots of frozen pneumococci of known CFU were rapidly thawed and centrifuged at 4000 x g in a bench-top micro centrifuge (Sigma). The supernatant was aspirated, and the bacteria were re-suspended in 400 µl of PBS. Then, based on the required CFU, a predetermined volume of this stock was mixed with PBS. When required, 50 µM NE was added to a separate portion of this preparation.

2.23.3. Colonisation experiments

Female CD1 outbred mice (Charles River, UK) at 9-11 weeks of age were used for colonisation models. The standardised inocula were prepared as described in 2.23.1. To determine the potential effects of hormones on pneumococcal colonisation state, the mice were moderately anaesthetised in an anaesthetic box with 2.5% (v/v) Isoflurane (Isocare, UK) over oxygen (1.5 litres/min). To prevent the inoculum disseminating into the lower respiratory tract, mice were held horizontally while 10 µl of PBS containing 5 x 10^5 CFU of *S. pneumoniae* were administered intranasally. Then, the mice were kept lying on their backs for recovery. After infection, the inoculum was promptly plated out on blood agar plates for viable counting. During the course of 7 days, the animals were monitored daily for disease signs such lethargy, hunched or piloerection. To determine the pneumococcal growth in the nasopharynx, the previously described protocol of Richards et al., ((Richards et al., 2010) was followed.

Following intranasal infection, set groups of mice were killed under deep anaesthesia with 5% (v/v) isoflurane over oxygen at day 0, 2 and 7. Then nasopharyngeal wash, bronchoalveolar lavage (BAL) and lung tissue samples were obtained. Nasopharyngeal wash samples were done using 500 µl of tryptic soy broth (TSB) while BAL samples

were collected by using 700 μl of the TSB medium. Lung tissues samples, however, were weighed before subjecting them into homogenization (Ultra Turrax, UK). The viable counts in the samples were determined through serial dilution in sterile PBS followed by inoculation on blood agar plates. All samples were plated out on blood agar plates with or without 5 μg/ml of gentamicin to prevent contamination with mice normal flora.

2.24. Statistics

All Statistical analysis were determined via Graphpad Prism software 6.0f (Graphpad, California, USA) and all experiments were performed in triplicate on at least 3 separate occasions, unless stated otherwise. Data were expressed as mean +/− standard error of the mean (SEM). Where appropriate, statistical analysis was first performed using an unpaired t-test or one-way ANOVA followed by Dunnett's multiple comparison tests. For metabolomics analysis, a PCA model was performed “Multivariate Data Analysis” (Camo software). Significance was defined as * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$.

Chapter 3. Results

Section A: Construction of genetically modified strains

3.1. Construction of isogenic mutants

The aim of constructing isogenic mutants was to examine the potential role of pneumococcal two-component systems (TCS) in *S. pneumoniae*-stress hormone interactions. Bacterial adaptation, by which they perceive and respond to environmental changes including host stress hormones levels, is often regulated by such essential mechanisms (Hoch, 2000;Stock et al., 2000). For many years, bacterial TCS systems have caught scientist attention due to the fact that they have not been found in their mammalian host rendering them a potential antibacterial target (Barrett and Hoch, 1998). Moreover, additional to bacterial adaptation to environmental changes within their host, they also contribute to pneumococcal competence, virulence and viability (Paterson et al., 2006). More importantly, their role in stress hormone-bacteria interactions has been identified in some Gram-negative bacteria (Hughes and Sperandio, 2008). Since it is plausible that they are also important in pneumococcus-catecholamine hormone interactions, pneumococcal TCS mutants were successfully constructed and analysed for their response to catecholamines. The genes encoding for these regulators were deleted by allelic replacement mutagenesis. The resulting mutant strains were used to evaluate the contribution of each TCS in *S. pneumoniae*-stress hormone interactions by growth studies, radio labelled hormone uptake, gene reporter and biofilm formation assays. Using the gene splicing overlap extension mutagenesis **(**SOEing), the mutations were introduced to all TCS except TCS02. Two attempts to mutate TCS02 (*vicR*) were not successful since it is the only pneumococcal TCS essential for pneumococcal viability (Paterson et al., 2006). Eventually, all known TCSs in *S. pneumoniae,* except TCS02, were mutated successfully using the SOEing PCR method and designated as TCS12, TCS13, TCS05, TCS08, TCS04, TCS03, TCS07, RitR, TCS06, TCS10, TCS01, TCS09*,* and TSC11.

3.1.1. Mutagenesis by gene Splicing overlap extension (SOEing) PCR

SOEing PCR was done according to the protocol originally described by (Horton, 1995;Song et al., 2005), which has been illustrated in Figure 3.1. This method offers an advantage over other strategies such as plasmid-based mutation techniques where cloning and propagation of recombinant plasmid in surrogate host is required. As a result, the SOEing PCR is rapid and less laborious (Horton, 1995), and can also assist in introducing desired mutations to well-defined locations within the gene of interest unlike mariner mutagenesis, which leads to random insertional mutagenesis by transposons (Akerley *et al.*, 2002). Moreover, the SOEing is an appropriate method to introduce mutations within the pneumococcal genome, as this organism is naturally competent, a physiological state by which the microbe is able to take up external DNA from the environment and incorporate it into its genome (Seitz and Blokesch, 2014). Using this method, the target genes were deleted and replaced by homologous recombination with the *aadA* coding for aminoglycoside-3-adenylyltransferase, which confers resistance to spectinomycin. The strategy for SOEing mutagenesis relies on two PCR steps. The first is the amplification of the *aadA* gene (spc^R) as well as the left and right flanking regions of target genes. The other PCR involve the fusion of these fragments together by the LF/F and RF/R primers. Ultimately, the fused product is introduced into the pneumococci by transformation.

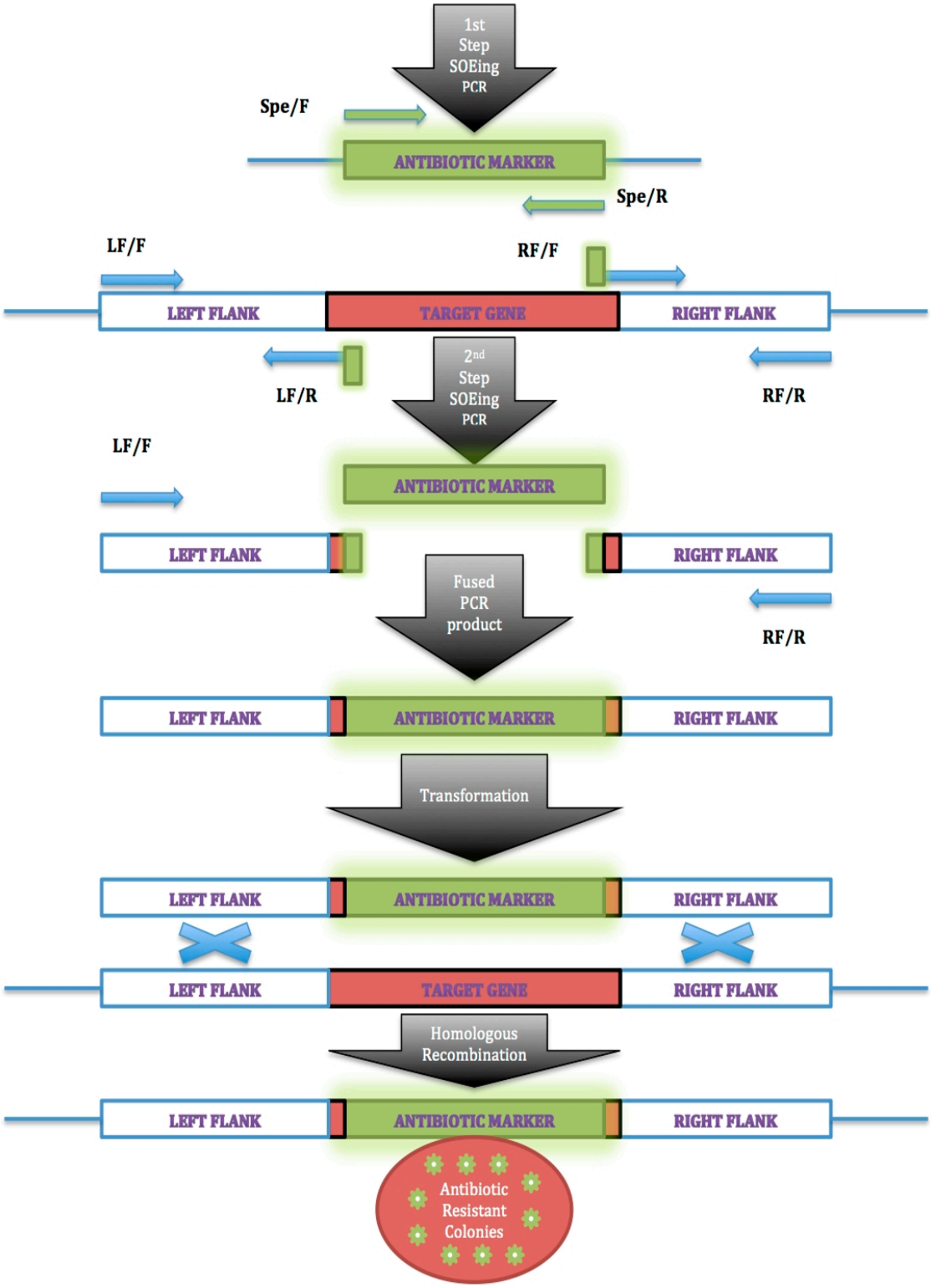


Figure 3.1. Illustration of SOEing mutagenesis steps. This schematic representation demonstrates the steps used for the allelic replacement mutagenesis and gene deletion by homologous recombination. LF and RF stand for left and right flanks respectively. Figure is made based on Horton (1995) and Song et al. (2005).

3.1.2. Amplification of SOEing fragments

As described in Figure 3.1, SOEing PCR is composed of two steps in order to replace the gene of interest with an antibiotic marker that is spectinomycin cassette gene *aadA* which is designated as *spc*^{R}. First was the amplification of left and right (~800 bp each) flanking regions of each targeted gene as well as *spc*^{R} gene (1158 bp) (shown in Figure 3.2., lanes 1, 3 and 2 respectively). The figure is an example of the first step of SOEing PCR showing the amplification of TCS12 left and right flanks and *spc*^{R} cassette gene. All flanking regions were incorporated compatible DNA ends with the spectinomycin cassette using modified primers.

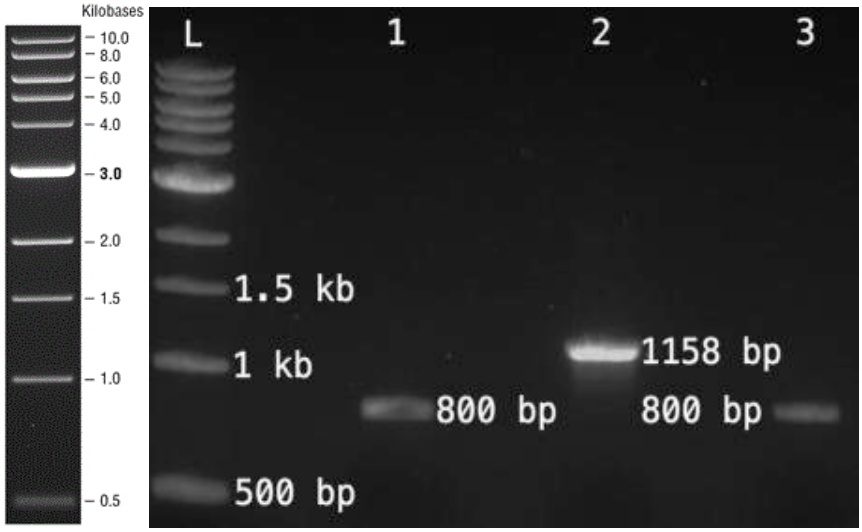


Figure 3.2. Agarose gel electrophoresis showing the amplification of TCS02 left and right flanks, and *spc*^{R} cassette gene. Lanes 1 and 3 contain the expected size (800 bp) of the amplified left and right flanking regions of TCS12, respectively. Lane 2 contains the expected size (1158 bp) of the amplified *spc*^{R} cassette gene. L contains 500 ng 1 kb DNA size marker (NEB, UK). The approximate fragment sizes have been indicated.

3.1.3. Fusion of SOEing fragments and transformation

The second SOEing PCR step resulted in joining the three amplified fragments (left flank, *spc*^{R} and right flank) to produce a fused DNA product by using LF/F-X and RF/R-X primers (where X indicates gene code) as explained in 2.14. As a result, each gene-specific fused DNA product was produced with a size of approximately ~2758 bp

(~800 bp + 1158 bp + ~800 bp). Figure 3.3 represents examples of the second SOEing PCR step showing the fused product of TSC12, TSC13, TSC05, TSC08, TSC04, TSC03, TSC07 and RitR. The linear fused DNA products were then introduced to *S. pneumoniae* D39 by transformation as previously described in section 2.13. The transformants were isolated and selected on BAB plates containing spectinomycin. All TCS mutants created are listed in Table 2.1.

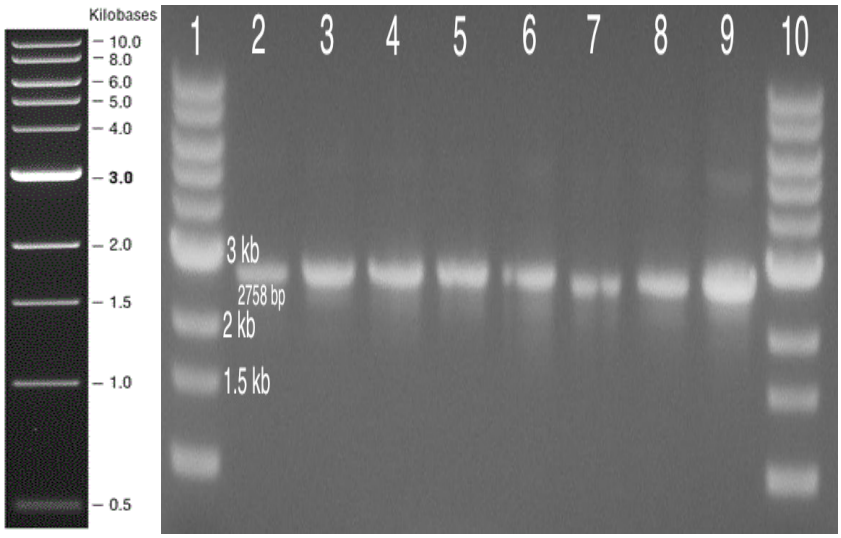


Figure 3.3. Agarose gel electrophoresis shows a representative example of fused products for TSC12, TSC13, TSC05, TSC08, TSC04, TSC03, TSC07 and RitR. Lanes 2, 3, 4, 5, 6, 7, 8 and 9 contain the fused and purified DNA for TSC12, TSC13, TSC05, TSC08, TSC04, TSC03, TSC07 and RitR products, respectively, with the predicted size of between 2635-2758 bp. Lanes 1 and 10 contain 500 ng 1 kb DNA size marker (NEB, UK). The approximate fragment sizes have been indicated.

3.1.4. PCR Confirmation strategy of mutation

Genomic DNA was extracted and analysed by agarose gel electrophoresis, as previously described in sections 2.4-2.8 from selected spectinomycin resistant transformants after growing them in BHI supplemented with the spectinomycin overnight at 37°C. Various sets of gene-specific primers were used to confirm the successful mutation events as illustrated in Figure 3.4. DNA of wild type strain (as a control) and mutants were amplified using LF/F-X and RF/R-X primers. The PCR products of the wild type DNA contained both the target gene and its flanking regions whereas the products of the

mutants contained the gene flanks and the inserted *spc*^{R} gene instead of the target gene (gene left flank 800 bp + *spc*^{R} gene 1158 bp + gene right flank 800 bp). The deletion of the target genes and the insertion of the *spc*^{R} gene created relatively similar size products. Therefore, additional sets of primers were used to interrogate the mutated region. LF/F-X and Spe/R primers amplified the left flank of the targeted genes and the inserted *spc*^{R} cassette with a total predictive size of 1958 bp while Spe/F and RF/R-X amplified the cassette and the right flanking regions of each target gene. The *spc*^{R} cassette (1158 bp) was also amplified by Spe/F and Spe/R primers (Figure 3.5. A, B, C, D, E, F, G, H, I, J, K, L, M and N) in the mutants but no amplification product could be obtained from D39 as expected (data not shown). Lane 2 in each panel represents the amplified PCR products using the gene specific LF/F-X and RF/R-X primers from the wild type DNA while lane 3 represents amplified PCR products by the same primers from each mutant DNA. Lanes 4 and 6 represent amplified PCR products by LF/F-X with Spe/R and Spe/F with RF/R-X primers, respectively, from each putative mutant. Lane 5 represents amplified PCR products by Spe/F and Spe/R primers from each putative mutant DNA.

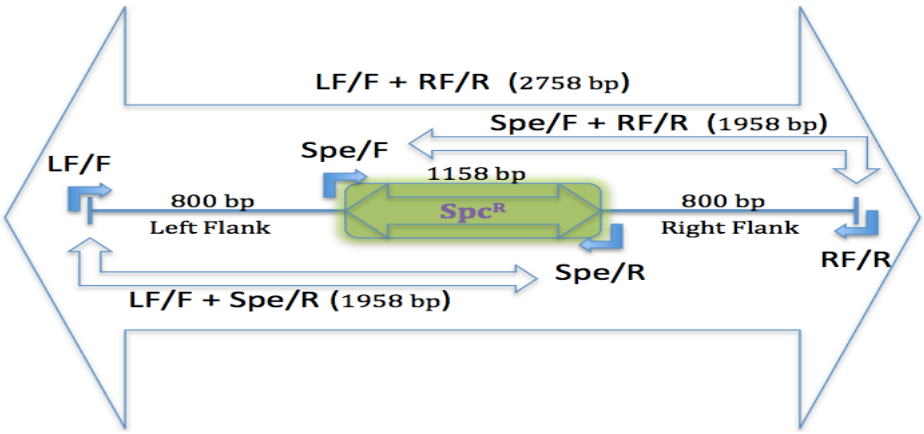
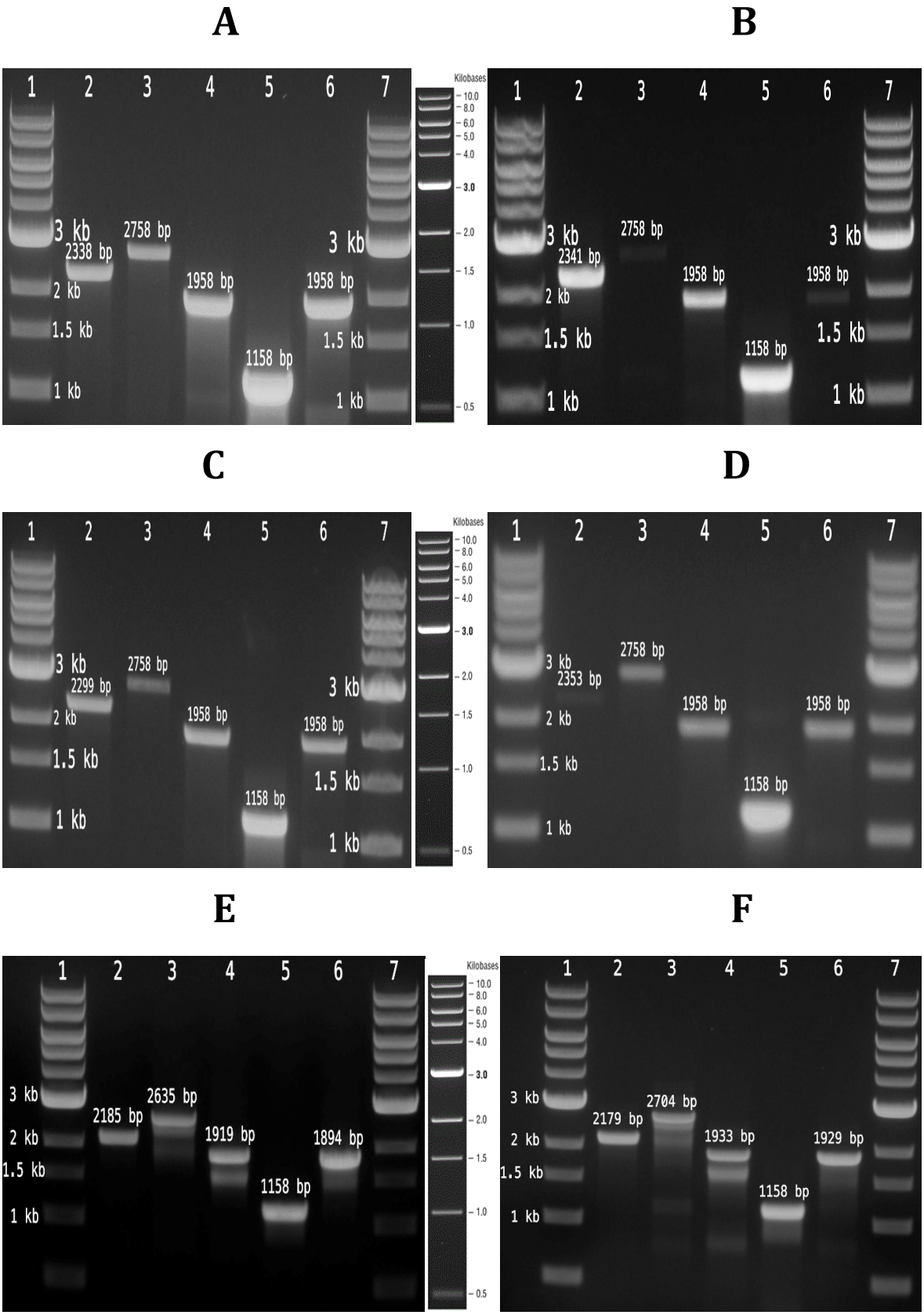


Figure 3.4. Diagram showing the strategy used for mutation confirmation by PCR. LF/F and RF/R primers were designed to amplify the target gene (wild type) or the *spc*^{R} gene (mutants) along with the 800 bp of the left and 800 bp of the right flanks (up and down-stream of the mutated region) with product size of around 2758 bp depending on the size of the target gene and flanks. LF/F-X and Spe/R primers were designed to interrogate the mutated region by amplifying the left flank of the targeted gene and the inserted *spc*^{R} cassette with a total predictive size of 1958 bp while Spe/F and RF/R amplified the cassette and the right flanking regions of the targeted gene with a total predictive size of 1958 bp. The *spc*^{R} cassette (1158 bp) was also amplified using Spe/F and Spe/R primers.


A
1 2 3 4 5 6 7
3 kb
2338 bp
2758 bp
2 kb
1958 bp
1.5 kb
1158 bp
1 kb
3 kb
1958 bp
1.5 kb
1 kb
Kilobases
10.0
8.0
6.0
5.0
4.0
3.0
2.0
1.5
1.0
0.5
B
1 2 3 4 5 6 7
3 kb
2341 bp
2758 bp
2 kb
1958 bp
1.5 kb
1158 bp
1 kb
3 kb
1958 bp
1.5 kb
1 kb
C
1 2 3 4 5 6 7
3 kb
2299 bp
2758 bp
2 kb
1958 bp
1.5 kb
1158 bp
1 kb
3 kb
1958 bp
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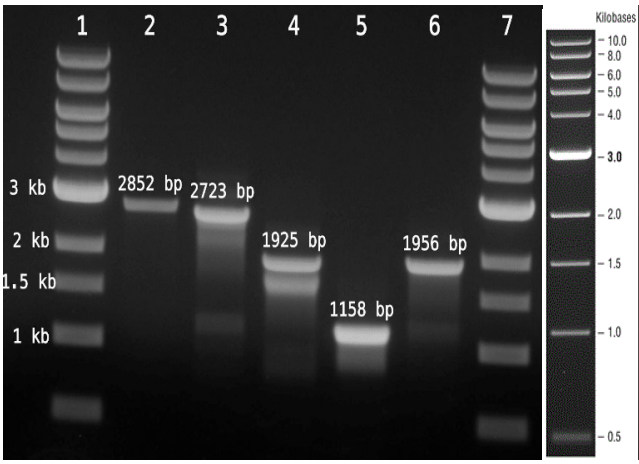
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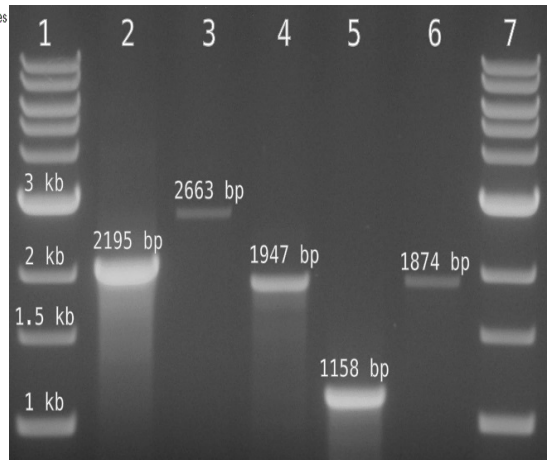
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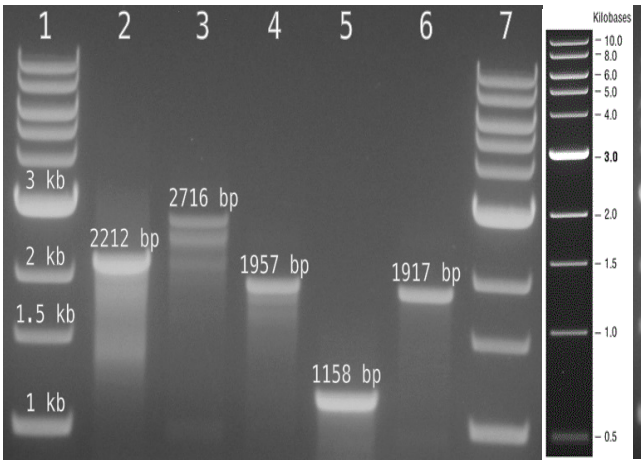
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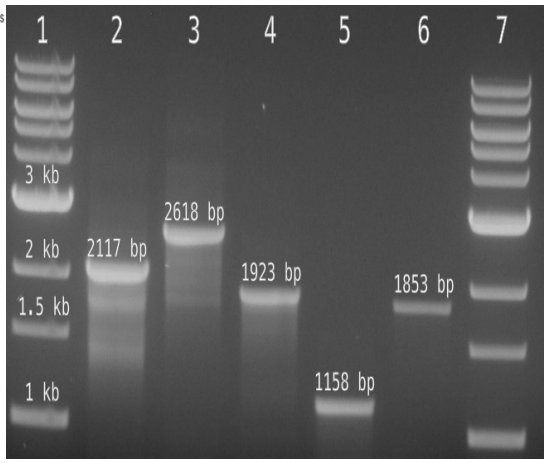
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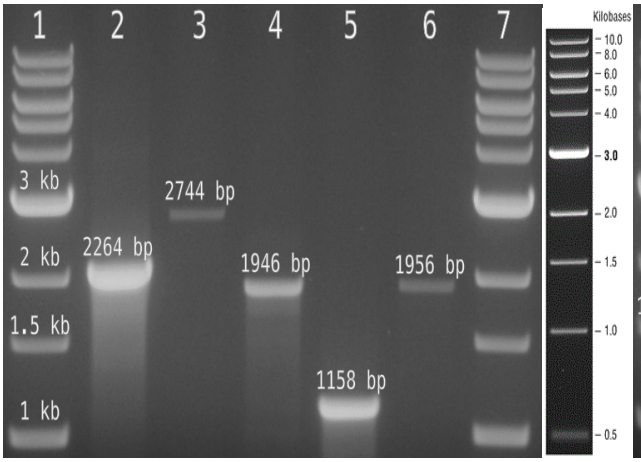
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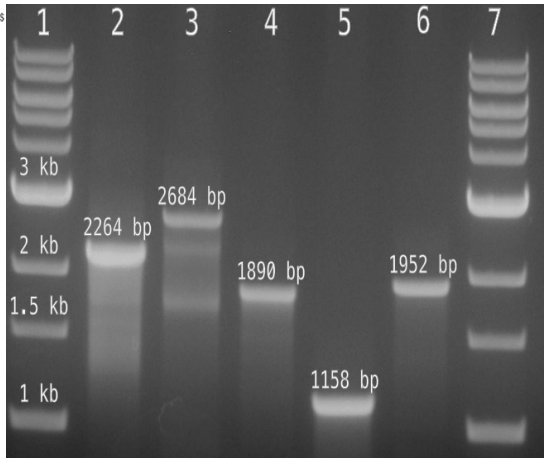
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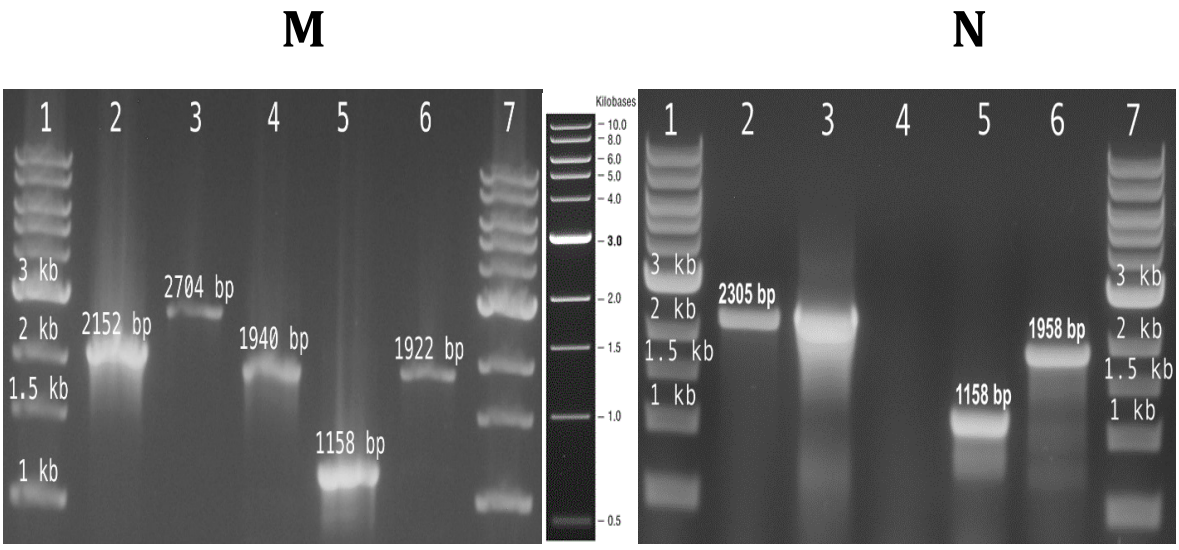


Figure 3.5. Agarose gel electrophoresis confirming the TCS13 (A), TCS05 (B), TCS08 (C), TCS12 (D), TCS04 (E), TCS03 (F), TCS07 (G), RitR (H), TSC06 (I), TCS10 (J), TCS01 (K), TCS09 (L) and TCS11 (M) mutants while N shows the unsuccessful TCS02 mutant. Lanes 1 and 7 contain 500 ng 1 kb DNA size marker (NEB, UK). The lanes contain the amplification products of the following primers sets using either wild type (WT) or mutant (M) DNA. Lanes 2: WT + LF/F + RF/R, 3: M + LF/F + RF/R, 4: M + LF/F + Spe/R, 5: M + Spe/F + Spe/R, 6: M + Spe/F + RF/R. The approximate fragment sizes have been indicated.

3.1.5. Mutation status confirmation by DNA sequencing

In addition to PCR, the mutated regions were sequenced to confirm that the successful gene replacement had taken place without undesired mutations. Mutant lacking all TCS genes were confirmed by sequencing using additional set of sequencing primers as previously described in section 2.14.4 and illustrated in Figure 3.6 A. Figure 3.6 B shows a representative example of the amplified PCR products of the TCS12 mutant which were purified using Wizard® SV Gel and PCR Clean-Up System from Promega before sending them along with the sequencing primers to PNACL, University of Leicester (UK). The results were analysed by BLAST software in NCBI and confirmed that the mutations were successful, and no unwanted mutations had taken place.

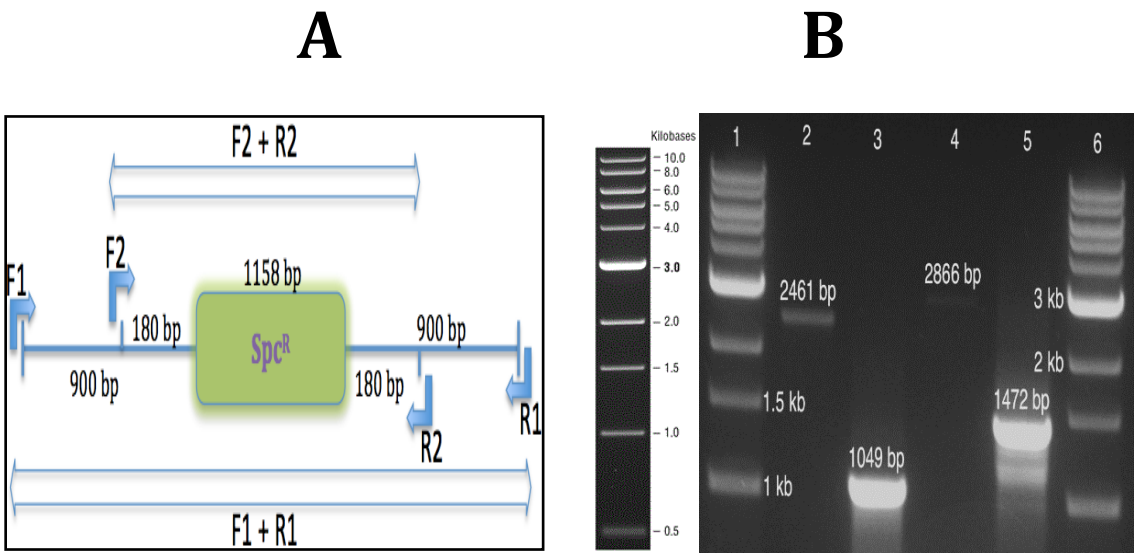


Figure 3.6. Diagram showing the strategy used for mutation confirmation by PCR and DNA sequencing (A). Agarose gel electrophoresis shows a representative example of the amplified products of the wild type and TCS12 mutant using sequencing primers (B). Lanes 1 and 6 contain 500 ng 1 kb DNA size marker (NEB, UK). The lanes contain the amplification products of the following primers sets using either wild type (WT) or mutant (M) DNA. Lane 2: WT + F1+ R1, 3: WT + F2 + R2, 4: M + F1 + R1, 5: M + F2 + R2. Lane 2 (2461 bp): the gene size of TCS012 in WT (753 bp) + left flank (889 bp) + right flank (819 bp). Lane 3 (1049 bp): TCS12 + LF (163 bp) + RF (151 bp). Lane 4 (2866 bp): *spc*^{R} gene (1158 bp) + LF (889 bp) + RF (819 bp). Lane 5 (1472 bp): *spc*^{R} gene + LF (163 bp) + RF (151 bp). The approximate fragment sizes have been indicated.

3.2. Construction of *lacZ*-fusions

The construction of transcriptional *lacZ*-fusions to promoter region of the regulators under study was achieved to examine their response to stress hormones. Transcriptional fusions are typically utilised to determine regulation of genes in eukaryotic and prokaryotic cells. To evaluate a transcriptional activity, many reporter systems are available such as, the green fluorescent protein gene *gfp* of the jelly fish *Aequorea victoria* (Prasher et al., 1992) and the *E. coli* β-galactosidase gene *lacZ* (Shapira et al., 1983). The later reporter gene was used in this project due to the fact that *lacZ* activity can be easily and cheaply tested using the relatively simple β-galactosidase assays (Miller, 1972).

The *lacZ* constructs were constructed by pPP2 plasmid, which is a promoter probe vector (Figure 3.7 A). This vector has been commonly used in several pneumococcal gene expression studies in response to glutamate, copper, and ammonium (Shafeeq et al., 2011;Kloosterman et al., 2006b). The expression of promoterless *lacZ* gene is driven by an insert carrying a putative promoter under investigation. The pPP2 plasmid containing the reporter gene is designed to integrate the reporter construct into the chromosome of the host cell by homologous recombination (Halfmann et al., 2007a). The plasmid contains a homologous region to *bgaA* (SPD_0562, encoding for native β-galactosidase in D39 strain) as well as SPD_0561, which facilitates the genomic integration of *tetM*-promoter-*lacZ* fusions. Once the integration occurs, the *bgaA* gene is disrupted leading to reduction of endogenous β-galactosidase activity (Figure 3.7 C). Furthermore, *tetM* gene and β-lactamase gene (*bla*) in the pPP2 plasmid confer tetracycline and ampicillin resistance in *S. pneumoniae* and *E. coli,* respectively. Hence, the recombinant plasmid can then be propagated in *E. coli* for genetic engineering and sequence analysis.

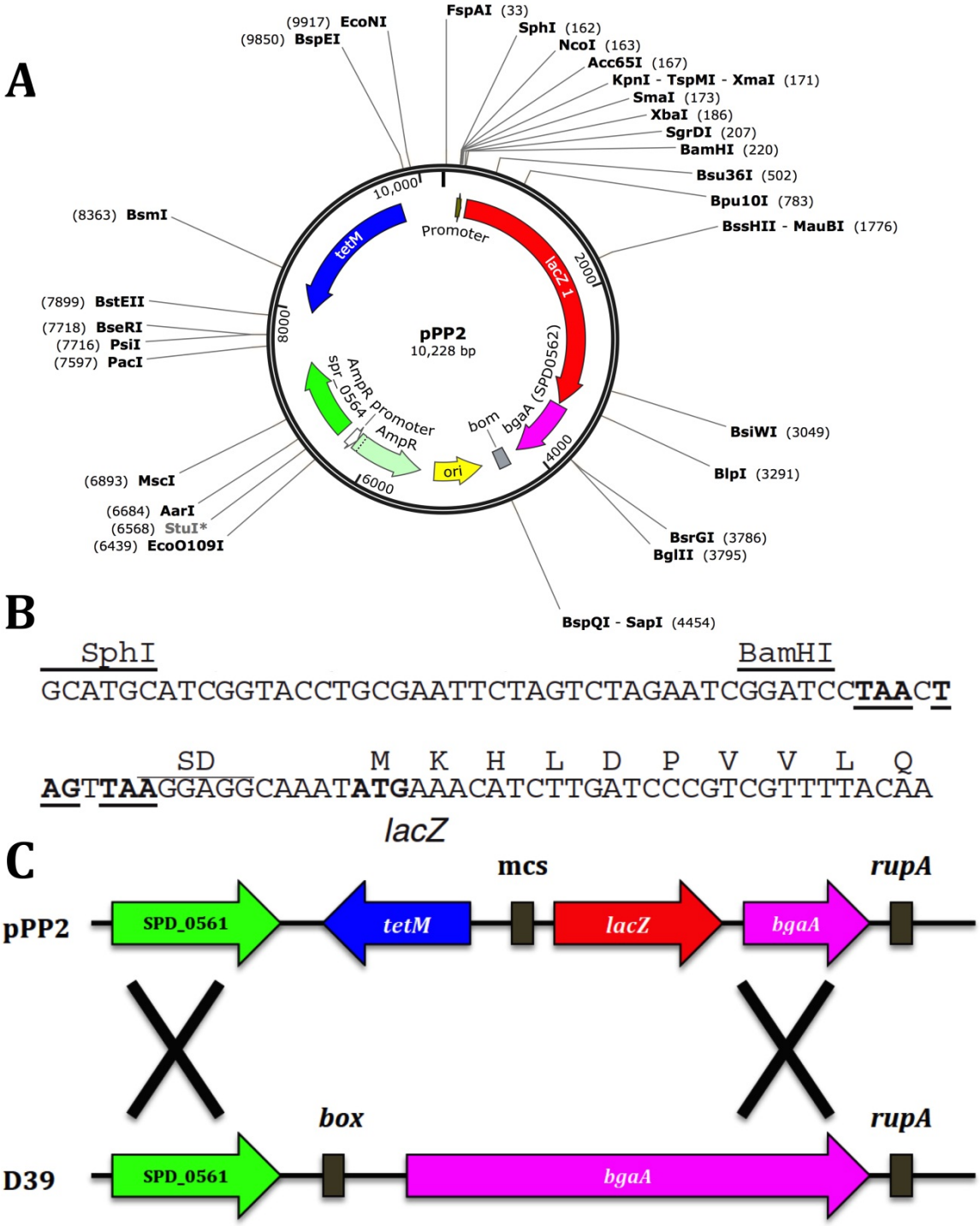


Figure 3.7. A: Illustration of genetic map of the integrative promoter probe pPP2. B: Illustration of the start of *lacZ* gene, *SphI* and *BamHI* restriction sites and the nucleotide sequence of the multiple cloning site (MCS). The location of *lacZ* start codon and the Shine–Dalgarno sequence (SD) (Shine & Dalgarno, 1974) are indicated. **C: Illustration of recombinant pPP2 and *bgaA* region of D39 replacement by homologous recombination.** Once integrated, the endogenous *bgaA* gene is deleted along with flanking repetitive elements (*box*, *rupA*).

3.2.1. Extraction and digestion of pPP2

The plasmid pPP2 was successfully extracted and double digested by *SphI* and *BamHI* restriction enzymes as mentioned in 2.5.3. The digestion of the plasmid was confirmed by agarose gel electrophoresis (Figure 3.8) and compared with an undigested pPP2. Lane 2 shows bands of different forms of undigested pPP2 plasmid whereas lane 3 shows about 10 kb of linearized pPP2.

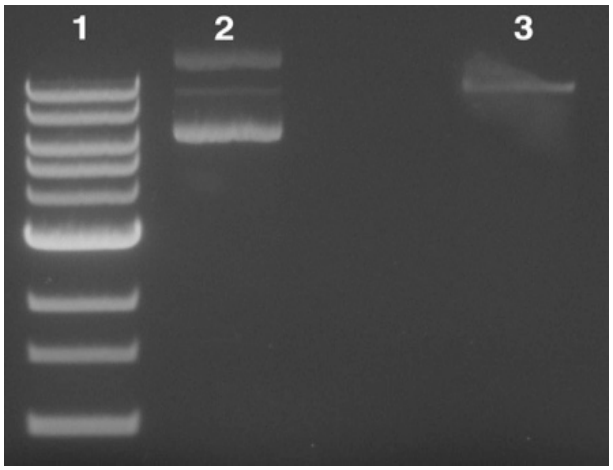


Figure 3.8. Agarose gel electrophoresis showing the amplification of undigested and digested pPP2. Lane 1 contains 500 ng 1 kb DNA size marker (NEB, UK). Lane 2 and 3 contain undigested and digested pPP2 plasmid, respectively.

3.2.2. Amplification of promoter regions

Based on the analysis of hormone-induced growth assays on section 3.5, the promotors of TCS03, TCS05 and TCS09 were further investigated for their potential direct or indirect role in *S. pneumoniae*-stress hormone interactions. The sequence regions upstream of the corresponding TCS genes were evaluated for the existence of promoter sequences via Softberry software; prediction of bacterial promoters (BPROM). The program detects potential sigma70 promoter recognition sites with approximately 80% specificity and accuracy.

The putative promoter regions of TCS03, TCS05 and TCS09 contained a potential sigma70 binding site with 28 bp coding sequence of each target gene were amplified using primers modified to incorporate *SphI* and *BamHI* sites (Table 2.5). The PCR products were purified by the QIAquick PCR purification kit and analysed by agarose gel electrophoresis (Figures 3.9). The results indicated that all DNA bands were of the expected sizes.

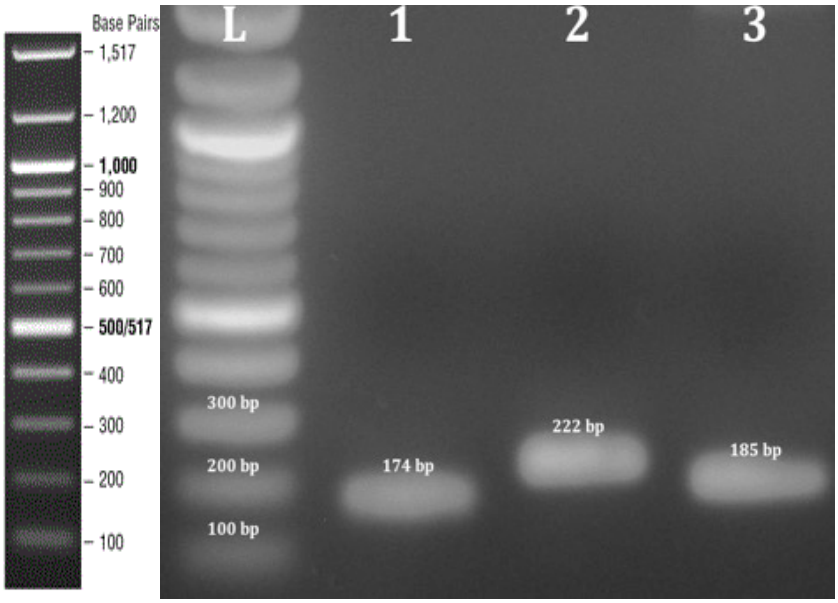


Figure 3.9. Agarose gel electrophoresis showing the amplified putative promoter regions of TCS03, TCS05 and TCS09. Lane 1: promoter regions of TCS03 (174 bp), lane 2: TCS05 (222 bp), lane 3: TCS09 (185 bp) while L: 500 ng of 100 bp DNA ladder (NEB). The approximate fragment sizes have been indicated. The approximate fragment sizes have been indicated.

3.2.3. Cloning of promoters to pPP2

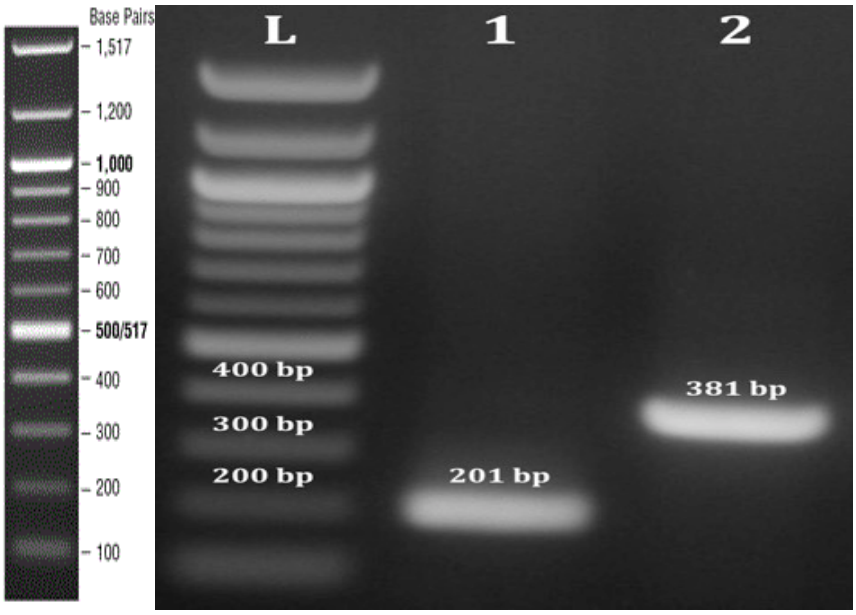
The putative promoter regions were amplified, double digested with *SphI* and *BamHI,* and ligated to the digested pPP2 as explained in section 2.5.3. Prior to introducing the cloned promotors to *S. pneumoniae*, the fused ligation mixtures were firstly introduced into *E. coli* TOP10 chemically competent cells. Agarose gel electrophoresis analysis of colony PCR and the purified DNA extracted from the *E. coli* confirmed the successful cloning as previously described in sections 2.4-2-8. Furthermore, the cloned regions

were sequenced to confirm the successful integration of the promotor within the plasmid using Fusion-Seq-UF and Fusion-Seq-DR primers and analysed as previously described section 2.15.2. The results showed that the constructs possessed the correct sequence of the putative promoters and therefore, the cloned promotors were suitable for insertion into *S. pneumoniae*.

3.2.4. Transformation of fusion constructs to *S. pneumoniae* D39

The sequenced recombinant pPP2 plasmids containing the correct inserts were transferred into wild type background as described in section 3.1. The transformants, were selected on BAB containing tetracycline. The reporter strains were designated as PTCS03::*lacZ*-wt, PTCS05::*lacZ*-wt and PTCS09::*lacZ*-wt (where P refers to promoter region). The PCR amplified products using the Fusion-Seq-UF and Fusion-Seq-DR sequencing primer confirmed the successful integration of the fusion constructs into the pneumococcal genome (Figure 3.10 A and B) and the PCR strategy is illustrated in Figure 3.11.

A



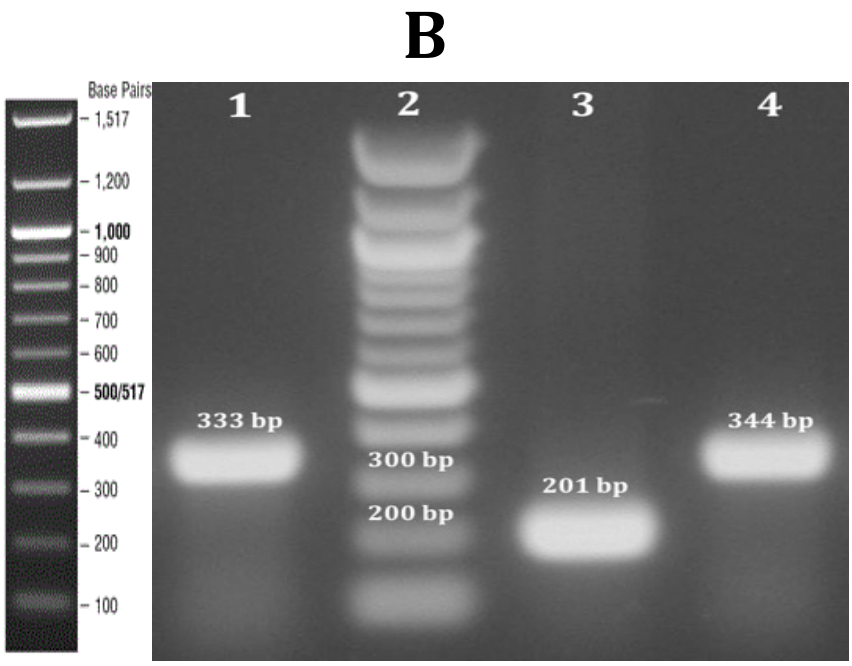


Figure 3.10. Agarose gel electrophoresis confirming the successful integration of the recombinant plasmid pPP2 within the chromosomal DNA of pneumococci. For confirmation, Fusion-Seq-UF and Fusion-Seq-DR primers were used. **Panel A.** L: 500 ng of 100 bp DNA ladder (NEB). Lanes 1 and 2: show a native fragment of the pPP2 plasmid (201 bp) and amplified promoter regions of TCS05 (381 bp), respectively. **Panel B.** Lane 2: 500 ng of 100 bp DNA ladder (NEB). Lanes 1, 3 and 4: show amplified PCR products of promoter regions of TCS03 (333 bp), the native pPP2 plasmid (201 bp) and promoter regions of TCS09 (344 bp), respectively. The approximate fragment sizes have been indicated.

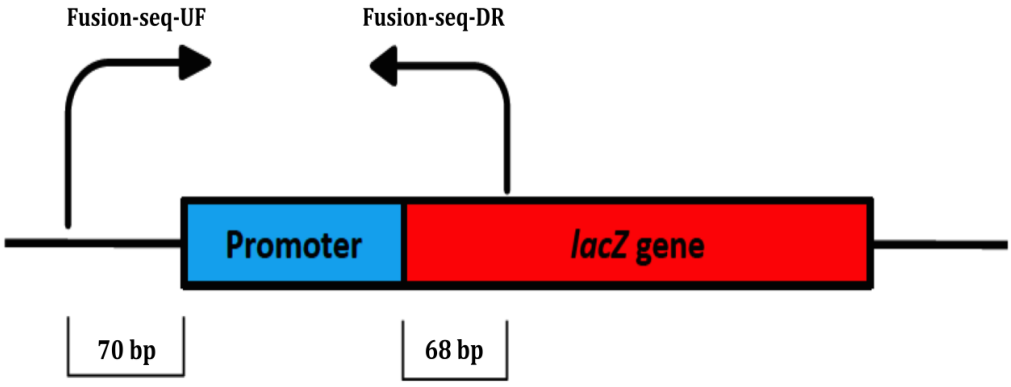


Figure 3.11. Illustration of the strategy used for confirming the successful integration of the transcriptional *lacZ* fusions within the genome of pneumococci. Primers, Fusion-Seq-UF and Fusion-Seq-DR were used to amplify the inserts containing putative promoter regions.

SUMMARY

1. All the known TCSs, except TCS02, in *S. pneumoniae* wild type D39 (TCS12, TCS13, TCS05, TCS08, TCS04, TCS03, TCS07, RitR, TCS06, TCS10, TCS01, TCS09, and TSC11) were successfully mutated using the SOEing PCR method and confirmed by PCR and DNA sequencing.
2. Using the pPP2 plasmid and *E. coli*, the construction of transcriptional *lacZ*-fusions to promoter region of the regulators TCS03, TCS05 and TCS09 was successfully achieved, and the reporter strains were designated as PTCS03::*lacZ*-wt, PTCS05::*lacZ*-wt and PTCS09::*lacZ*-wt.

Section B: Establishment of the optimum conditions to investigate *S. pneumoniae*-stress hormone interactions

3.3. Growth optimisation

3.3.1. Choice of growth medium

The conventional enriched media used for bacterial propagation, which normally facilitate good growth, do not regularly induce strong expression of virulence factors. In fact, the expression of adhesins, toxins or invasions of many bacteria with pathogenic potential, will only occur under the same environment (temperature, pH, nutrition, etc.) that they would experience upon entrance to the host (Lyte and Freestone, 2010). Thus, as far as microbial endocrinology experiments are concerned, exploring the effects of stress hormones on bacterial growth and virulence requires thoroughly mimicking those stressful conditions with hormones in a similar manner that bacteria would naturally face in the host. A minimal salts medium supplemented with 30% adult serum (serum-SAPI) developed by (Lyte and Ernst, 1992;Lyte and Ernst, 1993), is typically used when undertaking microbial endocrinology work. It is a highly stressful bacteriostatic medium, which has been reported to provide host-like conditions due to the presence of immune defence serum proteins, such as complement and antibodies as well as its limited nutrient availability and iron restriction (Freestone and Lyte, 2008). Without supplementation, most bacteria grow very poorly in this medium, which forces many researchers to modify serum-SAPI to meet the requirements of some fastidious pathogens (Roberts et al., 2002). Furthermore, to meet the complex nutritional needs of some bacteria, others have used rich iron-chelated media (e.g. Tryptic Soy Broth, Mueller Hinton Broth, etc.) with added serum to render them bacteriostatic (Coulanges et al., 1997;Anderson and Armstrong, 2006;Cogan et al., 2007). Therefore, it was essential to test whether this media that almost resembled the host environment, would be appropriate for the pneumococci while investigating the effects of stress hormones on pneumococcal growth and virulence. Since it has never

been tested before, many attempts were made in this study to reach the best possible environmental conditions *in vitro* to test pneumococcal responsiveness to the catecholamine hormones.

Initially, Sicard minimal medium specifically designed to meet the basic nutritional needs of the pneumococcus (Sicard, 1964) was added to the serum-SAPI medium (50/50%). However, the addition of the Sicard medium greatly diluted the serum-containing medium, minimised, and even eliminated the predicted growth stimulating effects of hormones on pneumococcal growth (data not shown). Thus, serum-SAPI was supplemented with only trace elements, glucose and vitamins of the Sicard medium to support the growth of the fastidious pneumococci. Combination of 20 µM glucose and vitamin solution of Sicard medium caused the best growth profile of the pneumococci on serum SAPI medium in response to NE and ferric nitrate Fe $(NO_3)_3$. The Fe $(NO_3)_3$ was included to prove that growth failure in serum containing medium resulted from a lack of an iron uptake system in the pneumococcus, and not from sensitivity to serum.

The highest OD_{600} in serum-SAPI supplemented with 20 µM glucose only in the presence of 50 µM Fe and NE were 0.20 ± 0.01 ($P < 0.001$) and 0.15 ± 0.00 ($P < 0.05$), respectively, compared to Fe/NE-free culture which was 0.12 ± 0.01, (n=3 each) (Figure 3.12 A and B). With vitamins only supplementation, the highest OD_{600} in the presence of 50 µM Fe and NE were 0.27 ± 0.00 ($P < 0.01$) and 0.25 ± 0.01 ($P < 0.05$), respectively, compared to Fe/NE-free culture which was 0.20 ± 0.00, (n=3 each) (Figure 3.12 C and D). Combination of 20 µM glucose and vitamins, on the other hand, produced the highest yields in the presence of 50 µM Fe and NE were 0.41 ± 0.04 ($P < 0.0001$) and 0.39 ± 0.02 ($P < 0.0001$), respectively, compared to Fe/NE-free culture 0.19 ± 0.01, (n=3 each) (Figure 3.12 E and F). Based on these data, additional glucose and vitamins of Sicard's had to be added to the serum-SAPI medium in order to improve the growth and responsiveness of the pneumococcus to the catecholamine hormones. Overall, the results show that the pneumococcus is stress hormone responsive and its response to Fe was strikingly similar to NE which mechanistically indicated the involvement of iron delivery from serum transferrin mediated by the catecholamine.

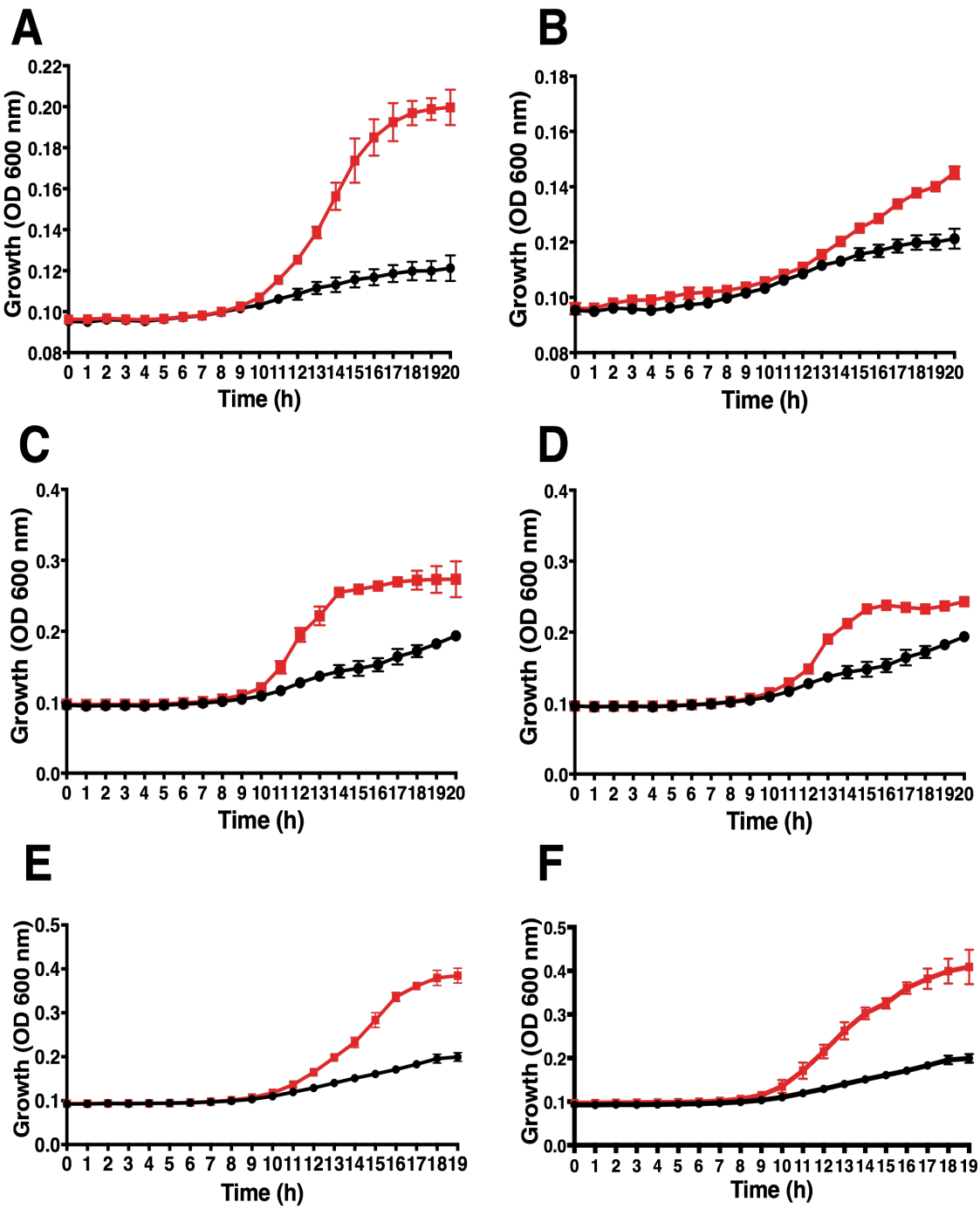


Figure 3.12. Optimising pneumococcal growth by adding glucose and/or vitamins in serum-SAPI medium. Panels **A** - **F** show the time course of growth of wild type *S. pneumoniae* strain D39 in serum-medium with (red line) and without (black line) the addition of 50 µM Fe and 50 µM NE, respectively. Panels **A** and **B** show Fe-and NE-induced growth with glucose only) while panels **C** and **D** show Fe-and NE-induced growth with vitamins only. Panels **E** and **F** show Fe-and NE-induced growth with both glucose and vitamins. The values shown are means of at least three independent experiments and error bars represent standard error of means (SEM).

3.3.2. Choice of inoculum size

The choice of a bacterial inoculum that precisely reflect the infectious dose that occurs *in vivo* is another essential element when undertaking microbial endocrinology experiments (Freestone and Lyte, 2008). Specifically, the typical starting inoculum used in microbial endocrinology-related studies is usually very low approximately 10^1–10^2 CFU/ml, which is selected to reflect the amounts of pathogenic bacteria likely to initiate the infection (Lyte and Freestone, 2010). However, for technical reasons, some researchers have used high numbers of bacteria, about 10^8 CFU/ml, to examine their response to hormones (Sperandio et al. 2003, Vlisidou et al. 2004). Additional microbial endocrinology-related experiments have shown that cell density of microbes have a great impact on specificity reaction to the catecholamine hormones (Freestone and Lyte, 2008). A study investigating the growth responses of *E. coli* O157:H7, *S. enterica* and *Y. enterocolitica* to different catecholamines using 8-log diluted inoculum sizes demonstrated that the effect catecholamine-induced growth in serum media becomes apparent at less than 10^4 CFU/ml and is greatest at around 10^2 CFU/ml cell densities (Freestone et al., 2007c). Since the catecholamine responsiveness of *S. pneumoniae* has never been examined *in vitro* using serum-SAPI, the exact cell densities that will exhibit the greatest impact of hormones on the growth and behaviour of the pneumococcus was unknown.

In this study, in which 5-log diluted inoculum sizes were used (1:100,000-fold dilutions), the pneumococcal inoculum size that caused the maximum catecholamine-stimulated growth was determined. Overall, it can be seen from Figure 3.13 A-E that the effect of catecholamine NE on pneumococcal growth in the serum medium became evident at 10^4 CFU/ml and lower cell densities with the greatest differences being observed at 10^3 CFU/ml. The pneumococcal culture using an inoculum of $1x10^7$ CFU/ml showed no significant growth response to NE compared to cultures that did not contain NE; $\log_{10}$ 6.13 ± 0.64 and 5.99 ± 0.00, respectively, (n=3 each), ($p > 0.05$). However, the pneumococcal culture using an inoculum of $1.2x10^6$ CFU/ml showed significant growth differences between control and NE-treated cultures; $\log_{10}$ 7.07 ± 0.06 and 6.59 ± 0.29

(n=3 each), respectively ($p < 0.05$). Essentially, the responsiveness of *S. pneumoniae* to NE increased progressively by reducing the inoculum size. For example, at initial inoculum sizes of $8x10^4$, $1.4x10^4$ and $9x10^2$ CFU/ml the growth yields were $\log_{10}7.53 \pm 0.09$ ($p < 0.05$), 8.11 ± 0.06 ($p < 0.0001$) and 7.82 ± 0.10 ($p < 0.001$), (n=3 each), in comparison with their NE-free cultures; 6.99 ± 0.01, 6.15 ± 0.08 and 6.99 ± 0.00, (n=3 each), respectively (Figure 3.13 F). Notably, both NE-free and NE-treated bacterial cultures initiated by smaller inoculum sizes had significantly longer lag phases associated with smaller starting inoculum sizes. For instance, NE-free bacterial cultures inoculated with $1x10^7$, $1.2x10^6$, $8x10^4$, $1.4x10^4$ and $9x10^2$ CFU/ml had lag phases of 170 ± 10 min, 310 ± 20 min, 450 ± 0 min, 580 ± 26.46 min and 780 ± 17.32 min, (n=3 each), respectively, whereas NE-supplemented cultures had lag phases of 170 ± 10 min, 290 ± 10 min, 390 ± 30 min, 500 ± 26.46 min and 530 ± 20 min, (n=3 each), respectively. Importantly, the inoculum size that resulted in a significant difference of the lag phases between the two groups was $9x10^2$ CFU/ml, which took the NE-free bacterial cultures 780 ± 17.32 min, n=3 to start the growth in the serum medium compared with 530 ± 20 min, n=3 of the NE-treated cultures ($p < 0.0001$). As a result, 10^2-10^3 CFU/ml cell density was chosen as the starting inoculum size of choice, for subsequent investigations on *S. pneumoniae* - stress hormone interactions.

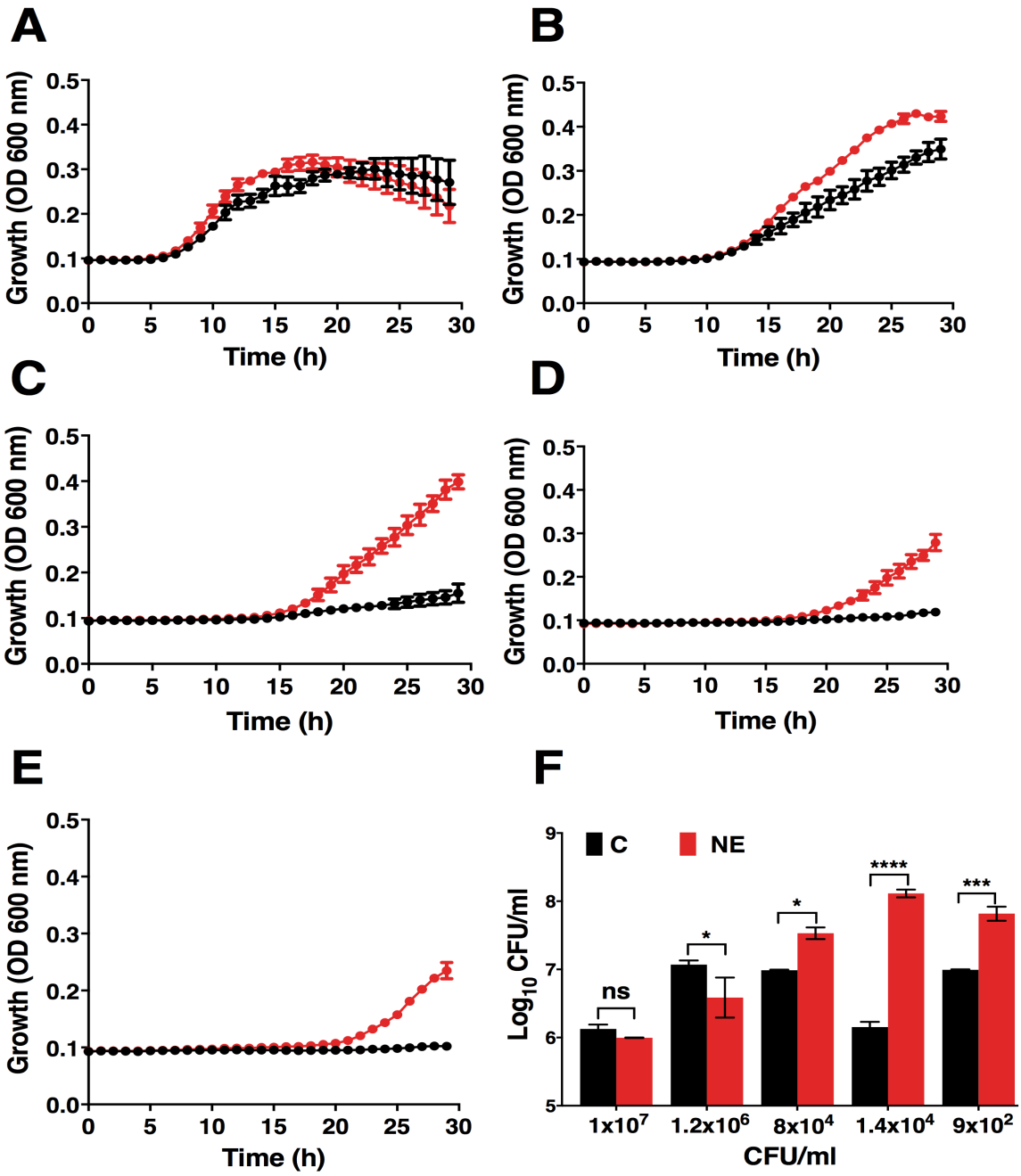


Figure 3.13. Determining starting inoculum size for pneumococcal growth in serum-SAPI medium. Panels **A** - **E** show the time course of growth of wild type *S. pneumoniae* strain D39 in serum-medium with (red line) and without (black line) the addition of 50 µM NE using different starting inoculum sizes; $1x10^7$, $1.2x10^6$, $8x10^4$, $1.4x10^4$ and $9x10^2$ CFU/ml, respectively. Panel **F** represent viable counts in cfu/ml for each group at the end of experiment. The values shown are means of at least three independent experiments and error bars represent standard error of means (SEM). The significant differences were seen comparing the stress hormone response of each group to its control using one-way ANOVA and Dunnett's multiple comparisons test. (* $p<0.05$, *** $p<0.001$ and **** $p<0.0001$).

3.3.3. Choice of temperature and aeration conditions

During infection, pathogenic bacteria typically encounter various host signals and conditions including changes in temperature and variation in the levels of oxygen, hormones, pH, etc. Such environmental signals not only stimulate adaptive responses in bacteria to these specific situations but can also affect virulence genes expression at the right place and time (Freestone et al., 2008a). Specifically, pathogenic bacteria encounter stressful conditions as soon as they contact their host, and for most, this involves a change in temperature (Fang et al., 2016). In addition, although the upper respiratory tract environment is rich with oxygen, the facultative anaerobic pneumococcus has mechanisms to withstand oxygen and its reactive derivatives. (Bortoni et al., 2009). In other locations within the respiratory tract, some pathogens encounter rather oxygen limitation (Worlitzsch et al., 2002). The pneumococci must overcome such environments from the moment they enter and establish colonisation in the nasopharynx of the host to the stage where they become invasive and cause diseases in the lungs, ears and blood. Therefore, pneumococcal hormone-induced growth was also studied under different conditions such as temperatures of 34, 37 and 39°C and in aerobic, micro-aerobic and anaerobic environments. These temperatures represent different host state and locations in health and disease within the host (White et al., 2011). Overall analysis of pneumococcal growth yield incubated in serum medium containing NE under different aeration conditions showed that the pneumococci were growing significantly better in both anaerobic and micro-aerobic environments while growing poorly in aerobic environment. More specifically, pneumococci grown under anaerobic condition yielded OD_{600} of 0.42 ± 0.025 ($P < 0.01$) when NE was present compared with NE-free culture which was 0.24 ± 0.008, (n=3 each). Similarly, pneumococci grown in a micro-aerobic environment yielded 0.41 ± 0.026 ($P < 0.01$) in the NE presence compared with NE-free culture which was 0.20 ± 0.008, (n=3 each). Incubating the pneumococcus in an aerobic environment, on the other hand, resulted in an extremely poor growth yield which was significantly measured when NE was present which was 0.12 ± 0.001 ($P < 0.0001$), compared with NE-free culture which was 0.09 ± 0.01, (n=3 each) (Figure 3.14 A).

Under different temperature conditions, the analysis of pneumococcal growth yield incubated in serum medium containing NE showed that the pneumococci were growing significantly better at both 34 and 37°C while almost no growth was seen at 39°C. More specifically, pneumococci incubated at 34°C yielded OD_{600} of 0.40 ± 0.018 when NE was present compared with NE-free culture which was 0.19 ± 0.020 ($P < 0.01$) whereas 37°C yielded 0.41 ± 0.026 when NE was present compared with NE-free culture which was 0.20 ± 0.008 ($P < 0.01$), (n=3 each) (Figure 3.14 B). Based these results, a micro-aerobic environment and temperature of 37°C were chosen for the following experiments.

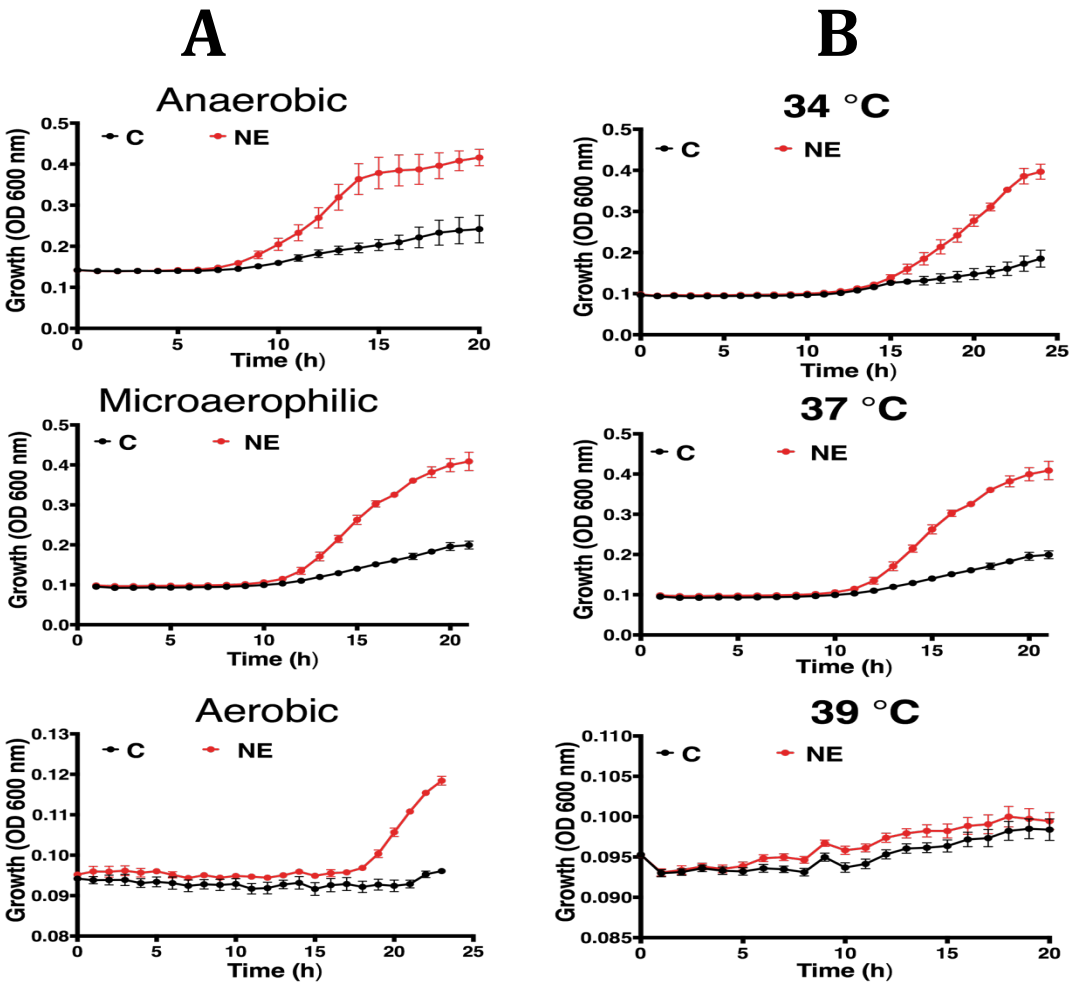


Figure 3.14. Catecholamine-induced growth in different oxygen levels (A) and temperatures (B). Pneumococcal cultures treated with 50 µM NE (red line) and control (black line) showed various growth levels in serum medium under aerobic, micro-aerobic and anaerobic conditions at 37°C **(A)** whereas panel (**B)** represents pneumococcal cultures incubated in serum medium at 34, 37 and 39°C under micro-aerobic condition. The values shown are means of at least three independent experiments and error bars represent standard error of means (SEM).

3.3.4. Choice of appropriate hormone concentrations

Similar to the choice of growth medium, inoculum size and environmental conditions, the selected concentration of hormones that will be tested in an *in vitro* assay should represent those that are likely to be encountered by bacteria in the host. Since it is stated to be difficult to determine the accurate hormone concentrations at body sites in addition to the fact that pathogenic bacteria will occupy more than just a single site during the period of an infection, it is very likely that pathogens are exposed to various hormone concentrations (Lyte and Freestone, 2010). More importantly, most hormones in the body are located in the targeted tissue where they take action, and so it is worth emphasizing that the values of specimens detected in body fluids are often much lower than the actual levels at mucosal surfaces or within the tissues where pathogens interact (Leinhardt et al., 1993). Therefore, the selection of a reasonably wide range of stress hormone concentrations to undertake dose responses analyses is highly recommended (Lyte and Freestone, 2010). For example, dose-response effects of catecholamines (NE, Epi and Dop) ranging from 1 to 500 μM were previously investigated on *E. coli* O157:H7, *S. enterica* and *Y. enterocolitica* (Freestone et al., 2007c).

In this project, the impact of different concentrations of catecholmines on the growth of *S. pneumoniae* strain D39 was examined. Pneumococcal growth on serum-based culture was not only norepinephrine-induced, as seen in the previous results, but also increasing concentrations of dopamine and epinephrine ranging from 1 to 50 μM, some of which are attainable *in vivo* (Thompson et al., 1999), were also shown to be effective (Figure 3.15). Concentrations of 1, 2, 5, 10 and 50 μM of NE yielded growth OD_{600} 0.355 ± 0.017 ($p < 0.0001$), 0.350 ± 0.013 ($p < 0.0001$), 0.352 ± 0.003 ($p < 0.0001$), 0.369 ± 0.013 ($p < 0.0001$) and 0.390 ± 0.011 ($p < 0.0001$), respectively, compared with NE-free culture which was 0.210 ± 0.005, (n=3 each). Similarly, the concentrations of 1, 2, 5, 10 and 50 μM of Epi yielded growth of 0.362 ± 0.013 ($p < 0.0001$), 0.360 ± 0.003 ($p < 0.0001$), 0.351 ± 0.005 ($p < 0.0001$), 0.365 ± 0.012 ($p < 0.0001$) and 0.468 ± 0.004 ($p < 0.0001$), respectively, compared with Epi-free culture which was 0.210 ± 0.005, (n=3

each). Lastly, the concentrations of 1, 2, 5, 10 and 50 μM of Dop yielded growth of 0.355 ± 0.012 ($p < 0.0001$), 0.350 ± 0.007 ($p < 0.0001$), 0.374 ± 0.006 ($p < 0.0001$), 0.342 ± 0.027 ($p < 0.0001$) and 0.342 ± 0.010 ($p < 0.0001$), respectively, compared with Dop-free culture which was 0.210 ± 0.005, (n=3 each). Based on the growth yield but not rate of these experiments, concentrations of 50 μM NE, 50 μM Epi and 5 μM Dop were selected for testing out the pneumococcal adrenergic and dopaminergic antagonists as well as catecholamine responsiveness of the TCS mutants.

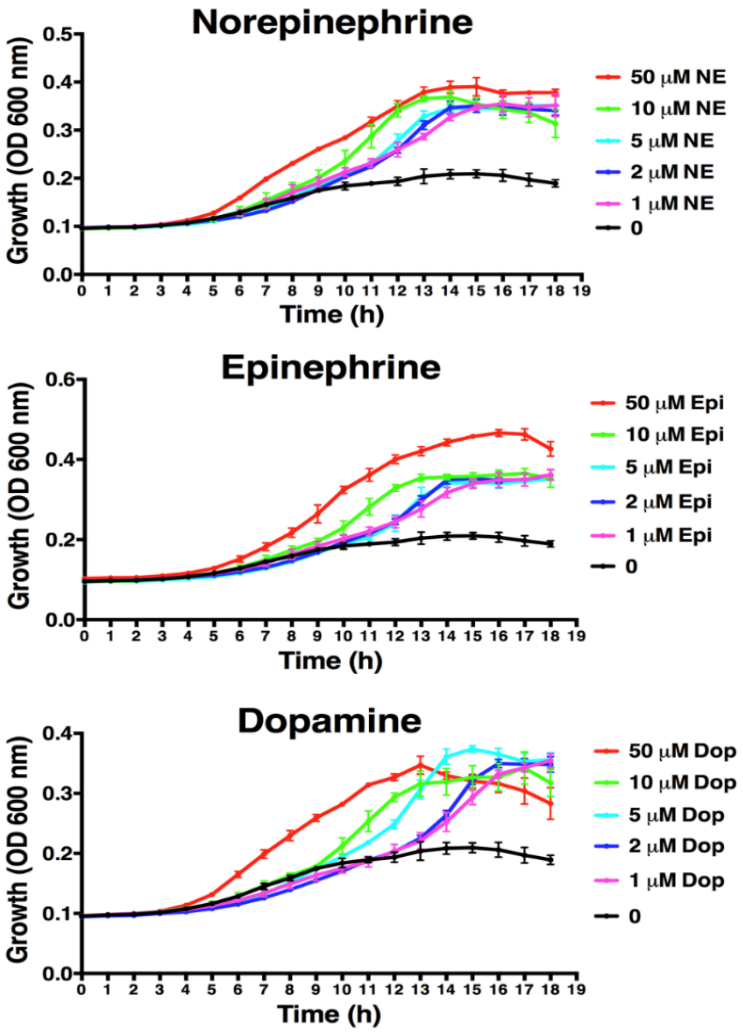


Figure 3.15. Dose-response effects of catecholamines on pneumococcal growth. *S. pneumoniae* strain D39 was inoculated at up to 10^3 CFU/ml into 1 ml aliquots of serum-SAPI containing the concentrations of the catecholamines shown and incubated for 20 h. Black lines are catecholamine-free cultures (control) while the coloured lines are catecholamine-treated cultures (1, 2, 5, 10 and 50 μM of NE, Epi & Dop respectively). The values shown are means of at least three independent experiments and error bars represent standard error of means (SEM).

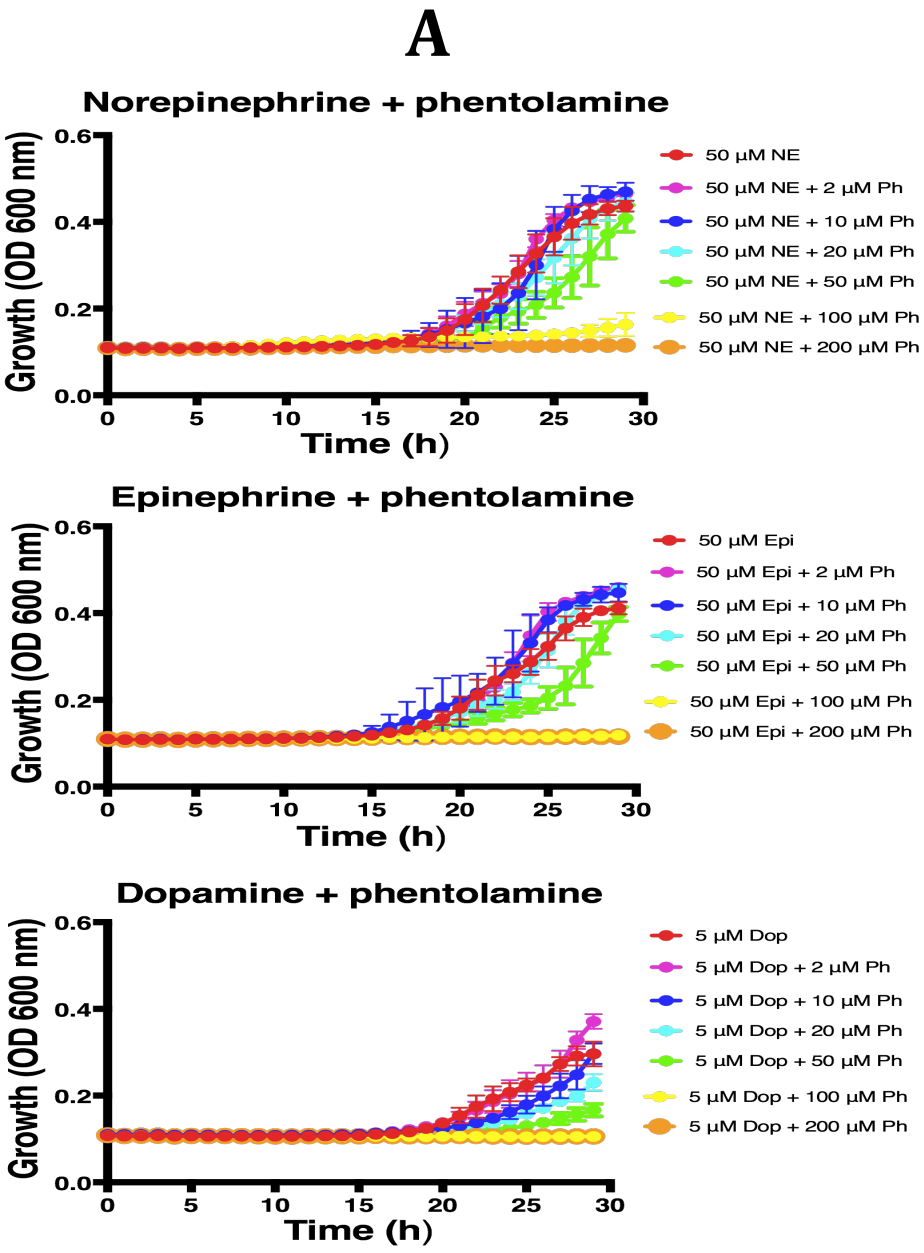
3.4. Catecholamine antagonism assays

It has always been a controversial question as to whether the interaction between microbes and catecholamines is mediated via specific receptors. The most probable answer is that the investigation of a putative receptor-associated process in bacteria stems from observations of mammalian systems where cellular catecholamine receptors have been identified, classified and subsequently led to the managements of various human diseases such as depression and hypertension (Freestone et al., 2007a). The biochemical pathway for the synthesis of catecholamines in mammals is *L*-dopa → Dop → NE → Epi. The sympathetic nerve terminals producing NE and Dop are widely distributed in the body covering the intestinal tract where they form part of the enteric nervous system (ENS) (Costa et al., 2000). In fact, 50% of NE in mammals is produced and used in the ENS whereas Epi (primarily synthesized in the renal adrenal glands) is not present within the ENS as the entire GI tract has never been found to have Epi biosynthetic pathways (Costa et al., 2000). Nevertheless, both NE and Epi bind to adrenergic-type receptors which are classified into 2 major families, α and β, whereas Dop binds to dopaminergic-type receptors which are at least 5 recognised receptor types (Bergson et al., 2003). Essentially, both NE and Epi are capable of interaction and stimulation of more than one receptor of the adrenergic family. For instance, NE is able to stimulate both α and β_1, but not β_2, adrenergic receptors while Dop, on the other hand, can interact with any of the D_1–D_5 receptor subtypes (Freestone et al., 2007a). Although the accessibility of a range of very specific antagonists has shed some light on the physiological role of the different receptor types and subtypes, it has been mainly studied in model systems of the mammals (Freestone et al., 2007a). The first attempt to identify a catecholamine receptor in bacteria was done by Lyte and Ernst, who investigated the power of some α and β adrenergic receptor antagonists using various concentrations to block the NE-induced growth of a number of Gram-negative enteric bacteria (Lyte and Ernst, 1993). Ten years later, an α and β adrenergic antagonist was found to inhibit NE and Epi-induced locus of enterocyte effacement (LEE) and flagella expression in *Escherichia coli* O157:H7 (Sperandio et al., 2002) followed by the identification of the putative adrenergic receptors; the QseBC and QseEF two

component regulators, which control flagella and LEE expression, respectively (Sperandio et al., 2003). A more recent study employing a pharmacological approach of a wide range of adrenergic antagonists was carried out to explore the specificity of catecholamine responsiveness in three main enteric pathogenic bacteria (Freestone et al., 2007a). The last approach has been used in this study since no antagonist has ever been utilized to examine the specificity of catecholamine responsiveness in pneumococci. To investigate the nature of the putative pneumococcal adrenergic and/or dopaminergic receptor(s) by which catecholamines bind and exert their effects, the previously published protocol was used (Freestone et al., 2007a). Catecholamine antagonism experiments were performed identically to catecholamine pneumococcal growth assays as in serum-based media containing catecholamines described in section 2.4. Three different types of antagonists, phentolamine, propranolol and chlorpromazine with concentrations ranging from 2 to 200 μM were used (α-Adrenergic, β-Adrenergic and Dopaminergic, respectively).

The use of specific catecholamine receptor antagonists in this study revealed that α and β adrenergic as well as dopaminergic antagonists were not capable of blocking norepinephrine -, epinephrine- or dopamine-induced growth of the pneumococcus (Figure 3.16 A, B and C) and (Table 3.1). For instance, no blockage was observed in the 50 μM-supplemented NE and Epi-induced growth despite using 2, 10, 20 and 50 μM of phentolamine (α), propranolol (β) adrenergic and chlorpromazine (D) dopaminergic antagonists. Moreover, 100 and 200 μM of phentolamine and propranolol have non-specific blocking effects on the growth induced by all catecholamines. Furthermore, 20 μM of phentolamine, propranolol and chlorpromazine did not reduce the NE-induced growth yield; OD_{600} 0.455 ± 0.018, 0.432 ± 0.022 and 0.468 ± 0.012 ($p > 0.05$ each) compared to their antagonists-free cultures; 0.439 ± 0.007, 0.472 ± 0.004 and 0.518 ± 0.015, (n=3 each), respectively. Similarly, 50 μM of phentolamine, propranolol and chlorpromazine did not reduce the Epi-induced growth yield; 0.398 ± 0.016, 0.449 ± 0.021 and 0.450 ± 0.004 ($p > 0.05$ each) compared to their antagonist-free cultures; 0.414 ± 0.007, 0.455 ± 0.005 and 0.493 ± 0.004, (n=3 each) respectively (Figure 3.16 A, B and C). In addition, all concentrations of chlorpromazine (except 200 μM) had no

impact on the catecholamine-enhanced growth (Figure 3.16 C). Interestingly, dopamine-induced growth appeared to be affected by concentration of phentolamine and propranolol as low as 50 µM, which are adrenergic antagonists, whereas all concentrations, except 200 µM, of the dopaminergic antagonist chlorpromazine did not attenuate the dopamine-induced growth. The results suggest that these different antagonists are not catecholamine-specific on the pneumococcal hormone-induced growth. Table 3.1 shows all growth yields of catecholamine-treated pneumococci with and without the addition of different concentrations of the antagonists.



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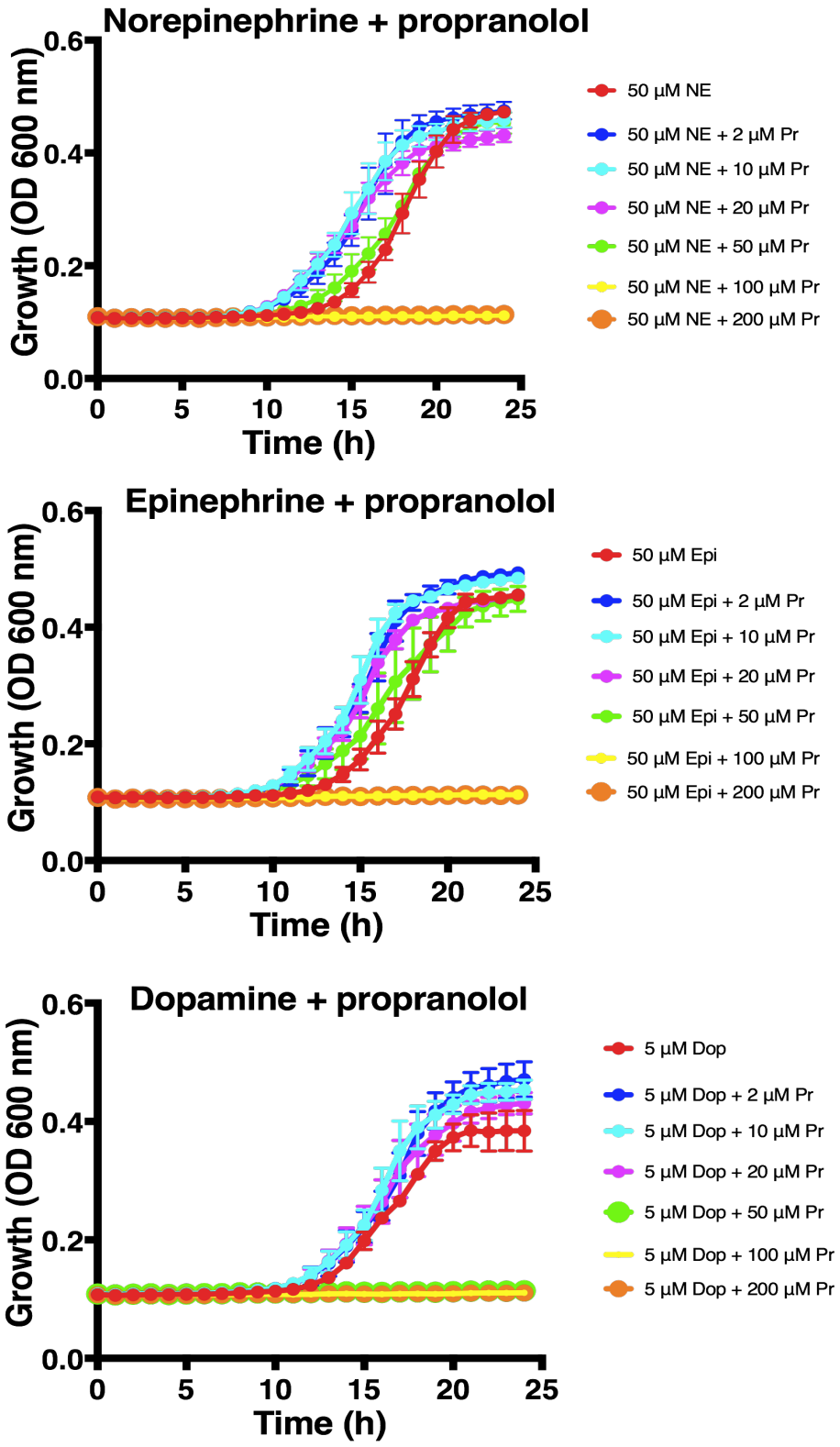

Norepinephrine + propranolol
Growth (OD 600 nm)
0.0
0.2
0.4
0.6
0
5
10
15
20
25
Time (h)
50 µM NE
50 µM NE + 2 µM Pr
50 µM NE + 10 µM Pr
50 µM NE + 20 µM Pr
50 µM NE + 50 µM Pr
50 µM NE + 100 µM Pr
50 µM NE + 200 µM Pr
Epinephrine + propranolol
Growth (OD 600 nm)
0.0
0.2
0.4
0.6
0
5
10
15
20
25
Time (h)
50 µM Epi
50 µM Epi + 2 µM Pr
50 µM Epi + 10 µM Pr
50 µM Epi + 20 µM Pr
50 µM Epi + 50 µM Pr
50 µM Epi + 100 µM Pr
50 µM Epi + 200 µM Pr
Dopamine + propranolol
Growth (OD 600 nm)
0.0
0.2
0.4
0.6
0
5
10
15
20
25
Time (h)
5 µM Dop
5 µM Dop + 2 µM Pr
5 µM Dop + 10 µM Pr
5 µM Dop + 20 µM Pr
5 µM Dop + 50 µM Pr
5 µM Dop + 100 µM Pr
5 µM Dop + 200 µM Pr

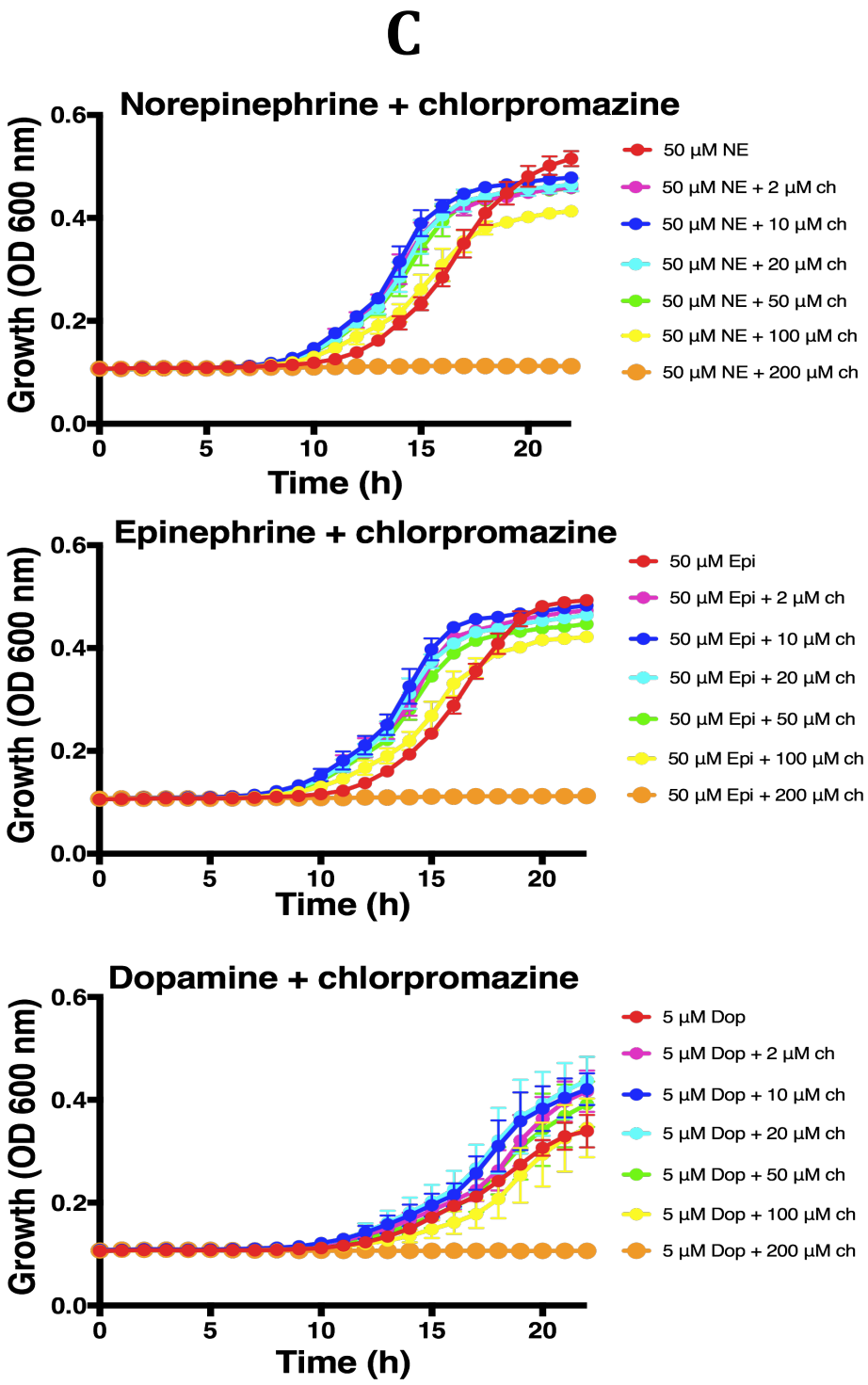


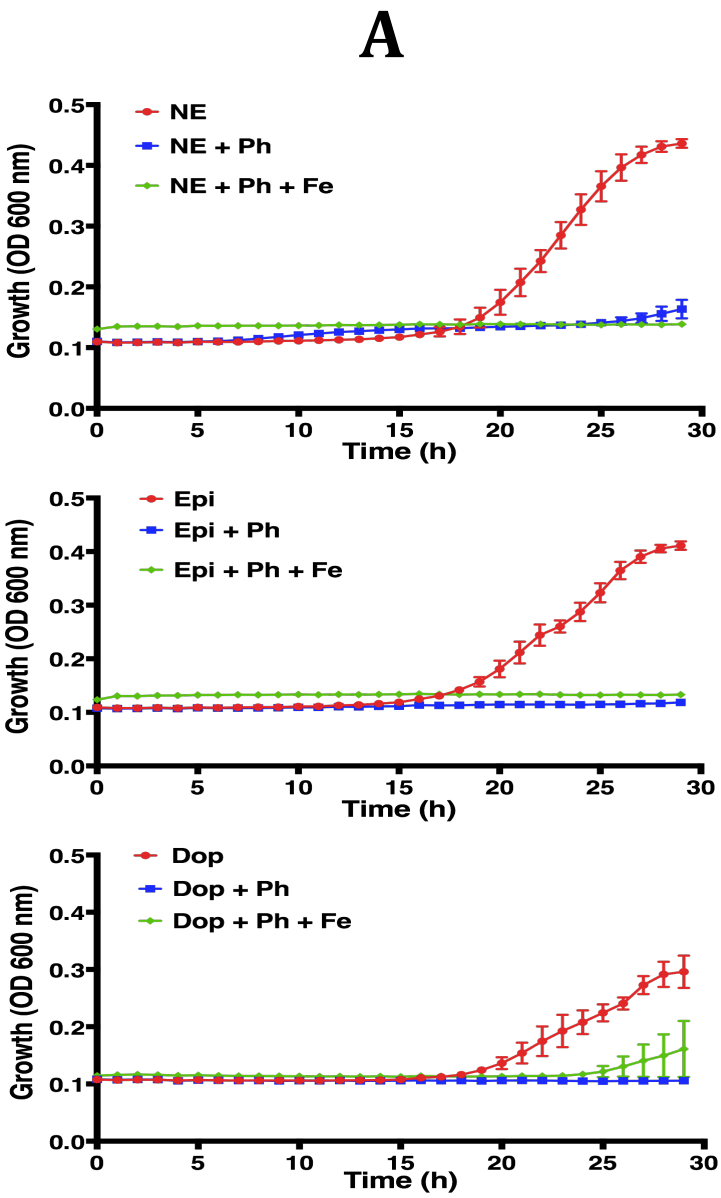
Figure 3.16. Antagonism of catecholamine-induced growth by (A) phentolamine (Ph), (B) propranolol (Pr) and (Ch) chlorpromazine (C). Antagonist supplemented cultures showed different growth levels using concentrations of antagonists ranging from 2 to 200 µM. Control is antagonist only-supplemented cultures. The values shown are means of at least three independent experiments and error bars represent standard error of means (SEM).

Table 3.1. Growth yields (maximal OD_{600}) of pneumococcal wild type strain grown in serum-based media containing hormones and antagonists. Values are average of three independent experiments. '±' indicates standard error of means (SEM).

Antagonist concentration	50 µM Norepinephrine	50 µM Epinephrine	5 µM Dopamine
0 µM Phentolamine	0.439 ± 0.007	0.414 ± 0.007	0.296 ± 0.028
0 µM Propranolol	0.472 ± 0.004	0.455 ± 0.005	0.385 ± 0.027
0 µM Chlorpromazine	0.518 ± 0.015	0.493 ± 0.004	0.344 ± 0.033
2 µM Phentolamine	0.469 ± 0.010	0.462 ± 0.005	0.387 ± 0.021
2 µM Propranolol	0.476 ± 0.016	0.494 ± 0.008	0.472 ± 0.031
2 µM Chlorpromazine	0.461 ± 0.007	0.476 ± 0.004	0.422 ± 0.041
10 µM Phentolamine	0.474 ± 0.013	0.450 ± 0.013	0.331 ± 0.008
10 µM Propranolol	0.459 ± 0.013	0.485 ± 0.007	0.455 ± 0.017
10 µM Chlorpromazine	0.482 ± 0.006	0.487 ± 0.007	0.425 ± 0.029
20 µM Phentolamine	0.455 ± 0.018	0.457 ± 0.010	0.258 ± 0.015
20 µM Propranolol	0.432 ± 0.022	0.455 ± 0.003	0.431 ± 0.018
20 µM Chlorpromazine	0.468 ± 0.012	0.467 ± 0.009	0.444 ± 0.047
50 µM Phentolamine	0.408 ± 0.031	0.398 ± 0.016	0.172 ± 0.014
50 µM Propranolol	0.453 ± 0.011	0.449 ± 0.021	0.114 ± 0.001
50 µM Chlorpromazine	0.460 ± 0.011	0.450 ± 0.004	0.402 ± 0.039
100 µM Phentolamine	0.170 ± 0.019	0.120 ± 0.004	0.106 ± 0.001
100 µM Propranolol	0.111 ± 0.001	0.113 ± 0.001	0.111 ± 0.001
100 µM Chlorpromazine	0.414 ± 0.005	0.425 ± 0.005	0.360 ± 0.049
200 µM Phentolamine	0.115 ± 0.001	0.116 ± 0.001	0.106 ± 0.001
200 µM Propranolol	0.113 ± 0.001	0.111 ± 0.001	0.110 ± 0.001
200 µM Chlorpromazine	0.113 ± 0.002	0.113 ± 0.001	0.107 ± 0.002

It appears that higher concentrations of these antagonists have rather toxic effects on pneumococcal growth in spite of the availability of catecholamines. Moreover, it became evident from viable counts that the highest concentrations of all antagonists under investigation are in fact bactericidal as there was no bacterial growth was recovered during or after the end of experiments. Moreover, the additional of iron did not recover bacterial growth from 100 µM of phentolamine (Figure 3.17 A),

propranolol (Figure 3.17 B) and chlorpromazine (data not shown). For instance, 50 µM of NE/E and iron combined did not cause the pneumococci to recover from the impact of 100 µM phentolamine and propranolol. Furthermore, antagonist only-supplemented cultures showed decreasing growth profiles corresponding with increasing concentrations of the antagonists. A representative example of chlorpromazine is shown in Figure 3.18. In conclusion, no blocking specificity on catecholamine-stimulated growth was observed despite using wide range concentrations of three different types of antagonists (α-Adrenergic, β-Adrenergic and Dopaminergic antagonists).



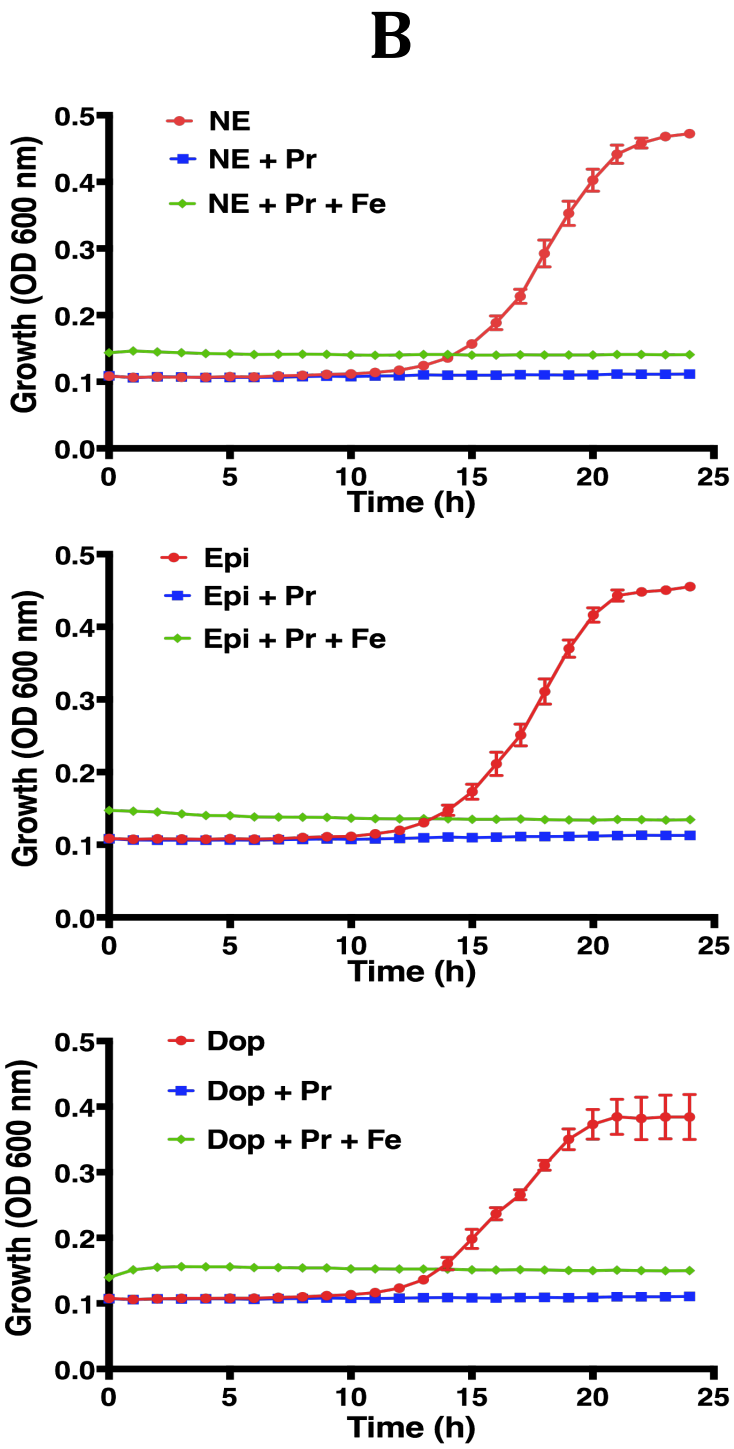


Figure 3.17. Toxic effect of phentolamine (A) and propranolol (B) on catecholamine-induced growth. Catecholamines (50 µM) and iron (50 µM) supplemented cultures showed almost no recovered growth after the addition of 100 µM of phentolamine (Ph) or propranolol (Pr). Control is catecholamine only supplemented cultures. The values shown are means of at least three independent experiments and error bars represent standard error of means (SEM).

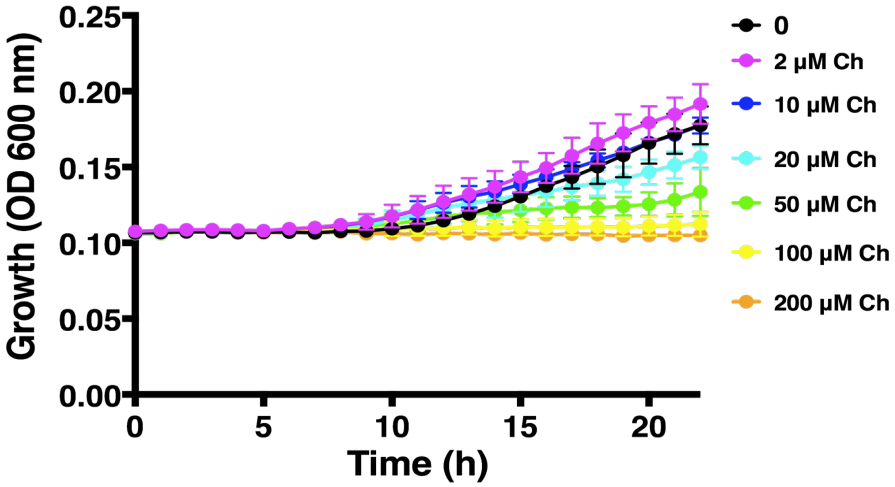


Figure 3.18. Toxic effect of chlorpromazine (Ch) on catecholamine-free cultures. Concentrations ranging from 2 to 200 µM of chlorpromazine showed progressively reduced growth while control is chlorpromazine-free culture. The values shown are means of at least three independent experiments and error bars represent standard error of means (SEM).

SUMMARY

1. Glucose and vitamins of Sicard's medium were added to the serum-SAPI medium to improve the growth and responsiveness of the pneumococcus to the hormones.
2. 10^2-10^3 CFU/ml cell densities was chosen as the starting inoculum size of choice.
3. The micro-aerobic environment and temperature of 37°C were chosen for investigation on *S. pneumoniae* interactions - stress hormones.
4. Concentrations of 50 µM NE, Epi and 5 µM Dop were appropriate concentrations for testing out the putative pneumococcal adrenergic and/or dopaminergic receptor as well as catecholamine responsiveness of the TCS mutants.
5. No blocking specificity on catecholamine-stimulated growth was observed despite using wide range concentrations of three different types of antagonists (α-Adrenergic, β-Adrenergic and Dopaminergic antagonists).
6. It appears that higher concentrations of the antagonists have rather toxic effects on pneumococcal hormone-induced growth.
7. Chlorpromazine (dopaminergic) was the least toxic antagonist compared with phentolamine (α-Adrenergic) and propranolol (β-Adrenergic).

Section C. Investigating the role of TCS systems in *S. pneumoniae*-stress hormone interactions and the mechanisms of hormone-induced growth

The aim the experiments described in this section was to evaluate the potential contribution of TCS regulators in *S. pneumoniae*-stress hormone interactions and to investigate the mechanism of hormone-mediated pneumococcal growth by growth studies, gene reporter assays, radio labelled hormone and iron uptake, and biofilm formation assays.

3.5. The role of TCS in catecholamine-induced growth

Bacterial adaptation, by which bacteria perceive and respond to environmental changes including host stress hormones levels, is often regulated by key mechanisms called two-component regulatory system or two-component signal transduction system (TCS) (Hoch, 2000;Stock et al., 2000). For many years, bacterial two-component systems (TCS) have caught scientist attention due to the fact that they have not been found in their mammalian host rendering them a potential antibacterial target (Barrett and Hoch, 1998). Moreover, they enable bacteria to sense and react to environmental changes within their host and contribute to bacterial competence, virulence and viability (Paterson et al., 2006). More importantly, their role in stress hormone-bacteria interactions has been identified in some Gram-negative bacteria (Hughes and Sperandio, 2008). Therefore, it is plausible that they are also important in pneumococcus-catecholamine hormone interactions as microarray analysis indicate TSC03 and TSC06 involvement in promoting pneumococcal growth in the presence of norepinephrine (Gonzales et al., 2013). Hence, pneumococcal TCS mutants were successfully constructed and analysed for their response to catecholamines.

Prior to testing the responsiveness of the TCS knockout strains to the catecholamines in serum-SAPI medium, the growth of the mutants was tested in BHI medium and the results showed that they were able to grow similar to the wild type. The growth yields of ΔTCS12, ΔTCS13, ΔTCS05, ΔTCS08, ΔTCS04, ΔTCS03, ΔTCS07, ΔRitR, ΔTSC06, ΔTCS10, ΔTCS01, ΔTCS09 and ΔTCS11 mutants were OD_{600} 1.407 ± 0.028, 1.427 ± 0.011, 1.337 ± 0.008, 1.401 ± 0.010, 1.400 ± 0.007, 1.393 ± 0.023, 1.391 ± 0.016, 1.397 ± 0.012, 1.377 ± 0.013, 1.349 ± 0.006, 1.354 ± 0.007, 1.340 ± 0.008 and 1.348 ± 0.007, (n=3 each), respectively, compared to that of wild type strain 1.378 ± 0.019, n=3 ($p > 0.05$) (Figure 3.19). In addition, when growing in the serum-SAPI, all hormone-free cultures of the mutants have shown a wild type-like growth yield and rate. In details, the growth yields of ΔTCS12, ΔTCS13, ΔTCS05, ΔTCS08, ΔTCS04, ΔTCS03, ΔTCS07, ΔRitR, ΔTSC06, ΔTCS10, ΔTCS01, ΔTCS09 and ΔTCS11 mutants were 0.199 ± 0.006, 0.196 ± 0.008, 0.201 ± 0.015, 0.190 ± 0.004, 0.182 ± 0.005, 0.192 ± 0.014, 0.206 ± 0.001, 0.213 ± 0.006, 0.200 ± 0.003, 0.197 ± 0.012, 0.209 ± 0.003, 0.173 ± 0.009 and 0.206 ± 0.017, (n=3 each), respectively, compared to that of wild type strain 0.210 ± 0. 0.005, n=3 ($p > 0.05$) (Figure 3.20 A). Furthermore, the growth rates (μ) of ΔTCS12, ΔTCS13, ΔTCS05, ΔTCS08, ΔTCS04, ΔTCS03, ΔTCS07, ΔRitR, ΔTSC06, ΔTCS10, ΔTCS01, ΔTCS09 and ΔTCS11 mutants were 0.107 ± 0.005 h^{-1}, 0.103 ± 0.005 h^{-1}, 0.127 ± 0.035 h^{-1}, 0.089 ± 0.004 h^{-1}, 0.082 ± 0.003 h^{-1}, 0.100 ± 0.013 h^{-1}, 0.134 ± 0.007 h^{-1}, 0.146 ± 0.009 h^{-1}, 0.116 ± 0.005 h^{-1}, 0.070 ± 0.012 h^{-1}, 0.122 ± 0.001 h^{-1}, 0.080 ± 0.007 h^{-1} and 0.162 ± 0.029 h^{-1}, (n=3 each), respectively, and the rates were not significantly different to that of wild type which was 0.109 ± 0.011 h^{-1}, n=3 ($p > 0.05$) (Figure 3.20 B). This shows that the mutation of TCS genes did not lead to growth defect neither in BHI, nor in serum-SAPI broth medium.

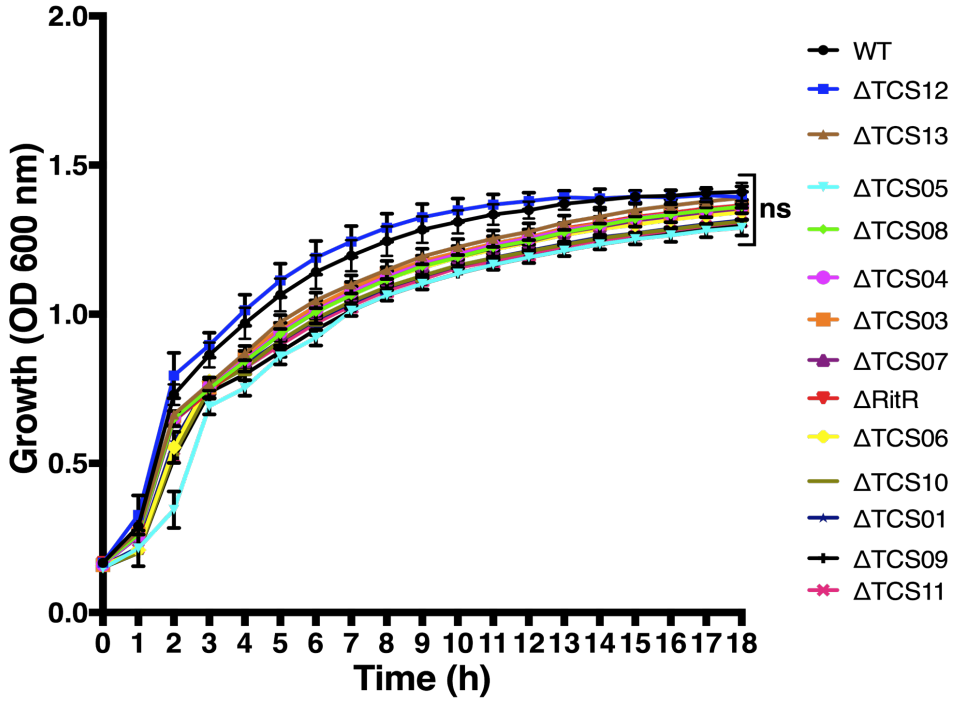
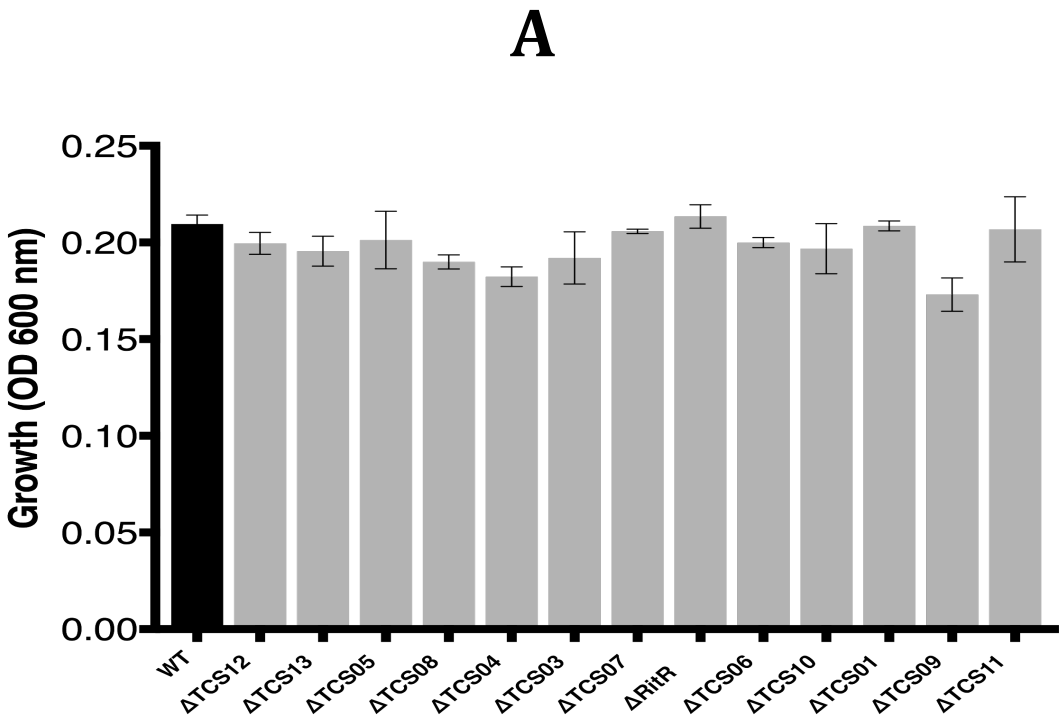


Figure 3.19. Growth curves of pneumococcal strains incubated in BHI. The values shown are means of at least three independent experiments and error bars represent standard error of means (SEM). No significant differences were observed when comparing the growth of wild type D39 to mutant strains using one-way ANOVA and Dunnett's multiple comparisons test ($p > 0.05$).



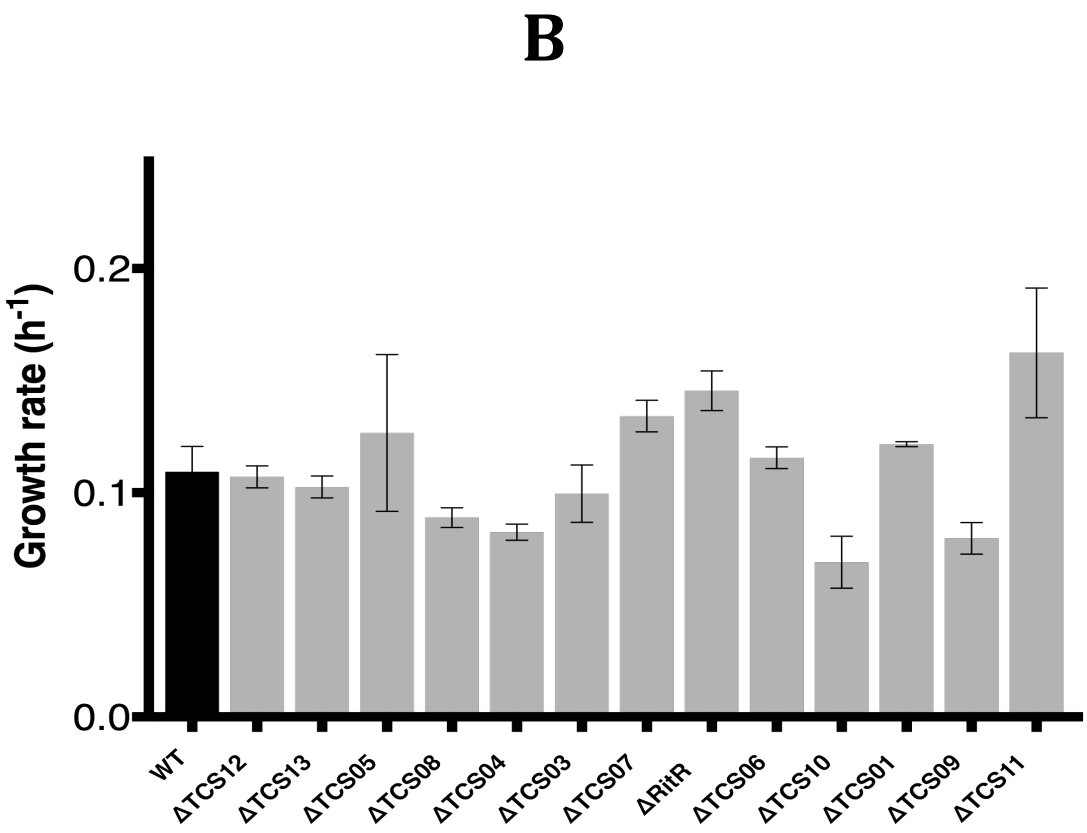
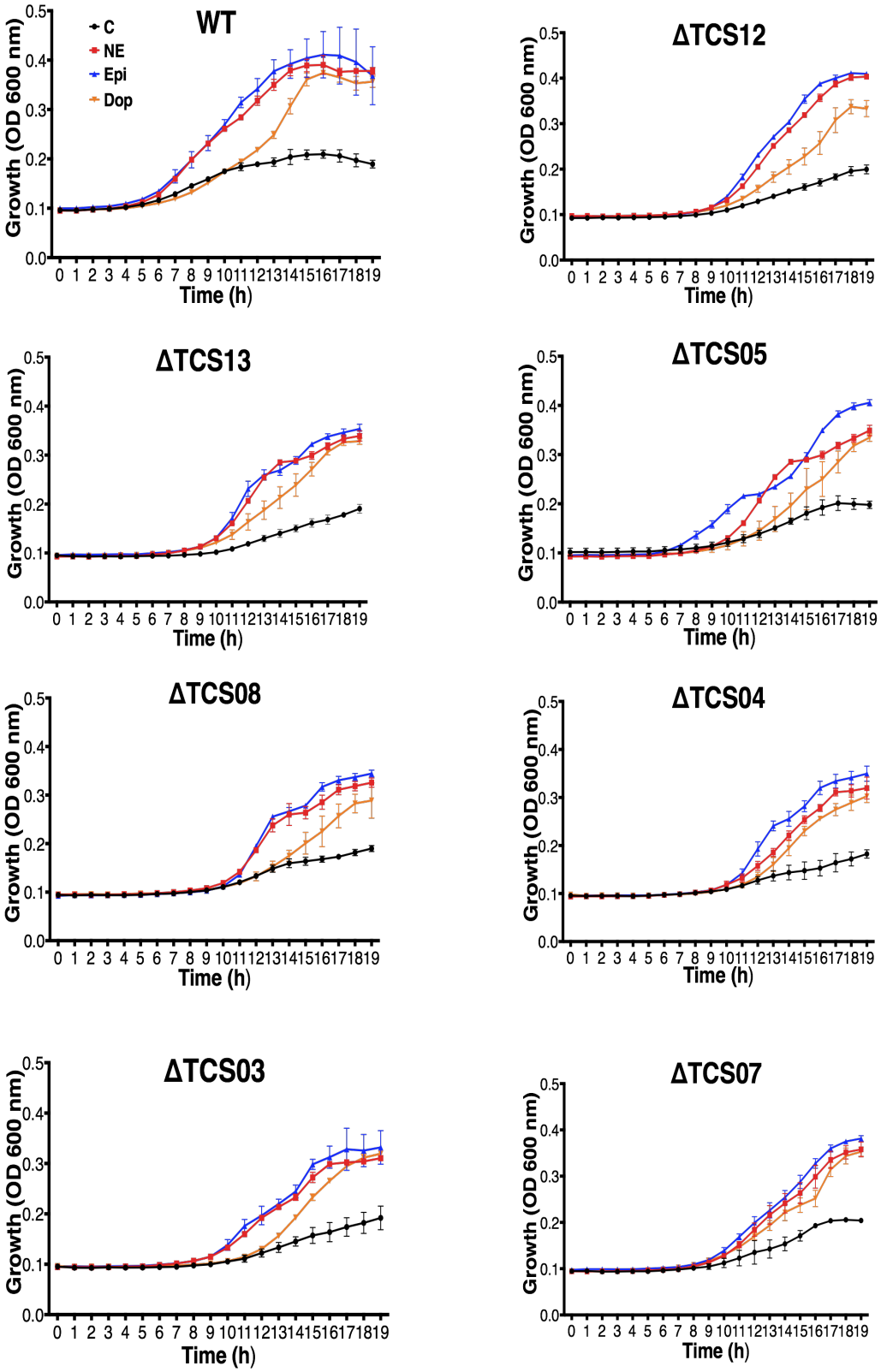


Figure 3.20. Growth yields (A) and rates (B) of pneumococcal strains in serum medium without hormones. It shows growth yields and rates of wild type *S. pneumoniae* strain D39 (black bar) and TCSs mutants (grey bar) in the serum-SAPI medium without the addition of hormones. The values shown are means of at least three independent experiments and error bars represent standard error of means (SEM). No significant differences were seen when comparing the growth yields and rates of mutant strains to the wild type D39 using one-way ANOVA and Dunnett's multiple comparisons test ($p > 0.05$).

When the hormones were present in the serum-SAPI, all mutants were seen to be stress-hormone-responsive compared to their controls, which did not contain the hormone, except the response to the dopamine in ΔTCS09 mutant. The growth yield and rate of the ΔTCS09 culture grown without dopamine was OD_{600} 0.17 ± 0.001, and 0.08 ± 0.007 h^{-1}, respectively, compared to the dopamine-treated culture which yielded just 0.19 ± 0.011 with growth rate 0.11 ± 0.006 h^{-1} ($p > 0.05$, n=3 each), showing that TCS09 is important for pneumococcal growth responses to dopamine. The growth rate and yield of all mutant strains as well as those of wild type that are presented in Figures 3.21-3.23.


WT
C
NE
Epi
Dop
ΔTCS12
ΔTCS13
ΔTCS05
ΔTCS08
ΔTCS04
ΔTCS03
ΔTCS07
Growth (OD 600 nm)
Time (h)

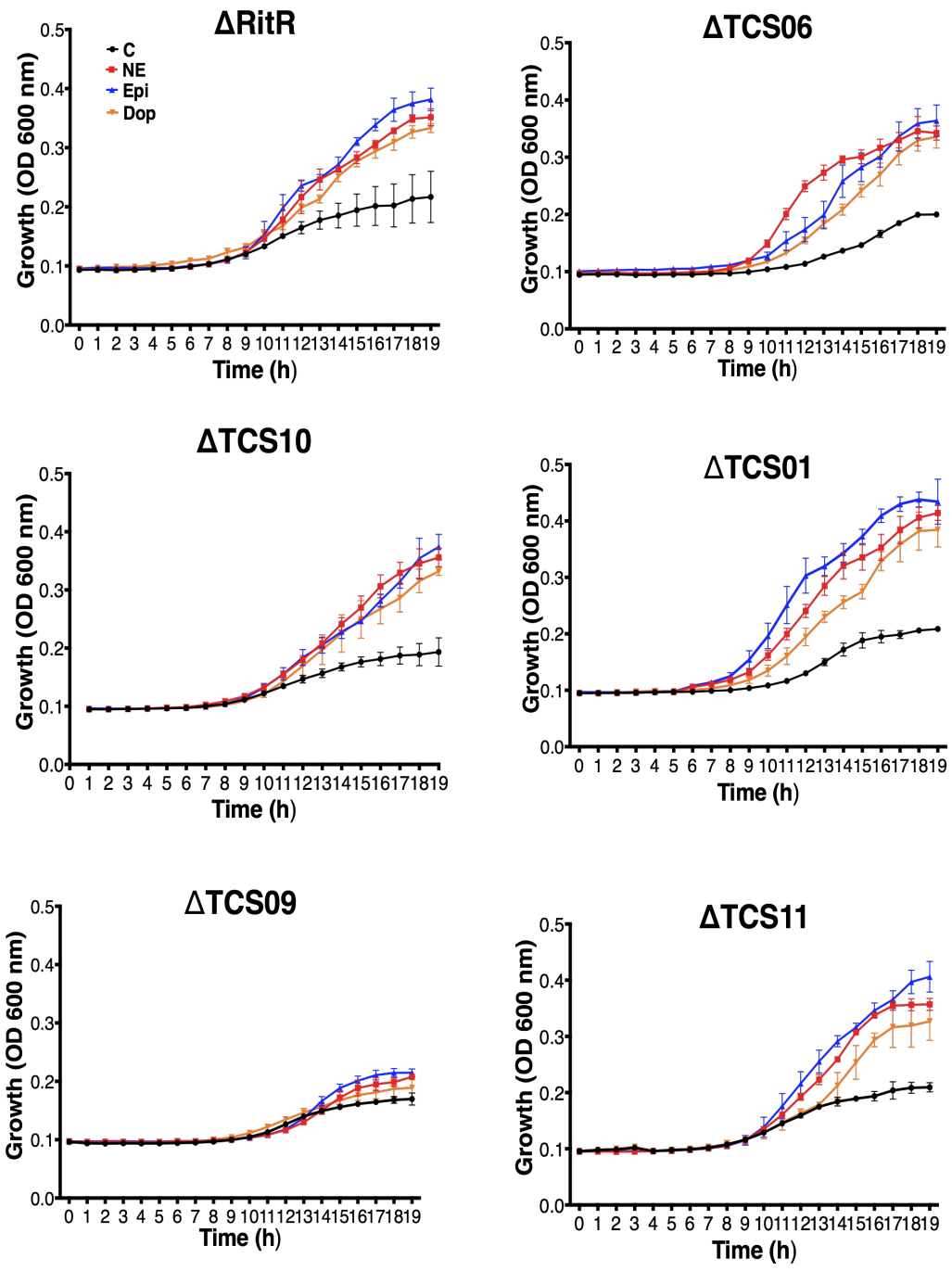


Figure 3.21. Catecholamine hormones effects on the growth of pneumococcal strains. The panels show the time course of growth of wild type (WT) and TCSs mutants (ΔTCS12, ΔTCS13, ΔTCS05, ΔTCS08, ΔTCS04, ΔTCS03, ΔTCS07, ΔRitR, ΔTSC06, ΔTCS10, ΔTCS01, ΔTCS09 and ΔTCS11) in serum-medium with the addition of 50 μM NE (red line), 50 μM Epi (blue line), 5 μM Dop (orange line) and without hormones (black line). The values shown are means of at least three independent experiments and error bars represent standard error of means (SEM).

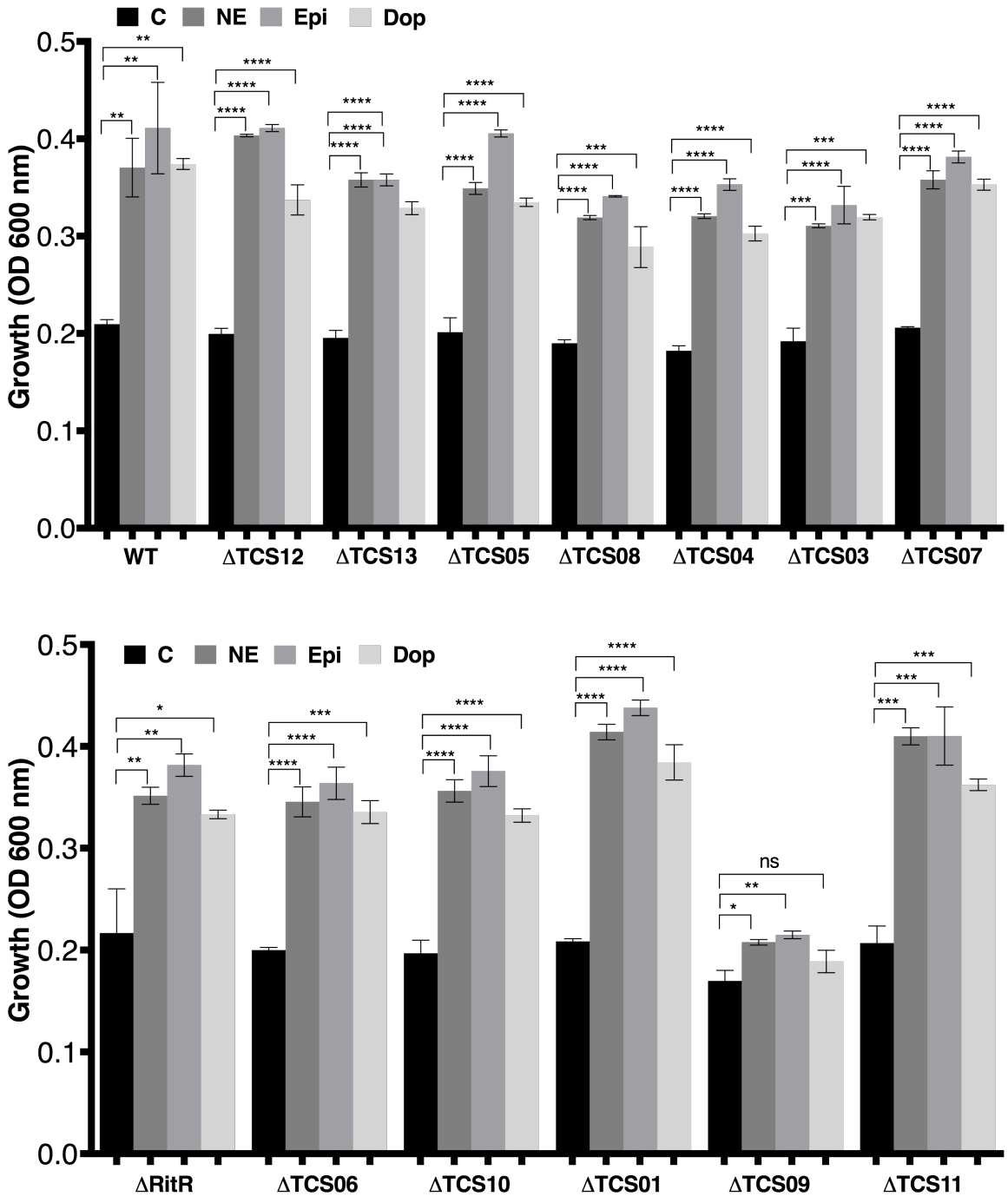


Figure 3.22. Growth yields of hormone treated pneumococcal WT and TCS mutant strains. The growth yields of wild type *S. pneumoniae* strain D39 and TCS mutants in serum-medium without (black bar) and with the addition of 50 µM NE, 50 µM Epi and 5 µM Dop, respectively, are shown. The vertical bars indicate the standard error of means (SEM), (n = 3). The significant differences were seen comparing the stress hormone response of each strain to its control using one-way ANOVA and Dunnett's multiple comparisons test. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$).

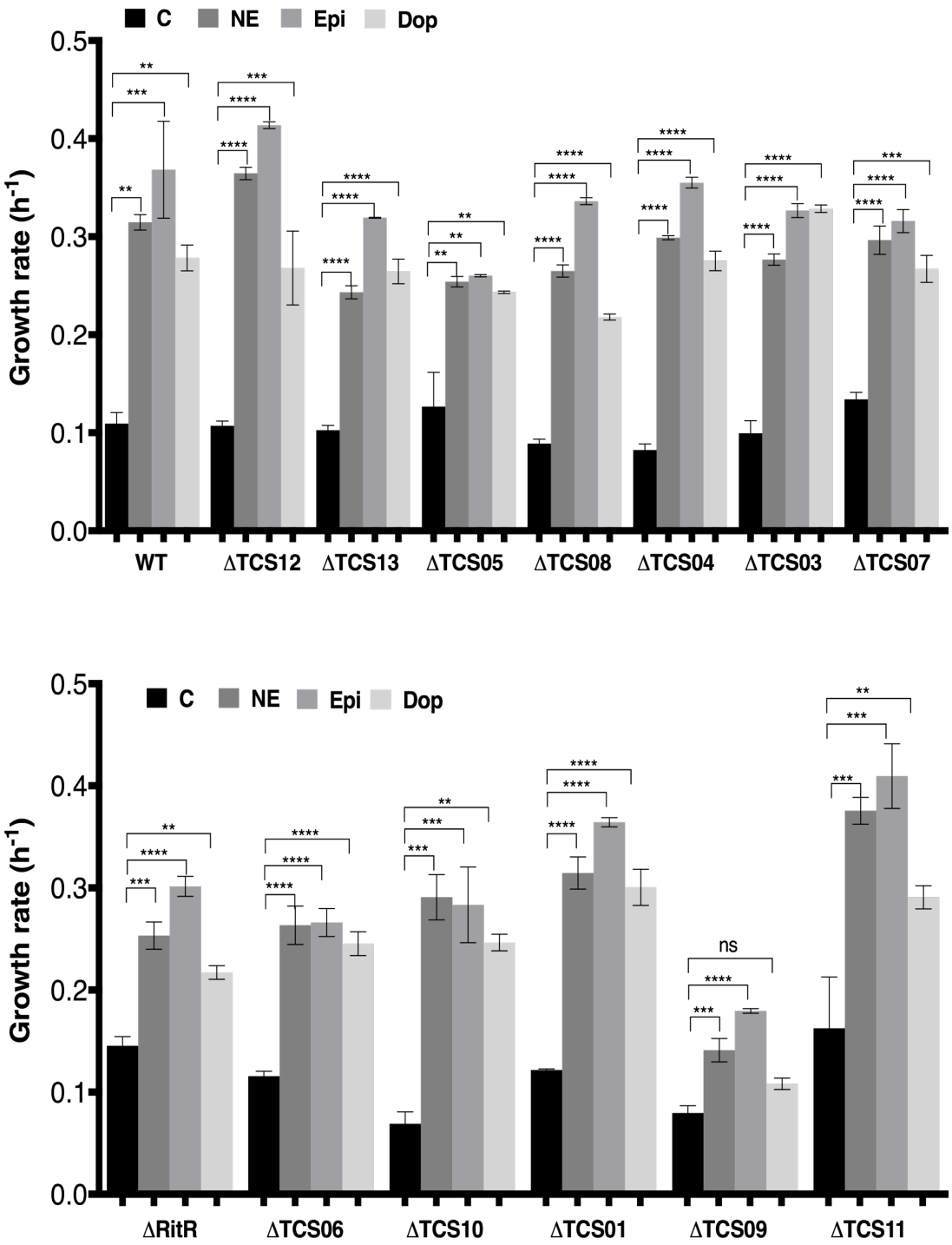
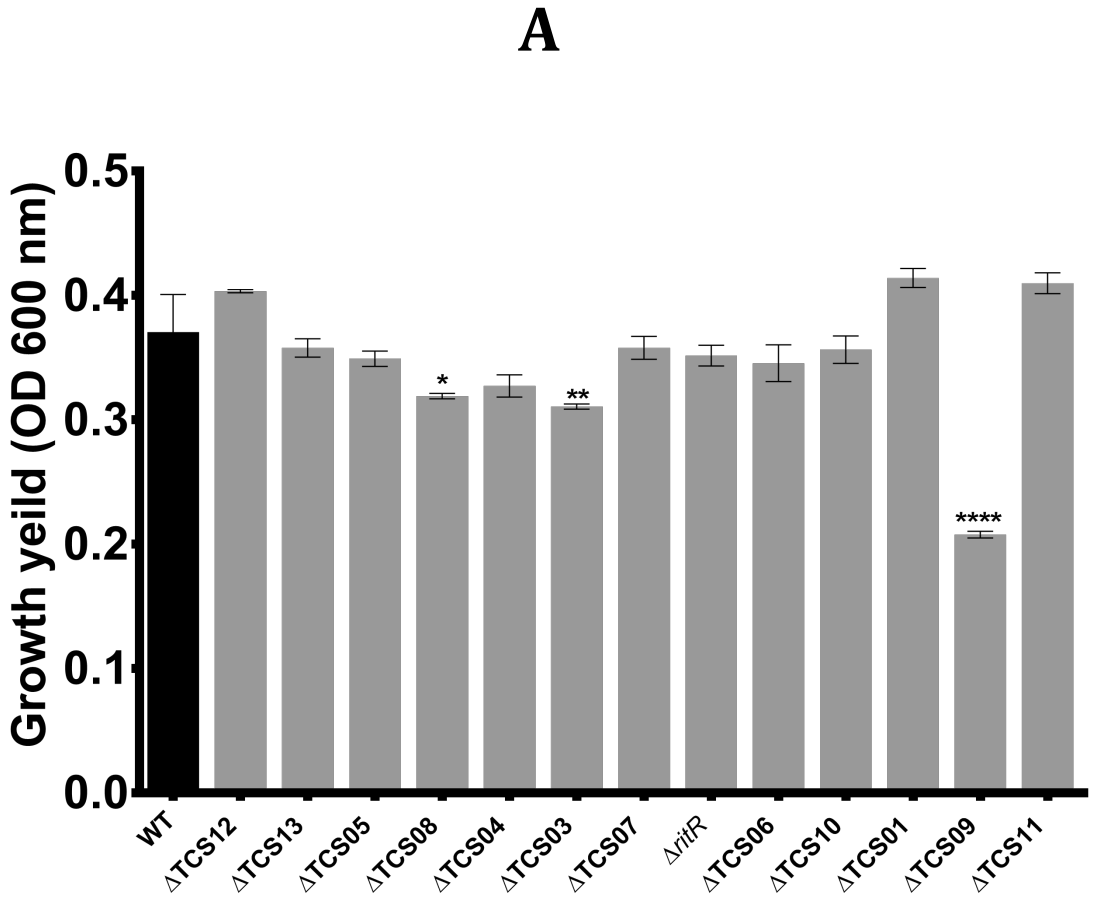


Figure 3.23. Growth rates of hormone treated pneumococcal strains. The figures show growth rates of wild type and TCS mutants in serum-medium without (black bar) and with the addition of 50 µM NE, 50 µM Epi and 5 µM Dop, respectively. The vertical bars indicate the standard error of means (SEM), (n = 3). The significant differences were seen comparing the stress hormone response of each strain to its control using one-way ANOVA and Dunnett's multiple comparisons test. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$).

Although, all the mutants were responsive to the hormones, the responsiveness of ΔTCS09, ΔTCS03, ΔTCS04 and ΔTCS08 in response to one or more of the catecholamines in terms of growth yields was significantly lower than the wild type. In details, mutant lacking TCS09 and TCS03 showed consistently reduced growth yields in response to NE, Epi and Dop than the wild type. For example, ΔTCS09 yielded OD_{600} = 0.21 ± 0.003 ($p < 0.0001$), 0.22 ± 0.004 ($p < 0.0001$) and 0.19 ± 0.011 ($p < 0.0001$) while ΔTCS03 yielded 0.31 ± 0.002 ($p < 0.01$), 0.33 ± 0.019 ($p < 0.05$) and 0.32 ± 0.003 ($p < 0.05$) respectively, compared to the wild type strain; 0.39 ± 0.011, 0.41 ± 0.047 and 0.37 ± 0.006, (n=3 each), respectively. Moreover, the growth yields of ΔTCS04 and ΔTCS08 strains in response to Dop were 0.30 ± 0.008 ($p < 0.05$) and 0.29 ± 0.021 ($p < 0.01$) respectively, compared to the wild type strain; 0.37 ± 0.006, (n=3 each). In addition, the growth yield of ΔTCS08 in response to NE was 0.33 ± 0.005, n=3, against that of wild type (0.39 ± 0.011, n=3) ($p < 0.05$) (Figures 3.24 A, B and C).



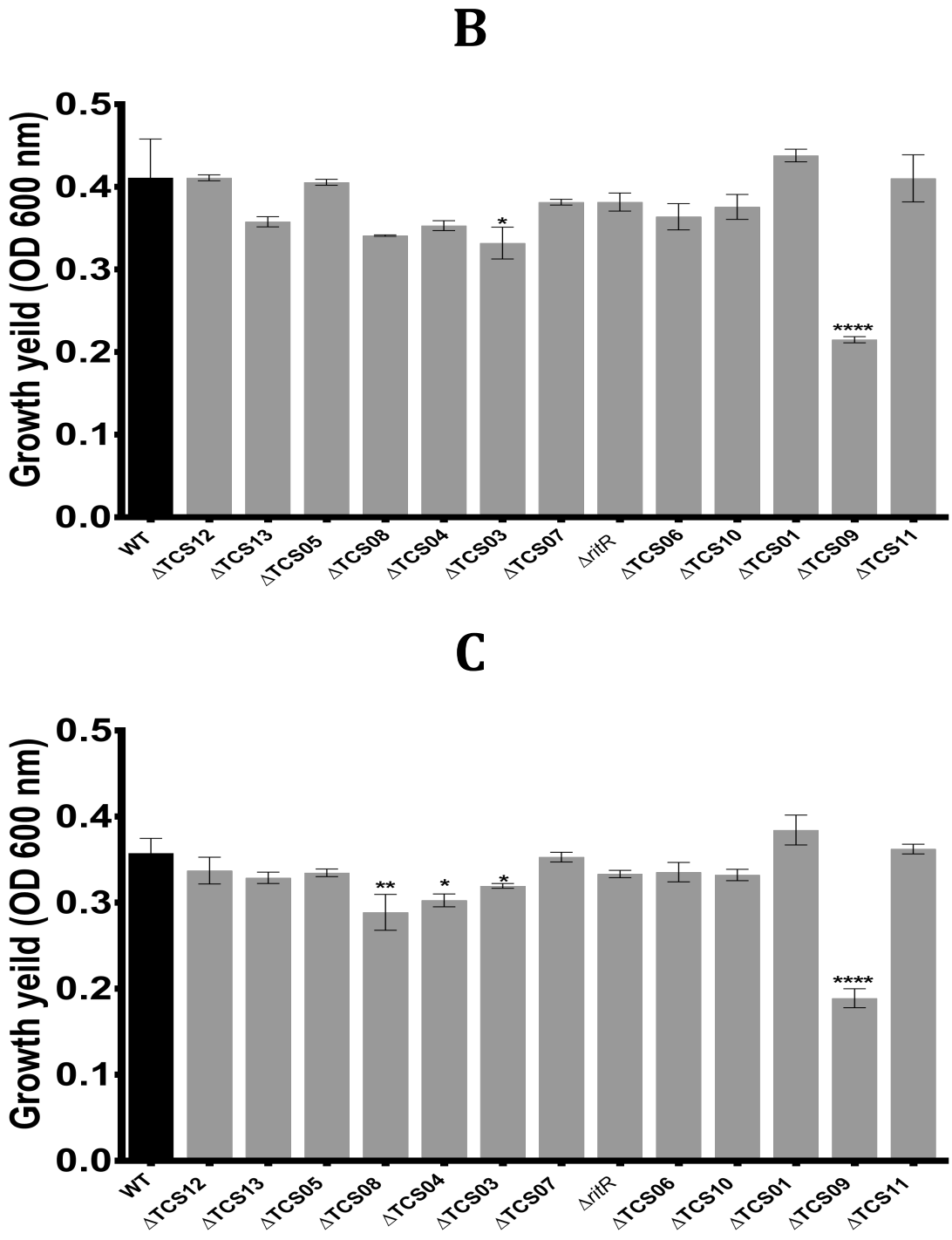
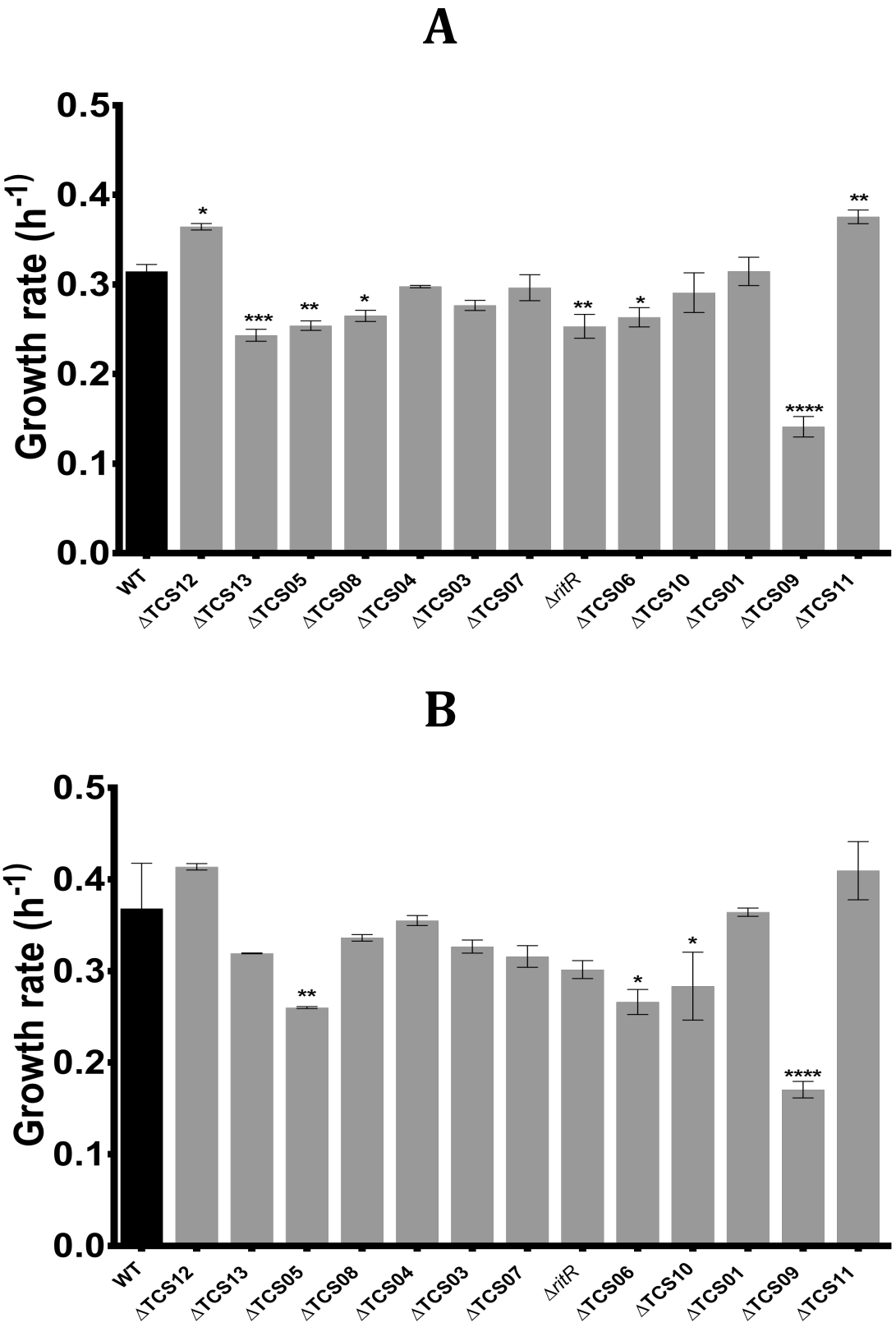


Figure 3.24 A, B and C. Growth yields of hormones treated pneumococcal strains. The graphs show growth response of wild type *S. pneumoniae* strain D39 (black bar) and TCSs mutants to 50 µM NE (**A)**, 50 µM Epi (**B)** and 5 µM Dop (**C)**. The values shown are means of at least three independent experiments and error bars represent standard error of means (SEM). The significant differences were seen comparing the stress hormone response of each strain to its control using one-way ANOVA and Dunnett's multiple comparisons test. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$).

In terms of growth rates, ΔTCS09, ΔTCS13, ΔTCS05, ΔTCS08, ΔRitR and ΔTCS06 mutants showed significant slower growth rates in response to one or more of the catecholamines in comparison with the wild type behaviour. For example, the growth rate of ΔTCS09 to NE, Epi and Dop was 0.14 ± 0.011 h^{-1} ($p < 0.0001$), 0.17 ± 0.009 h^{-1} ($p < 0.0001$) and 0.11 ± 0.006 h^{-1} ($p < 0.0001$) respectively, whereas the wild type strain was 0.32 ± 0.008 h^{-1}, 0.37 ± 0.049 h^{-1} and 0.28 ± 0.013 h^{-1}, (n=3 each), respectively. Moreover, ΔTCS13, ΔTCS05, ΔTCS08 and ΔRitR and ΔTCS06 mutants also showed a reduction in the growth rate in the presence of NE; 0.24 ± 0.007 h^{-1} ($p < 0.001$), 0.25 ± 0.005 h^{-1} ($p < 0.01$), 0.27 ± 0.006 h^{-1} ($p < 0.05$), 0.25 ± 0.013 h^{-1} ($p < 0.01$) and 0.26 ± 0.011 h^{-1} ($p < 0.05$) respectively, while ΔTCS12 and ΔTCS11 displayed a fast growth rate; 0.36 ± 0.004 h^{-1} ($p < 0.05$) and 0.38 ± 0.008 h^{-1} ($p < 0.01$), respectively, compared to the wild type which was 0.32 ± 0.008 h^{-1}, (n=3 each). The growth rates in the presence of Epi for ΔTCS05, ΔTCS06 and ΔTCS10 were 0.26 ± 0.001 h^{-1} ($p < 0.01$), 0.27 ± 0.014 h^{-1} ($p < 0.05$) and 0.28 ± 0.037 h^{-1} ($p < 0.05$) respectively, compared to the wild type 0.37 ± 0. 0.049 h^{-1}, (n=3 each). Lastly, the growth rate in the presence of Dop was for ΔTCS08 and ΔRitR; 0.22 ± 0.003 h^{-1} ($p < 0.05$) and 0.22 ± 0.007 h^{-1} ($p < 0.05$) respectively, compared to the wild type's; 0.28 ± 0.013 h^{-1}, (n=3 each) (Figures 3.25 A, B and C). Figure 3.26 summarises those mutants that showed significant reduction in growth yields and/or rates to at least two hormones or more compared to the hormone response of the wild type strain. The growth rate and yield of all mutant strains as well as the wild type are presented in Table 3.2.

The analysis of growth data in this study showed that all the 13 TCS mutants were responsive to the catecholamine hormones, except ΔTCS09, ΔTCS03, ΔTCS08, ΔTCS05, ΔRitR and ΔTCS06 mutants with significant reduced growth rates and/or yields to at least two hormones compared to the hormone response of the wild type strain (Figure 3.26). Specifically, mutant lacking TCS09 displayed a consistent reduction of growth yield and rate in the presence of all hormones in the serum containing media compared to the wild type which indicates a strong involvement of this TCS in *S. pneumoniae* - stress hormone interactions.


A
B
Growth rate (h-1)
0.5
0.4
0.3
0.2
0.1
0.0
*
***
**
*
**
*
**
****
**
WT
ΔTCS12
ΔTCS13
ΔTCS05
ΔTCS08
ΔTCS04
ΔTCS03
ΔTCS07
ΔritR
ΔTCS06
ΔTCS10
ΔTCS01
ΔTCS09
ΔTCS11

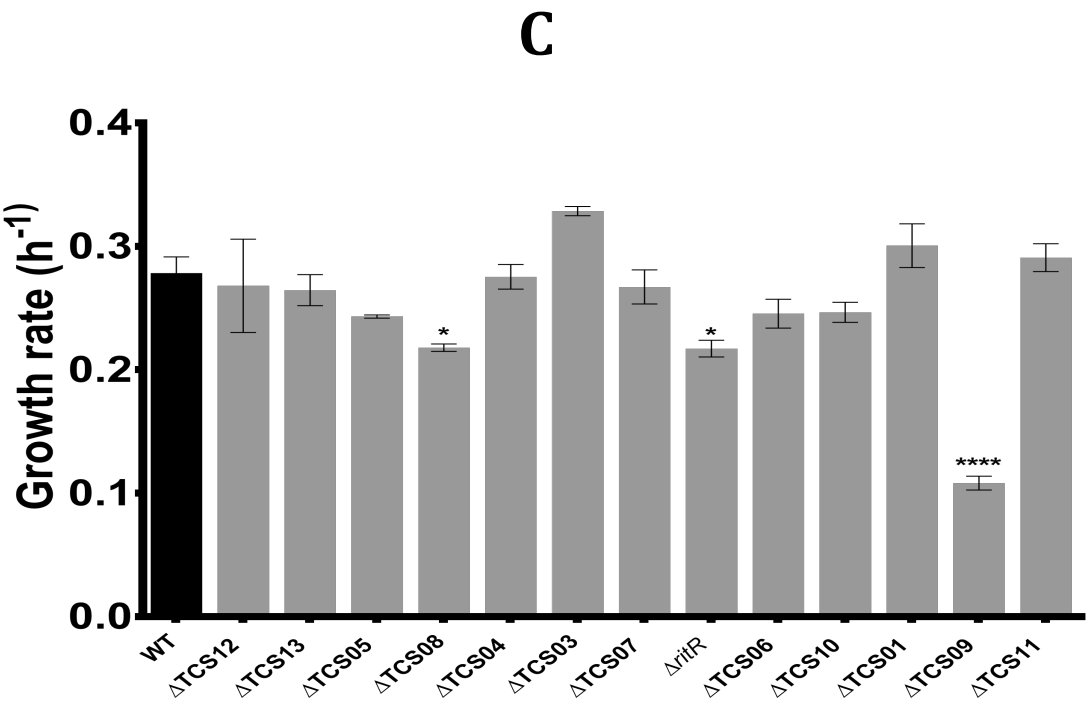


Figure 3.25 A, B and C. Growth rates of pneumococcal mutant strains grown in the presence of different hormones. The graphs show growth response of wild type *S. pneumoniae* strain D39 (black bar) and TCSs mutants to 50 µM NE (**A)**, 50 µM Epi (**B)** and 5 µM Dop (**C)**. The values shown are means of at least three independent experiments and error bars represent standard error of means (SEM). The significant differences were seen comparing the stress hormone response of each strain to its control using one-way ANOVA and Dunnett's multiple comparisons test. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$).

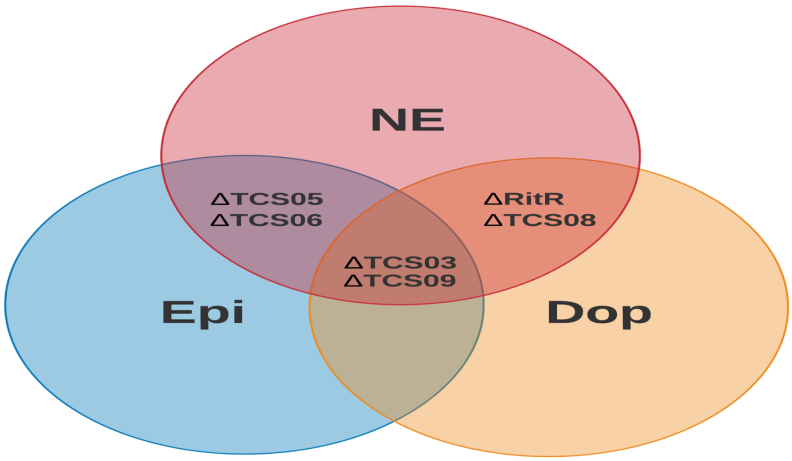


Figure 3.26. Summary of mutants' response to hormones. This chart shows mutants with significant reduced growth rates and/or yields to at least two hormones compared to the hormone response of the wild type strain.

Table 3.2. Growth rates (μ) and yields (maximal OD_{600}) of pneumococcal strains grown microaerobically in serum-based media using 50 μM of NE, Epi and 5 of μM Dop. Values are average of three independent experiments with three replicates. '±' refers to standard error of means (SEM).

Strains	Control (no hormones)		Norepinephrine		Epinephrine		Dopamine	
	μ (h^{-1})	Max OD_{600}	μ (h^{-1})	Max OD_{600}	μ (h^{-1})	Max OD_{600}	μ (h^{-1})	Max OD_{600}
WT	0.11±0.011	0.21±0.005	0.32±0.008	0.39±0.011	0.37±0.049	0.41±0.047	0.28±0.013	0.37±0.006
ΔTCS012	0.11±0.005	0.20±0.006	0.36±0.004	0.40±0.001	0.41±0.003	0.41±0.004	0.27±0.038	0.34±0.016
ΔTCS013	0.10±0.005	0.19±0.005	0.24±0.007	0.35±0.006	0.32±0.004	0.35±0.006	0.27±0.013	0.33±0.007
ΔTCS05	0.13±0.035	0.20±0.015	0.25±0.005	0.34±0.012	0.26±0.001	0.40±0.003	0.24±0.001	0.34±0.004
ΔTCS08	0.09±0.004	0.19±0.004	0.27±0.006	0.33±0.005	0.34±0.004	0.34±0.001	0.22±0.003	0.29±0.021
ΔTCS04	0.08±0.004	0.18±0.005	0.30±0.001	0.32±0.011	0.36±0.005	0.35±0.009	0.28±0.010	0.30±0.008
ΔTCS03	0.10±0.013	0.19±0.014	0.28±0.006	0.31±0.002	0.33±0.007	0.33±0.019	0.33±0.004	0.32±0.003
ΔTCS07	0.13±0.007	0.21±0.001	0.30±0.015	0.36±0.009	0.32±0.012	0.38±0.004	0.27±0.014	0.35±0.008
ΔRitR	0.10±0.048	0.22±0.043	0.25±0.013	0.35±0.008	0.30±0.010	0.38±0.011	0.22±0.007	0.33±0.004
ΔTCS06	0.12±0.005	0.20±0.003	0.26±0.011	0.34±0.007	0.27±0.014	0.36±0.016	0.25±0.012	0.34±0.011
ΔTCS10	0.07±0.012	0.19±0.013	0.29±0.022	0.36±0.011	0.28±0.037	0.38±0.015	0.25±0.008	0.33±0.014
ΔTCS01	0.12±0.001	0.21±0.003	0.23±0.016	0.42±0.008	0.36±0.005	0.44±0.008	0.30±0.018	0.38±0.017
ΔTCS09	0.08±0.007	0.17±0.001	0.14±0.011	0.21±0.003	0.17±0.009	0.22±0.004	0.11±0.006	0.19±0.011
ΔTCS11	0.16±0.029	0.21±0.002	0.38±0.008	0.41±0.008	0.41±0.032	0.41±0.029	0.29±0.011	0.36±0.006

SUMMARY

1. All TCS knockout strains were tested in BHI medium and the results showed that they were able to grow similar to the wild type.
2. In the serum-SAPI media, hormone-free cultures of the mutants have also shown a wild type-like growth yield and rate.
3. When the hormones were present in the serum-SAPI, all mutants have shown to be stress-hormones responsive compared to their controls (without the hormones).
4. Analysis of growth data in this study showed that all 13 TCS mutants were responsive to the catecholamine hormones, except ΔTCS09, ΔTCS03, ΔTCS08, ΔTCS05, ΔRitR and ΔTCS06 mutants with significant reduced growth rates and/or yields to at least two hormones compared to the response of the wild type strain.
5. ΔTCS09 displayed a consistent reduction of growth yield and rate in the presence of all hormones in the serum media compared to the wild type suggesting an involvement of this TCS in *S. pneumoniae*-stress hormone interactions.

3.6. Growth of pneumococcal strains in CDM containing different sugars

Since the ΔTCS09 mutant displayed a consistent reduction of growth yield and rate in the presence of all hormones in the serum containing media compared to the wild type, the question was whether this could be attributed to a defect in the metabolism. A previous study, for instance, indicated that mutant lacking TCS09 resulted in 102 genes differentially expressed compared to the wild-type strain D39 (Hendriksen et al., 2007). The set of genes down-regulated in the ΔTCS09 mutant were found to be encoding proteins involved in carbohydrate metabolism and facilitate the uptake of sugars such as mannose and fructose (Hendriksen et al., 2007). Serum-SAPI medium contains approximately 20 mM of glucose as well as transferrin, which also contains carbohydrates such as mannose and galactose (Talwar G.P. , 2003). As a result, it was essential to determine whether the reduction of growth response seen particularly in ΔTCS09 mutant was due to defect in sugar metabolism or due to reduction in NE and/or transferrin uptake or metabolism.

The results showed that the ΔTCS09 mutant as well as the ΔTCS03, ΔTCS05 and ΔRitR mutants did not show impairment of growth on CDM medium supplemented with either glucose or galactose relative to the wild type. In CDM medium containing glucose, the growth yield of ΔTCS09, ΔTCS03, ΔTCS05 and ΔRitR mutants OD_{600} = 0.91 ± 0.012, 0.85 ± 0.030, 0.79 ± 0.035 and 0.90 ± 0.042, respectively (n=3 each) were similar to that of wild type which was 0.90 ± 0.031, n=3 ($P > 0.05$). Similarly, in CDM medium containing galactose, the growth yield of ΔTCS09, ΔTCS03, ΔTCS05 and ΔRitR mutants were 0.56 ± 0.027, 0.55 ± 0.026, 0.55 ± 0.023 and 0.55 ± 0.026, respectively (n=3 each) were similar to that of wild type which was 0.61 ± 0.034, n=3 ($P > 0.05$) (Figure 3.28). This indicates that ΔTCS09 as well as the selected mutants were not affected in growth on glucose or galactose. Therefore, this result shows that the impairment in the growth yield and rate in the presence of stress hormones in serum medium is due to the potential involvement of TCS09's in stress hormone sensing, uptake or processing.

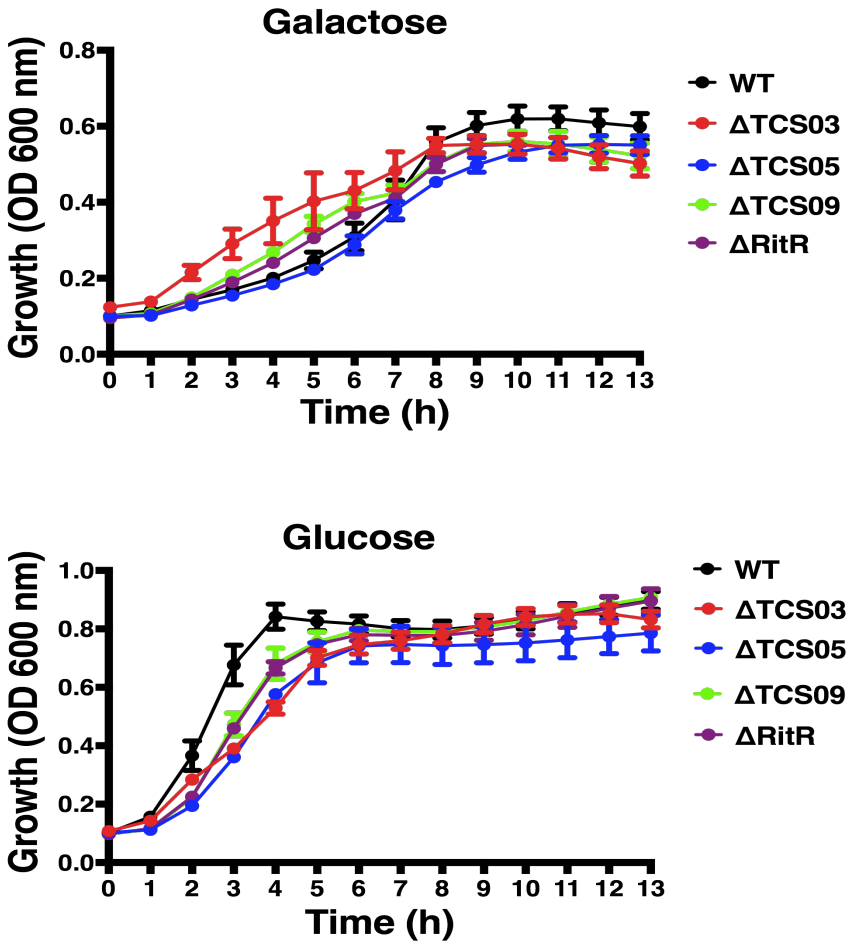


Figure 3.28. The effect of galactose and glucose on growth of selected TCS mutants. Pneumococcal strains; wild type (black), ΔTCS03 (red), ΔTCS05 (blue), ΔTCS09 (green) and ΔRitR (purple) mutants were inoculated at approximately 10^6 CFU/ml into 1 ml aliquots of CDM media containing galactose or glucose and incubated for 16 h. No significant differences were observed when comparing the growth rates of wild type D39 to the mutant strains using one-way ANOVA and Dunnett's multiple comparisons test ($p > 0.05$). The values shown are means of at least three independent experiments and error bars represent standard error of means (SEM).

SUMMARY

1. ΔTCS03, ΔTCS05, ΔTCS09, and ΔRitR mutants did not show impairment of growth in CDM medium supplemented with either glucose or galactose.
2. The result indicates that the consistent impairment in the growth yield and rate in the presence of stress hormones seen in serum medium is due to the potential involvement of TCS09 system in stress hormone sensing, uptake or processing.

3.7. Assessment of inducibility of selected TCS by stress hormones

The ΔTCS03, ΔTCS05 and ΔTCS09 mutants specifically displayed significant reduced growth rates and/or yields to at least two hormones in serum-containing medium. In addition, their roles in pneumococcal virulence have been reported in several studies. For example, a recent study found that TCS03 deletion resulted in absence of NE effect on adherence (Gonzales et al., 2013). TCS05, on the other hand, has also been shown to be an essential response regulator, which influences competence, virulence and antibiotic resistance in *S. pneumoniae* (Guenzi et al., 1994;Guenzi and Hakenbeck, 1995;Ibrahim et al., 2004;Throup et al., 2000;Zahner et al., 2002). TCS09 is yet another vital component of the pneumococcal TCSs system as mutation in *rr09* displayed avirulent behaviour in mouse models of pneumonia and bacteraemia resulting in the survival of all infected mice and rapid clearance of the bacteria (Blue and Mitchell, 2003). Therefore, based on the findings of this study and other studies, only promotors of genes encoding for TCS03, TCS05 and TCS09 were selected for testing using β-galactosidase assays.

The activity of β-galactosidase was determined as an indirect measure of promoter involvement in stress hormone responsiveness. The native pPP2 was integrated into pneumococcal wild type DNA to inactivate the endogenous *bgaA* and used as experimental control. As a result, any β-galactosidase activity detected with the promoterless pPP2 was very likely coming from the second pneumococcal β-galactosidase gene, *bgaC.* All reporter strains were microaerobically grown to mid exponential phase in serum medium supplemented with and without 50 μM NE, and β-galactosidase activity was determined to evaluate the responsiveness of the given promoters to the hormone. The results showed that there was no difference in activity between the hormone-treated and untreated cultures of the reporter strains and the promoters were not directly induced by stress hormones (Figure 3.27). Notably, PTCS05::*lacZ* fusion strain generated the highest β-galactosidase activity, assessed in Miller Units on both control and treated samples in this serum-containing medium as well as CDM and BHI (data not shown). Although an unidentified stimulus in serum and

other media induced the promoter of TCS05, studying the role of this TCS system without stress hormones intervention is beyond the scope of this project. Collectively, reporter strains were constructed in wild type background and their promoters' responsiveness to the hormone was tested. It was revealed that the putative promoters of these selected TCSs were not directly induced by stress hormones.

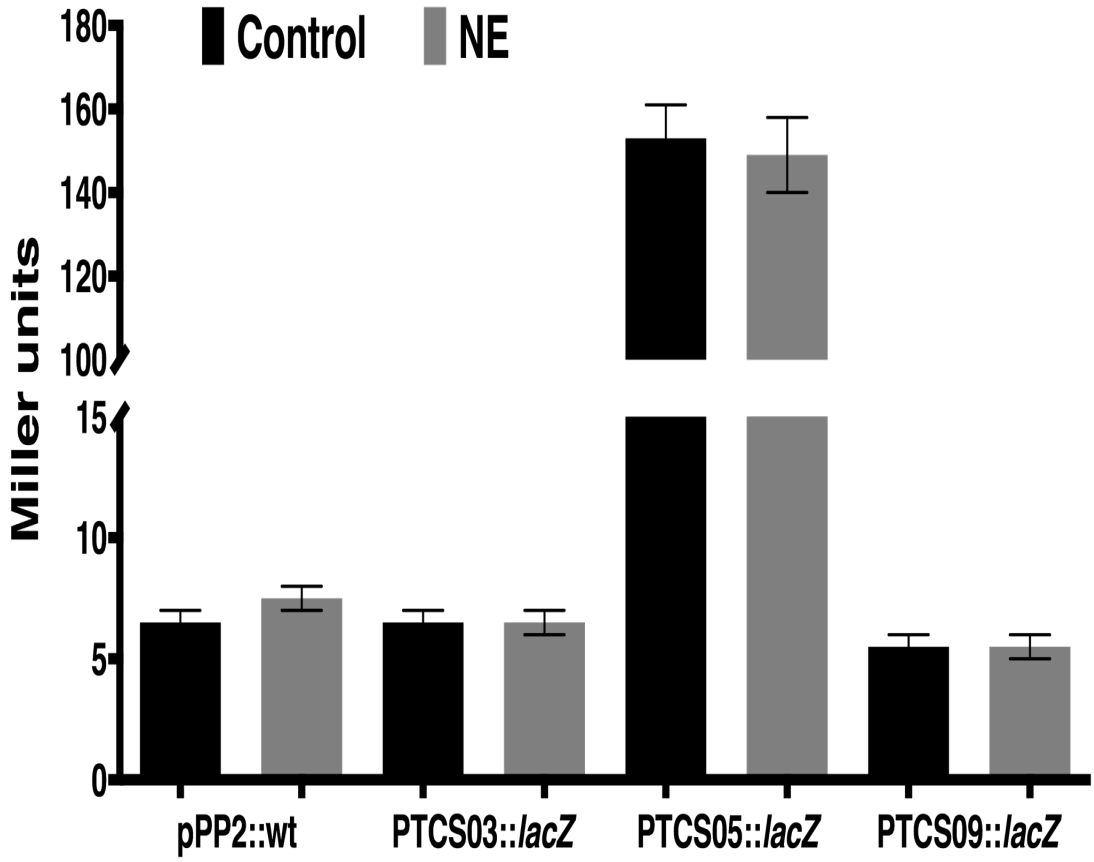


Figure 3.27. Expression levels (in Miller Units) of pneumococcal *lacZ*- fusions to the promoter of TCS03, TCS05 and TCS09 in wild type background. The activity is expressed in nmol p-nitrophenol/min/ml when using 50 μM of NE. The values shown are means of at least three independent experiments and error bars represent standard error of means (SEM). There was no significant difference when comparing the stress hormone response of each strain to its control (hormone-free culture) using unpaired t test analysis ($p > 0.05$).

SUMMARY

1. Using vector plasmid pPP2, 3 reporter strains containing putative promotors of TCS03, TCS05 and TCS09 were constructed in wild type background.
2. The putative promoters of the selected TCSs were not directly induced by stress hormone NE suggesting an indirect role of these systems in stress hormone interactions.
3. PTCS05::*lacZ* fusion strains generated the highest β-galactosidase activity by unidentified stimuli or possible due to high promoter strength.

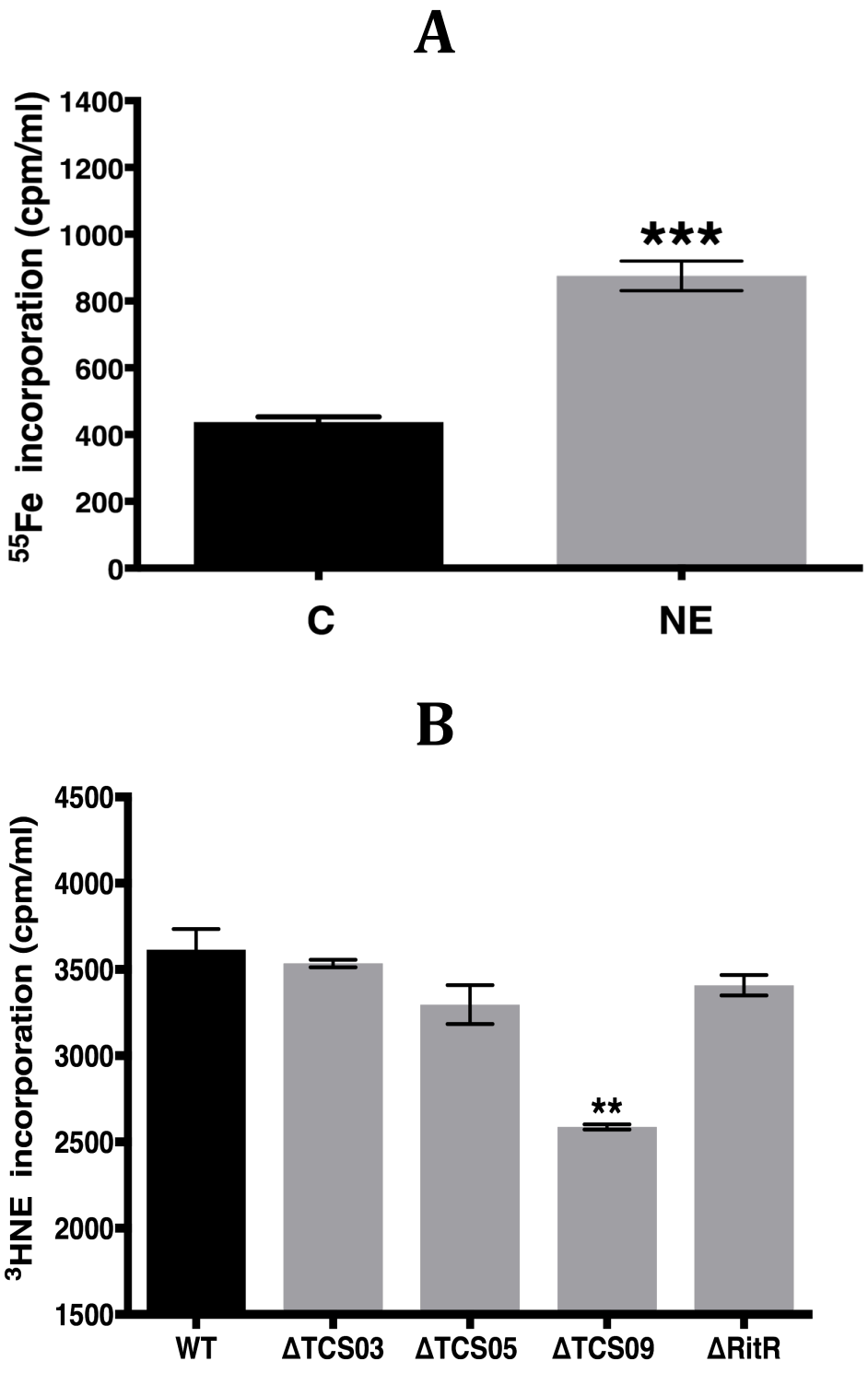
3.8. Radiolabelled Norepinephrine and transferrin uptake assays

Catecholamine hormones can enhance the growth of bacteria by mediating iron uptake from host Fe-binding protein such as transferrin. Hormones achieve this by assistance of their catechol moieties (Figure 1.3), which are able to bind and reduce ferric iron Fe (III) to ferrous iron Fe (II). This weakens the iron binding affinity of the transferrin, enabling bacteria to uptake the unrestricted iron via Fe (III)- or Fe (II)-uptake systems (Freestone et al., 2008a;Sandrini et al., 2010;Freestone et al., 2000). The pneumococci are able to utilise ferric and ferrous iron salts as well as host iron sources including Tf iron, haemin and haemoglobin (Brown et al., 2002;Brown et al., 2004;Tai et al., 1993). Although it has been shown that hormones such as NE can allow removal of iron from Tf, has not been identified the mechanism by which *S. pneumoniae* takes up iron from the NE-Tf complex. There are two possible models by which bacteria take up iron from NE-Tf complex, one is simply taking up the released iron via bacterial iron acquisition systems, and the second is NE acting like siderophores by chelating iron and transporting it to microbes where both NE-and Tf-derived iron are internalised. The iron-associated catechol moiety present in siderophores such as ferrichrome and enterobactin, is also found in catecholamine stress hormones as well as their related inotropes (Lyte and Freestone, 2010), (Figure 1.3). This understanding has shed some light on the underlying mechanism by which stress hormones stimulate bacterial

growth (Freestone et al., 2002). Since ΔTCS03, ΔTCS05, ΔTCS09, and ΔRitR mutants specifically displayed significant reduced growth rates and/or yields to at least two hormones in serum-containing medium, their potential involvements in stress hormone sensing, uptake or processing need more investigation. Therefore, it was essential to investigate Tf and NE uptake by pneumococcal strains following catecholamine encounter by including radiolabelled ^{55}Fe-Tf or ^{3}H-NE in the serum containing medium to explore the mechanism of stress hormone induced growth.

Radio-labelled iron transferrin (^{55}Fe-Tf) was used to elucidate the mechanism of catecholamine growth induction by NE. Data in Figure 3.29 A indicates that incubating *S. pneumoniae* wild type in serum-supplemented media with ^{55}Fe-Tf allowed it to acquire normally sequestered host Fe in higher amounts when NE was present 875.3 ± 44.24 cpm/ml, n=3 compared with NE-free culture 437.4 ± 9.18 cpm/ml, n= 3, ($P < 0.001$). Investigations were also undertaken to determine whether the NE was taken up by wild type *S. pneumoniae* during catecholamine growth induction by adding radio-labelled NE (^{3}H-NE) to the serum-medium. Figure 3.29 B shows that ^{3}H-NE was also internalised by the wild type pneumococcus (3545 ± 138.50 cpm/ml, n=3). Mechanistically, the results indicate that hormone-mediated iron delivery from Tf and NE internalisation probably explains growth enhancement by the catecholamine hormones in the serum medium seen in section B (3.3) and section C (3.5). In addition, Figure 3.29 B shows that the ^{3}H-NE was equally internalised by the wild type (3545 ± 138.50 cpm/ml, n=3), ΔTCS03 (3542 ± 14.38 cpm/ml, n=3), ΔTCS05 (3231 ± 130.30 cpm/ml, n=3) and ΔRitR (3374 ± 68.29 cpm/ml, n=3) strains while the uptake of the radiolabelled NE was significantly reduced in ΔTCS09 mutant (2592 ± 9.89 cpm/ml, n=3) compared to the wild type NE uptake ($P < 0.01$). Since the ΔTCS09 mutant showed reduction in ^{3}H-NE uptake, the mutant's ability for ^{55}Fe-Tf uptake was further tested in the presence and absence of cold not radiolabelled NE. Figure 3.29 C shows that the addition of NE significantly increased uptake of ^{55}Fe-Tf in both wild type and ΔTCS09 from 437.4 ± 9.18 cpm/ml to 875.3 ± 44.24 cpm/ml ($p < 0.001$) and from 356.9 ± 32.63 cpm/ml to 662.2 ± 6.47 cpm/ml, (n=3 each) ($p < 0.001$), respectively. More importantly, there was also a significant difference in the uptake of the ^{55}Fe-Tf uptake

between the wild type (875.3 ± 44.24 cpm/ml, n=3) and ΔTCS09 (662.2 ± 6.47 cpm/ml, n=3) in the presence of the cold NE ($p < 0.01$). Collectively, this data suggests that both NE and Tf-derived iron are internalised by pneumococci and TCS09 is important in mediating uptake of the catecholamine and catecholamine-released iron from Tf.



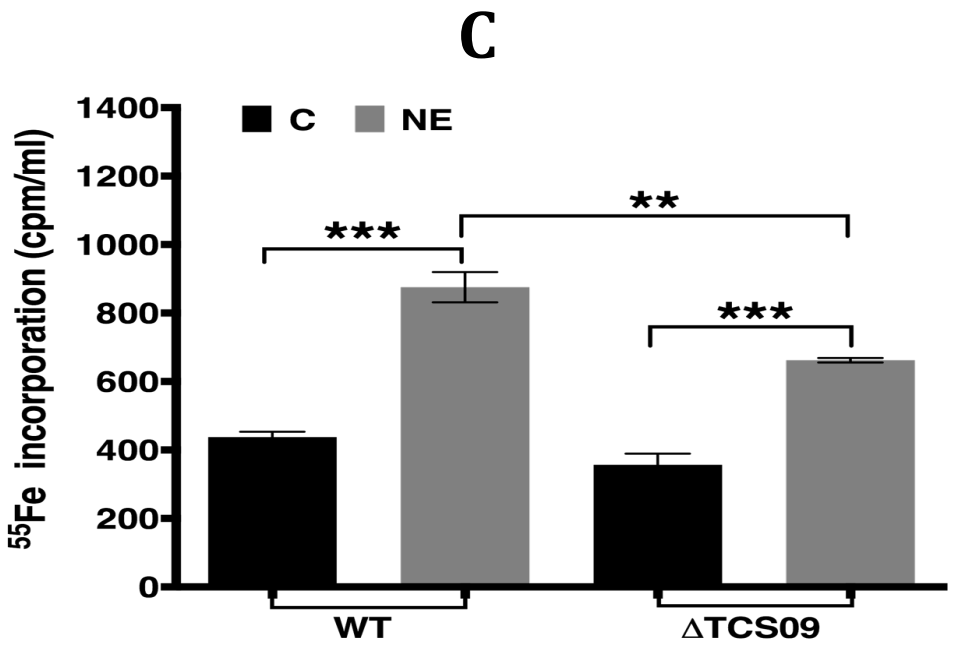


Figure 3.29. ^{55}Fe-Tf uptake by D39 (A). Transferrin uptake (in the form of ^{55}Fe) increased in the presence of 50 µM NE (grey bar) for wild type.**^{3}H-NE uptake by D39 and mutants (B).** ^{3}H-NE uptake of pneumococcal wild type (black bar), ΔTCS03, ΔTCS05, ΔTCS09 and ΔRitR mutants (grey bars) after 24 h incubation in serum-medium. **^{55}Fe-Tf uptake by D39 and ΔTCS09 (C).** Transferrin uptake (in the form of ^{55}Fe) increased in the presence of 50 µM NE (grey bars) for both wild type and ΔTCS09. The values shown are means of three experiments and error bars represent standard error of means (SEM). Unpaired t test was used for analysis of **A** and **C** while one-way ANOVA and Dunnett's multiple comparisons test was used for **B** (** $p<0.01$) and (*** $p<0.001$).

SUMMARY

1. NE and Tf-derived iron are internalised by *S. pneumoniae*.
2. *S. pneumoniae* acquires sequestered host Fe in higher amounts when NE was present suggesting a role of NE in Tf-derived iron delivery mechanism.
3. The radiolabelled NE (^{3}H-NE) was equally internalised by the wild type, ΔTCS03, ΔTCS05 and ΔRitR mutants indicating that TCS03, TCS05 and RitR are not involved in NE and Tf-iron uptake.
4. The uptake of both radiolabelled ^{3}H-NE and ^{55}Fe-Tf were significantly reduced in ΔTCS09 mutant compared to the wild type strain suggesting a role of TCS09 in mediating the uptake of the NE and NE-released iron from Tf.

3.9. Investigating the impact of stress hormones on pneumococcal biofilm formation

Bacterial ability to attach to different surfaces and form biofilms is an important aspect of virulence, particularly in the development of respiratory infections. The main characteristic of biofilms is the antibacterial resistance and the evasion of host immune responses due to the protective biofilm matrix, enabling bacterial persistence and dissemination (Costerton et al., 1999, Donlan and Costerton, 2002; Chole and Faddis, 2003; Lewis, 2008; Sanchez et al., 2011b). The clinical significance of *S. pneumoniae* biofilms is poorly understood despite the evidence of pneumococcal biofilm detection *in vivo* during infection courses of chronic rhinosinusitis, otitis media and pneumonia (Hall-Stoodley et al., 2006; Sanderson et al., 2006; Hoa et al., 2009; Reid et al., 2009; Sanchez et al., 2011b). Essentially, catecholamine-derived inotropes therapeutically used in the intensive care unit (ICU) are reported to increase the growth and biofilm formation within in-dwelling medical devices (e.g. intravenous lines) such as the case of the skin-dwelling *S. epidermidis*, which leads to subsequent infections in hospitalised patients (Lyte et al., 2003). Moreover, catecholamines as inotrope agents are given to approximately half of patients in the ICU and about 56% of pneumococcal-infected patients are admitted to the ICU (Smythe et al., 1993). More importantly, hospital-acquired pneumonia associated with ventilator known as ventilator-associated pneumonia (VAP) represents a major challenge in controlling bacterial pneumonia. VAP is defined as pneumonia occurring within 48-72 hours of endotracheal intubation and accompanied by signs of systemic infection, distinctive sputum characteristics and detection of an etiologic agent (Kalil et al., 2016). It accounts for approximately 50 % of all hospital-associated pneumonia cases and occurs in about 9-27% of all mechanically ventilated patients (Kalil et al., 2016;Vincent et al., 1995;Chastre and Fagon, 2002). In the ICU, VAP is the second most common nosocomial infection mainly affecting mechanically ventilated patients (Hunter, 2012;Afshari et al., 2012). Typically, *S. pneumoniae* is one of the causative agents of early-onset VAP along with *H. influenzae*, *S. aureus*, and some other antibiotic-sensitive enteric Gram-negative bacilli while late VAP are frequently caused by drug resistant bacteria including *P. aeruginosa,*

Acinetobacter and methicillin-resistant *S. aureus* (MRSA) (Hunter, 2012). A recent study found that NE treatment along with other host signals (such as ATP and glucose) on biofilms formed *in vitro,* and *in vivo* using biotic surfaces led to dispersion of the pneumococcus from the biofilm, and those dispersed cells were different in phenotype from those of both biofilm and broth-grown planktonic bacteria (Marks et al., 2013). In addition, a recent study demonstrated that the pneumococcal biofilm is also present during nasopharyngeal colonisation. (Marks et al., 2012a). Potentially, endogenous host stress hormones or exogenous catecholamine inotropes may stimulate biofilm formation during pneumococcal colonisation as well as invasive diseases. Therefore, the impact of stress hormones on pneumococcal biofilm formation was investigated.

This study analysed the effects of catecholamine hormones on biofilm formation of wild type D39 and selected mutants. Bacterial cultures were inoculated at a higher level of 10^7-10^8 CFU/ml to ensure that growth levels of control and catecholamine-treated cultures were the same. Firstly, the attachment of wild type strain was analysed and shown to be significantly enhanced after 16 hrs incubation in serum-medium in the presence of 2, 5, 10 and 50 μM of NE, Epi and Dop compared with hormone-free bacterial cultures (Figure 3.30). For instance, the figure shows that the biofilm of the pneumococcal wild type cells not treated with NE resulted in OD_{595} = 0.241 ± 0.041 whereas rising concentrations of NE considerably improved the biofilm for 2, 5, 10 and 50 μM of NE were 0.581 ± 0.019 ($p < 0.001$), 0.657 ± 0.046 ($p < 0.001$), 0.850 ± 0.054 ($p < 0.0001$) and 0.929 ± 0.043 ($p < 0.0001$), respectively (n =3 each). Similarly, it also reveals that the biofilm formation of the pneumococcal wild type cells not treated with Epi was 0.205 ± 0.017 while same concentrations of Epi significantly increased the biofilm which were 0.501 ± 0.007 ($p < 0.01$), 0.623 ± 0.049 ($p < 0.001$), 0.838 ± 0.048 ($p < 0.0001$) and 0.913 ± 0.063 ($p < 0.0001$), respectively (n =3 each). Lastly, the same figure shows that the biofilm of the pneumococcal wild type cells not treated with Dop was 0.291 ± 0.035 whereas the rising concentrations of Dop considerably improved the biofilm, which were 0.590 ± 0.047 ($p < 0.01$), 0.623 ± 0.051 ($p < 0.001$), 0.739 ± 0.041($p < 0.0001$) and 0.960 ± 0.026 ($p < 0.0001$), respectively (n =3 each).

After analysis of wild type, 10 μM of NE was chosen to investigate the biofilm formation of the selected mutants, which also displayed increased levels of biofilm formation in response to the hormone compared to their hormone-free culture (Figure 3.31). The OD_{595} of NE treated ΔTCS03, ΔTCS05, ΔTCS09 and ΔRitR mutants were 1.425 ± 0.117 ($p < 0.01$), 0.952 ± 0.076 ($p < 0.001$), 0.830 ± 0.029 ($p < 0.0001$) and 0.860 ± 0.121 ($p < 0.05$), relative to their hormone-free cultures which were 0.518 ± 0.051, 0.191 ± 0.003, 0.251 ± 0.019 and 0.305 ± 0.012, respectively (n =3 each). When comparing the response to 10 μM NE of the mutants to that of the wild type, all mutants displayed wild type-like biofilm formation. However, it should be particularly emphasised that ΔTCS03 strain produced a greater amount of biofilm compared to that of wild type which were 1.424 ± 0.117, n =3 and 0.746 ± 0.081, n =3, respictively, ($p < 0.01$). As a result, increasing concentrations of NE (2, 5, 10 and 50 μM) were used to compare the biofilm formation of the wild type strain to that of the ΔTCS03 mutant (Figure 3.32). It shows that the biofilm formation of the pneumococcal wild type cells not exposed to NE resulted was 0.241 ± 0.042, n=3 whereas the same concentrations of NE remarkably elevated the biofilm which were 0.581 ± 0.020 ($p < 0.001$), 0.657 ± 0.046 ($p < 0.001$), 0.850 ± 0.054 ($p < 0.0001$) and 0.929 ± 0.043 ($p < 0.0001$), respectively (n =3 each). Interestingly, the same concentrations of NE resulted in a much greater biofilm formation by the ΔTCS03 mutant, which were 0.963 ± 0.102 ($p < 0.0001$), 1.322 ± 0.119 ($p < 0.0001$), 1.486 ± 0.136 ($p < 0.0001$) and 1.792 ± 0.108 ($p < 0.0001$), respectively, relative to its control (hormone-free culutre of ΔTCS03) which was 0.285 ± 0.044 (n =3 each). The distinct phenotype of this mutant was not related to NE exposure in serum medium since this mutant also showed enhanced biofilm formation in BHI medium compared to wild type behaviour suggesting a role of this TCS in mediating biofilm formation independent of its involvement in stress hormone recognition and processing (data not shown). Collectively, different levels of stress hormones do have impact on the biofilm formation of *S. pneumoniae* by enhancing the attachment on abiotic surfaces and none of the selected mutants showed a reduction in their biofilm forming ability in response to the stress hormone NE.

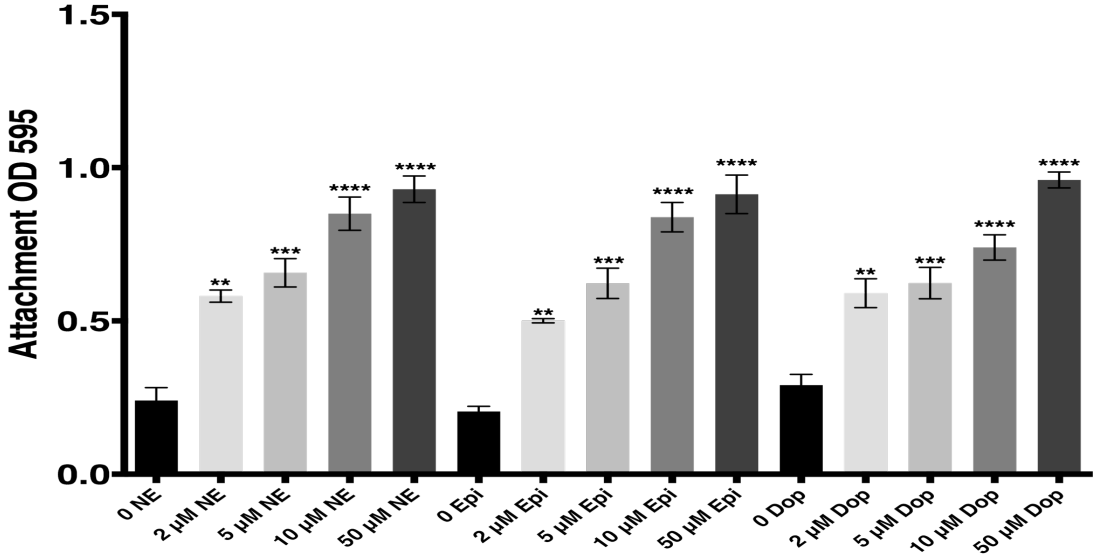


Figure 3.30. Catecholamine hormones stimulate pneumococcal biofilm formation. The attachment of wild type D39 after 16 hrs incubation in the serum-medium −/+ 2, 5, 10 and 50 µM of NE, Epi and Dop respectively. Black bar represents the attachment of wild type in the absence of the hormones (control). The columns shown are means of at least three independent experiments and error bars represent standard error of means (SEM). The significant differences were seen comparing the stress hormone response of each hormone to its control using one-way ANOVA and Dunnett's multiple comparisons test (** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$ relative to hormone-free sample).

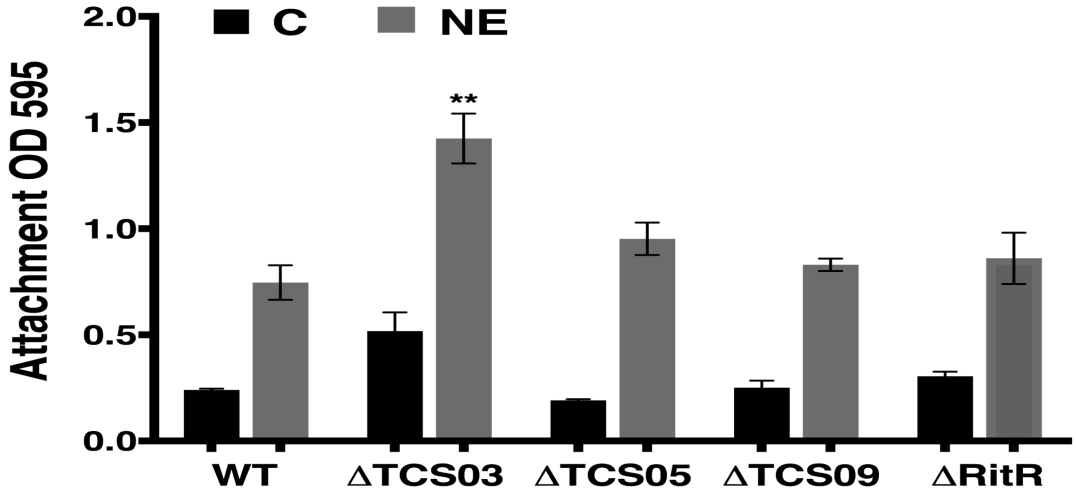


Figure 31. NE enhances biofilm formation of WT and mutant strains. The attachment of wild type D39 after 16 hrs incubation in the serum-medium with (grey bar) or without (Black bar) 10 µM of NE. The values shown are means of at least three independent experiments and error bars represent standard error of means (SEM). The significant differences seen were analysed by comparing stress hormone response of the wild type strain to those of mutant strains using one-way ANOVA and Dunnett's multiple comparisons test.

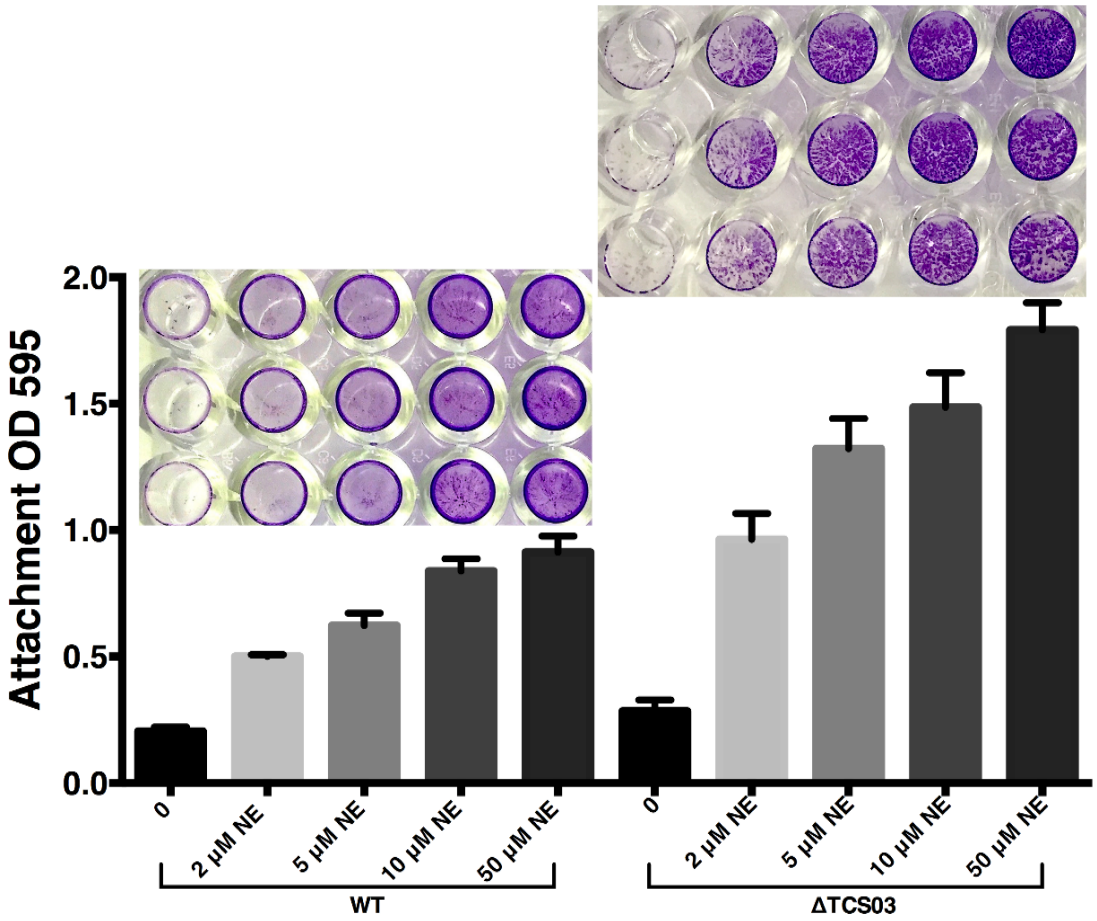


Figure 3.32. Demonstration of biofilm formation of the wild type strain and ΔTCS03 mutant. The attachment of wild type D39 and ΔTCS03 mutant after 16 hrs incubation in the serum-medium −/+ 2, 5, 10 and 50 µM of NE. Black bar represents the attachment in the absence of the hormones (control). The vertical bars shown are means of at least three independent experiments and error bars represent standard error of means (SEM).

SUMMARY

1- Various concentrations of NE, Epi and Dop enhanced pneumococcal biofilm formation.

2- ΔTCS05, ΔTCS09 and ΔRitR mutants displayed WT-like biofilm suggesting that they have no involvement in stress hormone-stimulated biofilm formation.

3- ΔTCS03 displayed the greatest biofilm formation in response to NE indicating a role of this TCS in mediating biofilm formation independent of its involvement in stress hormone recognition and processing.

Section D: Impact of stress hormones on cell morphology and metabolism

Since stress hormones have greatly affected pneumococcal growth yields and rates as well as biofilm formation in nutrient-limited serum medium as seen in section B (3.3) and C (3.5 and 3.9), it was not known whether hormones would also have an impact on pneumococcal morphology and metabolism.

3.10. Effect of NE on cell arrangement and size

Growth rate and nutrient availability are well-recognised factors that can influence bacterial cell size (Chien et al., 2012). Over 50 years ago, Schaechter, Maaløe, and Kjeldgaard were the first to observe that bacterial size is correspondent with growth rate depending on nutrient condition. They noted that Salmonella cells were double the size when incubated under conditions where growing rate was rapid in rich medium compared to the cells incubated at slower growth rates in a poor nutrient source (Schaechter et al., 1958;Kjeldgaard et al., 1958). Later research found that this nutrient-dependent control of cell size is also the case for many bacteria including Gram positive-bacteria such as *B. subtilis* (Pierucci et al., 1987;Sargent, 1975) as well as *S. aureus*, where glucose limitation leads to 40% reduction in cell size (Watson et al., 1998). Stress hormones have never been tested to establish if they exert their effect on bacterial size additional to their direct impact on growth and virulence. Therefore, pneumococcal cells of the wild type D39 strain were examined under the light microscope in the presence and absence of 50 μM NE in serum-containing media. This study has found that *S. pneumoniae* displayed a distinctive cell morphology after treatment with NE compared to the untreated cultures. Light microscopy images of both sample groups showed significant changes in bacterial cell arrangement as well as cell size. Cell to cell association has clearly transformed from short chains in the control sample, which was not treated with NE, into diplococci in the hormone treated sample (Figure 3.33 A and B). Furthermore, pneumococcal cell size became

significantly smaller after hormone treatment relative to untreated cultures (0.46 µm ± 0.007 and 0.79 µm ± 0.013, n=78 each), respectively. The difference was statistically significant ($p < 0.0001$) (Figure 3.34 A, B and C). In conclusion, this study found that the pneumococcus response to the catecholmine hormone NE resulted in a remarkable change in pneumococcal morphology from chains into diplococci with a significant reduction in cell size.

Figure 3.33.A

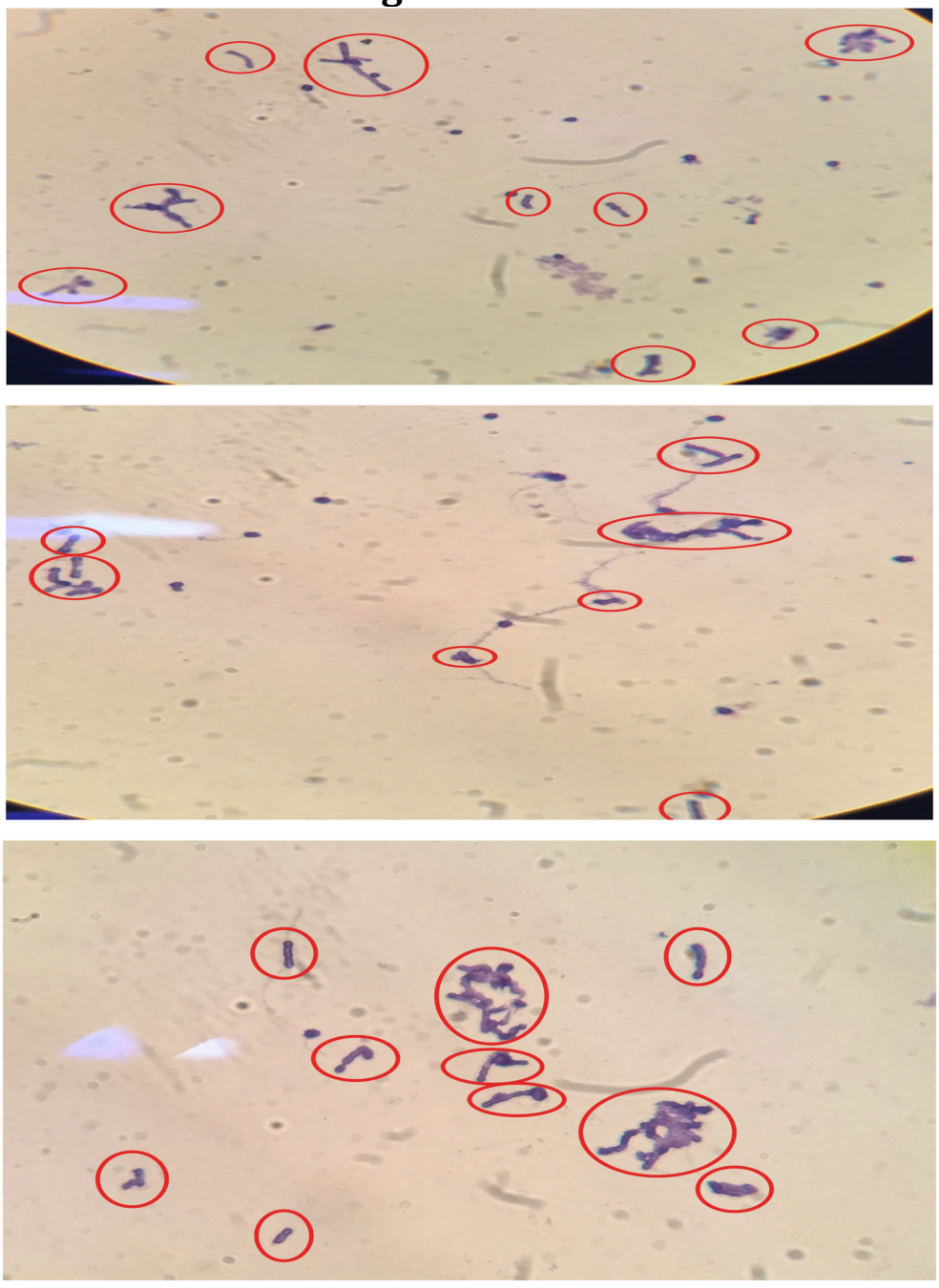


Figure 3.33.B

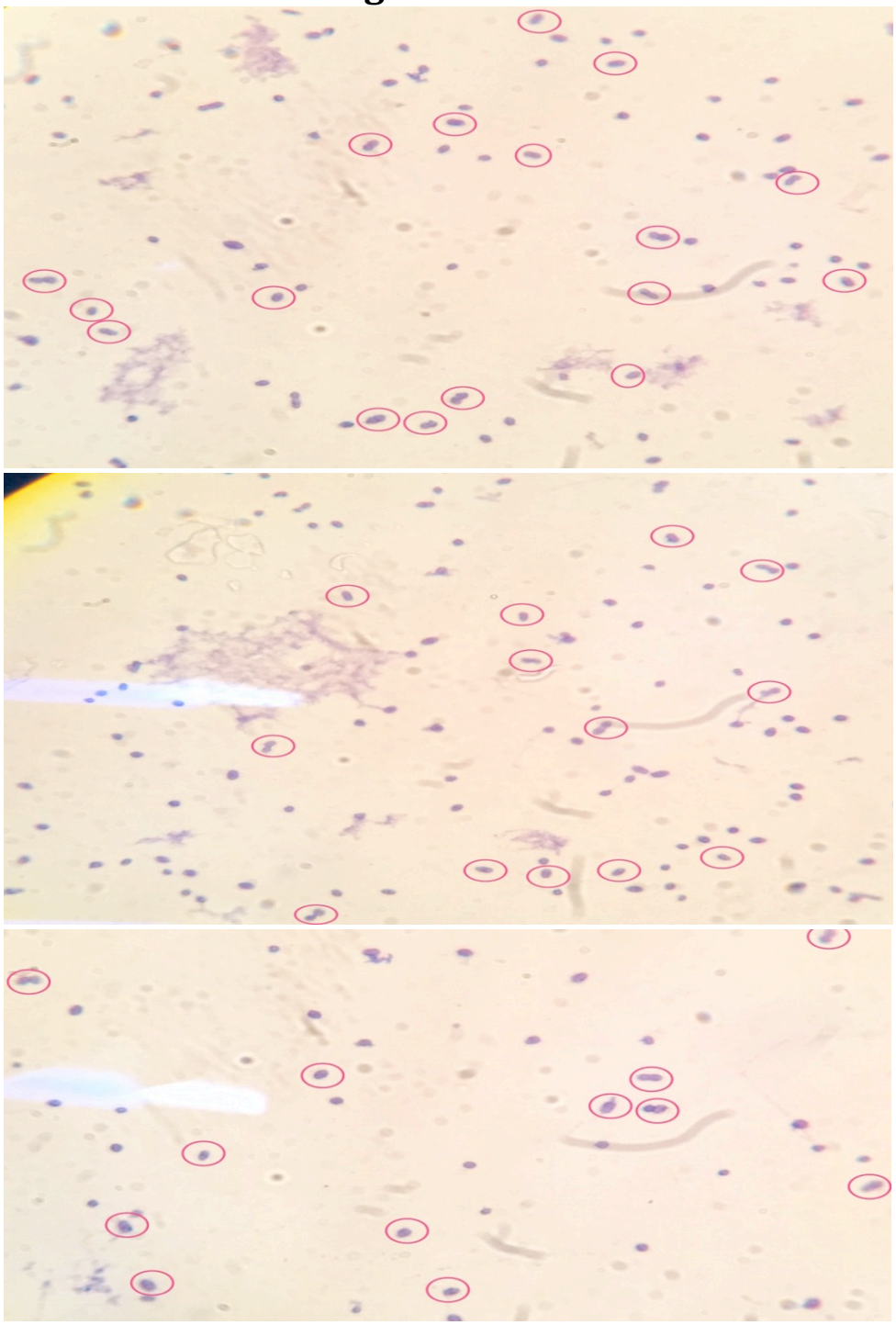


Figure 3.33. Phenotypic characterization of norepinephrine-exposed pneumococci. Cell to cell associations before (**A)** and after (**B**) NE exposure. Light microscopy images of Gram stained *S. pneumoniae* strain D39 (x100 magnification) after growth overnight in serum-SAPI with no additions appearing mostly in chains (**A**) or growth in serum-SAPI in the presence of 50 μM NE appearing mostly in diplococci (**B**); n=3.

Figure 3.34.A

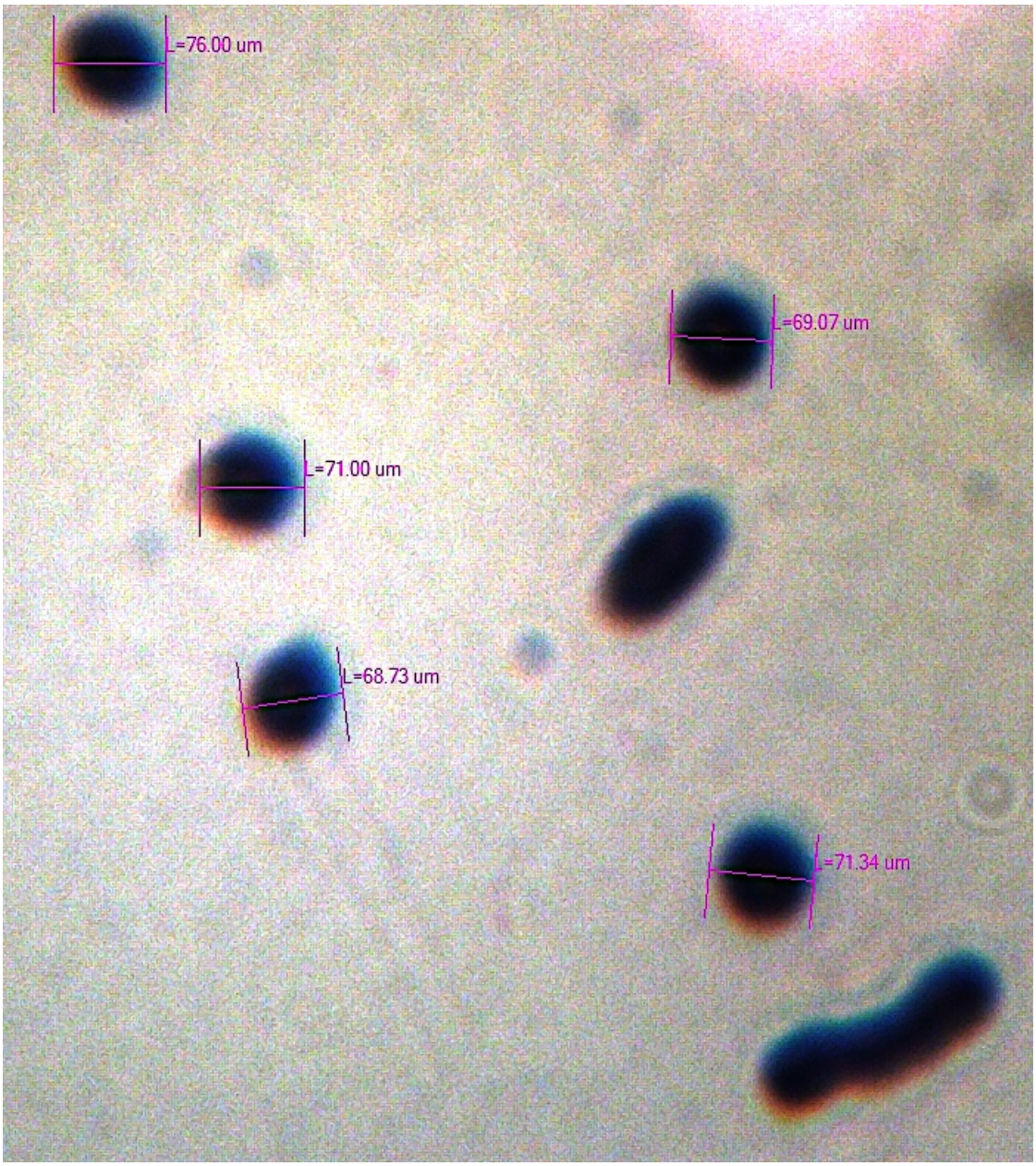


Figure 3.34.B

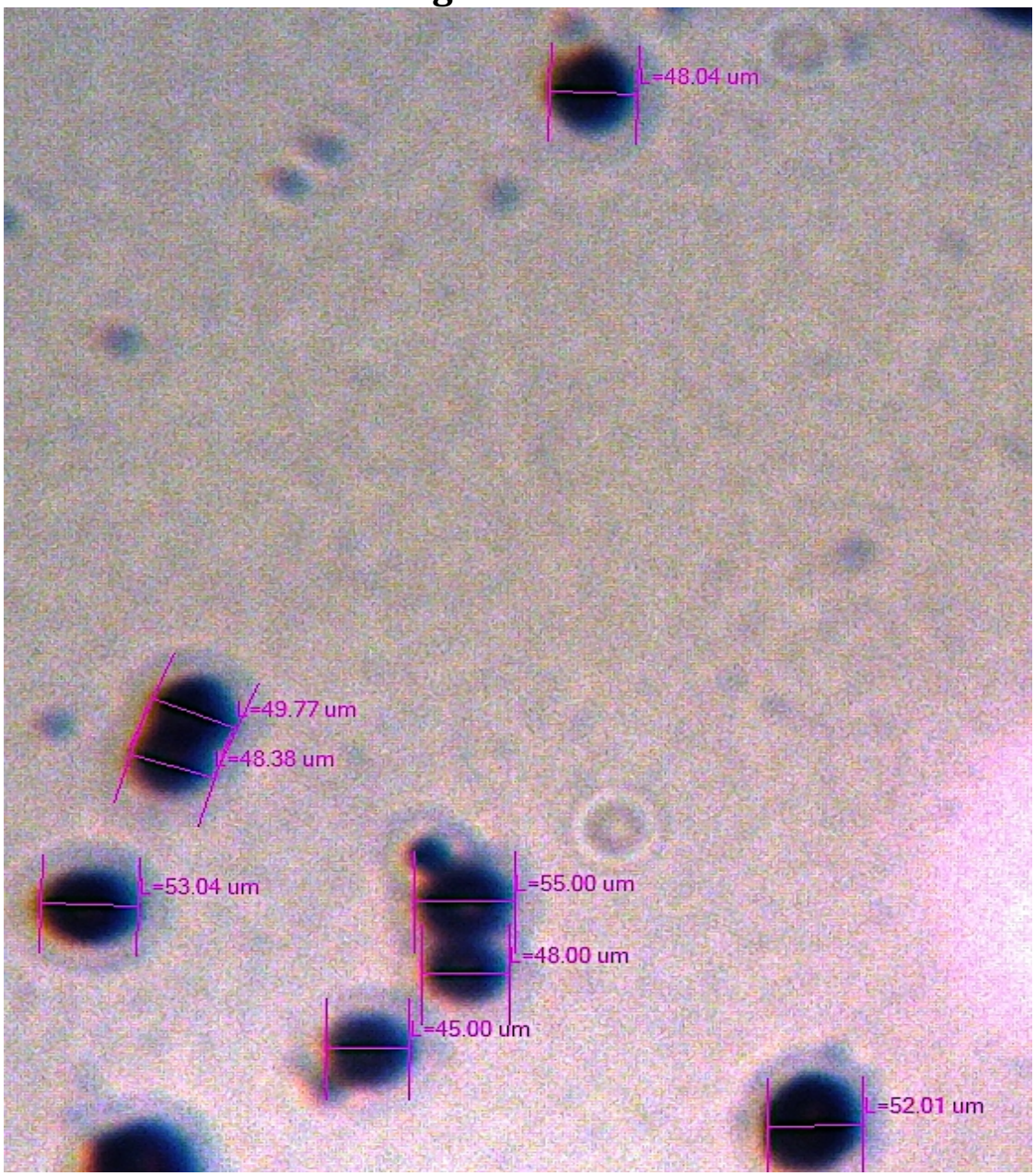

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Figure 3.34.C

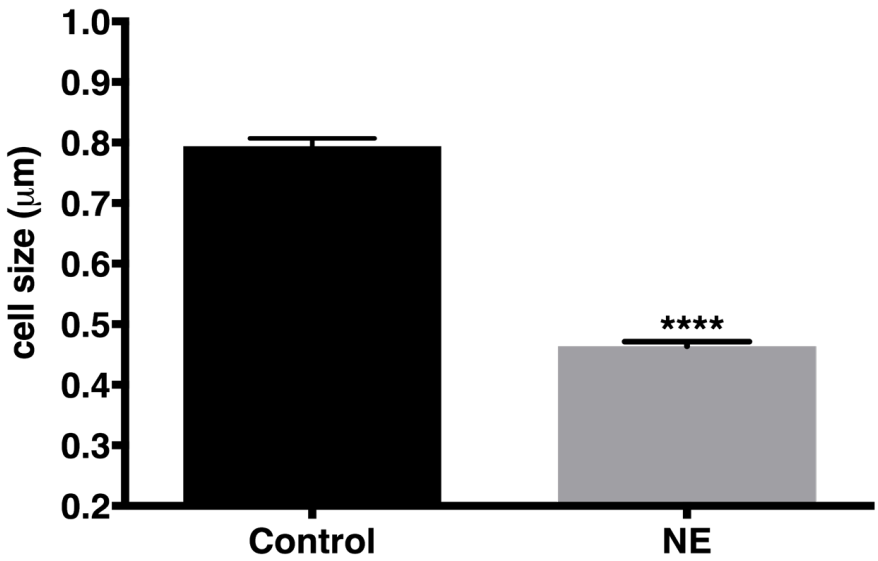


Figure 3.34. Phenotypic characterization of norepinephrine-exposed pneumococci. A and **B** demonstrate representative examples of *S. pneumoniae* strain D39 cell size after growth overnight in serum-SAPI with no additions **(A)** or in the presence of 50 μM NE (**B**). The vertical bars in **(C)** show the mean size of 78 cells (each) measured from the cultures shown in **A** and **B** using INFINITY 1 camera and INFINITY ANALYZE imaging software. The error bars represent standard error of means (SEM). Unpaired t test was used for analysis (**** $p < 0.0001$).

3.11. Effect of NE on capsular polysaccharides

The question that arose after finding that NE can significantly enhance biofilm formation and decreased pneumococcal cell size was would the capsular polysaccharide be also affected by hormone exposure? Clinical and experimental studies have shown that pneumococcal capsular polysaccharide is the most important virulence determinant for the pneumococcus. It is essential for colonisation, invasion, and dissemination from the respiratory tract. The capsule also protects the pneumococcus against mechanical clearance by mucous secretion (Nelson et al., 2007). There are over 90 serotypes of *S. pneumoniae* based on the capsule and those with higher carriage prevalence produce thicker capsules than low carriage prevalence serotypes (Weinberger et al., 2009). In addition, it has been shown that pneumococci

not in contact with the epithelial cells did not alter their capsular polysaccharide. However, when infecting the cells, the pneumococcus exhibited up-to-10^5 enhanced capacity to adhere and to invade the epithelial cells, and scanning electron microscopy revealed a reduction in pneumococcal capsular materials (Hammerschmidt et al., 2005). The influence of hormones on pneumococcal capsule has never been studied before, and to further investigate the cell size reduction and to determine whether NE would affect capsule synthesis differently, glucuronic acid was quantified. Analysis of *S. pneumoniae* type 2 capsular polysaccharides indicates that it is composed of D–glucuronic acid, D-galactose and L-rhamnose residues (Jansson et al., 1975). Therefore, glucuronic acid concentration was measured as an indicator component of pneumococcal capsular polysaccharide production. Consistent with the cell size reduction, NE-treated pneumococci had significantly lower glucuronic acid levels (46.94 ± 0.59 μg/$\log_{10}$ 6 CFU, n=3) compared to untreated pneumococci (59.80 ± 2.02 μg/ $\log_{10}$ 6 CFU, n=3) ($P < 0.01$) (Figure 3.35). Collectively, the results suggested that there is an influence of hormones over the synthesis of the pneumococcal capsular polysaccharides, and the decrease observed in cell size due to hormone exposure was also affecting glucuronic acid content within the capsular polysaccharides.

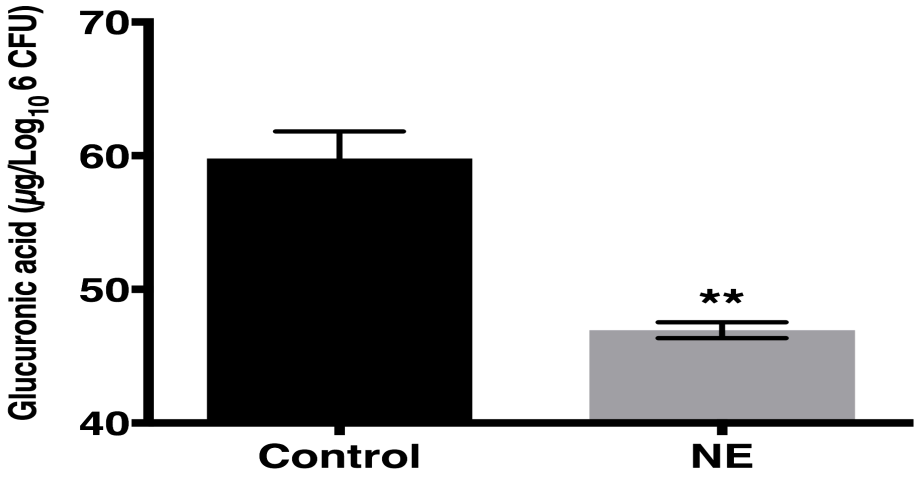


Figure 3.35. Pneumococcal glucuronic acid before and after NE treatment. Analyses of *S. pneumoniae* strain D39 glucuronic acid after growth overnight in serum-SAPI with no additions (black bar) or in the presence of 50 μM NE (grey). NE exposed pneumococcal cultures showed significantly lower glucuronic acid levels compared with the untreated samples in serum medium. The values shown are means of three experiments and error bars represent standard error of means (SEM). Unpaired t test was used for analysis (** $p < 0.01$).

3.12. Effect of NE on cell metabolism

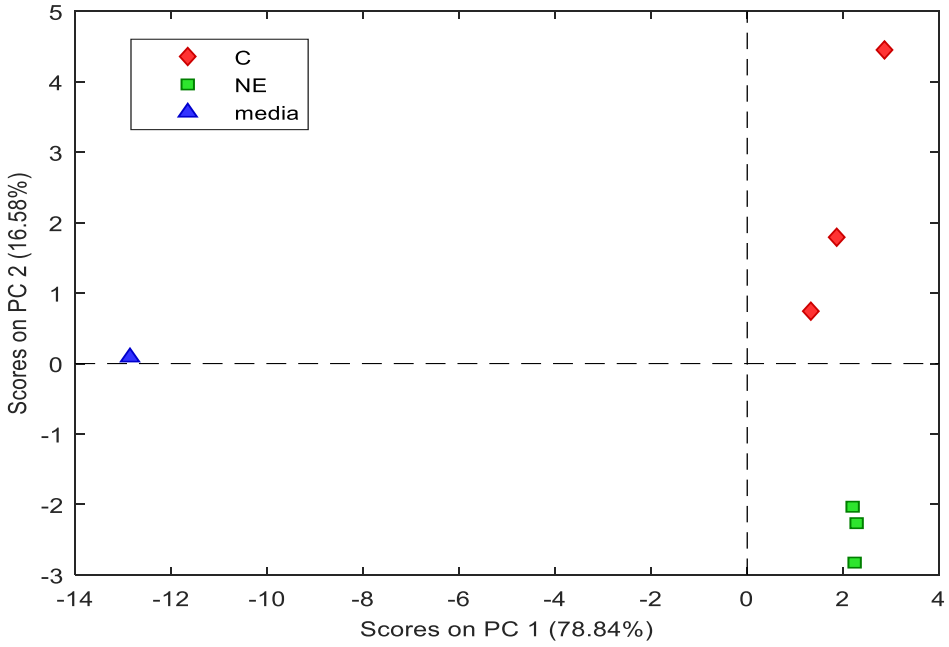
The investigations of pneumococcal metabolism before and after hormone interaction was carried out for many reasons. Firstly, it has been reported that therapeutic levels of noradrenaline markedly alter expression of genes involved in metabolism and virulence (Sandrini et al., 2014). Secondly, hormones have been shown to increase pneumococcal growth by iron delivery mechanism as seen in NE and Tf uptake assays. Iron plays an essential role for growth within the host and for cellular processes such as energy production, oxygen transport and DNA replication (Skaar, 2010). Iron availability by the hormone in poor nutrient environment such as the serum medium used in growth assays may alter pneumococcal metabolism accordingly. The third reason is that the change of pneumococcal growth, cell size, capsule and biofilm formation suggest that hormones could have broader effects that would include cell metabolism. In addition, since cell size (determined by growth rate depending on nutrition availability) was reduced due to hormone exposure in serum nutrition-limited medium, investigating pneumococcal metabolites under such conditions was carried out with and without NE addition. To date, hormone effects on pneumococcal metabolism have never been tested using metabolomics analyses.

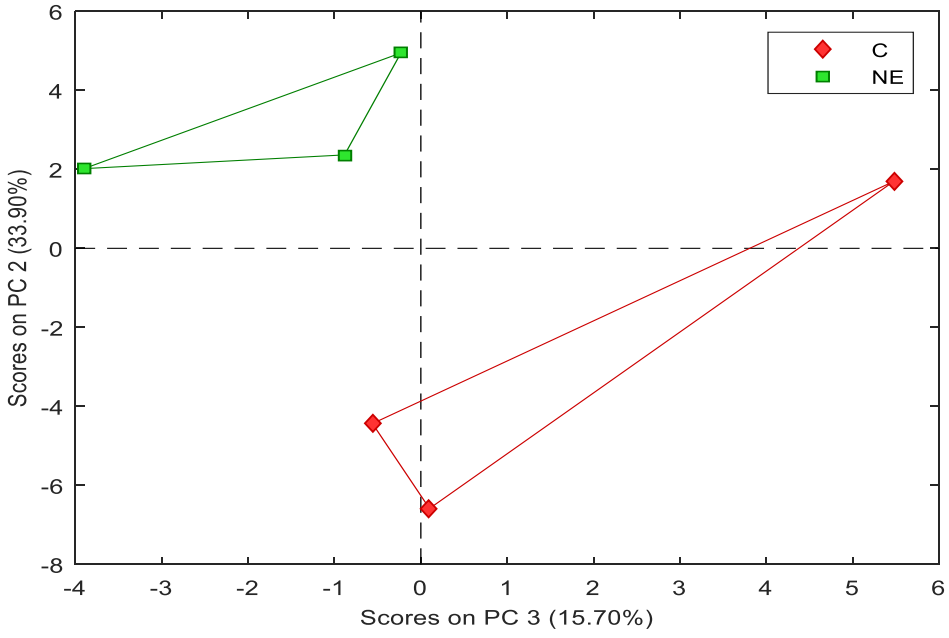
The pneumococcal metabolites were analysed using Gas Chromatography – Mass Spectrometry (GC-MS), which is one of the most applied analytical tools in metabolomics. GC-MS is capable of identifying hundreds of metabolites due to its high separation power. The intracellular and extracellular metabolites of *S. pneumoniae* grown in the presence and absence of NE have for the first time been profiled using GC-MS. Figure 3.36 A shows the summary data analysis score of the levels of 94 metabolites and reveals a clear difference between the compounds present in the control and NE-treated cultures. Figure 3.36 B shows a comparison of the levels of some key metabolites. Interestingly, relative to controls pyruvic acid and alanine levels were consistently lower in the NE-treated *S. pneumoniae* metabolite pool while the fumaric acid level was higher.

More detailed analysis of identified tricarboxylic acid (TCA) amongst extracellular metabolites (Figure 3.36 B) revealed that pyruvic acid, for example, was significantly consumed from the serum-SAPI medium (0.979 ± 0.006 mM) by both control and the NE-treated group; 0.748 ± 0.027 mM ($p < 0.001$) and 0.578 ± 0.012 mM ($p < 0.0001$), respectively (n = 3 each). Surprisingly, fumaric acid was significantly produced by both NE-treated and control groups compared to the media with higher production of NE-treated group which was 0.008 ± 0.001 mM ($p < 0.0001$) while the control group was 0.006 ± 0.001 mM ($p < 0.0001$), relative to the medium (0.004 ± 0.001 mM) (n = 3 each). Predictably, lactic acid was produced in high quantities by both NE-treated and control groups, which was 2.069 ± 0.027 mM ($p < 0.0001$) and 2.112 ± 0.048 mM ($p < 0.0001$), respectively, relative to the medium (1.275 ± 0.058 mM) (n = 3 each). This was also seen with malic acid which was significantly produced by both NE-treated and control groups compared to the media, which was 0.027 ± 0.001 mM ($p < 0.0001$) and 0.026 ± 0.001 mM ($p < 0.0001$), respectively, relative to the medium (0.015 ± 0.001 mM) (n = 3 each). In addition, alpha-ketoglutaric acid, was significantly consumed from the serum-SAPI medium (0.091 ± 0.001 mM) by both control and NE-treated group; 0.052 ± 0.005 mM ($p < 0.001$) and 0.044 ± 0.004 mM ($p < 0.001$), respectively (n = 3 each). Analysis of amino acids also showed clear differences between both groups. For example, alanine was significantly consumed in both NE-treated and control cultures compared to the media with lower consumption within NE-treated group which was 0.118 ± 0.001 mM ($p < 0.0001$) while the control group was 0.136 ± 0.004 mM ($p < 0.001$), relative to the medium (0.158 ± 0.001 mM) (n = 3 each). This was also seen in glycine, which was significantly consumed in both NE-treated and control cultures compared to the media with slightly lower consumption within NE-treated group which was 0.017 ± 0.001 mM ($p < 0.0001$) while the control group was 0.032 ± 0.001 mM ($p < 0.0001$), relative to the medium (0.092 ± 0.001 mM) (n = 3 each). More analysis of amino acid release and other components of extracellular and intracellular metabolites can be found in appendix in page 158. In conclusion, the metabolites of *S. pneumoniae* grown in the presence and absence of NE have for the first time been profiled using GC-MS. The analysis score of the levels of 94 metabolites revealed a clear difference between the compounds present in the control and NE-treated cultures. As expected

from a lactic acid-producing microorganism, lactic acid was significantly produced by both NE-treated and control groups compared to the media. Interestingly, relative to controls pyruvic acid and alanine levels were consistently lower in the NE-treated *S. pneumoniae* metabolite pool while the fumaric acid level was higher.

A





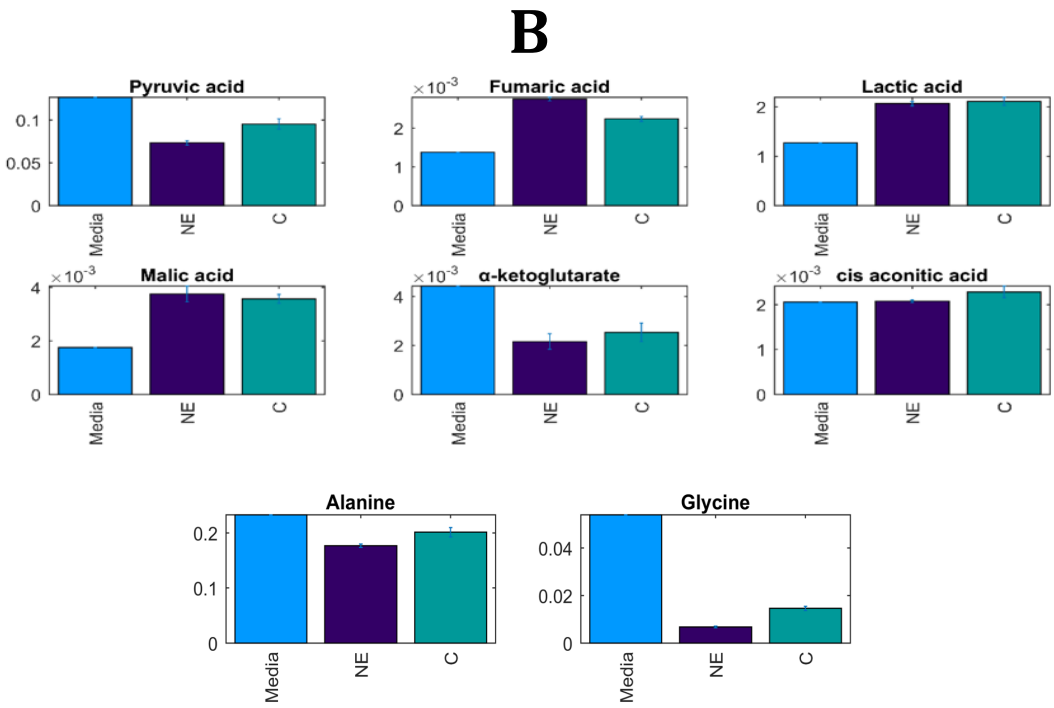


Figure 3.36. Metabolomics profiling of *S. pneumoniae* strain D39. Figure **A** shows the summary data analysis score of analysis of the levels of 94 extracellular and intracellular metabolites, respectively, of *S. pneumoniae* strain D39 after growth overnight in serum-SAPI (Media) with no additions (C) or in the presence of 50 µM NE (NE); n=3 (Score plot from principal component analysis (PCA) model calculated on the relative concentrations of the variables in the reduced dataset). **B** histograms shows a comparison of the levels of some externally released key TCA and amino acid metabolites; n=3.

SUMMARY

1. The pneumococcal response to NE resulted in a remarkable change in cell-cell association from chains into diplococci and reducing cell size and the capsule.
2. The metabolites of *S. pneumoniae* grown in the presence and absence of NE have for the first time been profiled using GC-MS.
3. The analysis of 94 metabolites revealed a clear difference between the compounds present in the control and NE-treated cultures.
4. Lactic acid was significantly produced by both NE-treated and control groups.
5. Pyruvic acid and alanine levels were lower in the NE-treated *S. pneumoniae* metabolite pool while the fumaric acid level was higher relative to the control.

Section E. *in vivo* study

3.13. Role of NE in pneumococcal invasiveness

Having used *in vitro* models to test the effects of catecholamine hormones on pneumococcal phenotype, it was next tested whether the hormones would also have an influence on *in vivo* phenotype using established mouse models for pneumococcal colonisation and disease (Al-Bayati et al., 2017;Kahya et al., 2017). Although the serum media that reflects the same environment that bacteria must survive in was used *in vitro* assays, the mechanisms underlying the bacteriaum's ability to cause disease will never be fully understood without host factors *in vivo* experiments. The increase of growth yield and rate while reducing cell size and capsule materials as well as enhanced biofilm formation after NE exposure *in vitro* would suggest that the hormone may also potentially initiate pneumococcal pathogenesis and trigger its transition from colonisation of the nasopharynx into invasion of the lungs and deeper tissues. Due to the reports that TCS mutants had attenuated virulence as reported in (Paterson et al., 2006), they were not tested in the animal study. Hence, a stock of serotype-2, wild type D39, was investigated for the role of stress hormone NE in pneumococcal transition from colonisation to invasive state in mice.

Prior to *in vivo* experiments, bacterial inoculum was prepared in two ways for infection to explore the effect of short and long-term exposure of NE on the pneumococcus. The first one was prepared by exposing the bacterial inoculum to NE or PBS for short time prior to the administration of infection dose into the mice. This inoculum was designated as "NE-short". The second bacterial inoculum, on the other hand, was prepared by exposing the pneumococci to NE or PBS for a much longer period in order to investigate the long-term effects of the hormone on pneumococcal behaviour. This inoculum was designated as "NE-long". Hence, the inoculum was prepared by growing the bacteria overnight with and without the hormone on serum-SAPI as previously described in section 2.3.

Pneumococci pre-treated with NE or PBS as a control were administered intranasally into CD1 mice strains and over time the numbers of bacteria were determined by serial dilution of nasal wash, bronchoalveolar lavage (BAL) wash and homogenized lung tissue of infected mice at the time of infection, 2 and 7 days after infection. Nasopharyngeal samples were analysed to assess the effect of NE on pneumococcal colonisation while samples of BAL fluids and lung tissues, the "gold standard" for pneumonia diagnosis in microbiological studies (Song et al., 2013), were analysed to evaluate the pneumococcal presence in mucosal surfaces of bronchoalveolar lavage and lung tissues. All mice were infected intranasally with 10 µl of PBS or NE containing 4.5×10^5 to 5×10^5 CFU / mouse. At different time intervals, mice were scarified, nasopharyngeal, BAL and lung samples were collected, and analysed. Then, viable plate counts were obtained, and Log_{10} CFU / sample was determined. The number of colony forming unit of the lung samples was assessed per mg of homogenized tissue.

Immediately after intranasal infection, the bacterial load in the nasopharyngeal wash samples for NE-short (Log_{10} 4.13 ± 0.07 CFU/ml, n=4) and control groups (Log_{10} 4.18 ± 0.12 CFU/ml, n=4) were the same ($p > 0.05$) confirming that both groups received similar inoculum size (Table 3.3 A and Figure 3.37 A). The analysis of samples obtained from the nasopharynx revealed that there were no significant differences of bacterial load between the two groups at all time points (D0: at zero time, D2: 2 days post-infection and D7: 7 days post-infection) under short exposure of NE. This indicates that pre-treatment of the pneumococcus with NE for short time prior to the infection did not affect the number of bacteria colonising the nasopharynx compared with the control bacteria. For example, the bacterial load in the nasopharynx at day 2 was the same for the control and NE in the short exposure group, which was Log_{10} 3.97 ± 0.15 and 3.99 ± 0.19 CFU/ml, (n=4 each), ($p > 0.05$) respectively. In addition, although the bacterial load decreased at day 7 compared to day 0 and 2 in the control and NE-short groups, it remained the same, which was Log_{10} 3.81 ± 0.20 and 3.48 ± 0.19, CFU/ml, (n=4 each), ($p > 0.05$), respectively (Table 3.3 A and Figure 3.37 A).

With regards to BAL samples, few bacteria were detected in both groups at day zero with no significant difference while no bacteria were recovered in day 2 and 7 in both control and NE-short groups (data not shown). Interestingly, analysis of bacterial loads in the lung tissue samples of the NE-short group showed that bacterial presence in the lung at day 2 was detected in 1/4 of the mice whereas not at all in the control group. In addition, bacterial dissemination to the lung at day 7 was detected in 75% of mice infected with pneumococci exposed to NE compared with 50% in control group (Table 3.3 B and Figure 3.37 A).

On the other hand, the bacterial load for NE-long and control groups was Log_{10} 3.57 ± 0.11 and 3.48 ± 0.08 CFU/ml, (n=4 each), respectively ($p > 0.05$ confirming that both groups received similar inoculum size. (Table 3.3 A and Figure 3.37 B). The analysis of samples obtained from the nasopharynx revealed that there were no significant differences of bacterial load between the two groups at all time points (D0: at zero time, D2: 2 days post-infection and D7: 7 days post-infection) under long exposure of NE. This indicates that pre-treatment of the pneumococcus with NE for a long period prior to infection did not affect the number of bacteria colonising the nasopharynx compared with the control bacteria. For example, the bacterial load in the nasopharynx at day 2 was the same for the control and NE-long group, which was Log_{10} 3.94 ± 0.18 and 3.81 ± 0.16 CFU/ml, (n=4 each), ($p > 0.05$) respectively. In addition, the bacterial load at day 7 was similar in the control and NE-long group, which was Log_{10} 3.44 ± 0.52 and 3.43 ± 0.10 CFU/ml, (n=4 each), ($p > 0.05$), respectively (Table 3.3 A and Figure 3.37 B).

With regards to BAL samples, few bacteria were detected in both groups at day zero with no significant difference while no bacteria were recovered at day 2 and 7 in both control and NE- long groups (data not shown). Interestingly, analysis of bacterial loads in the lung tissue samples at day 7 indicated that long NE exposure rendered the pneumococci 50% more likely to disseminate into the lungs compared with the control group after one week of intranasal infection. (Table 3.3 B and Figure 3.37 B).

Table 3.3: NE impact on pneumococcal nasopharyngeal colonisation **(A)** and on dissemination to the lung **(B).** 48 mice were infected intranasally with 10 µl of PBS or NE containing 4.5-5×10^5 CFU/mouse. At different time intervals, mice were scarified, nasopharyngeal samples were analysed. Then, the viable plate counts were obtained, and Log_{10} CFU/sample was determined. Data are the mean of 4 mice and '±' represents the standard error of mean. D0: zero time, D2 and D7: 2 and 7 days post-infection.

A

Groups	D0	D2	D7
Control	4.18±0.12	3.97±0.15	3.81±0.20
NE-short	4.13±0.07	3.99±0.19	3.48±0.19
Control	3.57±0.11	3.94±0.18	3.44±0.52
NE-long	3.48±0.08	3.81±0.16	3.43±0.10

B

Groups	D0	D2	D7
Control	0.00±0.00	0.00±0.00	2.20±1.28
NE-short	0.00±0.00	0.92±0.93	2.84±0.96
Control	0.00±0.00	0.00±0.00	0.00±0.00
NE-long	0.00±0.00	0.00±0.00	1.67±0.097

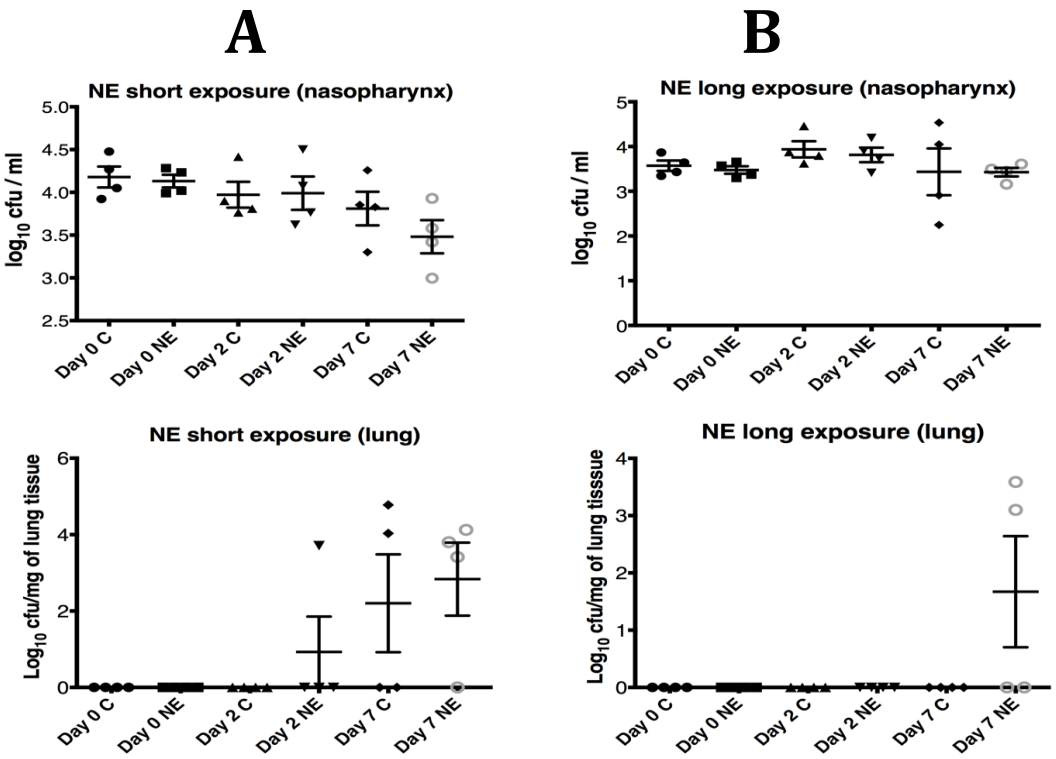


Figure 3.37. The effect of NE on pneumococcal colonisation in the nasopharynx and dissemination to the lung. A (NE short exposure) and **B** (NE long exposure) represent nasopharyngeal wash and lung tissue samples of control (C) and NE-short/long groups (NE) collected at three-time points (0, 2 and 7 days; n=4 each).

To confirm the finding, a second infection experiment was carried out only with NE-long treatment. The results 7 days post infection showed that bacteria pre-treated with NE were able to disseminate in the lung more efficiently than the control groups with 10/10 the group of mice infected with pneumococci exposed to NE compared with 4/10 of control group. Moreover, the mean of bacterial loads in the lung tissue of the group of mice infected with pneumococci exposed to NE was significantly higher than the mean of those infected with PBS; Log_{10} 2.34 ± 0.17, and 0.87 ± 0.36, mg^{-1} tissue, (n=10 each), respectively ($P < 0.01$) (Table 3.4 and Figure 3.38). In conclusion, although these results suggest that stress hormone exposure does not enhance pneumococcal colonisation in the nasopharynx over time, it clearly increases the likelihood of pneumococcal transition to the lungs, which is an important factor for the invasiveness of *S. pneumoniae.*

Table 3.4: The effect of NE on pneumococcal dissemination to the lung. In a second independent experiment, all mice were infected intranasally with 10 µl of pneumococci incubated overnight in serum media with PBS or NE containing 4.5-5×10^5 CFU / mouse. After 7 days of post-infection, 20 mice were scarified, lung tissues were dissected, and homogenised. Then, the viable plate counts of homogenates were obtained, and Log_{10} CFU mg^{-1} lung tissue was determined. Data are the mean of triplicate count per mouse and '±' represents the standard error of mean (SEM).

Number of mice	Control	NE
1	0.00±0.00	1.96±0.01
2	0.00±0.00	1.60±0.20
3	2.48±0.01	2.50±0.02
4	0.00±0.00	3.38±0.19
5	0.00±0.00	2.42±0.03
6	1.44±0.21	2.56±0.20
7	2.57±0.30	2.80±0.07
8	0.00±0.00	1.85±0.09
9	0.00±0.00	1.84±0.04
10	2.23±0.04	2.53±0.03

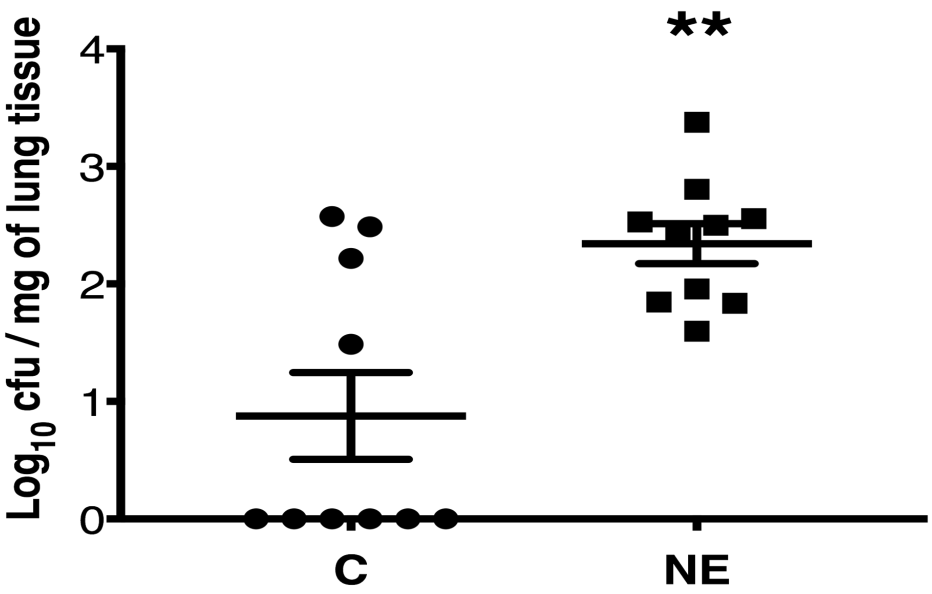


Figure 3.38. Norepinephrine increases pneumococcal invasiveness. The figure represents an independent experiment by collecting lung tissues samples of control and NE long exposed groups at day 7 (n=10 each). Unpaired t test was used for analysis (** $p < 0.01$).

SUMMARY

1. NE did not increase pneumococcal colonisation in the nasopharynx in both control/NE-short and control/NE-long groups suggesting that pre-treatment of the pneumococcus with NE prior to the infection did not affect the number of bacteria colonising the nasopharynx.
2. No bacteria were recovered in the BAL samples in both control/NE-short and control/NE-long groups.
3. Pneumococci exposed to NE for long time were highly likely to disseminate into the lungs.

Chapter 4. Discussion

4.1. General Discussion

S. pneumoniae is one of the most common causes of community and hospital-acquired pneumonia as well as otitis media, meningitis and septicaemia (Bogaert et al., 2004). In addition, pneumococci harmlessly colonise the upper respiratory tract in approximately 10% of healthy adults and 60% of children (Bogaert et al., 2004;Dudley et al., 2001). Despite pneumococcal carriage and range of diseases, fundamental aspects of its ability of transition from colonisation to invasive mode are not fully understood. It is reported that the first stage of invasive pneumococcal disease is nasopharyngeal colonisation (Bogaert et al., 2004). However, the trigger of transition from colonisation to invasiveness remains largely unknown. This is despite the indication that certain environmental factors such as sugar and metal composition in tissues as well as variable oxygen concentrations are known to have an impact on the virulence of the pneumococcus (Yesilkaya et al., 2013;Shafeeq et al., 2011). Despite the importance of these host environmental factors, they do not completely explain the pneumococcal ability to transit from colonisation to invasiveness mode. Hence, other host factors such as stress-associated catecholmine hormones (NE, Epi and Dop) have been investigated for their potential role in the transition from commensal to pathogenic existence. These catecholamine hormones are physiologically abundant when it comes to their signalling roles within tissues and organs of the mammalian body (Goldstein et al., 2003). As a result, it is predicted that *S. pneumoniae* will at some point encounter these catecholamines during colonisation and infection, and so is likely to have evolved sensory systems to monitor the levels of host stress hormones. While the importance of stress hormones is known for pathophysiology of various microbial diseases, knowledge on how the stress hormones affect the pneumococcal phenotype is currently limited.

Microorganisms can communicate with their hosts by the assistance of several host molecules, such as hormones that are released from eukaryotic cells and sensed by bacteria, events known as inter-kingdom signalling. Such hormonal communication is part of a relatively recent research discipline that has been named Microbial Endocrinology. This field of study aims to understand and examine the microorganism-host interaction in both health and disease (Lyte and Freestone, 2010). Host stress hormones can serve as environmental cues to initiate bacterial growth and pathogenic processes and their influence on the outcome of infection have been demonstrated in several studies. For example, it has been shown that catecholamine hormones increase growth of different, mainly Gram-negative, pathogenic bacteria such as *P. aeruginosa*, *S. typhi*, *E. coli* and *C. jejuni* (Freestone et al., 2008a). However, it is unknown how the Gram-positive *S. pneumoniae* senses and responds to the stress hormones, and what genetic cascades are responsible for recognition and processing of such signals.

The hypothesis of this study stemmed from the quick change in the concentrations of stress hormones caused by emotional, physical stress, or those hormone-derived inotropic drugs, from the adverse effect of stress hormones on the immune system, and from the ability of bacteria to sense, process and respond to host stress hormone signals (Reiche et al., 2005;Freestone et al., 2008a). Catecholamine-derived inotropes are given to approximately half of patients in intensive care unit (ICU) and about 56% of pneumococcal infected patients are admitted to the ICU (Smythe et al., 1993). Therefore, stress hormones are relevant stimuli for pneumococci, and a detailed understanding of their impact on pneumococcal biology is therefore important.

4.2. Establishment of the conditions to investigate *S. pneumoniae*-stress hormone interactions (Section B)

Before starting this project, there was little evidence that the host hormones could have an impact on the pneumococcus. In 2013 it had been shown that the pneumococci recognise host factors such as glucose, mammalian cell lysates, ATP and norepinephrine as signals to disperse from biofilms and to subsequently cause invasive

disease by using a tissue culture and animal biofilm model of infection (Marks et al., 2013). Therefore, for the current project there was a need to establish *in vitro* host-like conditions to investigate the effects of stress hormone on pneumococcal growth, morphology and virulence, and then examine the role of hormones on pneumococcal ability to transit from colonisation to invasiveness *in vivo* model of infection.

The first challenge was the choice of medium that can best reflect the host environment and simultaneously allow the growth of the fastidious pneumococcus. The conventional enriched media used for bacterial propagation, which normally facilitate fair growth, do not regularly induce strong expression of virulence factors. In fact, the expression of adhesins, toxins or invasions of many bacteria with pathogenic potential, will only occur under the same environment (temperature, pH, nutrition, etc.) that they would experience upon entrance to the host (Lyte and Freestone, 2010). Thus, when undertaking microbial endocrinology experiments, exploring the effects of stress hormones on bacterial growth and virulence required mimicking those stressful conditions with hormones in a similar manner that bacteria would naturally face in the host. A minimal salts medium supplemented with 30% adult serum (serum-SAPI) developed by (Lyte and Ernst, 1992;Lyte and Ernst, 1993), is typically used in microbial endocrinology studies. It is a highly stressful bacteriostatic medium, which has been reported to provide host-like conditions due to the presence of immune defence serum proteins, such as complement and antibodies as well as its limited nutrient availability and iron restriction (Freestone and Lyte, 2008). Without supplementation, most bacteria grow very weakly in this medium, which forces many researchers to modify serum-SAPI to meet the requirements of fastidious pathogens (Roberts et al., 2002). Furthermore, to meet the complex nutritional needs of some bacteria, others have used rich iron-chelated media (e.g. Tryptic Soy Broth, Mueller Hinton Broth, etc.) with added serum to render them bacteriostatic (Coulanges et al., 1997;Anderson and Armstrong, 2006;Cogan et al., 2007). Therefore, it was essential to first test whether this medium that resembled the host environment, would be appropriate for the pneumococcus. This study explored various environmental conditions *in vitro* to test pneumococcal responsiveness to the catecholamine hormones.

Since pneumococcal growth in serum-SAPI was extremely poor, Sicard minimal medium, which was specifically designed to meet the basic nutritional needs of the pneumococcus (Sicard, 1964), was added. Although the addition slightly enhanced the growth, it was not satisfactory as Sicard medium greatly diluted the serum within serum-SAPI medium, which minimised and even eliminated the growth stimulating effects of the stress hormones on the pneumococcus. Thus, serum-SAPI was supplemented with only trace elements, glucose and vitamins of the Sicard medium to support the growth of the fastidious pneumococci. Fe $(NO_3)_3$ was included to prove that growth failure in serum-containing medium resulted from a lack of iron uptake system in the pneumococcus, and not from sensitivity to serum. The use of modified serum-SAPI for the first time proved that the pneumococcus is actually a stress hormone-responsive pathogen and its response to Fe was strikingly similar to NE which mechanistically indicated the involvement of iron delivery mechanism (discussed in section 4.3) mediated by the catecholamine. The results demonstrated the importance of selecting an appropriate growth medium when undertaking microbial endocrinology experiments as recommended by Freestone and Lyte (2008). The use of rich media such as BHI was avoided since a previous study could not demonstrate growth stimulating effect of NE on *E. coli* and *P. aeruginosa* using a rich LB medium (Straub et al., 2005).

The choice of a bacterial inoculum that precisely reflects the infectious dose that occurs *in vivo* is another essential element when undertaking microbial endocrinology experiments (Freestone and Lyte, 2008). Typically, the starting inoculum used in microbial endocrinology-related studies is usually very low approximately 10^1–10^2 CFU/ml, which is proposed to reflect the amounts of pathogenic bacteria likely to initiate the infection (Lyte and Freestone, 2010). This study is in agreement with the previous studies as it showed that inoculum levels of 10^2-10^3 CFU/ml were the best for investigating hormone-induced growth of *S. pneumoniae*. The pneumococci appear to take advantage of the presence of hormones only when stressed and they are extremely low in number that reflect the infectious dose that occurs *in vivo* conditions. The reason they respond to stress hormones at low cell density could be mainly due to hormone-

induced iron uptake as demonstrated in other bacteria (Freestone et al., 2000;Sandrini et al., 2010). It is possible to propose that bacterial utilisation of the hormone-assisted iron uptake at low numbers might be due inadequate quorum sensing signals produced by the initial low bacterial population to increase their proliferation rate and numbers in the nutrient-limited serum medium.

In addition, this study also found that catecholamine effects were bacterial cell density-dependent; for instance, a pneumococcal culture using a starting inoculum of 10^6 CFU/ml showed no significant difference of growth response to NE compared with the NE-free cultures. Similarly, a study investigating the growth responses of *E. coli* O157:H7, *S. enterica* and *Y. enterocolitica* to different catecholamines using 8-log diluted inoculum sizes demonstrated that the effect of catecholamine-induced growth in serum media becomes apparent only at cell concentration less than 10^4 CFU/ml and greatest at around 10^2 CFU/ml initial cell densities (Freestone et al., 2007c). It must be considered that inoculating large numbers of bacteria in host-like media such as serum-SAPI, can lead to incorrect evaluation of the response to host hormones due to the cell mass overwhelming the bacteriostatic potential of such serum-containing media. For example, a study used large initial inoculum of a number of Gram-negative bacteria concluded that no significant effects of hormones were observed (Belay et al., 2003) contrary to the finding of earlier studies (Lyte and Ernst, 1992;Lyte and Ernst, 1993). In Belay's experiment, the starting bacterial numbers were so high that no lag phase was observed, and maximal growth was achieved in a very short time by both control and hormone-treated cultures (Belay et al., 2003). When the same researchers later carried out the same assays using a much lower initial inoculum, they observed a much longer lag phase and an increase of bacterial response to the hormone (O'Donnell et al., 2006) in line with other previous studies (Lyte and Ernst, 1992;Lyte and Ernst, 1993;Freestone et al., 2002;Freestone et al., 2003). This study also found that high inoculum sizes resulted in much shorter lag phases for adaptation to the serum media, which could be attributed to sufficient quorum sensing signals being secreted by the large number of bacterial cells to initiate growth before obtaining the hormone-assisted iron. Although this does not appear to affect the growth phenotype of the

pneumococcus, it does not rule out the potential impact of hormone on other pneumococcal phenotypes. Therefore, for technical reasons, some researchers have used high numbers of bacteria (10^8 CFU/ml) to examine their response to hormones (Sperandio et al., 2003;Vlisidou et al., 2004). In agreement with the previous studies, this study has also found that starting inoculum size of 10^6 CFU/ml and higher resulted in no significant differences in the maximum growth yields between the control and hormone-treated cultures. Hence, this inoculum size was used in biofilm formation, NE/Tf uptake, reporter assays, metabolomics and *in vivo* experiments to ensure that growth levels of control and catecholamine-treated cultures were the same.

During infection, pathogenic bacteria encounter various host signals and conditions including changes in temperature and variation in the levels of oxygen, hormones, and pH. Such environmental signals not only stimulate adaptive responses of bacteria to these specific situations but can also affect virulence gene expression at the right place and time. Specifically, pathogenic bacteria encounter stressful conditions as soon as they contact their host, and for most, this involves a change in temperature (Fang et al., 2016). In addition, although the upper respiratory tract environment is rich in oxygen, the facultative anaerobic pneumococcus has mechanisms to withstand oxygen and its reactive derivatives. (Bortoni et al., 2009). In other locations within the respiratory tract, some pathogens encounter rather oxygen limitation (Worlitzsch et al., 2002). The pneumococci must overcome such environments from the moment they enter and establish colonisation in the nasopharynx of the host to the stage where they become invasive and cause diseases in the lungs, ears and blood. Therefore, the pneumococcal hormone-induced growth in serum medium was also studied under different conditions such as temperature of 34, 37 and 39°C and in aerobic, micro-aerobic and anaerobic environments. These temperatures represent different host state and locations in health and disease within the host (White et al., 2011). This project found that the pneumococci were growing significantly better in both anaerobic and micro-aerobic environment while growing poorly in aerobic environments. This is not surprising as *S. pneumoniae* is a facultative anaerobic organism, and so it can naturally live in both micro-aerobic and anaerobic conditions. However, this may indicate that

the pneumococci are able to take advantage of host hormone availability independent of their location within various host sites during nasopharyngeal colonisation and during the infection of lungs, ears and blood.

The poor growth seen in oxygenated environment was significantly better when NE was present compared with NE-free culture suggesting that NE is not only important for enhancement of bacterial growth but probably aids bacterial survival when the pneumococci encountered such extreme conditions in the host, or in the environment between hosts. In addition, under different temperature conditions, the overall analysis of pneumococcal growth yield incubated in serum medium containing NE showed that the pneumococci were growing significantly better at both 34 and 37°C while almost no growth was seen at 39°C. The 39°C growth phenotype corresponds with fever of moderate degree may be beneficial to the host in pneumococcal pneumonia infection (Sebag et al., 1977). These temperatures represent different host state and locations in health and disease within the host since 34°C, for instance, is within the range of the airway temperatures that pneumococci may experience inside the host (White et al., 2011). Interestingly, Oxford et al. (1980) found that some influenza virus isolates, a recognised high-risk factor for invasive pneumococcal infections (van der Poll and Opal, 2009), produced 10 times more viral plaques at 34°C than at 39°C. Therefore, it is possible to speculate that pneumococcal infection is more likely to occur when associated with viral infection in the airway location where the temperature of the host drops to 34°C. Furthermore, when the host is infected with a respiratory virus, the levels of stress hormones may be elevated, which could trigger *S. pneumoniae* to initiate growth and infection.

Similar to the choice of growth medium, inoculum size and environmental conditions, the selected concentration of hormones tested in *in vitro* assays should represent those that are likely to be encountered by bacteria in the host. It has been stated to be difficult to determine the accurate hormone concentrations at body sites due the fact that pathogenic bacteria will occupy more than just a single site during the period of an infection. Thus it is plausible that pathogens are exposed to various hormone

concentrations (Lyte and Freestone, 2010). More importantly, most hormones in the body are located in the targeted tissue where they take action, and so it is worth emphasizing that the values of specimens detected in body fluids are often much lower than the actual levels at mucosal surfaces or within the tissues where pathogens interact (Leinhardt et al., 1993). Therefore, the selection of a reasonably wide range of stress hormone concentrations to undertake dose responses analysis is highly recommended (Lyte and Freestone, 2010). For example, dose-response effects of catecholamines (NE, Epi and Dop) ranging from 1 to 500 μM were utilized on *E. coli* O157:H7, *S. enterica* and *Y. enterocolitica* (Freestone et al., 2007c). In this current study, the impact of different concentrations of catecholmines on the growth of *S. pneumoniae* strain D39 was examined. Pneumococcal growth on serum-based culture was not only norepinephrine-induced, as seen in the previous results, but also increasing concentrations of dopamine and epinephrine ranging from 1 to 50 μM, some of which are attainable *in vivo* (Thompson et al., 1999), were also shown to be effective. This range is biologically relevant because the concentration of catecholamines at the site of action in the body is predictably variable or unknown.

In addition, it is important to consider that the levels of hormone exposure can vary between tissues as bacteria occupy and infect various location within the host. For example, it has been shown that 50 μM of NE or Dop and 100 μM of Epi are suitable for bacterial species such as *E. coli*, *Yersinia*, *Salmonella* and the coagulase-negative *staphylococci* (Freestone et al., 2007c) whereas this study found that 50 μM of NE or Epi and 5 μM of Dop was most suitable for the pneumococcus. This suggests that a low level of stress has a significant impact on pathogenesis of pneumococcal disease than diseases caused by other infectious agents. This result has also a clinical implication since up to 300 μM of Epi, for instance, could be directly administered to ventilated patients to decrease airway inflammation (Freestone et al., 2012), which can be detrimental for progression of pneumococcal diseases in these patients.

Another interesting observation was that, higher concentration of Dop did not result in maximal growth. This could be linked to the production of toxic free radicals, which are

chemical species containing unpaired or an odd number of electrons (mostly referred to a reactive oxygen species ROS) (Brand et al., 2004). Dopamine is an unstable hormone by nature, and it undergoes auto-oxidation leading to production of ROS (Slivka and Cohen, 1985). Hence, at high concentration, ROS produced through auto-oxidation of dopamine may be toxic to cells and attenuate growth.

The bacterial response to a given hormone can differ depending on their previous encounter and coexistence with that particular hormone when in their preferred host niche (Freestone et al, 2007). Bacterial catecholamine specificity has been investigated in some bacteria inhabiting the gut. For example, a study conducted on three enteric pathogens has revealed a distinct preference for NE and Dop over Epi. In fact, *Y. enterocolitica* (primarily gut inhabitant) did not show significant growth in response to Epi (Freestone et al., 2007c). This could be explained by the fact that epinephrine is not synthesized in the enteric nervous system (Costa et al., 2000) due to the lack of neurons containing phenylethanolamine N-methyltransferase. As a result, this particular species is perhaps less likely to encounter and develop systems to respond to this hormone in the gut. A similar situation was also demonstrated in *Mycoplasma hypopneumoniae* (Oneal et al., 2008). In contrast, in this study *S. pneumoniae* was found to be responsive to all three catecholamines tested suggesting that the microbe is very likely to encounter various stress hormones within the respiratory tract and infected organs of its host.

4.3. Role of pneumococcal TCS09 in stress hormone response (Sections A and C).

The question that arose after examining the responsiveness of *S. pneumoniae* to stress hormone was which pneumococcal genetic elements are responsible in the mechanism of catecholamine growth induction; hence the role of the pneumococcal two-component systems was investigated. For long time, bacterial two-component systems (TCS) have been in the focus of many researchers for some time due to the fact that the TCS systems have not been found in the mammalian host, rendering them a potential antibacterial target (Barrett and Hoch, 1998). Moreover, TCS enable bacteria to sense

and react to environmental changes within their host and contribute to bacterial competence, virulence and viability (Paterson et al., 2006). The role of TCS in stress hormone-bacteria interactions has been identified in some Gram-negative bacteria (Hughes and Sperandio, 2008). Therefore, it is plausible that they are also important in pneumococcus-catecholamine hormone interactions as microarray analysis indicate TSC03 and TSC06 involvement in promoting pneumococcal growth in the presence of norepinephrine (Gonzales et al., 2013).

The analysis of catecholamine-induced growth data in this study showed that all the 13 TCS mutants were as responsive as their wildtype parent to the catecholamine hormones, except the ΔTCS09, ΔTCS03, ΔTCS08, ΔTCS05, ΔRitR and ΔTCS06 mutants which displayed significantly reduced growth rates and/or yields to at least two hormones compared to the hormone response of the wild type strain. The involvement of TSC03 and TSC06 in stress hormone-*S. pneumoniae* interactions has been observed in a recent study using microarray analysis of pneumococcal cultures grown in rich-iron chelated media (Gonzales et al., 2013). The variation in response to hormones among those TCS mutants in this study might be attributed to their general role in regulating bacterial adaptation to recognise and respond to host signal like hormones in serum-SAPI media. Nevertheless, a mutant lacking TCS09 displayed a constant reduction of growth yield and rate in the presence of all hormones in the serum containing media compared to the wild type which indicates a strong involvement of this TCS in *S. pneumoniae* - stress hormone interactions. A previous study indicated that TCS09 mutant expressed down regulation of genes involved in carbohydrate metabolism and facilitate the uptake of sugars such as mannose and fructose (Hendriksen et al., 2007). Therefore, this study indicated that the reduction of growth response seen particularly in ΔTCS09 mutant was not due to a defect in sugar metabolism but possibly due to a reduction in NE and transferrin uptake. The growth of TCS09 mutant on CDM medium supplemented with either glucose or galactose was similar to wild type growth ruling out the possible sugar metabolism defect.

The reporter strains expressing *lacZ* under the control of putative promotors of TCS09, TCS03 and TCS05 did not show induction in the presence of stress hormones, ruling

out their direct involvement in stress hormone responsiveness. It is therefore likely that these TCS systems may have indirect involvement in stress hormone recognition, uptake, and processing. It is also possible that one or more of these regulatory systems are activated by assistance of other key elements such as the case of auto-inducer-3 (Al-3) in Gram-negative *E. coli.* It has been reported that Al-3 along with NE and Epi signals are collectively required in order to be sensed by the TCS QseC and then response is regulated by QseB, which in turn activates the expression of another TCS QseEF involved in the expression of virulence genes. However, such a complex signaling cascade has not been fully elucidated (Moreira and Sperandio, 2016) and several groups have found that deletion of QseC and QseE in either *E. coli* or *Salmonella* has no effect on catecholamine responsiveness (Sandrini et al, 2015).

None of the putative promoters were found to be induced specifically by stress hormones. This can be due to two possible reasons. One is that promoter identification software was not accurate, and the second is that the promoters are indeed unresponsive to the stress hormones tested. In this study, the putative promotors were identified using the BPROM software, which detects potential sigma70 promoter recognition sites within the sequence regions upstream of the corresponding TCS genes with approximately 80% specificity and accuracy. Although the likelihood of error in detection the TCS corresponding promotors is 20%, the high inducibility of PTCS05::*lacZ* fusion strain measured by β-galactosidase activity proved the successful promoter identification method. Nevertheless, further investigation is required to confirm the role of exact TCS promotors in *S. pneumoniae* - stress hormone interactions by utilising more accurate promoter identification method such as rapid amplification of cDNA ends (5′-RACE).

The putative promoters of the selected TCSs were apparently not induced by stress hormones, this suggests that TCS09, TCS03 and TCS05 are not involved directly in stress hormone recognition and processing. However, this does not rule out the possibility that the TCS system might be involved in stress hormone and iron uptake. The uptake of radio-labeled NE, and ^{55}Fe from ^{55}Fe-labelled transferrin (Tf) by the D39

strain and the selected mutants were therefore examined. To study the mechanism of stress hormone-induced growth, stress hormones role in iron uptake was investigated. Catecholamine hormones can enhance the growth of bacteria by mediating iron uptake from host Fe-binding protein such as Tf (Freestone et al., 2002). It has been reported that hormones can achieve this by assistance of their catechol moieties, which are able to bind and reduce ferric iron Fe(III) to ferrous iron Fe(II). This weakens the iron binding affinity of the Tf, enabling bacteria to uptake the unrestricted iron via Fe(III) or Fe(II) uptake systems (Freestone et al., 2008a;Sandrini et al., 2010;Freestone et al., 2000). This understanding has shed some light on the underlying mechanism by which stress hormones stimulate bacterial growth (Freestone et al., 2002). The pneumococcus is able to utilize ferric and ferrous iron salts as well as host iron sources including Tf iron, haemin and haemoglobin (Brown et al., 2002;Brown et al., 2004;Tai et al., 1993). In addition, it has been demonstrated in a collaborative study that *S. pneumoniae* is bound to host serum Tf (Sandrini et al., 2014). The current study also showed that *S. pneumoniae* was able to internalise NE and obtain normally sequestered host Fe in higher amounts in the presence of stress hormone. Mechanistically, the hormone-mediated iron delivery via Tf and NE internalisation probably explains growth enhancement by the catecholamine hormones in serum medium.

To confirm the possibility that the TCS system were involved in stress hormone and iron uptake, the uptake of radio-labeled NE, and ^{55}Fe from ^{55}Fe-labelled transferrin (Tf) by the D39 strain and the selected mutants were examined. The results revealed that the radiolabelled NE (^{3}H-NE) was equally internalised by the wild type, ΔTCS03, ΔTCS05 and ΔRitR strains while the uptake of ^{3}H-NE was significantly reduced in ΔTCS09 mutant. The mutant's ability to take up ^{55}Fe-Tf was also significantly attenuated in the presence of cold (not radiolabelled) NE. This is the first study that a direct role of TCS in pneumococcal iron uptake has been revealed, which emphasises the importance of studying host-pathogen interactions to produce novel data by applying host-like conditions. TCS09 has been shown to be a key component of the pneumococcal TCSs system as its mutation led to avirulent phenotype in mouse models

of pneumonia and bacteraemia resulting in the survival of all infected mice and rapid clearance of the bacteria (Blue and Mitchell, 2003).

For most pathogenic bacteria, iron plays an essential role for their growth within their host and for cellular processes such as energy production, oxygen transport and DNA replication (Skaar, 2010), and it is a key metal for viability and pathogenicity of *S. pneumoniae* (Throup et al., 2000). However, inside the mammalian host, iron is highly restricted representing an important innate immune defence against pathogenic bacterial infection. Approximately 70 % of iron is secured in red blood cells and much of remaining is tightly bound by high affinity iron-binding proteins; Tf in serum and Lf in extra-cellular fluids. Given the importance of iron for bacteria and the high restriction within the human body, iron provision by the assistance of stress hormone could play a major role in the ability of the pneumococcus to transform from colonisation to invasion. In conclusion, the data of this study suggest that TCS09 in particular is important in hormone-induced growth and mediating the uptake of the hormone and Tf-released iron. It also indicates that both NE and Tf-derived iron are internalised by the pneumococcus, which sheds some light on the mechanism by which *S. pneumoniae* interact with host stress hormones.

4.4. Catecholamines stimulate *S. pneumoniae* biofilm formation via capsule reduction (Section D)

The ability of bacteria to attach to surfaces and form biofilms is an important aspect of virulence particularly when developing a respiratory infection. The clinical significance of *S. pneumonia* biofilm is poorly understood despite the evidence of pneumococcal biofilm detection in *vivo* during the courses of infection in chronic rhinosinusitis, otitis media and pneumonia (Hall-Stoodley et al., 2006; Sanderson et al., 2006; Hoa et al., 2009; Reid et al., 2009; Sanchez et al., 2011b). In addition, catecholamine-derived inotropes therapeutically used in the ICU are reported to increase bacterial growth and biofilm formation within indwelling medical devices (e.g. intravenous lines) such as the case of the skin-dwelling *S. epidermidis* leading to subsequent infections in hospitalised

patients (Lyte et al., 2003). Essentially, hospital acquired pneumonia associated with ventilator known as ventilator-associated pneumonia (VAP) represents a major challenge in controlling bacterial pneumonia. VAP is defined as pneumonia occurring within 48-72 hours of endotracheal intubation and accompanied by signs of systemic infection, distinctive sputum characteristics and detection of an etiologic agent (Kalil et al., 2016). It accounts for approximately 50% of all nosocomial pneumonia cases and occurs in about 9-27 % of all mechanically ventilated patients (Kalil et al., 2016;Vincent et al., 1995;Chastre and Fagon, 2002). In the ICU, VAP is the second most common nosocomial infection mainly affecting mechanically ventilated patients (Hunter, 2012;Afshari et al., 2012). Typically, *S. pneumoniae* is one of the causative agents of early-onset VAP along with *H. influenzae*, *S. aureus* (Hunter, 2012). Importantly, catecholamine inotropes are administered to about 50% of ICU patients and 56% of pneumococcal infected patients are admitted to the ICU (Smythe et al., 1993). Hence, the pneumococcus is further exposed to external hormones in addition to the endogenously produced stress hormones, which may stimulate pneumococcal biofilm formation on indwelling medical devices.

The current definition of biofilm takes into account the evidence that bacterial extracellular polysaccharides are not constantly needed in order to develop biofilm, as seen in *S. aureus* (Toledo-Arana et al., 2005). More importantly, with regards to *S. pneumoniae*, the presence of the capsule decreased pneumococcal biofilm by more than 60% (Moscoso et al., 2006). Therefore, pneumococcal ability to regulate capsule expression could have a major impact on the transition from the carrier state to invasiveness (Waite et al., 2001). These findings are of a great importance for the pneumococci which are classified into more than 90 different types based on their capsular polysaccharides (Henrichsen, 1995). Interestingly, it is reported that the down-regulation of capsular polysaccharide production seems to increase pneumococcal attachment to host cells of the nasopharynx as well as invasion in the asymptomatic carrier state but the capsule has to be recovered for bacterial survival after the invasion (Hammerschmidt et al., 2005;Weiser and Kapoor, 1999). This study found that there is dual influence of NE over the synthesis of the pneumococcal capsular

polysaccharide and over biofilm formation by decreasing capsule synthesis, it enhances pneumococcal biofilm. The influence of NE over capsule synthesis may occur by affecting Cps2A, LytR and Psr, which are believed to maintain normal capsule levels and attachment at the cell wall in *S. pneumoniae* strain D39 (Eberhardt et al., 2012). Hormonal impact on pneumococcal capsule synthesis has never been reported in spite of the fact that the capsule is essential for colonisation, invasion, and dissemination from the respiratory tract. In addition, it has been shown that pneumococci not in contact with the epithelial cells did not alter their capsular polysaccharide. However, when infecting the cells, the pneumococcus exhibited 10000-fold enhanced capacity to adhere and to invade the epithelial cells, and scanning electron microscopy revealed a reduction in pneumococcal capsular materials (Hammerschmidt et al., 2005).

In biofilm formation assays, the results revealed that increasing concentrations of catecholamines progressively enhanced biofilm formation of the pneumococcal cultures. NE has previously been shown to increase attachment of pathogen and commensal *E. coli* to intestinal tissues (Lyte et al., 1997;Hendrickson et al., 1999). Moreover, catecholamine-derived inotropes markedly enhanced staphylococcal biofilm formation using plasma-based biofilm assays (Lyte et al., 2003). Although catecholamine and inotrope-induced biofilm formation has been reported in other bacteria, their effects on pneumococcal biofilm are controversial and not fully understood. For example, Marks et al(Marks et al., 2013) found that NE treatment of biofilms formed *in vitro,* and *in vivo* using biotic surfaces led to dispersion of the pneumococcus from the biofilm, and those dispersed cells were different in phenotype from those of both biofilm and broth-grown planktonic bacteria. Despite that the dispersion of the pneumococcus from the biofilm might subsequently lead to invasion, contrary to the Marks' finding, this study showed that in the serum-containing medium catecholamine exposure significantly enhanced biofilm formation on abiotic surfaces. The reason in part could be due to the obvious methodological variation and also to a possible dual effect of catecholamines on pneumococcal biofilm. Stress hormones could initially enhance pneumococcal biofilm formation and at some point in the infection process, depending on pneumococcal host state and growth phase of the bacteria,

might also induce dispersion of the biofilm as observed in the other study (Marks et al., 2013). Further work is required to elucidate the stress hormones effects of pneumococcal biofilm formation by considering the biofilm former cells and planktonic cells both *in vitro* and *in vivo.*

Similar to the wild type, the selected mutants, ΔTCS05, ΔTCS09 and ΔRitR also displayed increased levels of biofilm formation in response to NE compared to NE-free cultures. However, it should be particularly emphasised that ΔTCS03 strain had a greater amount of biofilm compared to that of wild type. The distinct phenotype of this mutant was not related to NE exposure in serum medium since this mutant showed also enhanced biofilm formation in BHI medium compared to wild type behaviour suggesting a role of this TCS in mediating biofilm formation independent of its involvement in stress hormone recognition and processing. In addition, TCS03 system (LiaFSR) has been reported to play a role in acid tolerance and biofilm formation in *S. mutants* (Chong et al., 2008;Li et al., 2002). However, studying the role of this system in *S. pneumoniae* biofilm independent of its involvement in stress hormone recognition and processing is outside the scope of this project. Overall, different levels of stress hormones do have impact on the biofilm formation of *S. pneumoniae* by enhancing the attachment on abiotic surfaces and none of the selected mutants reduced in their biofilm forming ability in response to the stress hormone NE.

4.5. Impact of NE on pneumococcal cell morphology (Section D)

Among the genus *Streptococcus*, the pneumococcal growth in the form of short chains or diplococci is a unique characteristic and the underlying cause of this phenomenon is unclear. A recent study indicated the importance of the length of bacterial chains amongst the pneumococci. They demonstrated both *ex vivo* and *in vivo* that decreasing bacterial chain length represents a complement evasion strategy, which is essential for resistance to opsonophagocytic killing in *S. pneumoniae* (Dalia and Weiser, 2011). In this study, the norepinephrine-treated wild type D39 appeared as diplococci under light microscopy while the control group, without hormone, were seen as short chains.

Interestingly, this is consistent with the fact that pneumococci from clinical samples normally exist in diplococcal form (Tomasz et al., 1964) while short chain phenotype is typical for asymptomatic colonisation which occurs within complex multicellular communities (Munoz-Elias et al., 2008;Marks et al., 2012a). The pneumococcal transformation from chains into diplococci might be due to the iron availability via the Tf-iron delivery mechanism mediated by the stress hormone. For example, pneumococci grown on CDM media supplemented with haemoglobin as a source of iron appeared as diplococci compared with un-supplemented cultures which formed long chains (Gupta et al., 2009).

Growth rate and nutrient availability are well-recognised factors that can influence bacterial cell size (Chien et al., 2012). Over 50 years ago, Schaechter, Maaløe, and Kjeldgaard were the first to observe that bacterial size is correspondent with growth rate depending on nutrient condition. They noted that Salmonella cells were double the size when incubated at fast growing rate in rich medium compared with the cells incubated at slower growth rates in a poor nutrient source (Schaechter et al., 1958;Kjeldgaard et al., 1958). Later research found that this nutrient-dependent control of cell size is also the case for many other species of bacteria including Gram positives such as *B. subtilis* (Pierucci et al., 1987;Sargent, 1975) as well as *S. aureus*, where glucose limitation leads to 40% reduction in cell size (Watson et al., 1998). Stress hormones have never been tested if they exert their effect on bacterial size additional to their direct impact on growth and virulence. Pneumococcal cells size are normally between 0.5 and 1 micrometers in diameter (Reid et al., 2009). This study has identified a significant reduction of pneumococcal cell size after NE treatment. This change in size perhaps might be an advantage for pneumococcal invasion in otherwise small and sterile sites during the infection. Moreover, by reducing its surface size in response to NE, less complement and antibody deposit on the surface of microbe, and in return less efficient clearance would take place by host immune system. More importantly, the reduction of glucuronic acid levels within the pneumococcal capsular polysaccharides after NE treatment (as discussed in section 4.4) may be associated to cell size reduction.

Bacterial pathogens that frequently cause invasive infections such as *N. meningitidis* and *H. influenzae* share a common characteristic, which is their small size. It has been observed on other pathogens that their cellular morphology has a great influence over their virulence (Justice et al., 2004;Okagaki et al., 2010;Zaragoza et al., 2010). It is possible to propose that hormone exposure, which cause a reduction in bacterial size and chain length may be an additional factor that promotes the invasiveness of *S. pneumoniae*. In conclusion, this study found that the pneumococcal response to the catecholamine hormone NE resulted in a remarkable change in pneumococcal morphology and size, from chains into diplococci as well as a significant reduction in cell size. As a result, pneumococcal reaction to hormone presence by changing phenotypes suggest that hormones may be the trigger of pneumococcal transition from harmlessly colonising bacteria into invasive and more aggressive pathogen.

4.6. Impact of NE on pneumococcal metabolism (Section D)

In recent years, metabolomics has become a key tool to investigate host-pathogen interactions as well as to uncover potential novel therapeutic targets. It has been reported that therapeutic levels of noradrenaline markedly alter expression of genes involved in metabolism and virulence (Sandrini et al., 2014). Additionally, the notable effects of hormones on pneumococcal growth, cell size, capsule and biofilm formation in this study suggest that hormones could have broader effect that would include cell metabolism. Since cell size can be influenced by growth rate depending on nutrition availability (Chien et al., 2012), and was reduced due to hormone exposure in serum nutrition-limited medium in this study, pneumococcal metabolites under such condition was determined with and without NE addition.

The intracellular and extracellular metabolites of *S. pneumoniae* grown in the presence and absence of NE have been profiled using GC-MS for the first time in this study. The analysis of 94 metabolites revealed a clear difference between the compounds present within the control and NE-treated cultures. Interestingly, relative to the control pyruvic acid and alanine levels were consistently lower in the NE-treated *S. pneumoniae*

metabolite pool while the fumaric acid level was higher. Analysis of extracellular metabolites showed that as predicted from a lactic acid-producing organism, lactic acid was highly produced by both NE-treated and control groups, relative to the medium.

Surprisingly, fumaric acid was significantly produced by NE-treated compared to control group and the media. Fumaric acid (also known as fumarate, (E)-2-butenedioic or trans-1,2-ethylenedicarboxylic acid) is an organic acid and a key intermediate in the tricarboxylic acid (TCA) cycle and produced by many microbes in small amounts (Roa Engel et al., 2008). Although the pneumococci are not known to produce fumaric acid, the medium used does contains pyruvate, which can be fermented into malate and then fumarate. This could explain the reduction in pyruvate content in both groups especially the NE-treated group where the fumarate and malate where produced in higher quantities than the control. Fumaric acid is structurally similar to succinic and malic acids in the metabolic pathways of different organisms. While bacteria commonly produce succinic acid, fumaric acid can be produced during anaerobic respiration (mainly in fungi) because it is an important intermediate in the citrate cycle (Roa Engel et al., 2008). It has been reported that pneumococcal diseases are associated with increased levels of TCA metabolites in the host fluids. For example, serum of mice infected with *S. pneumoniae* showed increased levels of metabolites related to TCA cycle intermediates including fumarate compared to infections with *E. coli, S. aureus* and *P. aeruginosa* (Hoerr et al., 2012). Moreover, unpredicted high levels of fumarate were detected in the urine of patients infected with *S. pneumoniae* (Slupsky et al., 2009). Currently, it is unknown whether the pneumococcus also contributes to this fumarate pool in host secretions.

The pneumococcal optimum pH range is 6.5-8, but during pneumococcal growth, lactic acid is produced decreasing the pH of the culture to approximately pH 5.0 (Manning, 1997). It is estimated that the human naso/oro pharynx can be inhabited by several hundreds of species of diverse microflora (Aas et al., 2005). The reduction in pH has been observed in *in vitro* experiments after growing the pneumococci in serum medium with or without the addition of NE. The pH of the uninoculated serum medium was 7.5

and dropped to 6.0 and 5.8 in control and NE treated samples, respectively. Therefore, it is possible that fumaric acid release by the pneumococcus after NE encounter could further lower the pH in the nasopharynx, which might be an exclusive advantage of the hormone growth-stimulating effect for the pneumococci over other microbes within this competitive environment. The low pH strategy is used by *Streptococcus mutans*, a major cariogenic microbe in the human mouth that produces high amount of acid, exceeding buffering capacity of salivary, which gives this species an advantage to outcompete noncariogenic commensals at low pH conditions. This is one of the key components by which this organism is able to survive on acidic environments by modulating sugar metabolic pathways (Loesche, 1996).

S. pneumoniae uses strategies to outcompete other species in upper respiratory tract, such as the production of hydrogen peroxide (H_2O_2), which is lethal for most bacteria. It is a well-studied mechanism used by bacteria to compete with rival species (Bosch et al., 2013). The pneumococcus is extraordinarily tolerant to H_2O_2, and release bactericidal levels even for the H_2O_2-neutralizing enzyme, catalase-producing bacteria like *H. influenzae* (Pericone et al., 2000) and *S. aureus* (Regev-Yochay et al., 2006). Therefore, similar to hydrogen peroxide production, I propose that lowering the pH by producing lactic and fumaric acid during hormone exposure are key mechanisms for pneumococcal growth and pathogenicity. To conclude, the metabolites of *S. pneumoniae* grown in the presence and absence of NE have for the first time been profiled using GC-MS. The analysis revealed a clear difference between the compounds present with in the control and NE-treated cultures. Although it is not understood how hormones alter metabolism, it has become evident that hormone exposure has a great impact on the pneumococcus by changing its metabolic profile, which could lead to more competitive and virulent phenotype when infecting a stressed or inotrope-receiving host.

4.7. NE triggers pneumococcal transition from colonisation to invasiveness (Section E)

After *in vitro* test of catecholamines' effect on the pneumococcus, NE effect on pneumococcus *in vivo* using a mouse model of pneumococcal colonisation was examined. Due to the evidence that TCS mutants had attenuated virulence as described in (Paterson et al., 2006), they were not tested in the animal study. Hence, a stock of serotype-2, wild type D39, was investigated for the role of stress hormone NE in pneumococcal transition from colonisation to the invasive state in mice. It is worth mentioning that direct administration of hormones into mice was avoided partially due to the extremely rapid hormone elimination rate (Ginn and Vane, 1968) from the circulation requiring multiple hormone administration or via continue intravenous administration both of which are inappropriate. Secondly, hormone administration into mice would have massive side effects due to stimulation of sympathetic system (e.g. tachycardia) (Kulka and Tryba, 1993).

Creation of equal conditions between hormone-treated and control cultures has been stated to be extremely difficult due to the poor growth of control cultures caused by bacteriostatic nature of serum-SAPI medium (Lyte and Freestone, 2010). To bypass this challenge, a recent study used Luria broth media, which is a rich microbiological medium to support the growth of the control culture whereas the test culture was grown on serum-SAPI supplemented with 2 mM NE. The study used pre-treated *S. typhimurium* with NE *in vitro* prior to infecting young pigs, which resulted in increased virulence and numbers of bacteria with greater distribution in the gut tissues compared to the control bacteria (Toscano et al., 2007). The current study has also used *in vitro* pre-treatment of *S. pneumoniae* with 50 μM NE prior to infection of mice, which resulted in increased numbers of bacteria in the lung tissues compared to the control bacteria. However, instead of using rich media to support the control culture, this study inoculated both cultures grown on serum-SAPI media with high numbers of bacteria to circumvent the bacteriostatic nature of the serum media as suggested by Lyte and Freestone (2010). In addition, the pneumococcal pre-treatment with NE was

performed in two ways to evaluate the impact of exposure time of NE on the pneumococcus and the media used.

Both bacterial inoculum preparation in BHI (NE-short exposure) and in serum-SAPI (NE-long exposure) resulted in increased dissemination into the lungs of mice with a better outcome in the NE-long group. The second animal study using only NE-long exposure in serum-SAPI confirmed that pneumococci exposed to NE rendered them more likely to invade the host by transition from the nasopharynx into the lungs. The enhanced dissemination into the lungs after NE exposure could be attributed to the previously discussed hormone effects on the pneumococcal cells, such as increase in growth yields and rates as well as reduction in cell size and capsule content, which enhances binding to epithelial cells. These factors might improve the invasiveness of the pneumococcus into deep sterile sites within the respiratory tract of the host.

The overall analysis of *in vivo* experiments indicates for the first time that the hormone is an important factor for pneumococcal transition from commensalism to parasitism. While the results showed that stress hormone exposure did not enhance pneumococcal numbers in the nasopharynx over time, the colonising strain exposed to the hormone became more invasive, suggesting that pneumococci acquired from a stressed host would spend less time for colonisation than those from non-stressed host. This is of great importance in hospitals, where patients are more exposed to endogenous and exogenous hormones particularly those receiving hormone-derived inotropes in the ICU. Therefore, extra measurements and precaution must be taken to ensure that individuals receiving catecholamines do not spread aerosols, perhaps by wearing face masks. Many clinical and laboratory studies have identified a number of risk factors for pneumococcal pneumonia and invasive pneumococcal disease. The high-risk factors for IPD include children under 2 years and adults older than 65 of age, alcoholism, asplenia, HIV and influenza infection as well as diabetes mellitus and defect in humoral immunity (van der Poll and Opal, 2009). This study suggests that catecholamine administration should also be recognised as an additional predisposing factors for developing invasive pneumococcal infections.

In summary, this study found that the pneumococcus has mechanisms to recognise and process host stress hormones by Tf-iron delivery mechanism, which might be mediated via TCS09 system. In addition, the pneumococcal response to NE resulted in a remarkable change in cell-cell association from chains into diplococci and cell morphology by reducing cell size and the capsule. Furthermore, pneumococcal exposure to NE remarkably increased biofilm formation and altered metabolism. The overall analysis of *in vivo* experiments indicates that NE encounter might trigger transition from colonising the nasopharynx to infecting the lungs. The infographic figure 4.1 summarises the major findings of this study.

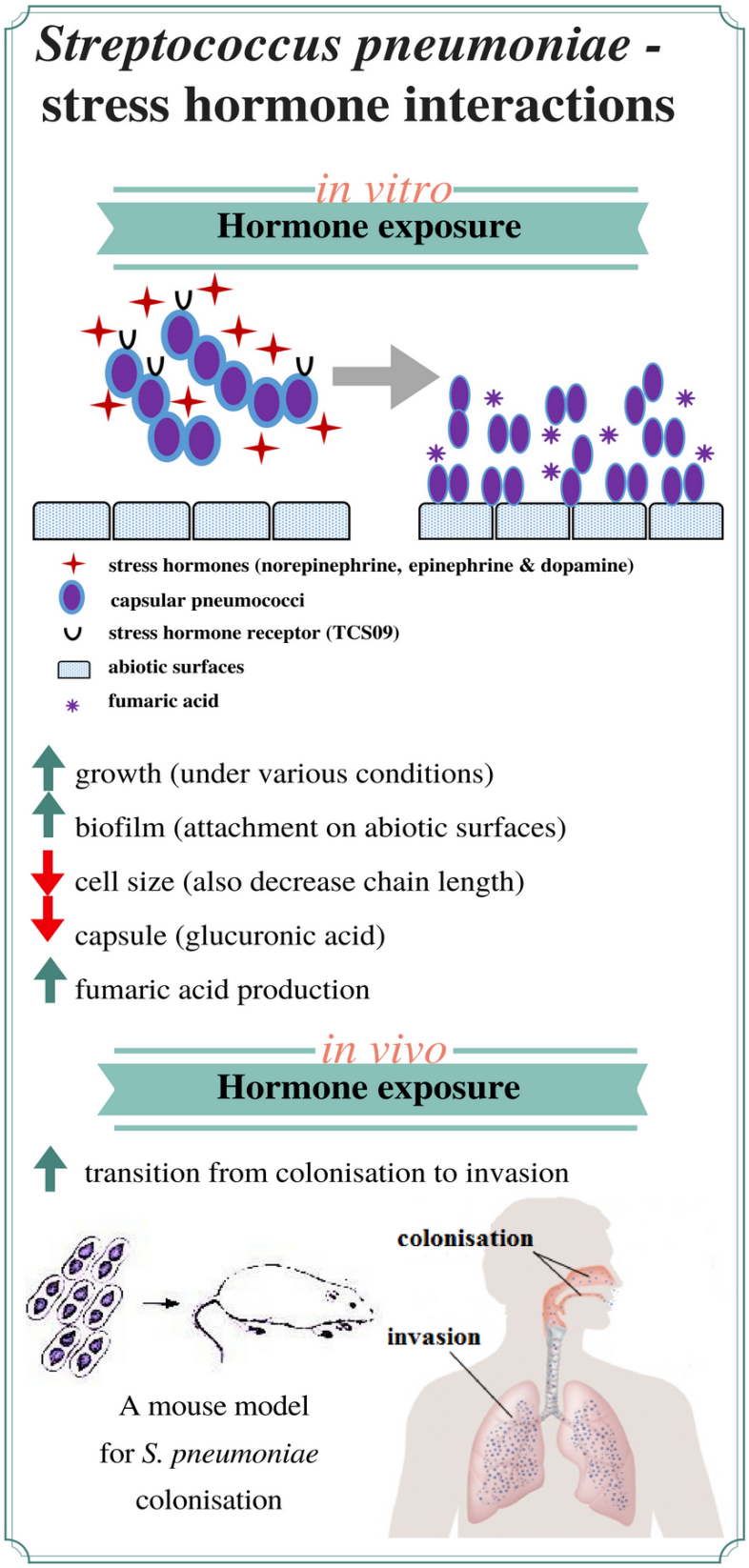


Figure 4.1. Summary of the major findings of this study.

Final remarks

By successful establishment of growth media conditions necessary to investigate different aspects of *S. pneumoniae*-stress hormone interactions, this study has found that clinically attainable levels of stress hormones increased pneumococcal growth and biofilm formation. In addition, the growth induction mechanism involved inotrope-delivery of transferrin-iron and internalisation of the catecholamine hormones, and also included the involvement of pneumococcal TCS09 in mediating the recognition and progressing of the stress hormone. The pneumococcus response to the catecholamine hormone NE also resulted in a notable change in pneumococcal appearance from chains into diplococci as well as a significant reduction in cell size. Moreover, this study also found that there is an influence of hormones over the synthesis of the pneumococcal capsular polysaccharides, and the decrease observed in cell size due to hormone exposure was also affecting glucuronic acid content within the capsular polysaccharides. The metabolites of *S. pneumoniae* grown in the presence and absence of NE have for the first time been profiled using GC-MS. The analysis score of the levels of 94 metabolites revealed a clear difference between the compounds present with in the control and NE-treated cultures. The high fumaric acid production in NE-treated *S. pneumoniae* metabolite pool, which has also been detected in clinical samples of pneumococcal infections suggest that this is a key strategy of the pneumococcus to survive and compete rival species within the nasopharyngeal host. The analysis of *in vivo* experiments indicated that a stress hormone encounter might trigger translocation from the nasopharynx into the lungs, which may enhance *S. pneumoniae* in its transition from commensal to pathogen. Therefore, the pneumococcal ability to respond to host stress signals may be key to its capacity to cause life-threatening pneumonia, septicaemia and meningitis.

Future work

This study investigated the impact of catecholamine hormones using one of the most virulent pneumococcal strains serotype 2 D39, which is able to cause life-threatening pneumonia, otitis media, septicaemia and meningitis (Brueggemann et al., 2003;Kronenberg et al., 2006;Hausdorff et al., 2000). However, it would be interesting to investigate the effects of hormones on less virulent strains such as the most commonly isolated colonising serotypes 6, 19, 14 and 23 (Meats et al., 2003;Soininen et al., 2001;Goldblatt et al., 2005). It is not fully understood why different pneumococcal serotypes can naturally colonise the nasopharynx in otherwise healthy people, and when they manage to translocate from the mucosal barrier to disseminate inside the host, they, then, cause severe damage and diseases (Gillespie, 1989). Approximately 20 out of over 90 serotypes are able to cause serious invasive diseases (Mulholland, 1999;Musher, 1992). This is due to pneumococcal ability to adhere to and invade various epithelial and endothelial cells via cell-associated mechanisms for internalisation (Adamou et al., 1998;Cundell et al., 1995;Elm et al., 2004;Ring et al., 1998;Talbot et al., 1996;Zhang et al., 2000). It possible to speculate that host factors such as stress hormones are involved in process of translocation from colonised niches into sterile sites. This work supported by future investigation could reveal that human stress increases susceptibility to pneumococcal infection and increases survival of the bacteria as it moves between hosts. Although it is unknown whether pneumococcal strains from a stressed individual are more infectious or better at colonising a new host, the work in this thesis supports the idea that stress hormone exposed pneumococci are more infectious and invasive. This can be answered by further investigation using animal infection models that focus on colonisation. However, testing hormone effects in mouse models is challenging, as stressing the control group of animals when administering the bacterial dose is almost inevitable which may influence the overall outcome. The stress hormone effects on pneumococcal behaviour inside the host also needs further investigation particularly in virulence model of infection. This can be

achieved *in vivo* model of pneumonia or bacteraemia infection by evaluating signs of disease, bacterial load in blood and tissue samples and survival assays.

Catecholamine hormones are widely distributed throughout mammalian body; however, future investigations should include other important groups of host hormones such as glucocorticoids (GCs), group of steroids which are lipophilic molecules, and the cortisol being one of the most important GCs human and animal hormones. It plays an essential role in various systems including immunological, homeostatic, cardiovascular and metabolic systems (Padgett and Glaser, 2003). Levels of cortisol increase during a stressful situation leading to an increase of both blood sugar and pressure but a decrease in the immune response. (Haas and Schauenstein, 2001;Morale et al., 2001;Padgett and Glaser, 2003;Reiche et al., 2004). Future studies *in vitro* might also cover the impacts of host hormones on various aspects of pneumococcal virulence. The most studied pneumococcal virulence factors include the polysaccharides capsule (PSC), pneumolysin (Ply), choline-binding proteins (CbpA) also called pneumococcal surface protein C (PspC), neuraminidases (NanA and NanB), hyaluronate lyase (Hyl), pneumococcal surface protein A (PspA), autolysin (LytA), pneumococcal surface antigen A (PsaA), enolase (Eno) and pneumococcal adhesion and virulence A protein (PavA) (Jedrzejas, 2001;Kadioglu et al., 2008). The spatiotemporal importance of these virulence elements during the pneumococcal infection can vary, although the maximum virulence of the pneumococcus involves the collective action of all these factors (Kadioglu et al., 2008). This study showed that PSC material was reduced after NE encounter, so it would be interesting to see if stress hormones can exert their effect on other pneumococcal virulence factors.

Although, the role of TCS09 in pneumococcal growth and NE uptake *in vitro* have been demonstrated, additional assays such as real time PCR, microarrays and metabolomics analysis are required to confirm to what extent this TCS does in pneumococcal regulation when exposed to host stress hormones. Additionally, Pneumococcal Surface Protein A (PspA) has been identified as lactoferrin-binding protein (Hammerschmidt et al., 1999). Thus, it is possible to investigate if TCS09 and PspA are collectively

involved in Tf/Lf pneumococcal binding and hormone-iron uptake. Currently, it is unknown whether the response regulator TCS09 is involved in regulating such surface proteins in the events of *S. pneumoniae* - stress hormone interactions. Furthermore, the role of this important gene has not been tested *in vivo* since mutant lacks this protein became nonvirulent in pneumonia and bacteraemia model of infection. Therefore, colonisation models of infection using the TCS09 knockout mutant might shed some light in its involvement in the pneumococcal-hormone interaction.

In addition, given there are multiple levels of genetic control, studying the impact of hormones on pneumococcal epigenetic profile is an important aspect since epigenetic mechanisms in the hormonal regulation have shown to involve reversible epigenetic modifications to chromatin and DNA methylation profiles. Methylation of the genomic DNA is vital for various aspects of fundamental biological processes including genome stability, imprinting, aging, differentiation and development (Kouzmenko et al., 2010). This would provide much deeper understanding of hormone effect on bacterial DNA. Other classes of hormones such as glucocorticoid particularly cortisol as well as catecholamine inotropes might also be tested in pneumococcal-host interaction both *in vitro* and *in vivo.*

Clinicians should extend their view of the side effects of drugs such as inotropes to also include their patient's microbiome. This is of great importance in hospitals, where patients are more exposed to endogenous and exogenous hormones particularly those receiving hormone-derived inotropes in the ICU. Therefore, extra measures and precaution should be taken to ensure bacteria like the *S. pneumoniae* is not under unnecessary constant hormone exposure, which could contribute to the risk of a serious pneumococcal infection.

Appendix

The pneumococcal metabolites were analysed using Gas Chromatography – Mass Spectrometry (GC-MS), which is one of the most applied analytical tools in metabolomics. GC-MS is capable of identifying hundreds of metabolites due to its high separation power. The intracellular and extracellular metabolites of *S. pneumoniae* grown in the presence and absence of NE have been profiled using GC-MS. Data revealed 94 compounds in total. Two reduced datasets where created from this: one for the extracts (containing 59 compounds) and one for the media samples (containing 41 variables).

Principal component analysis (PCA) model

Simply, PCA is a method of extracting key variables (e.g. components) from a large data set. For information about PCA models, the chapter two in "Multivariate Data Analysis for Dummies" (Camo software) demonstrates a brief and simple read introduction (http://www.camo.com/par/MVA-For-Dummies/index.html).

Bar charts

Bar charts of the individual variables in the reduced dataset are provided both in this report and as individual figures in PNG format. The error bars represent the standard deviation between replica samples, n=3. In order to ease interpretation of the results the compounds are divided in to four compound classes in the bar charts:

Amino acids: Covers amino acids and peptides
TCA compounds: Covers compounds related to the tricarboxylic acid cycle
Fatty acids: Covers various fatty acids
Misc: All other compounds

Putatively annotated compounds will have a question mark in their name while unknown compounds appear as X in this report.

1. Media samples

1.1. PCA model

The score plot calculated on the reduced dataset for the media samples (Figure 1) shows a clear separation between the three different sample types (media control, C and NE)

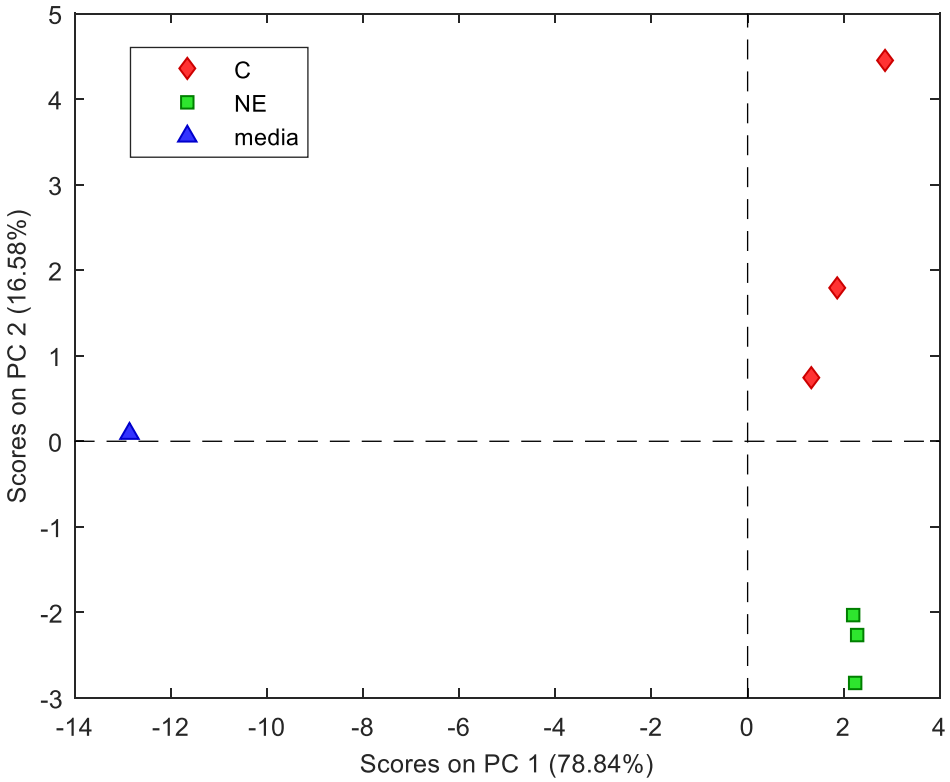


Figure 1. Score plot from PCA model calculated on the relative concentrations of the variables in the reduced dataset. Data has been autoscaled.

The loading plot (Figure 2) shows which variables that are responsible for the patterns observed in the score plot. Compounds located in the same location as a specific sample group is positively associated with that group and compounds oppositely located are

negatively associated (e.g. pyruvic acid and alanine are relatively low in NE samples while fumaric acid are relatively high).

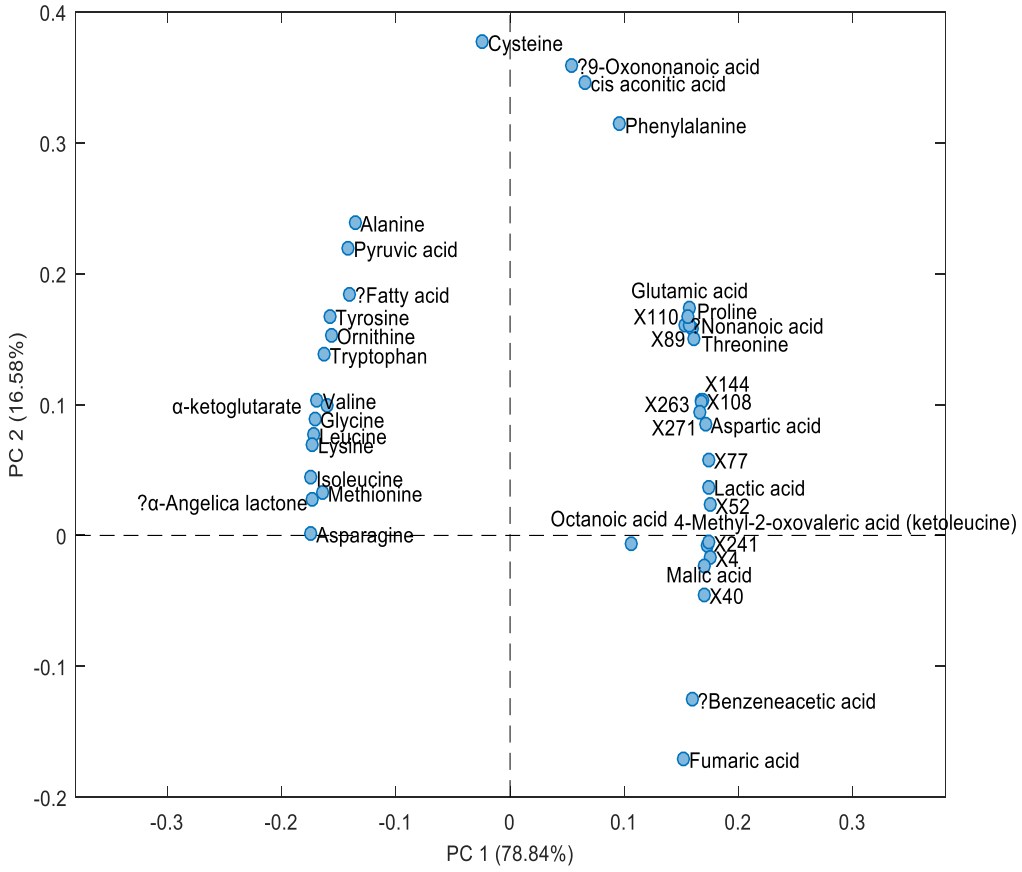
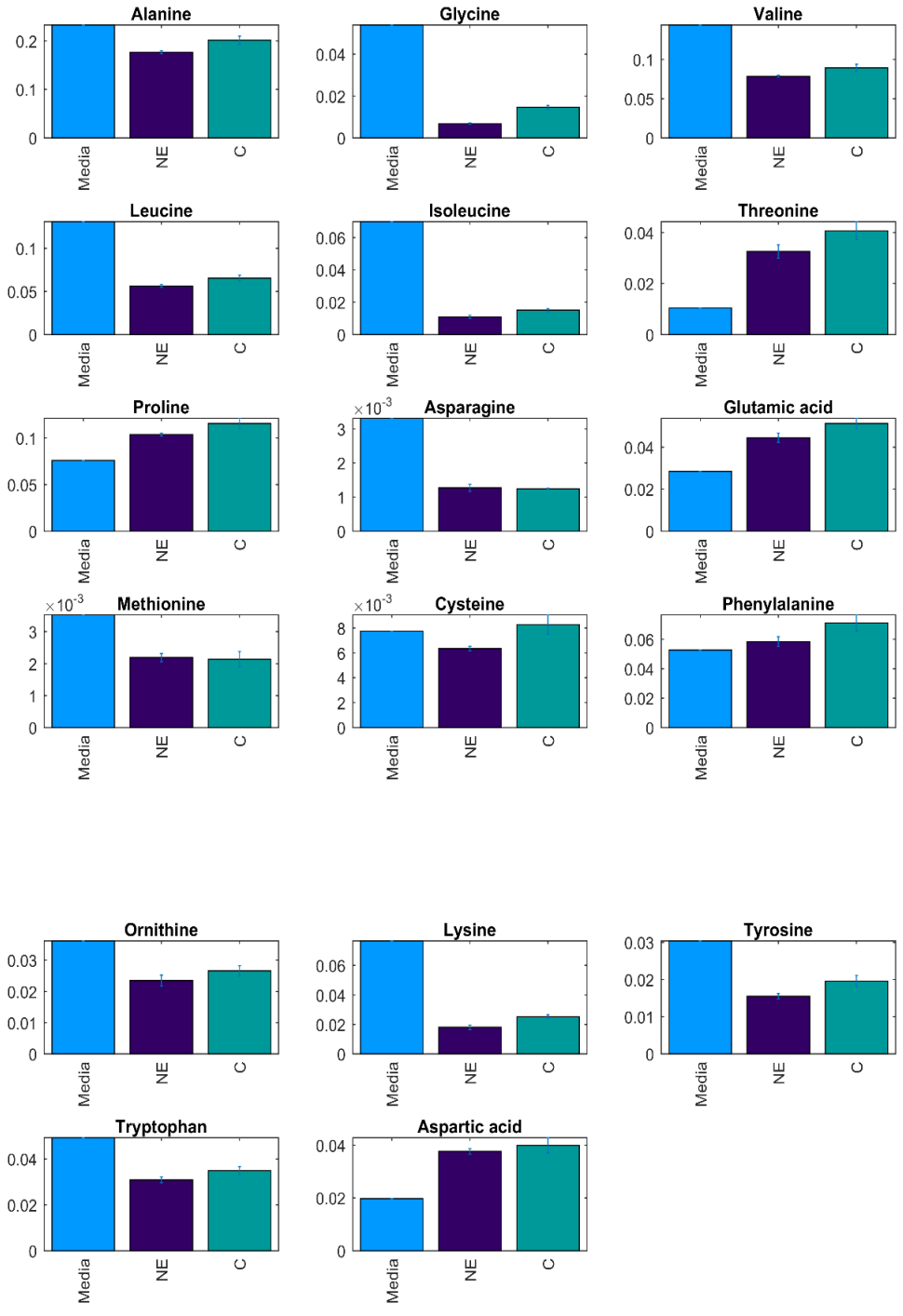


Figure 2. Loading plot from PCA model calculated on the relative concentrations of the variables in the reduced dataset. Data has been autoscaled.

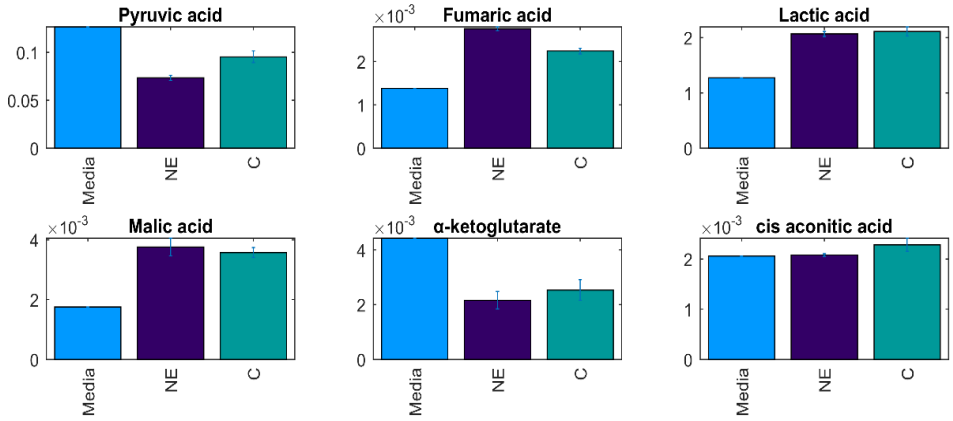
1.2. Bar charts

In the figure below bar charts are shown of the individual variables in the reduced dataset. Error bar represents the standard deviation between the three replicas.

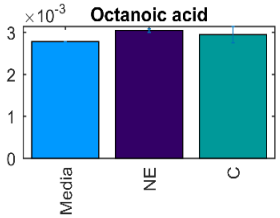
1.2.1. Identified amino acids



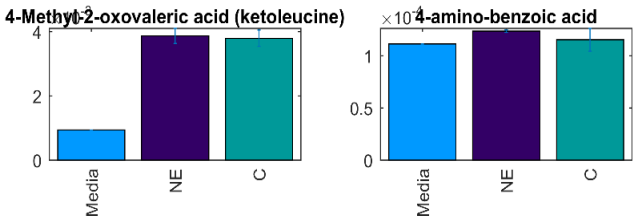
1.2.2. Identified TCA compounds



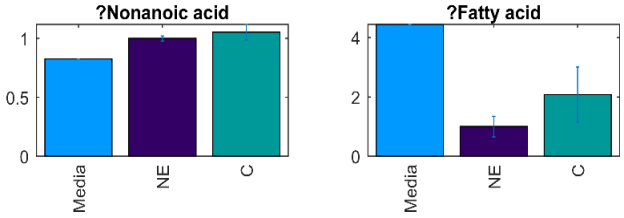
1.2.3. Identified fatty acids



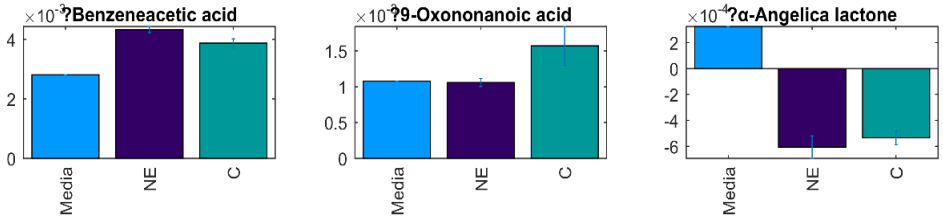
1.2.4. Identified misc.



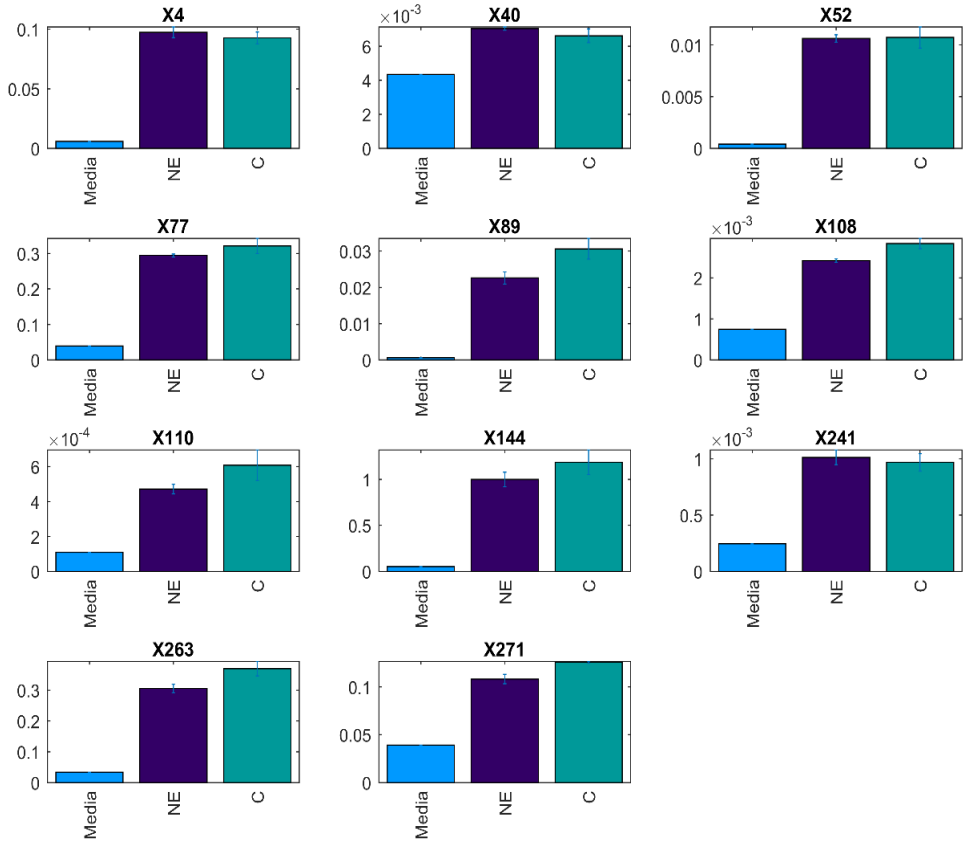
1.2.5. Annotated fatty acids



1.2.6. Annotated misc.



1.2.7. Unknown compounds



2. Intracellular compounds

2.1. PCA model

The score plot calculated on the reduced dataset for the extract samples does not show any clear grouping in PC 1 (not shown). However, in PC2 and PC3 (Figure 3) a clear separation between the two sample groups are observed (C and NE).

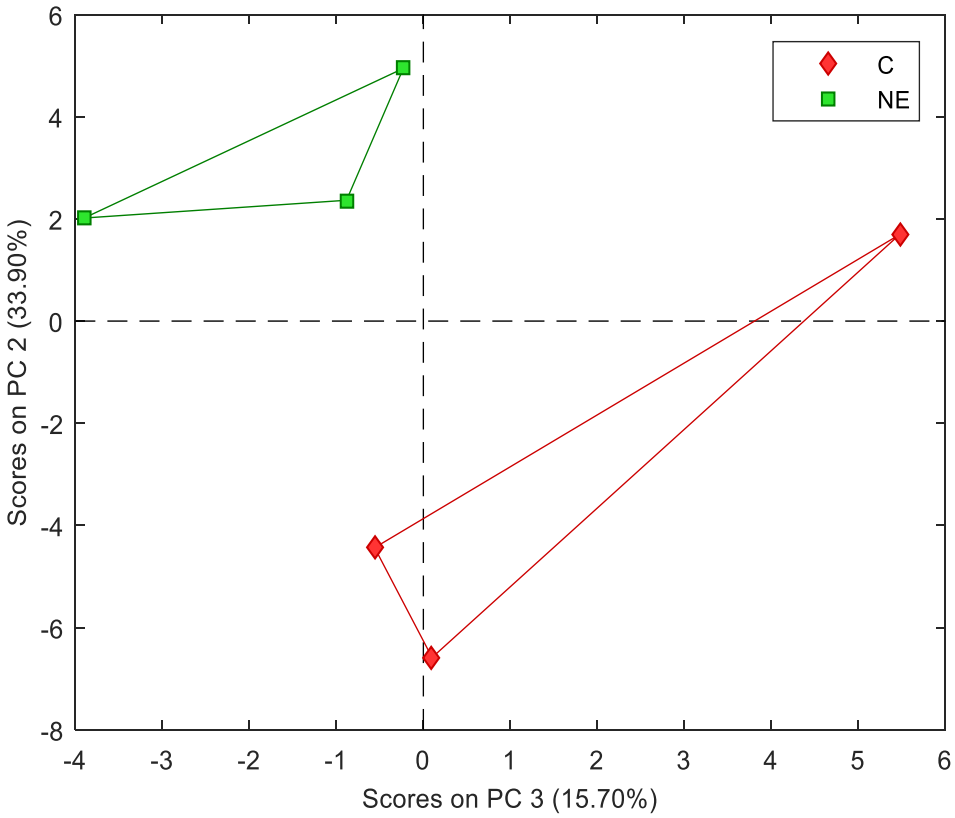


Figure 3. Score plot from PCA model calculated on the relative concentrations of the variables in the reduced dataset. Data has been autoscaled.

The loading plot (Figure 4) shows which variables that are responsible for the patterns observed in the score plot. Compounds located in the upper left quadrant are positively associated with NE samples while compounds located in the lower right quadrant are negatively associated with this samples group (and vice versa for samples group C).

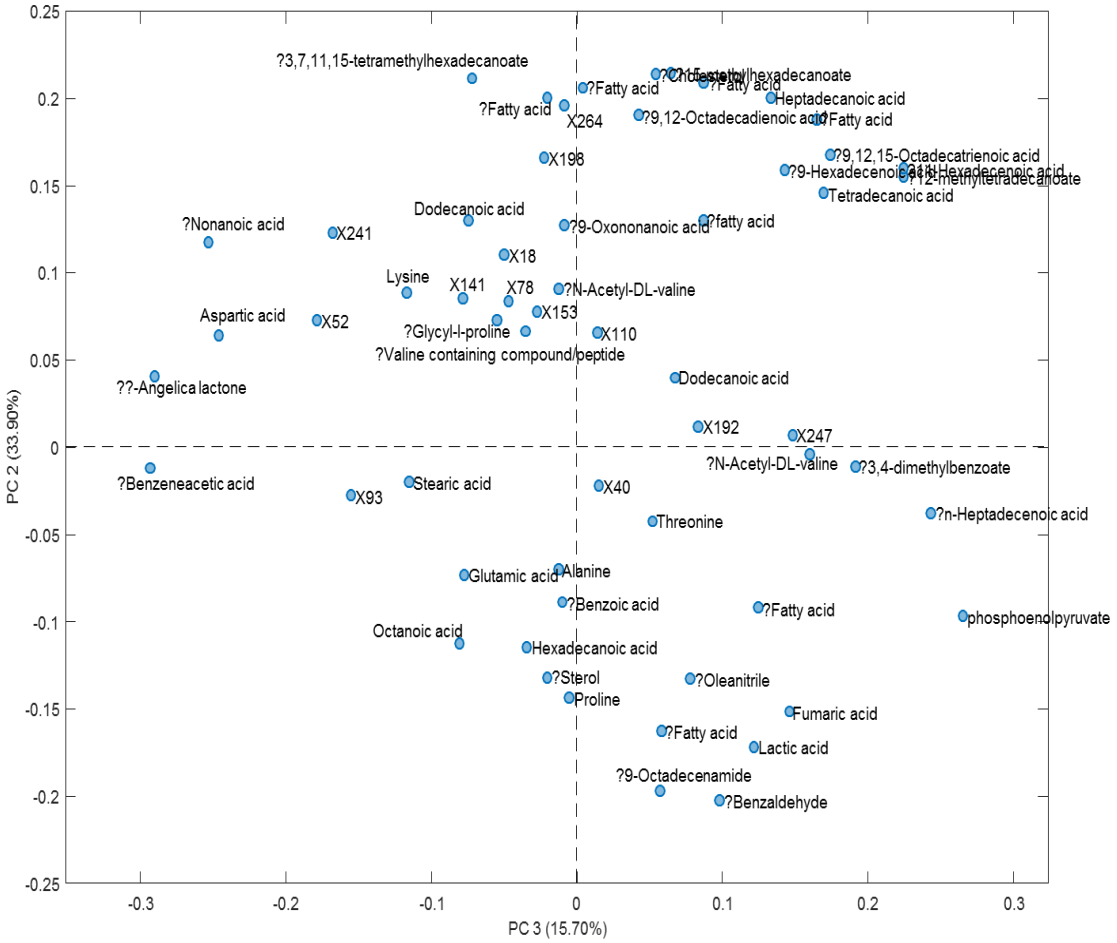
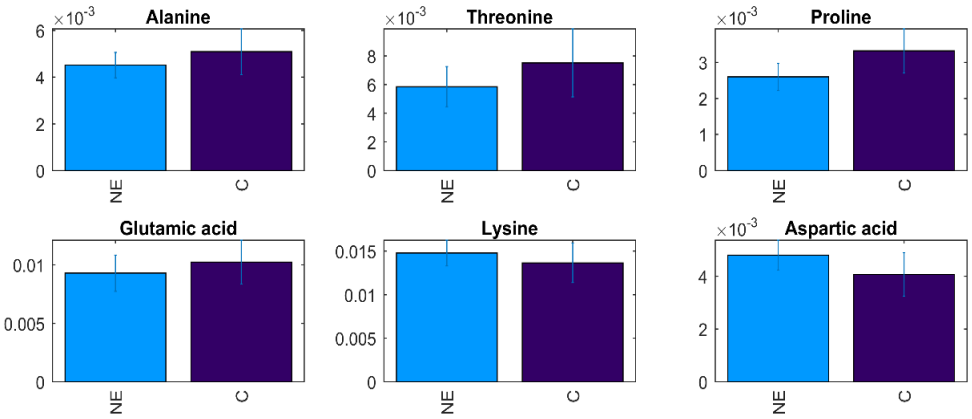


Figure 4. Loading plot from PCA model calculated on the relative concentrations of the variables in the reduced dataset. Data has been autoscaled.

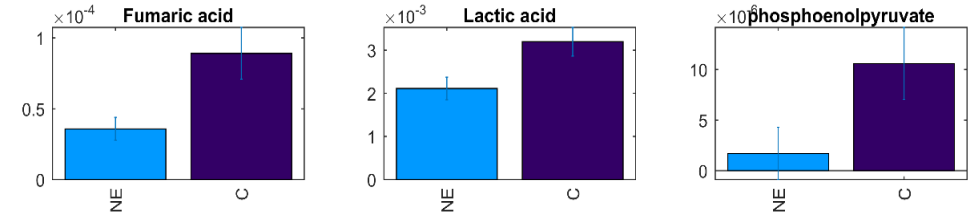
2.2. Bar charts

In the figure below bar charts are shown of the individual variables in the reduced dataset. Error bar represents the standard deviation between the three replicas.

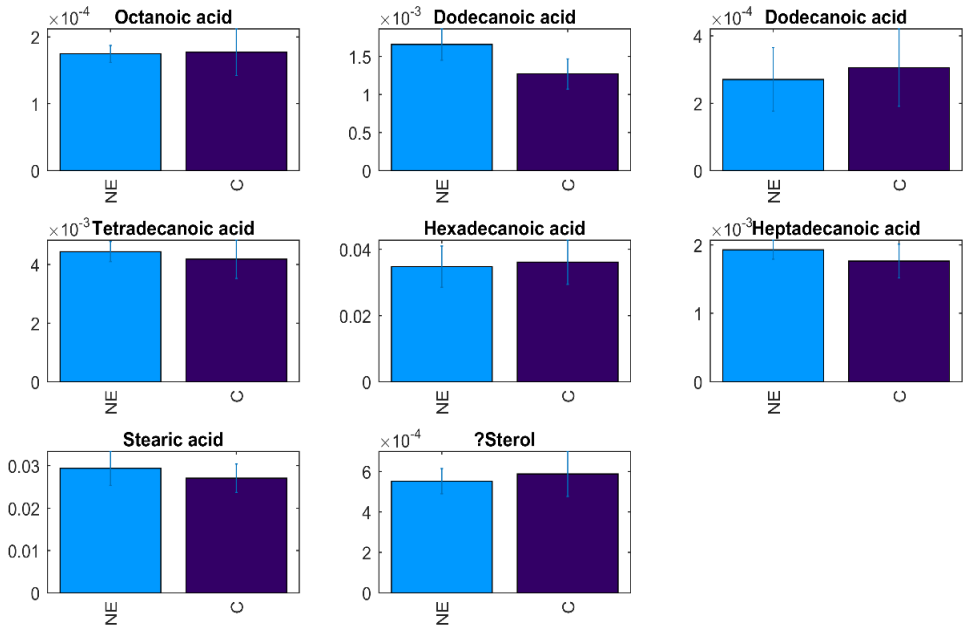
2.2.1. Identified amino acids



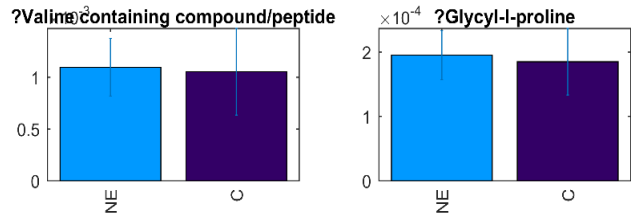
2.2.2. Identified TCA compounds



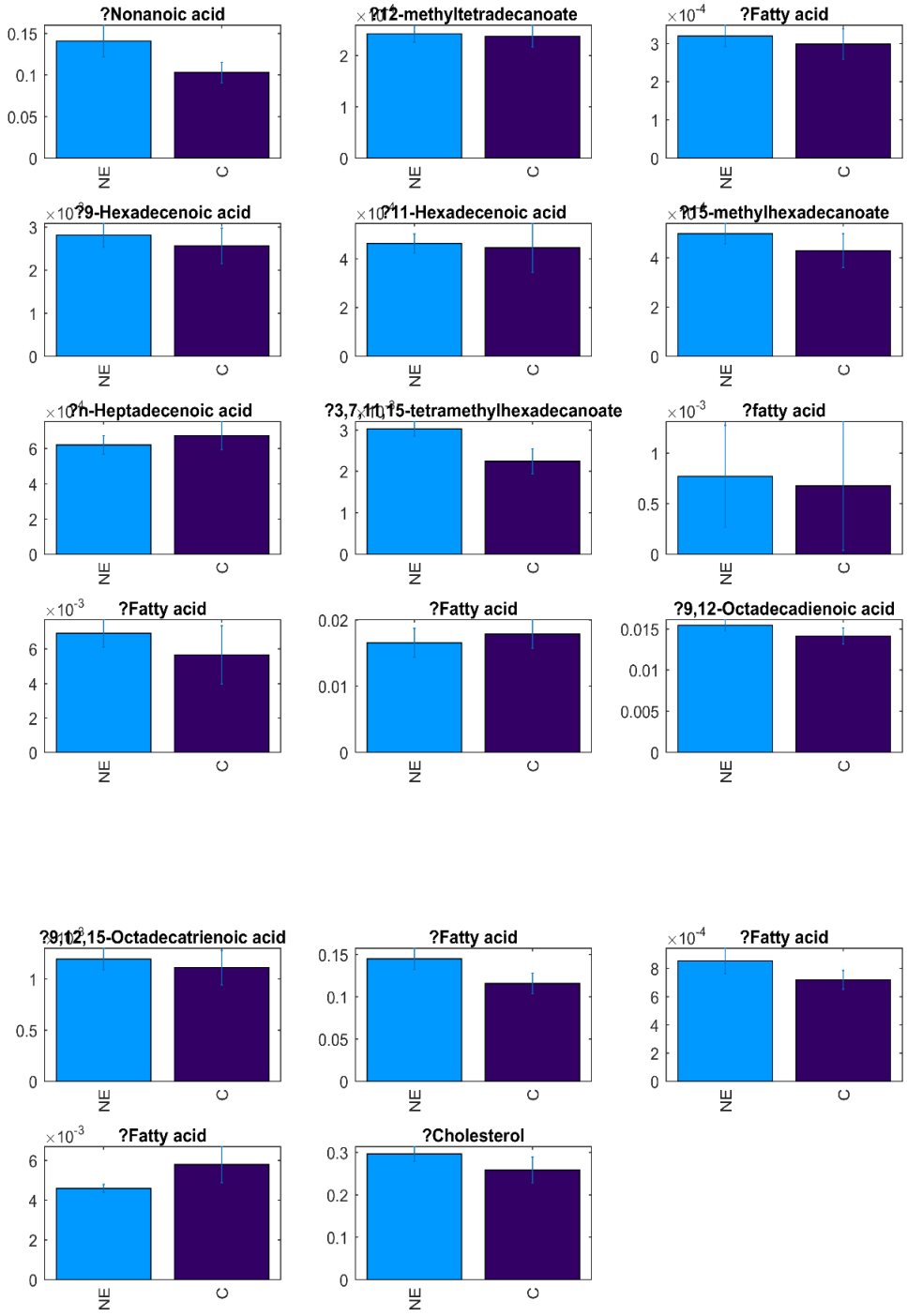
2.2.3. Identified fatty acids



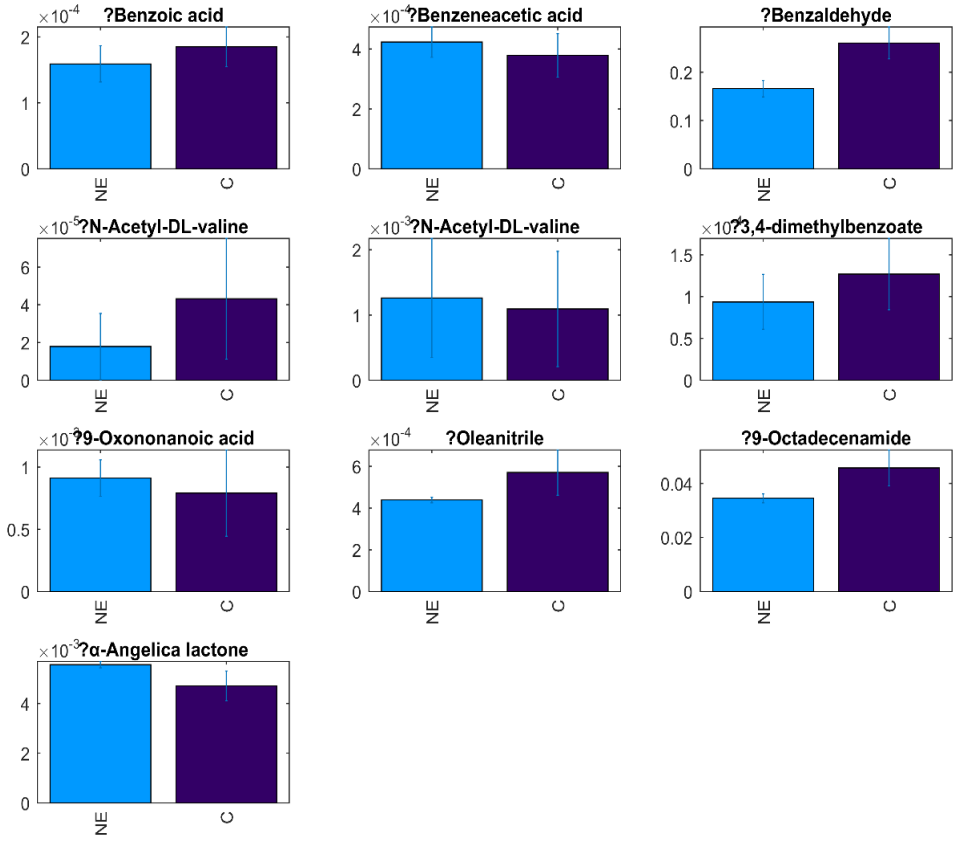
2.2.4. Annotated amino acids



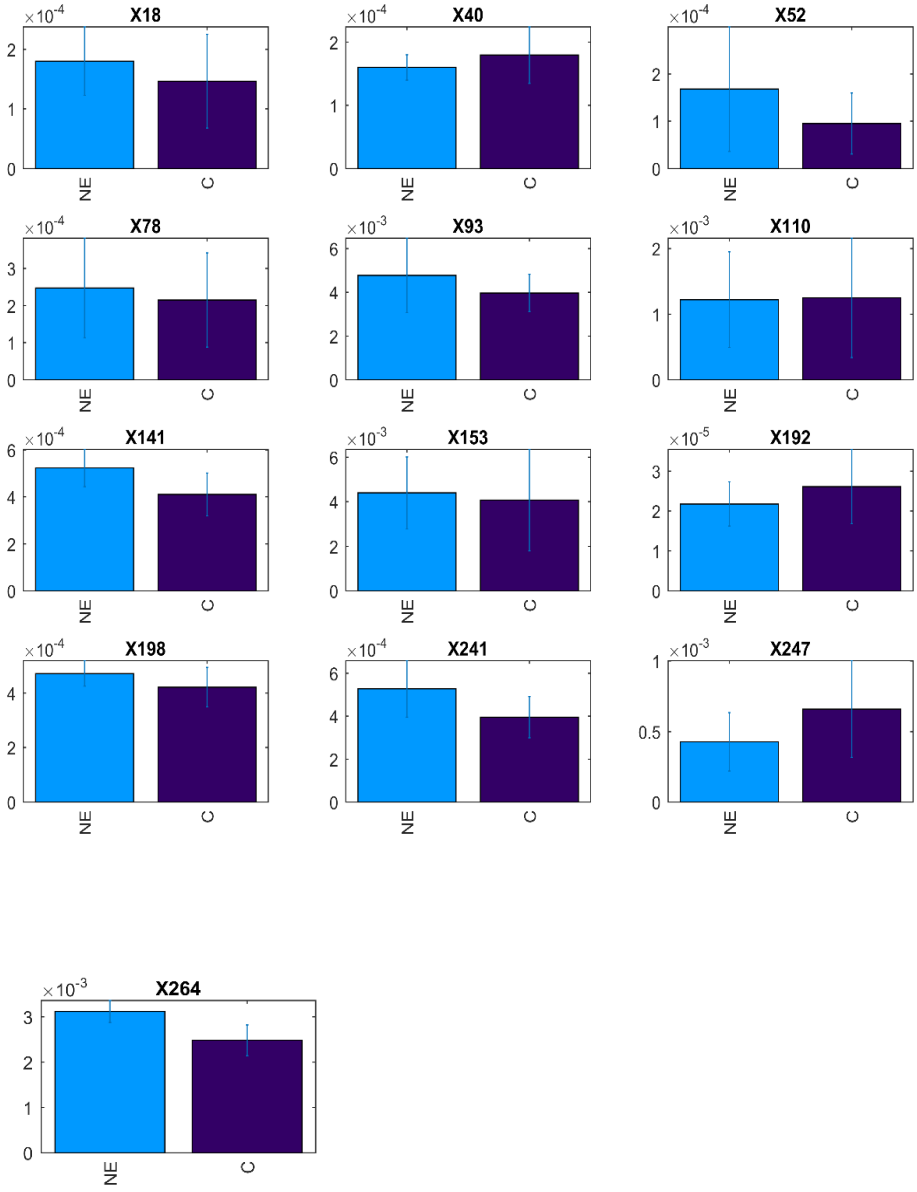
2.2.5. Annotated fatty acids



2.2.6. Annotated misc.



2.2.7. Unknown compounds



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