Genetic regulation of virulence in *Streptococcus pneumoniae*

A thesis submitted to the University of Glasgow for the degree of

Doctor of Philosophy

By

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Submitted September 2012

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September 2012

Acknowledgements

I would like to thank Professor Tim Mitchell for the opportunity to work in his lab and work with such an interesting pathogen. I have thoroughly enjoyed all four years of my PhD research and that is in part due to the wonderful people I have worked with, who have supplied endless knowledge, support and friendship, in particular Ashleigh Holmes, Carol McInally, Catherine Dalziel, Jiang Tao Ma, Ryan Ritchie, Andrea Mitchell, Matt Horsham, Kashif Mughal, Sultan Mansoor Al-Sharif, Yahya Noori and Kirsty Ross. I would like to also thank Denise Chandlish, June Irvine, Margaret Mullins and Diane Vaughan for invaluable help with scientific techniques and protocol development.

Most of all I would like to thank my parents and siblings Natalie, Becky, Nicky and Jonny for their support, for not having a clue what I do and providing the sometimes required non scientific conversation. And finally I thank Tom for his love and support throughout the good and the bad.

Abstract

S.pneumoniae is the leading cause of bacterial pneumonia and meningitis. Pneumonia alone has been estimated to kill more children under the age of five than that caused by AIDS, malaria and tuberculosis combined. The current vaccines which are used to prevent pneumococcal infection only protect against a small number of the 90+ serotypes currently identified. Current issues which may prevent the long term use of these vaccines is capsular switching, a phenomenon observed where some strains are able to escape the vaccine through switching their capsule genes. Further serotype replacement has been shown to occur since the introduction of the PCV7 vaccine, where serotypes not protected against by the vaccine have caused a higher incidence of invasive pneumococcal disease compared to the pre vaccine era. One strategy to avoid this is via the use of a multi-component protein based vaccine which is serotype independent.

The pneumococcus is normally found as a harmless commensal yet can also cause invasive disease as stated above, the pneumococcus is also the leading cause of otitis media. The ability for the pathogen to occupy a number of different niches and evade host defences is attributed to its large cache of virulence factors, including numerous cell surface adhesins. The ability of the bacteria to regulate genes required for adaptation to a specified niche is vital for survival. In this study a number of signalling systems that are able to modulate gene expression (specifically virulence factors) to facilitate adaptation to varying environmental conditions are assessed to determine the genes they regulate. Further key environmental signals are evaluated to determine the effect they have on regulation of important cell surface adhesins.

The main systems used to modulate global expression changes are two-component signal transduction systems (TCS). 13 TCS and one orphan response regulator are encoded in the pneumococcal genome. Little information is available with regards to the importance of each system, whether each system regulates its own separate collection of genes and the extent to which cross regulation may occur between these systems. This study used whole genome expression analysis data obtained through microarray analysis of single and double TCS mutants to assess the potential cross regulation of two chosen

systems. A number of the systems have also been shown to regulate the same islet, which encodes a pilus. Measuring expression of the islet itself enabled the role of the systems shown to regulate the islet to be assessed for potential interactions between the systems and whether a hierarchy exists.

The pneumococcus is highly genetically variable due to its ability to become naturally competent, taking up DNA from the environment and recombining it into its genomic DNA to aid genetic variation and survival. The new era of whole genome sequencing has begun to shed light on just how variable this pathogen is. Although a number of TCS have been shown to regulate pilus expression, with the use of whole genome sequencing of two closely related strains (one contains reduced pili expression levels) a number of other factors have also been identified which have been shown to alter pilus expression, this includes a serine/ threonine protein kinase, pyruvate oxidase and lactate oxidase. Further the pneumococcus has been shown to respond to exogenously added hydrogen peroxide which increases pilus expression levels. Levels of hydrogen peroxide may act as a key environmental cue to signal to the bacterium that they are present in the nasopharynx and require increased levels of cell surface adhesins.

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Abbreviations

%	Percentage
Δ	Deletion
∇	Insertion
°C	Degrees Celsius
μg	micro gram
μl	micro litre
μM	micro molar
aa	Amino acid
ATP	Adenosine-5'-triphosphate
BAB	Blood agar base
BHI	Brain heart infusion
BSA	Bovine serum albumin
CBP	Choline binding protein
cDNA	Complimentary DNA
CFU	Colony forming units
CPS	Capsule polysaccharide
CSF	Cerebrospinal fluid
CSP	Competence stimulating peptide
D39	*S.pneumoniae* serotype 2 strain
DNA	Deoxyribonucleic acid
E.coli	*Escherichia Coli*
ESTPK	Eukaryotic like serine/ threonine protein kinase

FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
$FMNH_2$	Reduced flavin mononucleotide
gDNA	Genomic DNA
GSP	General secretion pathway
H_2O_2	Hydrogen peroxide
HK	Histidine kinase
HRP	Horseradish peroxidase
I.N	Intranasal
I.P	Intraperitoneal
IPD	Invasive pneumococcal disease
I.V	Intravenous
Kb	Kilobase
kDa	Kilodalton
LB	Luria broth
LctO	Lactate oxidase
M	Mole
Mg	Milligram
Ml	Millilitre
mM	Millimolar
NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
ng	Nanogram

OD	Optical density
PASTA	Penicillin binding protein and serine/threonine kinase associated domain
PBP	Penicillin binding protein
PCR	Polymerase chain reaction
PI-1	Pilus islet 1
PI-2	Pilus islet 2
R6	*S.pneumoniae* serotype 2 strain
RNA	Ribonucleic acid
ROD	Region of diversity
RPM	Revolutions per minute
RR	Response regulator
S.pneumoniae	*Streptococcus pneumoniae*
SpxB	Pyruvate Oxidase
ST	Sequence type
STP	Protein phosphatase
StkP	Serine/ threonine protein kinase
TCS	Two-component signal transduction system
TIGR4	*S.pneumoniae* serotype 4 strain
UK	United Kingdom
USA	United States of America
VF	Virulence factor
WGS	Whole genome sequence
WT	Wild type

1 Introduction

1.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae is a gram positive diplococcus that was first isolated simultaneously by George Sternberg and Louis Pasteur in 1881 (Sternberg, 1881, Pasteur, 1881). In the majority of instances this organism is found as a coloniser in the nasopharynx of children and adults but can also cause invasive disease. *S.pneumoniae* can cause a number of diseases but is most commonly associated with causing pneumonia, septicaemia, meningitis and otitis media, with the highest disease burden seen in young children, the elderly and the immunocompromised.

The pneumococcus has a polysaccharide capsule, which is an important virulence factor and is also used as a classification system assigning each isolate into a serogroup. Over 90 pneumococcal capsular types (serotype) have currently been discovered, with serotype specific antibodies produced against each (Henrichsen, 1995, Park et al., 2007, van Dam et al., 1990, Bratcher et al., 2010). Some serotypes induce the production of antibodies that are cross reactive to other serotypes due to similar capsule structure and some strains express no capsule and are known as non typables (Smart, 1986). *S.pneumoniae* can be further phenotypically identified in a number of ways, though production of alpha haemolysis on blood agar, bile solubility and optochin sensitivity. Yet these tests do not necessarily distinguish this pathogen from other viridans group streptococci with some *S.pneumoniae* strains observed that are optochin resistant and bile insoluble (Kontiainen & Sivonen, 1987, Phillips et al., 1987, Fenoll et al., 1990). For more reliable classification multi locus sequence typing (MLST) is used which allows characterisation of pneumococcal isolates based on the sequence of seven housekeeping genes (*aroE*, *gki*, *gdh*, *xpt*, *recP*, *spi* and *ddl*), with variation in the alleles of these genes in different isolates used to assign a sequence type (ST), which can then be used as a gauge of relatedness to other isolates, there are currently over 4000 different ST identified (Enright & Spratt, 1998).

1.1.1 Disease burden

In 2000 there were roughly 14.5 million cases of invasive pneumococcal disease (IPD) encompassing meningitis, septicaemia and pneumonia (O'Brien et al.,

2009). *S.pneumoniae* is the leading cause of bacterial pneumonia and meningitis worldwide. Pneumococcal infections account for 11% of all child deaths between the age of 0-5 years old, which equates to roughly 800,000 deaths per year (Bryce et al., 2005). *S.pneumoniae* is the leading cause of bacterial pneumonia. WHO statistics state deaths from pneumonia equate to more deaths than that caused by tuberculosis, malaria and AIDS combined. Over 90% of deaths from pneumococcal infection occur in the developing world, with the highest burden present in Africa and Asia (O'Brien et al., 2009). The burden of pneumococcal disease is thought to be greatly underestimated due to less equipped diagnostics facilities in the developing world, inaccessibility of hospitals and potentially some deaths being attributed to alternative causes such as HIV or influenza, where pneumococci cause a secondary bacterial infection which is often the cause of death.

Although there are over 90 serotypes only a small proportion contribute to the majority of IPD cases, with the most common disease causing serotypes globally including serotypes 1, 5, 6A, 6B, 14, 19F and 23F prior to the introduction of the first pneumococcal conjugate vaccine (PCV7) in 2000, discussed further in section 1.4 (Johnson et al., 2010). Serotypes 1, 5 and 14 accounted for 30% of all cases of IPD in 20 of the poorest countries (Johnson et al., 2010). The major disease causing serotypes often vary between different countries making it hard to produce a vaccine that gives global protection. Introduction of PCV7 in 2000 gave protection against 49-82% of IPD causing serotypes with the greatest protection observed in Europe and North America where the disease burden in generally low comparatively to the developing world (WHO, 2012). Introduction of PCV10 and PCV13 however protects against roughly 70% of IPD causing serotypes globally (WHO, 2012).

1.2 Pneumococcal carriage

In most instances pneumococcal carriage in the nasopharynx does not lead to invasive disease. Yet the high instance of IPD is thought to be due to the high carriage rate of the pathogen, with 40% of children and 10% of adults colonised in the developed world, which can increase to 90% in the developing world (Regev-yochay et al., 2004, Austrian, 1986, Obaro & Adegbola, 2002). In children, carriage can occur from birth and may occur with more than one

serotype at once (Obaro & Adegbola, 2002). Carriage rates decrease over time with children thought to act as the main reservoir due to their high carriage rate (Gray et al., 1980, Bogaert et al., 2004, Leiberman et al., 1999). Vaccination of children promotes herd protection by removing the reservoir from which the elderly and at risk groups acquire pneumococcal carriage (Isaacman et al., 2008, O'Brien & Dagan, 2003).

Although colonisation is assumed to be required for progression to invasive disease some strains vary in their ability to cause disease with some serotypes more readily associated with carriage including serotype 6B, 9V, 19F and 23F. Whereas some were found more commonly in disease including serotypes 1, 4, 14 and 18C (Brueggemann et al., 2003). Carriage of one serotype can occur for a number of months. However this is not always the case and serotype 1 although causing a high incidence of invasive disease is almost never detected in carriage studies (Brueggemann et al., 2003, Brueggemann & Spratt, 2003), except in some indigenous Australian communities (Smith-Vaughan et al., 2009).

Other serotypes are opportunistic pathogens with invasive disease only occurring if the host is immunocompromised. One main factor predisposing to IPD is influenza infection with pneumococcal infection often occurring secondary to the viral infection. *S.pneumoniae* in this instance is often the cause of death (Short et al., 2012). The majority of deaths during the 1918 influenza pandemic were due to pneumococcal infection rather than the virus itself. This was also observed in 2009 where 29% of deaths caused by H1N1 showed signs of secondary bacterial infection (Morens et al., 2009, Chien et al., 2009). This is also the case for HIV infected patients with roughly 100,000 of the 800,000 IPD disease associated deaths In 2000 occurring in HIV positive children (Amdahl et al., 1995, O'Brien et al., 2009). This is also a problem in the developed world with immunosuppressed patients such as the elderly or cancer patients etc.

1.3 Pneumococcal disease

1.3.1 Pneumonia

S.pneumoniae is also termed the pneumococcus due to its ability to cause pneumonia, with *S.pneumoniae* being the commonest cause of community

acquired pneumonia accounting for 30-50% of hospitalisations due to pneumonia infections in Europe and America (WHO, 2012). Of the 800,000 deaths caused by IPD observed in 2000, 90% of these were caused by pneumococcal pneumonia (O'Brien et al., 2009). Pneumonia can often be treated with antibiotics, which upon their introduction has reduced the mortality rate of pneumococcal pneumonia from 60% to 9%, however antibiotic treatment in developing countries is still often unavailable (Flippin et al., 1951).

As mentioned already certain factors increase the risk of developing pneumococcal pneumonia such as immunosuppression which may be caused by influenza infection, HIV infection, cancer, diabetes, smoking, alcoholism etc (Bogaert et al., 2004, Amdahl et al., 1995, Herrero & Olivas, 2012). A large scale study has been done investigating the bacterial genes essential for lung infection using a murine model of pneumonia (Hava & Camilli, 2002). Included in these genes are a number of key virulence factors including PhtD, Ply, PspA, CbpA, a number of transcription regulators including that of the pilus islet (RlrA) and a number of TCS (two component signal transduction system) response regulators (RR) including RR01, RR07, RR09 and the histidine kinase (HK) of TCS12 (Hava & Camilli, 2002).

1.3.2 Bacteraemia

Bacteraemia is characterised by bacteria being present in the normally sterile blood stream, during pneumococcal pneumonia over 50% of patients are found to have pneumococci in the blood, thought to occur via direct transmission from the alveoli to the blood stream (Musher et al., 2000). Mortality rates caused by pneumococcal septicaemia can be high with up to 20% mortality in the developing world, with the rate highest in children under 2 years of age. Even in the developing world where antibiotics are prescribed as prophylaxis there still remains a high mortality rate due to pneumococcal septicaemia, which can range from 15-20% in adults and 30-40% in the elderly (WHO, 2012).

Studies into bacterial genes potentially important during bacteraemia have been performed looking at the gene expression changes of bacteria isolated from the blood of mice with septicaemia compared to growth in broth (Orihuela, Radin,

et al., 2004). This included up regulation of PspA and a number of choline binding proteins and up regulation of RR02 and RR10.

1.3.3 Meningitis

Meningitis can be caused by a number of bacteria however pneumococcal infection accounts for up to 37% of adult meningitis (Durand et al., 1993).This often occurs post bacteraemia or can also occur directly through the olfactory nerve from carriage bypassing pneumonia and bacteraemia (van Ginkel et al., 2003). Meningitis is often preceded by pneumonia and otitis media with 18% and 30% of meningitis sufferers having previously had one of these infections respectively (Østergaard et al., 2005). Meningitis is caused by the presence of bacteria in the cerebrospinal fluid and the meningeal covering of the brain, which leads to inflammation and swelling of the meninges. High morality rates are associated with meningitis which can be up to 50% in the developing world. As high as 58% of meningitis survivors suffer some form of neurological sequelae ranging from hearing loss, seizures to brain damage (Goetghebuer et al., 2000).

Studies have been performed into the genes that are important for bacterial replication during meningitis using a rat model of meningitis (Molzen et al., 2011). However none of the genes found to be important for this represent the key adhesins or TCS regulators other than that of the capsule. A number of ABC transporters were also shown to be important here which may be required due to the limited nutrients available in the CSF (Molzen et al., 2011). However, studies looking at the expression changes in a rabbit meningitis model relative to growth in broth show some virulence factors may be important during meningitis but perhaps non essential (Orihuela et al., 2004). Again although none of the well studied cell surface adhesins were differentially regulated in this study a down regulation of TCS03 and virulence factors SpxB, Ply and LytA was observed (Orihuela et al., 2004).

1.3.4 Acute otitis media (AOM)

Acute otitis media also known as earache is the most common form of pneumococcal infection, where pneumococci are present in the middle ear (Hausdorff et al., 2002). Although a generally mild infection the sheer number of

cases cause a huge burden on the health services worldwide. In American alone 7 million cases are reported each year with a cost of treatment estimated to be around $5 billion (Bondy et al., 2000). Roughly 10% of AOM cases result in relapse and require further treatment (Sox et al., 2008). Introduction of PCV7 has helped reduce the AOM cases as roughly 60-70% and 40-50% of the serotypes responsible for disease in children aged 6 months- 5 years old and of 0-6 months old are included in the vaccine respectively (Hausdorff et al., 2002). However further studies have indicated that although there has been a reduction in AOM cases caused by vaccine serotypes there has been no overall reduction in the total number of cases. Post PCV7 introduction Increasing numbers of AOM cases have been caused by non vaccine types, *Haemophilus Influenzae* and *Moraxella catarrhalis* (see review Dagan, 2004).

Again studies have been performed into assessing the genes required for AOM using a chinchilla ear infection model, a number of virulence factors were shown to be essential for ear infection including PspA, CbpA, PhtE, SrtD (pilin sortase), RlrA (pilus transcription regulator) and HK06 and RR10 (Chen et al., 2008).

1.3.5 Other

Some of the less commonly associated diseases caused by the pneumococcus includes endocarditis, empyema, brain abscesses and haemolytic uraemic syndrome, which are often found as complications of pneumonia and meningitis (Lindberg & Fangel, 1999, Gigliotti et al., 1981, “Proceedings of the Society,” 1929, Shayegani et al., 1982, Fincher, 1945, Byington et al., 2002).

1.4 Vaccines

1.4.1 Current vaccines in use

All current vaccines on the market target the pneumococcal polysaccharide capsule. As there are over 90 different serotypes all the vaccines include only a small number of these which are found to cause the highest incidence of disease (Henrichsen, 1995, van Dam et al., 1990, Bratcher et al., 2010, Park et al., 2007). Three vaccines are currently in the market PPSV23 (23-valent polysaccharide vaccine), PCV10 (10-valent polysaccharide conjugate vaccine) and PCV13 (13-valent polysaccharide conjugate vaccine) with one being phased

out PCV7 (7-valent polysaccharide conjugate vaccine). For information about vaccines below see review (Pittet & Posfay-Barbe, 2012).

1.4.1.1 PPSV23

PPSV23 (Pneumovax 23) was the first pneumococcal vaccine available (1983, Merck), which includes serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F, 33F. This vaccine consists of the capsular polysaccharide from each of the above serotypes and is currently used in the elderly and in some at risk groups and children over 2 years of age. However the vaccine is poorly immunogenic in children where a large incidence of disease occurs and this vaccine is therefore not used in the developing world.

1.4.1.2 PCV (pneumococcal conjugate vaccine)

Later came the pneumococcal polysaccharide conjugate vaccines which couple the capsular polysaccharide to that of a carrier protein used to elicit better immunity in children under the age of 2, as polysaccharide alone is poorly immunogenic in this at risk group.

The first manufactured conjugate vaccine in use was PCV7 (Prevnar 7), made available in 2000 (Wyeth), which includes the polysaccharide from serotypes 4, 6B, 9V, 14, 18C, 19F, 23F conjugated to a diphtheria toxoid. However although this vaccine has made a huge impact on the incidence of IPD as stated earlier the serotypes covered by this vaccine are more common in Europe and America where disease burden is much lower than that of the developing world. Due to this PCV7 is currently being phased out and replaced by PCV10 and PCV13, which protect against roughly 70% of all serotypes causing the highest incidence of IPD globally.

PCV10 (Synflorix) was next to be made available in 2009 (GlaxoSmithKline), which again contains the capsular polysaccharide of 10 serotypes conjugated to a carrier protein. This includes serotypes 1, 4, 5, 6B, 7F, 9V, 14 and 23F conjugated to protein D (outer membrane protein of *H.influenzae*), serotype 19F conjugated to a diphtheria toxoid and serotype 18C conjugated to a tetanus toxoid.

Finally PCV13 (Prevnar 13) made available in 2010 (Pfizer), which contains the capsule polysaccharide of serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F and 23F conjugated to a non-toxic diphtheria carrier protein, CRM 197.

These conjugate vaccines are currently only used in children and have shown to provide good protection from IPD, with a reduction in overall IPD observed by roughly 70%-90%, and 20% reduction in AOM cases caused by vaccine types in the years following PCV7 introduction (Bennett et al., 2003, Grijalva et al., 2006, Hennessy et al., 2005). Childhood vaccination with these vaccines has also shown benefits to the incidence of IPD in the elderly and immunocompromised as childhood vaccination removed the reservoir for adult infection (Isaacman et al., 2008, O'Brien & Dagan, 2003). Nevertheless the cost of these vaccine are high which limits their use in the developing world and due to this only a small number of serotypes are included in the vaccine, because of this and the reasons stated below these vaccines may be limited in their long term efficiency.

1.4.2 Serotype replacement

One phenomenon which will limit the long term use of the conjugate vaccines is serotype replacement, which has been studied since the introduction of PCV7. This has indicated that although the serotypes that cause the highest incidence of IPD have been removed, and the overall incidence of IPD dropped since PCV7 introduction there has been an increase in the incidence of IPD caused by non-vaccine serotypes. In America between 1998-2004 incidence of invasive disease caused by non vaccine serotypes increased post PCV7 vaccination in both children under 5 and adults. This increase was mainly due to serotypes 3, 15, 19A, 22F and 33F (Hicks et al., 2007), with the main disease causing serotype being 19A. In Alaska alone studies showed an increase in IPD in children under 2 by 140%-180% caused by non vaccine serotypes from the pre vaccine era to 2004. Of this increase around 30% was caused by serotype 19A (Singleton et al., 2007, Kellner et al., 2009). Although overall the IPD disease still remained lower in 2004 than in the pre vaccine era. Worryingly an increase in serotype 19A in AOM cases have also been observed post PCV7 introduction which is a multidrug resistant strain (Pichichero & Casey, 2007). Whether the post vaccine IPD incidence will rise to that of the pre vaccine incidence due to non-vaccine

serotypes remains to be seen. Some of the serotypes causing IPD in the PCV7 era are now protected against in the new vaccine compositions (PCV10/PCV13).

1.4.3 Capsular switching

Another phenomenon that may hinder the effectiveness of all the current pneumococcal vaccines is capsular switching. Due to the pneumococcus being naturally competent it is able to take up DNA out of the environment and recombine it into its own, switching genes with other strains. The ability of the pneumococcus to do this with the genes of the capsule locus means that strains protected against by the vaccines are able to change their capsule for that of one not protected against by the vaccine and therefore evade death. This has occurred in a number of antibiotic resistant strains including serotype 9V clones becoming serotype 14 and identification of 19F, 23F and 19A serotypes of the same clone, with evidence indicating this may be a frequent occurrence in the pneumococcus (Coffey et al., 1998, Coffey et al., 1999, Croucher et al., 2011).

1.4.4 New vaccine development

New efforts are focusing on development of a multi-component protein based vaccines, which will be immunogenic in children under 2 years of age. In most instances a single protein would not give protection as a number of the key virulence factors are only present in a proportion of strains including the pneumococcal pilus, PsrP etc. Using more than one protein also reduces the likelihood of the pneumococcus being able to evade the vaccine by removing this factor as they can do for the capsule (Blomberg et al., 2009). Current proposed vaccines include use of a RrgB (pilus backbone protein) fusion protein joining the three different variants of this pilin (Harfouche et al., 2012). StkP a serine/ threonine protein kinase, PcsB a murine hydrolase and a number of other surface associated virulence factors including PspA, CbpA, PhtD and the toxin pneumolysin are all included in multi-component protein based vaccine currently under investigation (Olafsdottir et al., 2012, Denoël et al., 2011, Ogunniyi et al., 2007, Xin et al., 2009, Cao et al., 2007, Douce et al., 2010).

1.5 Genetic variation

S.pneumoniae is one of the viridans group streptococci that is naturally competent. *Streptococcus oralis* and *Streptococcus mitis* can also become naturally competent (Håvarstein et al., 1997). This enables the bacteria to take up DNA out of the environment and recombine it into its genome, which can be used to acquire new genes which may be beneficial for survival or repair of genes made non functional via spontaneous mutations etc (Johnsborg, et al., 2008, Steinmoen, et al., 2002).

1.5.1 Development of natural competence

The development of natural competence is controlled by TCS12 also known as ComDE. ComDE is one of the TCS that the extracellular stimulus is known, this TCS is activated by a small peptide known as CSP which is encoded by *comC* located directly upstream of the genes encoding the TCS (Havarstein et al., 1995, Havarstein et al., 1996, Pestova et al., 1996). Activation of the TCS via binding of CSP leads to the regulation of two distinct sets of genes known as the early and late competence induced genes. The early competence induced genes include ComC/D/E as upon activation of ComDE a positive feedback occurs positively regulating the expression of all three gene, which increases the level of CSP and the TCS which are activated (Ween et al.,1999, Peterson et al., 2004). Other early competence genes include ComA/B which are required for the maturation and export of pre-CSP. ComX is also an early competence gene which is an alternative sigma factor and is required for induction of expression of the late competence genes, which include genes required for the uptake and processing of DNA for recombination into the transformants (Lee & Morrison, 1999, Luo, Li, & Morrison, 2003, Luo & Morrison, 2003). The ability of the pneumococcus to do this is the main reason for such high genetic variability, some of the studies elucidating this are discussed below.

1.5.2 Regions of diversity (ROD)

A number of studies have been performed looking into the regions that are diverse between different strains. Using comparative genome hybridisation, large scale studies have been performed looking at regions between strains of the same ST and serotype that are divergent. This kind of analysis would detect

large chunks of DNA that are missing/ present in certain strains but would not reveal whether the genes contain small changes such as SNPs or indels.

The current methods used to assign pneumococci into groups that show high similarity is based on their serotype (capsule genes) and sequence type (based on 7 housekeeping genes), which has been described above. However recent studies have indicated that although these genes may be identical the rest of the genome may not with strains of the same ST and serotype varying greatly in their ability to cause disease (Silva et al., 2006, Sandgren et al., 2005). A total of 25 ROD were observed by Silva et al., 2006 between the 14 strains assessed. In this study three serotype 14 strains ST124 which were all isolated from the blood of infected patients in 2000/2001 in Scotland showed a number of variable genes between the three strains and each strain also showed varied levels of bacteraemia after 6 hours post infection (Silva et al., 2006). Obert et al (2006) analysed a total of 72 strains for RODs that may be associated with virulence, with comparison performed between 42 strains taken from patients with invasive disease and 30 from non invasive disease. The accessory genome in this study equated to 27% of the genome, 153 genes were found to be associated with invasive disease and 176 with non invasive disease causing strains (Obert et al., 2006). Further analysis of some of the ROD identified in this study was performed in Embry et al., 2007. Another larger scale study was done using a similar technique assessing the variable regions (accessory regions) between 47 different strains of varying serotype and ST (some shared the same ST and serotype), which were taken in some instances from different origins of infection (Blomberg et al., 2009). A total of 41 accessory regions were identified containing 95 genes, roughly 34% of the genome was shown to contain genes that are non essential and therefore vary between different strains (Blomberg et al., 2009).

Some smaller scale studies have also been performed focusing on the distribution of the known virulence factors in different pneumococcal strains. A study looking a the presence of virulence factors in strains causing community acquired pneumonia in Japan indicated *eno*, *pavA*, *piuA*, *cbpA* and *cbpG* were in all strains and therefore may be important for this disease. Whereas other virulence factors were variable among strains including *hly*, *piaA*, *rlrA*, *psrP*, *nanC* and *pspA* (Imai et al., 2010). Along with the presence of virulence factors

assessed in different pneumococcal isolates this has also been performed looking at their presence in other viridans group streptococci, specifically *S.oralis*, *S.mitis* and *Streptococcus pseudopneumoniae* (Johnston et al., 2010). Interestingly a large number of the key pneumococcal virulence factors are also found in these species including NanA, NanB, PhtA,B,D, a large cache of choline binding proteins, penicillin binding protein and some of the regulatory genes including TCS05 and TCS12. However again their presence seem to vary depending on the strain (Johnston et al., 2010). This indicated DNA is also swapped between different streptococci.

1.5.3 Whole genome sequencing

Another way to assess genome diversity between different isolates is by the use of whole genome sequencing, which has revolutionised the ability to assess genetic variability and evolution of bacteria pathogens. The progress in technology and reduced cost of genome sequencing has made it accessible to the wider scientific community and permits larger scale studies to be performed enabling comparison of the whole genome of a number of different strains. The use of whole genome sequencing not only allows identification of large ROD between strains but also small changes such as SNPs and indels can be identified. This technology has been applied to include large scale genome comparisons to assess the conservation of genes between different strains which can be used to identify potential vaccine candidates, this approach has been used in *Streptococcus agalactiae* assessing the genes conserved in this pathogen and within other related streptococci (Tettelin et al., 2005). These studies have also linked genomic factors to invasive disease, for instance the association of a mutation in a transcription regulator in *Staphylococcus aureus* was shown to be key in progression from carriage to disease (Young et al., 2012). Similarly in *Neisseria meningitidis* the presence of a prophage was linked to the strains ability to cause meningitis (Bille et al., 2005). This technology was also used to assess the genotype of the *Escherichia coli* strain which caused an outbreak in Germany in summer 2011, the genome sequencing of this strain showed this strain has acquired a prophage which carried a shiga toxin responsible for its increased virulence (Rasko et al., 2012). Whole genome sequenceing has also been used to follow the evolution of a bacterium over time, which was performed on *Vibrio cholerae* strains isolated during a pandemic outbreak

(Mutreja et al., 2011). This technology has also proven to be useful in assessing the origin of infection of some bacterial pathogens such as that observed for *Mycobacterium leprae* which was found in wild Armadillos and was identified as a zoonotic source of human infection (Truman et al., 2011). This technology has also been used for metagenomic studies assessing the bacteria and viral flora that Is present in different host (human) niches (Lysholm et al., 2012, Nelson et al., 2010). Finally this technology will also pave the way for the potential for discovery of new microbial and viral species which are currently not identified as they cannot be cultured by standard methods (Hongoh & Toyoda, 2011).

A large number of pneumococcal genome sequences are now available. The first pneumococcal genome sequences were made available in 2001 which was that of a serotype 4 strain (TIGR4), this was shortly followed by serotype 2 strain (R6), and then another serotype 2 strain (D39) in 2007 (Tettelin et al., 2001, Hoskins et al., 2001, Lanie et al., 2007). Findings from multiple genome comparisons have further confirmed the high genetic variability between different pneumococcal strains with genomes ranging from 2000-2200 kb, number of predicted coding sequences ranging between 2200-2800 and variations in the percentage of the core genome ranging from 21-33% deduced from comparing the genomes of 17 strains with different ST and serotype (Hiller et al., 2007). A further large scale study was performed comparing the whole genome sequence of 240 isolates of the PMEN1 serotype 23F multidrug resistant clone taken from Europe, South Africa, America and Asia between 1984-2008, to assess over time how the strain had evolved. This study further validates the genome plasticity of the pneumococcus, with multiple recombination events observed over a short period of time with some allowing development of antibiotic resistance and capsular switching to enable vaccine escape (Croucher et al., 2011).

1.5.4 Phase variation

Genetic variation can also be observed in the pneumococcus within the same bacterial population. In a single population of pneumococci there are often two colony types present, encompassing opaque and transparent phenotypes, this phenomenon can also be observed in other bacteria (Weiser, 1993, Weiser et al., 1994). The two colony types can be distinguished between one another using a light microscope (Weiser, 1993, Weiser et al., 1994, Weiser et al., 1996). Both

phenotypes vary in a number of different ways with both showing different surface protein profiles, different membrane fatty acid compositions, different capsule levels, different teichoic acid levels, different bacteriocin activity and both also vary in the *in vivo* niche in which they are able to survive (Aricha et al., 2004, Overweg et al., 2000, Benisty et al., 2010, , Kim et al., 1998, Kim et al., 1999, Weiser et al., 1996, Dawid et al., 2009). Studies into what causes the difference between the two phenotypes have shown that mutations in a number of genes can lead to a switch from transparent to an opaque phenotype, including genes involved in glycerol metabolism, mutations in the capsule genes and mutations in pyruvate oxidase (McEllistrem et al., 2007, Saluja & Jeffrey, 1995, Ramos-Montañez et al., 2008).

1.6 Virulence factors (VF)

1.6.1 Overview

The pneumococcus contains a large number of VF which are important for its survival, which aid in adherence to host cells and help evade the host defences. An overview of the most important are shown in Figure 1-1. In large scale virulence screens a large number of genes are classed as virulence factors yet are not present on the cell surface or secreted and therefore are unlikely to interact directly with host factors. In this instance deletion of these genes may have a knock on effect to the expression of cell surface VF or alter the fitness of the cell through altered metabolism/ make required nutrients unavailable (Hava & Camilli, 2002). Below only the virulence factors found on the cells surface of the bacteria or secreted will be discussed in more detail with regards to their role in virulence.

Protein VF present on the cell wall are initially targeted for secretion by the GSP (general secretion pathway), with the majority containing the typical cell wall targeting signal peptide, once secreted the VF can be retained in the cell wall via three mechanisms, see review Pérez-Dorado et al., 2012. LPXTG anchored proteins are covalently attached to the cell wall/ peptidoglycan via specific sortase enzymes, this includes the pneumococcal pilus, which contains three dedicated sortases for its attachment/ assembly, other LPXTG anchored surface proteins are attached via the house keeping sortase (SrtA) (Kharat & Tomasz,

2003, Paterson & Mitchell, 2006). The second class are retained in the cell wall via non-covalent attachment known as choline binding proteins of which most have a C-terminal choline binding domain which interacts with phosphorylcholine moiety in teichoic acid (TA) or lipoteichoic acid (LTA). The pneumococcus contains a large number of these proteins (19 TIGR4/ 13 D39) of which some will be discussed in more detail later (Tettelin et al., 2001, Lanie et al., 2007). The final group include proteins retained at the cell wall through weak interactions with lipids.

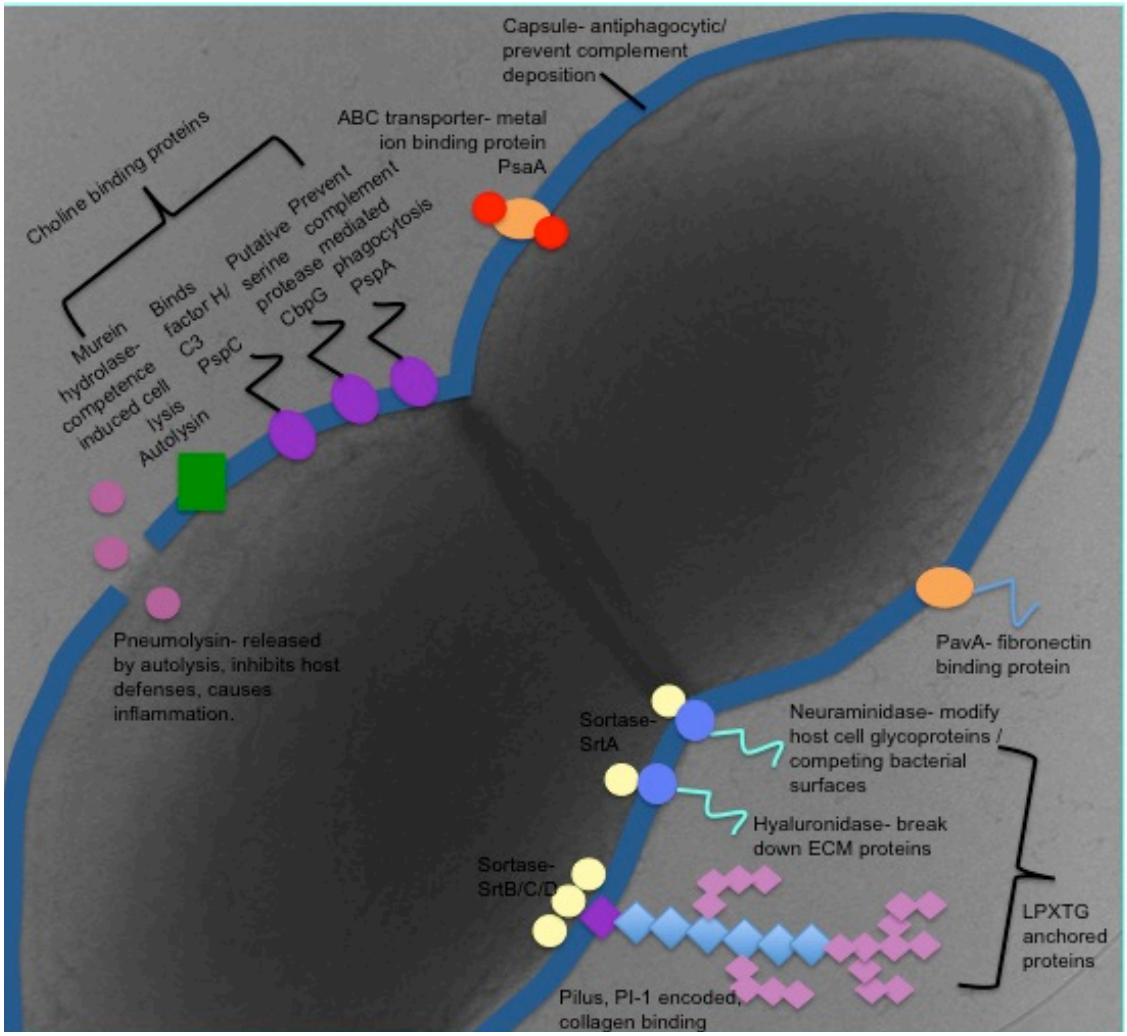


Figure 1-1: Pneumococcal virulence factors

Schematic diagram modified from (Mitchell, 2003) showing some of the known pneumococcal virulence factors and their function during pathogenesis.

1.6.2 Capsule

The capsule is perhaps thought to be the most important virulence factor as strains with no capsule are avirulent, and vaccination with capsular polysaccharide can protect from IPD (Watson & Musher, 1990, Brown et al., 1983, MacLeod,. 1945). As described previously there are over 90 different capsule types currently known, due to the large number they cannot all be included in a vaccine (Henrichsen, 1995, van Dam et al., 1990, Bratcher et al., 2010, Park et al., 2007). The genes which are required for capsule synthesis are always found to lie between *dexB* and *aliA* genes with the size of the intervening region varying between them depending on the serotype (Bentley et al., 2006, Jiang et al., 2006). The first four genes of the capsule are almost always conserved (*cpsA-D*), adjacent to these genes are varying numbers of glycosyltransferases which create the serotype specific capsule (Bentley et al., 2006). Other genes present here are also involved in transport of the oligosaccharide repeat units to the cell surface and their polymerisation (Wzy polymerase/ Wzx flippase). Finally genes at the end of the locus are involved in synthesis of sugar precursors (Bentley et al., 2006, Morona et al., 2000, Kong et al., 2005).

Little is know about regulation of the capsule yet it has been associated with phase variation as described above, with opaque variants having increase capsule levels and are more virulent during invasive disease and transparent variants having less capsule and are better able to colonise the nasopharynx (Kim & Weiser, 1998, Weiser et al., 1994). Further confirmation the capsule plays an important role in IPD is shown by the fact *In vivo* the capsule is up regulated (Ogunniyi et al., 2002). The importance of the capsule during IPD fits in with the role of the capsule in preventing phagocytosis via blocking complement deposition/ antibody binding, preventing being snared in neutrophil extracellular traps, it provides some resistance to antibiotic mediated cell lysis and reduces pneumococcal mucus mediated clearance, all features which would aid survival in the host (Wartha et al., 2007, Abeyta et al., 2003, Fernebro et al., 2004, Nelson et al., 2007, Hardy et al., 2000, Hyams et al., 2010). Further studies have linked expression of capsule genes to regulation of carbon catabolism with mutants in RegM (transcription regulator) showing reduced capsule expression (Giammarinaro & Paton, 2002).

Although virulence has been linked to the amount of capsule, more recent findings clearly show other factors are also important for virulence as strains of the same serotype can vary greatly in their disease causing potential, some of these other factors are described below (MacLeod & Krauss, 1950, Briles et al., 1992, Harvey et al., 2011).

1.6.3 Pneumolysin (Ply)

Ply is a pore forming toxin which is included in the family of cholesterol dependant cytolysins (CDCs) found in a number of bacteria, see review Gilbert, 2010. These toxins are able to form pores in the host cell membranes by binding cholesterol and inserting into the membrane forming large pores lysing the host cell (Tilley et al., 2005). Since its initial discovery a large number of studies have been performed assessing the role of Ply in virulence and how it get outside the cell. Ply unlike other CDC does not contain a signal sequence targeting it for secretion and is thought to be released during autolysis, caused by autolysin (LytA) which is described below (Guiral et al., 2005). However more recent studies have suggested that Ply is secreted and this function lies within the gene located upstream of Ply, yet this is still widely contested (Price et al., 2012). In the pneumococcus Ply has been well characterised as an important virulence factor and is associated with increased inflammation during meningitis and increased virulence in a pneumonia and bacteraemia model of infection (Rubins et al., 1996, Jounblat et al., 2003, Berry et al., 1995, Alexander et al., 1998, Alcantara et al., 1999). Ply is generally conserved between strains with only small nucleotide changes between serotypes, however some strain that remain virulent have a non haemolytic Ply (Kirkham et al., 2006, Jefferies et al., 2007). Interestingly Ply has also been characterised to be a potential anti-virulence factor with low haemolytic variants giving a selective advantage for growth during bacteraemia and higher bacterial numbers are tolerated in the blood (Harvey et al., 2011, Benton & Everson, 1995). Some well characterised functions of Ply include induction of phagocyte respiratory burst plus cytokine release, CD4+ T-cell activation, activation of the classical complement pathway, cell lysis via pore formation inducing inflammation etc (McNeela et al., 2010, Kadioglu et al., 2004, Mitchell et al., 1991, for an overview see Marriott et al., 2008).

1.6.4 Pilus

More information will be given on the pneumococcal pilus as this will be a major focus within this thesis.

Pili or fimbriae were first identified in 1949 in gram negative pathogens (Anderson, 1949). Pili in gram negative pathogens can be categorised into four groups: pili assembled by the chaperone usher pathway, pili assembled by the alternative chaperone usher pathway, Type IV pili and pili assembled by the nucleation/precipitation. Pili have been shown to play roles in biofilm formation, twitching motility, host cell invasion/ adherence, DNA transfer and phage binding (see review Proft & Baker, 2009). Pili in gram negative bacteria will not be discussed further unless comparisons made to gram positive pili. Pili in gram positive pathogens were discovered much later than gram negative pili, where in 1968 surface rods were observed on the cell surface of *Corynebacterium renale* via electron microscopy (Yanagaw et al., 1968). However a better understanding of the assembly, structure and importance of pili to virulence was not assessed till some time later, with interest in these structures initiated by Schneewind and colleagues (Ton-That & Schneewind, 2003, Ton-That, et al., 2004, Ton-That et al., 2004, Budzik & Schneewind, 2006).

Since the initial discovery many gram positive species have been shown to contain surface exposed pili, two types have been indentified: short thin rods (*Streptococcus salivarius*/ *S.oralis*) and longer flexible pili (*S.pneumoniae*/ *S.agalactiae*/ *Streptococcus pyogenes*) (Pauline et al., 1984, Komiyama & Gibbons, 1984, Barocchi et al., 2006). Long flexible gram positive pili have been shown to be composed of a long backbone reaching around 3μm beyond the cell, which is composed of the major pilin covalently linked to itself. Linkage of the pilins it catalysed by the sortase enzymes (transpeptidase reaction) encoded on the same genetic islet as the pilins (Ton-That & Schneewind, 2003). Minor pilins may also be encoded on the islet which can be present along the backbone or at the tip and often act as adhesins for host cells (Ton-That & Schneewind, 2003). Some streptococci contain more than one pilus, this includes 9 in *S.pyogenes* (FCT 1-9), 3 in *S.agalactiae*, 4 in *Streptococcus suis* and two in *S.pneumoniae* (Jacques et al., 1990, also see review Kreikemeyer et al., 2011). Between the

different pilus islets there are variations with regards to the number of pilins (range 2-3), number of sortase enzymes (1-5) and whether a positive transcription regulator is encoded on the islet (Kreikemeyer et al., 2011). The islets themselves are thought to have arisen from the same ancestor and diverged depending on the pathogen. Some islets also include a small signal peptidase gene, which is thought to act as a chaperone for the pilin proteins.

Regulation of these operons is often complex and involves a number of transcription regulators and in the pneumococcus a number of two-component signal transduction systems (TCS) have also been shown to alter pilus expression (Haas et al., 2004, Hemsley et al., 2003, Hendriksen et al., 2007, Rosch et al., 2008, Song et al., 2009, Sebert et al., 2002). . Regulation of pili has also been linked to certain physiological conditions, which may give a clearer idea of the importance of these adhesins in virulence/ survival. For instance in *S.pyogenes* pili regulation has been linked to changes in pH with increase levels of pili observed at pH 6.4 than at higher pH which is thought to be representative of the environment on the skin (low pH), enabling the bacteria to colonise (Manetti et al., 2010). Links in *S.pyogenes* have also been made to pili being regulated upon variations in hydrogen peroxide levels, with wild type (WT) strains showing increased expression of pili genes upon treatment with hydrogen peroxide, which may represent being present in and aerobic environment such as in the throat where colonisation is important (Grifantini et al., 2011).

There are currently two islets identified in the pneumococcus which encode pili, PI-1 (pilus islet 1) and PI-2 (pilus islet 2) (Bagnoli et al., 2008, Hava & Camilli, 2002). Both will be described here however the focus will be on PI-1 encoded pilus as this has been more widely studied and is the pilus that is studied further in this thesis.

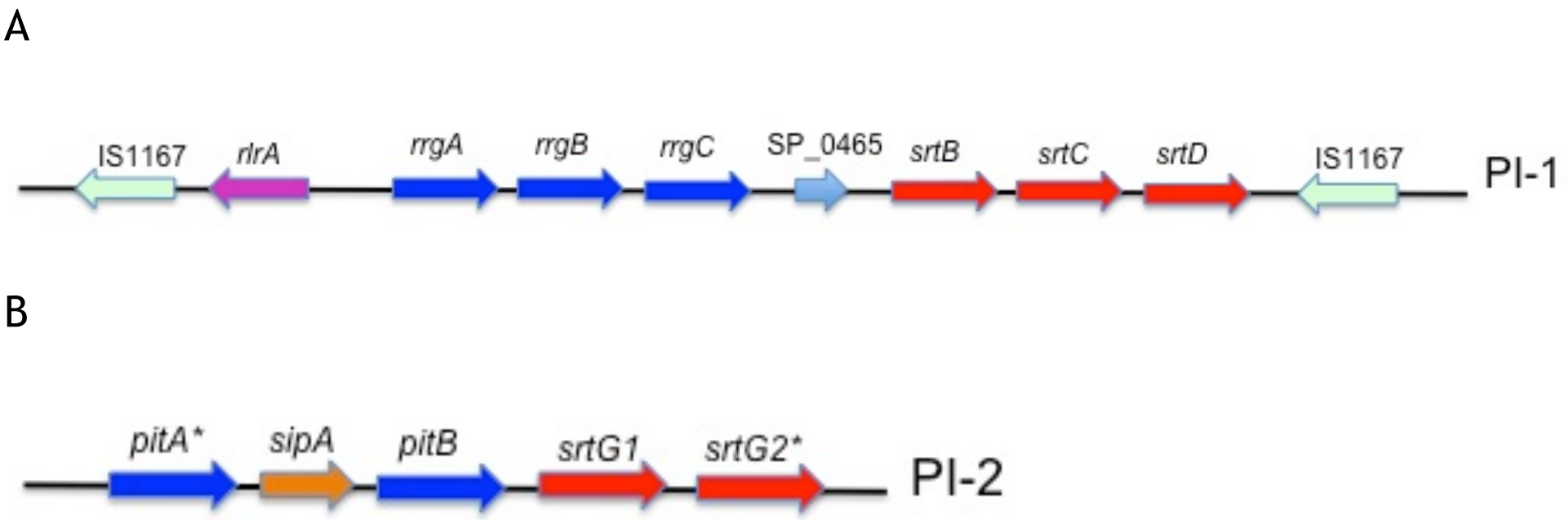


Figure 1-2: Genome organisation of islets encoding pili in the pneumococcus

Schematic diagram modified from Bagnoli et al., 2008, representing the genome organisation of pilus islet 1 (A) and pilus islet 2 (B) present in some pneumococcal strains. (A) Gene names are present above the gene, dark blue arrows represent the pilins, red arrows the sortase enzymes, pink arrow the transcription regulator, green arrows the flanking insertion sequences and in pale blue genes not related to the functioning of the islet. (B) Gene names are present above the gene, dark blue arrows represent the pilins, red arrows the sortase enzymes and orange arrow signal peptidase like gene. Star next to the gene name means it contains a frameshift in all strains or some strains and is therefore non functional.

1.6.4.1 Pilus islet 1 (PI-1)

Genes encoded on PI-1 (Figure 1-2) were first identified during a large scale virulence screen, which identified some of the pilus islet genes to be important in a pneumonia and colonisation model of infection (Hava & Camilli, 2002). One of these genes *rlrA* the positive transcription regulator of the operon was shown to have homology to the RofA and Nra transcription regulators in *S.pyogenes* (Fogg et al., 1994, Podbielski et al., 1999). Identified downstream of *rlrA* is 6 genes, three that encode pilins designated *rrgA*, *rrgB* and *rrgC* and three that encode sortase enzymes designated *srtB*, *srtC* and *srtD,* and all 7 genes are flanked by IS1167 elements (Figure 1-2). Since this initial study a large effort has been put into characterising this islet as to assess its role in virulence, epidemiology, structure, regulation and its potential as a vaccine candidate.

Epidemiological studies into the presence of the pilus islet in pneumococcal strains have shown that this islet is only present in a small proportion of strains depending on the study. The most encompassing study looking at the presence of the pilus in strains over the world found the PI-1 islet to be in 30% of strains (Moschioni et al., 2008). Whereas other studies looking at strains taken from one region have found a prevalence of around 21% of strains PI-1 positive in USA and 35% positive from strains isolated from the Thailand/ Burma 2011 (Basset et al., 2007, Turner et al., 2011). Basset et al., 2007 also assessed the levels of PI-1 pili positive strains prior to and after the introduction of PCV7, which showed a reduction in PI-1 positive strains from 40% to 21% post PCV7 introduction, this is not surprising as the PI-1 islet has been found to be more highly associated with serotypes present in the current vaccines (Basset et al., 2007). However the association of the PI-1 islet correlated better to the ST of the strain rather that the serotype, which has been observed in a number of studies (Basset et al., 2007, Moschioni et al., 2008, Moschioni et al., 2010).

Although PI-1 has been found to be more highly associated with vaccine serotypes no link has been made between strains being pili positive and causing invasive disease (Basset et al., 2007). Studies looking at the transmission of pili positive strains from mother to child showed that the pilus was also not associated with strains with increased carriage durations and increased transmission rates (Turner et al., 2011). However it has been hypothesised the

pilus must confer some selective advantage as re-emergence of the PI-1 islet in non vaccine serotypes has been observed in Massachusetts (Regev-Yochay et al., 2010). The presence of the P1-1 islet in antibiotic resistant strains has also been observed in a number of studies but why this would be the case is unknown (Moschioni et al., 2010, Regev-Yochay et al., 2010). Carriage of strains that are PI-1 positive also seems to inversely correlate to carriage of *S.aureus*, suggesting these strains may confer an advantage during colonisation (Regev-Yochay et al., 2009). However the PI-1 islet has been found to be more associated with strains causing AOM confirmed via *in vivo* studies using a chinchilla model where RlrA and SrtD have been found to be vital for middle ear infection (Vainio et al., 2011, Chen et al., 2008).

Studies into the genetic variation within the pilus islet have found three different variants of the islet exist classed clade I, II and III. Within the clades there is high sequence similarity between all the genes. However between the different clades there can be large variation in % homology (Moschioni et al., 2008). The most conserved gene between the clades is *rlrA,* which shows 100% identity in all three followed by the three sortase enzymes which share 93-99% homology. The three pilins show the most sequence diversity between the three clades with 97-99% diversity between *rrgC*, 84-99% diversity of *rrgA* and 49-67% diversity observed in *rrgB*. The highest diversity being observed in *rrgB* is thought to be due to the fact that this pilin being the pilus backbone protein is likely more accessible by the host immune system and therefore the islet has undergone positive selection to escape this (Muzzi et al., 2008).

Three structures of the PI-1 encoded pilus have currently been proposed, the first put forward in 2008 was that the main pilus shaft was composed of two RrgB profilaments arranged in a coiled-coil structure which contained along the length RrgA and RrgC minor pilins acting as the adhesins (Hilleringmann et al., 2008). A revised structure was published in 2009 by the same group which showed only a single RrgB backbone shaft and RrgA at the tip of the pilus acting as the adhesin and RrgC at the bottom anchoring the pilus to the cell wall (Hilleringmann et al., 2009). Two years later a further structure was published based on careful assessment of the role of the sortase enzymes in attachment of the pilins to themselves/ each other (El Mortaji et al., 2012). Being the most recent structure this is diagrammatically represented below (Figure 1-3) with the current known

roles of the sortase enzymes. Time will tell whether other revisions will be made to this model. This model retains that RrgC acts as the anchor attaching the RrgB shaft to the cell wall/ peptidoglycan, this attachment although not designated to any of the three sortases currently is independent of the house keeping sortase (SrtA), which is not required for pilus assembly (LeMieux et al., 2008). A certain amount of redundancy occurs within the three sortase enzymes encoded on PI-1 so this function will likely lie with one of these (LeMieux et al., 2008). This model also retains that the backbone is composed of RrgB monomers and that RrgA is found at the tip and acts as an adhesin. One of the main variation within this structure is that RrgA is also able to polymerize forming long branches of RrgA adhesins coming from the tip of the pilus and also at varying positions along the RrgB shaft, which has been shown to be catalysed by SrtC. SrtC also catalyses the association of RrgB to RrgA whereas SrtD catalyses the integration of RrgA into the pilus shaft (El Mortaji et al., 2012).

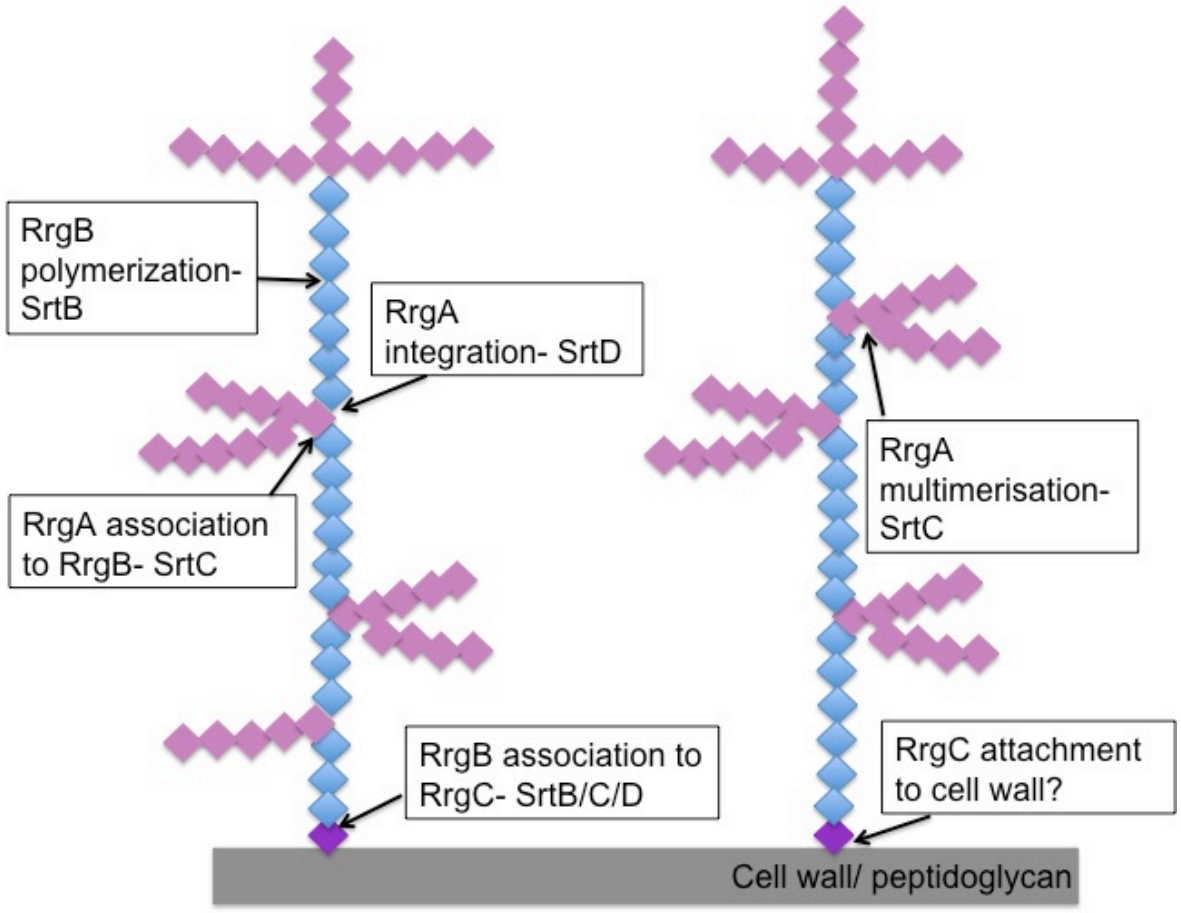


Figure 1-3: Structure of P1-1 encoded pilus

Schematic diagram of pilus structure modified from (El Mortaji et al., 2012). Purple triangle represent RrgC, Blue RrgB and pink RrgA, boxed is the proposed function of each sortase enzyme with regards to their ability to polymerise the different pilins.

RrgA has long been recognised to act as the main adhesin which is present at the tip of the pilus, studies in a RrgA knockout have shown a clear reduced adherence to A549 cell lines (respiratory epithelium) and upon its over expression increased adherence (Nelson et al., 2007). Interestingly upon deletion of RrgB and RrgC adhesion was similar to that of the WT suggesting only RrgA is important, yet RrgA mutants still produced full length pili. *In vivo* analysis in a murine colonisation model further confirmed the importance of RrgA in adherence but not RrgB and RrgC (Nelson et al., 2007). The crystal structure of RrgA has been solved which gives a further indication what the pilus may bind to on host cells (El Mortaji et al., 2012). RrgA was found to have four flexible domains, which may enable it to bind to a number of host components. One domains shares high homology to a eukaryotic like integrin I domain which are important receptors involved in attachment of the cells to the extracellular matrix molecules (ECM). RrgAs core structure shares high homology to other streptococcal adhesins such as in *S.pyogenes* which have been shown to have collagen binding capabilities, which is what RrgA is hypothesised to bind to, which is a highly abundant ECM protein (El Mortaji et al., 2012, Kreikemeyer et al., 2005).

A large number of studies have been performed into the role of the PI-1 encoded pilus in virulence, using the typical adherence assays and mouse models of infection. Yet as described above to date there is no evidence the pilus is more highly associated with strains that cause a higher incidence of disease or more severe disease. Upon its initial discovery all genes encoded on the PI-1 islet were assessed for their potential role in virulence in a murine model of infection assessing their ability to compete with their parent strain (Hava & Camilli, 2002). RrgA and SrtD were shown to be important for pneumonia. During colonisation RrgA and SrtB to a lesser extent were shown to be important. Strangely deletion of SrtC and RrgC led to a hypercolonisation phenotype relative to the parent strain, which was not assessed further. Later studies placing the whole pilus islet into a non piliated strain (D39) showed the pilus aids adherence as an increased adherence of this strain to A549 cells was observed and gave a competitive advantage *in vivo*, conversely deletion of this islet from a piliated strains showed a reduced adherence and was out competed by the WT strain in a colonisation, pneumonia and bacteraemia model of infection

(Barocchi et al., 2006, Maisey et al., 2009). A similar study to this deleting the whole pilus islet in a TIGR4 strains rendered the strain practically avirulent when given via the intratracheal route of infection, which is not observed when given via an alternative route (Rosch et al., 2008). Due to this it was hypothesised the pilus may help protect the bacteria from killing by macrophages and neutrophils found in high numbers in the lung (Rosch et al., 2008). The pilus was also shown to stimulate the host immune system with higher TNF-α levels observed in piliated strains (Barocchi et al., 2006). This has led to the idea that the pilins could be used as a component of a protein based vaccine, with further studies showing that all three PI-1 encoded pilins are immunogenic upon vaccination of mice and that this is able to protect the mice from normally lethal challenge (Gianfaldoni et al., 2007). Although there is variability between the sequence of the major pilin RrgB this has been overcome by creating a fusion protein consisting of the three varying RrgB clades, of which each can protect against clade specific RrgB (Harfouche et al., 2012, Moschioni et al., 2012). The pneumococcal pilus has also been linked to a potential involvement in biofilm formation which is not surprising as promoting biofilm formation is a key role of pili in other pathogenic streptococci (Sanchez et al., 2010, Munoz-Elias et al., 2008, Manetti et al., 2007).

Studies have also been performed into how the pneumococcal pilus is regulated which have elucidated a large number of factors that seem to alter pilus expression. Initial studies assessed the role of RlrA in pilus regulation which is encoded on the islet itself. Upon deletion of *rlrA* the expression of all the pilins and sortases is reduced suggesting RlrA positively regulates all the genes in the operon (Hava et al., 2003). RlrA was shown to bind directly to four promoters in the operon, which includes its own suggesting it positively regulates itself, it also binds upstream of *rrgA*, upstream of *rrgB* and upstream of *srtB*. Interestingly four RlrA promoter binding regions were observed between *rlrA*-*rrgA*, two upstream of *rlrA* and two upstream of *rrgA*, with one thought to perhaps compete with σ^{70} RNA polymerase binding, however this has not been further confirmed (Hava et al., 2003). No putative σ^{70} binding sites were observed upstream of genes *rrgA*, *rrgB* and *srtB* which led to the idea an alternative sigma factor may regulate these genes but again this has not been further assessed (Hava et al., 2003). Since this initial discovery a number of

other transcription regulators have been shown to alter pilus regulation such as MgrA which has been shown to act as a repressor of the pilus islet (Hemsley et al., 2003). To date six TCS have also been shown to alter expression of the pilus islet genes, this includes TCS03, TCS06, TCS08, TCS09 and TCS10 which all act as repressors of the islet shown by the fact that deletion caused an increase in pilus gene expression, and TCS05 acts as a positive regulator as its deletion reduced pilus expression (Rosch et al., 2008, Hendriksen et al., 2007, Haas et al., 2004, Song et al., 2009, Sebert et al., 2002). Both RR03 and RR06 have been shown to bind directly to the *rlrA* promoter region (Rosch et al., 2008). However this has not been assessed for the other four RR and they may also act directly at the *rlrA* promoter or alternatively regulate another factor which affects pilus regulation (Rosch et al., 2008). Pilus regulation has also been linked to the ability to regulate manganese levels as deletion of *mntE* (encoding a manganese efflux pump) or of *psaR* (encoding a manganese dependant regulator) an increase in pilus expression is observed (Hendriksen et al., 2009, Rosch et al., 2008, Rosch et al., 2009, Johnston et al., 2006). Pilus regulation is also linked to regulation of the divalent cation zinc through MerR which has been shown to regulate transport of this ion, as deletion of *merR* causes an increase in pilus expression (Rosch et al., 2008, Kloosterman et al., 2007). A summary of the transcription regulators currently known to alter expression of the genes encoded on PI-1 (directly or indirectly) can be seen in Figure 1-4.

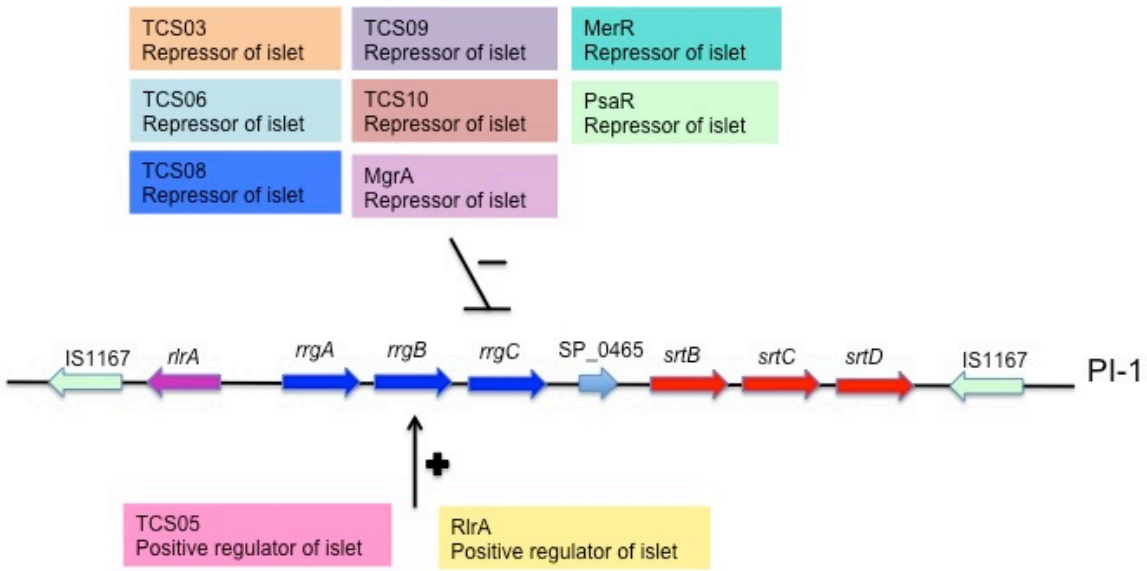


Figure 1-4: Summary of the transcription regulators known to alter pilus expression in the pneumococcus.

Diagram summarises the role of a number of pneumococcal transcription regulators with regards to their ability to alter pilus expression.

More recent findings have shown that within a growing bacterial population two populations exist, cells that are pili positive and cells that are pili negative. With a TIGR4 strains showing roughly 30% of cells pili positive (Basset et al., 2011, De Angelis et al., 2011). This has been shown in part to be regulated by the levels of RrgA, which can bind directly to RlrA, which prevents it from positively regulating itself and other genes present on the islet. Deletion of *rrgA* leads to almost all cells becoming pili positive (Basset et al., 2011, Basset et al., 2012). This bistable regulation of the pilus at the population level is also observed in *S.pyogenes* which has been shown to modulate the level of pilus expressed within the population depending on the temperature at which the bacteria are grown, thought to mimic conditions present during superficial skin infections (30°C) or systemic infection (37°C) (Nakata et al., 2009). However little is currently know about exactly how this phenomenon is modulated.

It is important for bacteria to be able to adapt to changing environments quickly. Heterogeneity within a bacterial population is often modulated through two-component signal transduction systems, some examples in the pneumococcus include natural competence development (as discussed in section 1.7.1.1.12). Where some bacterial cells become naturally competent and can take up DNA from the environment to aid genetic variation and therefore survival. Another TCS has also been shown to aid in protecting a proportion of the bacterial population from autolysis, which are the bacterial population though to lyse to create a DNA pool for the naturally competent population (as discussed in section 1.7.1.1.12).

As already stated a number of these TCS in the pneumococcus also regulate the pilus, it is currently unknown if these modulate the pilus at the population level though (as described above). If this was the case its likely that these systems detect an external stimulus that signals being in a certain niche within the host and modulates the levels of pilus required at the population level accordingly. Assuming that the pilus is present on the surface of more cells when found in an environment where adhesion is required (e.g. nasopharynx). This seemed to be the case for GAS regulation of the pilus which showed higher expression levels at

the population level when grown at lower temperatures, thought to mimic being present on the skin where adherence is important (Nakata et al., 2009). It was hypothesised that regulation at this level may allow some bacteria within the population to avoid detection by the immune system as the pilus is immunogenic. This may enable survival of some of the population as is observed during natural competence development and autolysis.

Heterogeneity can also be modulated through genome changes such as that observed for phase variants, which has been linked to SNPs etc in a number of pneumococcal genes (section 1.5.4). Changes at this level if beneficial to the bacterium would be positively selected for and spread through the population.

1.6.4.2 Pilus islet 2 (PI-2)

In 2008 a second pilus islet was identified in serotype 1 strain INV104 through analysis of its genome sequence, this islet showed high similarity to that of an islet encoding a pilus in *S.pyogenes* (Bagnoli et al., 2008, Mora et al., 2005). Further studies into this islet confirmed this was also the case in *S.pneumoniae* with this 6.6kb islet encoding 5 genes *pitA*, *pitB*, *sipA*, *srtG1* and *srtG2* (Figure 1-2). Further analysis revealed in all strain *pitA* contained a premature stop codon and in all but 1 strain *srtG2* is also non functional, yet their non functionality did not seem to effect pilus polymerisation and only *pitB*, *sipA* and *srtG1* were required for pilus polymerisation (Bagnoli et al., 2008). PitB was confirmed to be the only pilin and made up the whole length of the pilus, which was polymerised via SrtG1. The role of SipA is still unknown but it is required for polymerisation, and thought to encode a small peptidase (Bagnoli et al., 2008). Homologues of this gene in *S.pyogenes* pili islets are thought to potentially act as a chaperone to the pilin (Zahner & Scott, 2008).

Presence of P1-2 has been associated with serotypes 1, 2 7F, 11A, 19A, 19F however again its presence is more closely related to the ST of the strain (Bagnoli et al., 2008, Zähner et al., 2010). ST127 has been shown to encode both P1-1 and P1-2 however this does not seem to be common. Again like P1-1, P1-2 is not present in all strains with roughly 16% of strains containing this pilus islet, this is lower in AOM causing strains dropping to 7% (Moschioni et al., 2010, Bagnoli et al., 2008). This islet is also not associated with strains causing IPD yet

has been shown to aid in adherence to a number of cell lines including A549, Detroit 562 (nasopharyngeal epithelium), 16 HBE 14_{O}^{-} (bronchial epithelium) and Hep-2C (laryngeal epithelium) cell lines, which all showed a reduced adherence using a PI-2 mutant (Bagnoli et al., 2008). Little information is available about the structure of the PI-2 encoded pilus apart from data indicating PitB contains intramolecular isopeptide bonds stabilising them (Zähner et al., 2011). Even less is known about the regulation of this islet which encodes no transcription regulator unlike PI-1 and no studies have currently been performed to assess any other potential transcription regulator that may regulate this operon.

1.6.5 Pneumococcal serine rich repeat protein (PsrP)

PsrP is a very large cell surface protein present in the pneumococcus, this gene is encoded on a large islet spanning 37kb which consists of itself a large 4777 aa (amino acid) protein, 10 glycosyltransferase (function to glycosylate PsrP) and 7 genes thought to encode a sec translocase system (Obert et al., 2006). PsrP itself gained its name from its protein structure which contains two serine rich repeat regions (SRR1/ SRR2), SRR1 is short and contains only 8 repeats however SRR2 contains 539 SRR2 repeats (Rose et al., 2008). This protein is covalently attached to the cell surface via its LPXTG motif like the pilus and is present in roughly 50% of strains (Munoz-Almagro et al., 2010). Strains containing PsrP positively correlates with strains causing IPD (Munoz-Almagro et al., 2010). PsrP is able to bind directly to keratin 10 on lung cells and aid adherence (Shivshankar et al., 2009), and knockouts show a reduced virulence in a mouse model of infection (Munoz-Almagro et al., 2010, Obert et al., 2006). Further study has also indicated PsrP to play a role in intra strain adhesion and biofilm formation (Sanchez et al., 2010). This protein is also a potential vaccine candidate as it is immunogenic and can protect mice from lethal challenge (Rose et al., 2008).

1.6.6 Neuraminidase

One of the other well studied cell surface proteins is neuraminidase (NanA) present in 100% of strains, its role is still under scrutiny however is thought to act by enzymatically cleaving sialic acid off the surface of host cells revealing receptors for bacterial adherence and modifying competing bacterial cell

surface glycoproteins (Bergmann & Hammerschmidt, 2006, Pettigrew et al., 2006). More recently another role of NanA was proposed where it aids adherence to brain endothelial cells and can cause cytokine release followed by bacterial uptake (Banerjee et al., 2011, Uchiyama et al., 2009). Studies have shown varying roles of NanA in virulence, initial virulence screens implied no role for NanA in virulence using a intraperitoneal (I.P) and intranasal (I.N) route of infection yet later studies implied a role in nasopharyngeal colonisation and spread to the lungs and blood in a mouse model of infection (Berry & Paton, 2000, Manco et al., 2006). The pneumococcus also contains two further neuraminidases, NanB and NanC but these have been less well characterised, they are present in 96% and 51% of strains respectively (Pettigrew et al., 2006). NanB unlike NanA does not contain the C-terminal cell wall anchoring domain so is not retained at the cell surface, however is thought to play a similar role in virulence as NanA (Manco et al., 2006, Lock et al., 1988). More recently NanB has been hypothesised to play a role in obtaining nutrients and NanC is thought to regulate NanA function (Xu et al., 2011)

1.6.7 Choline binding proteins (CBPs)

CBPs attach to the cell wall through non covalent attachments via interactions through their C-terminal choline binding domains (CBDs) to phosphorylcholine residues present in LTA and TA (Gosink et al., 2000, Yother & White, 1994). Although the CBD are generally highly conserved (roughly 20aa) between CBPs the N-terminal regions are highly diverse, and in the pneumococcus CBPs have a number of different functions, including adhesins and autolytic enzymes, and the number of CBPs varies between the strains with roughly 10-15 in *S.pneumoniae* (Bergmann & Hammerschmidt, 2006). The same CBP can also vary in its structure between different strains (Brooks-Walter et al., 1999, Hollingshead et al., 2000). Some of these CBPs also have homologues in other closely related streptococci which may act as a pool for acquisition of variants enabling escape from the immune system (Johnston et al., 2010). Some of the key CBPs are described below.

1.6.7.1 Adhesins?

One well studies CBP is CbpA (PspC/ SpsA) which is known to act as an adhesin present on the surface of the pneumococcus, CbpA is highly variable and can be sorted into two clades (Brooks-Walter et al., 1999). Some variants attach to the cell wall not through interactions with phosphorylcholine but via LPXTG anchoring. This variant known as Hic shares homology to a group B streptococcal protein (Jarva et al., 2004, Janulczyk et al., 2000). This CBP has been shown to be important for virulence via knockouts showing a reduced ability to bind to epithelial cells, reduced nasopharyngeal colonisation and reduced survival in a mouse pneumonia and sepsis model (Rosenow et al., 1997, Iannelli et al., 2004, Quin et al., 2005, Quin et al., 2007). It functions in part through binding of polymeric immunoglobulin receptor, which transports secretory IgA, perturbing this function (Hammerschmidt et al., 2000, Zhang et al., 2000). CbpA is also able to bind factor H, which is important for activation of the alternative complement pathway leading to bacterial opsonophagocytosis (Dave et al., 2004, Cheng et al., 2000, Janulczyk et al., 2000, Quin et al., 2005, Quin et al., 2007)

PspA is another well studied CBP found on the pneumococcal cell surface, again this protein is highly structurally variable and can be divided into three families and further split into six clades (Hollingshead et al., 2000). Like CbpA this protein is able to evade host defences via preventing complement deposition on the bacterial cell surface by inhibiting activation of the classical and alternative complement pathway (Ren et al., 2003, Ren et al., 2004a, Ren et al., 2004b, Ochs et al., 2009, Tu et al., 1999). PspA is also able to bind lactoferrin, a host iron transport protein, and is thought to help protect from apolactoferrin bactericidal activity (Shaper et al., 2004, Hakansson et al., 2001). With regards to virulence the data is conflicting but it may be required for *in vitro* growth (McDaniel et al., 1987, Ren et al., 2004a, Berry & Paton, 2000, Orihuela et al., 2004, Hava & Camilli, 2002). PspA however is immunogenic and is a component in a number of protein based vaccine studies (Nguyen et al., 2011, Xin et al., 2009, Yamamoto et al., 1997)

1.6.7.2 Hydrolytic enzymes

The pneumococcus contains a number of cell wall hydrolytic enzymes of which the most well known are LytA/B/C. LytA has been shown to function as a N-acetylmuramoyl-L-alanine amidase cleaving N-acetylmuramoyl-L-alanine moieties bond of pneumococcal peptidoglycan (Howard, et al., 1974, Tomasz et al., 1988). Its main activity has been shown to cause bacterial cell autolysis but may also contribute to cell wall turnover and growth. Why autolysis is physiologically important is still debated, one function is though to be to release Ply which is a VF, however this is now contested. Another suggestion is that the lysed cells provide DNA for recombination into surviving bacterial cells to aid genetic variation and survival (Steinmoen et al., 2002, Guiral et al., 2005). Studies into its role in virulence show conflicting information with some strains showing mutants are as virulent as WT however some show deficiencies in virulence (Berry et al., 1989, Tomasz et al., 1988, Hirst et al., 2008, Canvin et al., 1995, Berry & Paton, 2000, Orihuela et al., 2004). LytB functions during cell division localising to the cell septum, mutants in this gene are unable to properly separate and form long chains (Garcia et al., 1999, Rudner & Jarvis, 1999). LytCs function is less well characterised and shows little phenotype change unless deleted alongside LytA. However both LytB and LytC have been shown to be important during colonisation (Gosink, et al., 2000).

On top of the CBPs discussed above a number of other choline binding proteins exist with varying functions, briefly this included CbpJ and CbpI of which their function is currently unknown (Paterson et al., 2006). CbpF is thought to modulate in some way LytC function (another CBP) (Molina et al., 2007, Molina et al., 2009). CbpD plays a role in competence induced cell lysis and has been characterised as another hydrolytic enzyme (Steinmoen et al., 2003, Guiral et al., 2005, Kausmally et al., 2005). CbpE is also thought to act as a cell wall hydrolase also known as lytD and Pce, and has been shown to play a role in virulence (Höltje & Tomasz, 1974, De las Rivas et al., 2001, Vollmer & Tomasz, 2001). PcpA is important for virulence in a mouse model of infection and is immunogenic (Glover et al., 2008, Hava & Camilli, 2002).

1.6.8 Lipoproteins

PsaA is one of the many pneumococcal cell surface lipoproteins currently identified, with 40-50 known, some with roles in virulence (Overweg et al., 2000, Hermans et al., 2006). PsaA will be the only example discussed here. PsaA has been shown to be important in pneumococcal adherence to mammalian cells *in vitro*, and antibodies raised against this protein prevent adherence (Briles et al., 2000, Romero-steiner et al., 2003, Romero-Steiner et al., 2006, Berry & Paton, 2000). Recent studies have indicated it binds directly to E-cadherin present on surface of nasopharyngeal cells (Anderton et al., 2007), and can also aid in cell invasion (Rajam et al., 2008). Yet its role as an adhesin is contested as structural analysis implies that this surface protein would not protrude beyond the cell wall. Deletion mutants of this gene are avirulent in a mouse model of colonisation, pneumonia and septicaemia (McAllister et al., 2004, Tseng et al., 2002, Lawrence et al., 1998). PsaA has been further characterised as a component of an ABC transporter specific for manganese yet can also bind Zinc, the PsaA component acts as the metal ion binding component (Dintilhac et al., 1997, McAllister et al., 2004, Lawrence et al., 1998), and has also been shown to play a role in resistance to oxidative stress (Johnston et al., 2004, Tseng et al., 2002).The other ABC transporter components include PsaB and PsaC, which also show a reduced adherence upon their deletion (Johnston et al., 2004). The final member of the operon is PsaD which is characterised as a putative thiol peroxidase, showing homology to an *E.coli* thiol peroxidase (Cha & Kim, 1996, Novak et al., 1998). This gene is also thought to play a role in resistance to oxidative stress as deletion mutants of this gene are hypersensitive to hydrogen peroxide (Johnston et al., 2004, Tseng et al., 2002).

1.6.9 Other

Pneumococcal adherence and virulence factor A (PavA) is found on the pneumococcal cell surface even though it contains no signal sequence or membrane anchoring motif (Holmes et al., 2001). One of its main functions has been shown to be binding of fibronectin which is found in the ECM surrounding host cells, of which It is though to contribute to 50% of the cells fibronectin binding capability (Holmes et al., 2001). A role in virulence has been assigned to

this protein with mutants showing a reduced virulence in a sepsis and meningitis model (Holmes et al., 2001, Pracht et al., 2005).

1.6.10 Hydrogen peroxide

The ability of the pneumococcus to produce high concentrations of hydrogen peroxide (H_2O_2) is used to its advantage, whereas in other bacteria H_2O_2 is produced merely as a by-product of aerobic metabolism and is degraded quickly via enzymes such as catalase etc to prevent toxic effects to the cell. In the pneumococcus H_2O_2 is produced as a by-product of the enzymatic reaction catalysed by SpxB (pyruvate oxidase), which converts pyruvate to acetyl phosphate during aerobic metabolism. SpxB produces over 90% of the total cellular H_2O_2 which can equate to 0.5-2mM (Spellerberg et al., 1996, Pericone et al., 2003). A small amount of H_2O_2 is also produced by LctO (lactate oxidase), which also functions under aerobic conditions converting lactate to pyruvate, which can then be processed by SpxB (Taniai et al., 2008).

Little is known about the role of LctO in virulence but SpxB has long been recognised as a very important virulence factor, partly through production of H_2O_2 and through energy production as this is the main pathway used during aerobic metabolism to obtain ATP. ATP is produced by conversion of acetyl phosphate (product of SpxB reaction) to acetate via acetate kinase (Pericone et al., 2003). The use of H_2O_2 alone has been shown to be important for killing off competition in the nasopharynx due to the high levels produced (Pericone et al., 2000). Bactericidal effects caused by H_2O_2 levels produced from the pneumococcus are high enough to kill other upper respiratory tract pathogens including *H.influenzae* and *N.meningitidis* (Pericone et al., 2000, Regev-Yochay et al., 2006). In a mouse model of infection *spxB* mutants are unable to colonise the nasopharynx and replicate in the blood and lungs in a mouse model. Analysis of *in vivo* gene expression also indicates *spxB* expression is higher in the nasopharynx therefore clearly plays an important role here (Spellerberg et al., 1996, Orihuela et al., 2004, LeMessurier et al., 2006).

Interestingly deletion of SpxB causes the loss of tolerance of the pneumococcus to H_2O_2 and therefore SpxB plays a currently unknown function in protecting itself from endogenously produced H_2O_2 (Regev-Yochay et al., 2007). This

phenomenon is thought to be in part due to the reduced ATP levels produced upon SpxB deletion which may be required to repair the damage caused by the H_2O_2. This can be backed up as upon deletion of acetate kinase (enzyme catalysing the last step in ATP production) spontaneous mutations are observed in SpxB (Ramos-Montañez et al., 2010). SpxB is also one of the genes important for phase variation as its deletion results in only opaque variants being observed, discussed above (Overweg et al., 2000, Ramos-Montañez et al., 2008).

Hydrogen peroxide has recently been shown to directly oxidise a protein involved in fatty acid biosynthesis (FabF), which is important in regulating the level of membrane fatty acid saturation (Benisty et al., 2010)).

1.7 Gene regulation

To be able to adapt to different environmental conditions and stresses genes required in different niches need to be tightly regulated to enable survival, this includes regulation of cell surface adhesins and other virulence factors. For this a number of different signalling systems and signalling molecules are available in bacteria to enable this to happen, some of which are discussed below.

1.7.1 Two component signal transduction systems (TCS)

TCS are one of the major signalling systems utilised in bacteria to respond to changing environmental conditions, see review Stock et al, 1989. These systems are present in almost all bacterial species (bar Mycoplasma species) and can also be found in some lower eukaryotes. A prototypical TCS consists of two proteins: a membrane bound HK (histidine kinase) and a cytoplasmic RR (response regulator). The HK contains a N-terminal sensor domain, which responds to certain environmental changes, this domain is often extracellular and is followed by a C-terminal kinase domain, which is located within the cell. The second protein in a TCS pairs is the RR which is found in the cytoplasm, this protein contains a N-terminal receiver domain followed by a C-terminal output domain (often DNA binding function) (Stock et al, 1989). Upon recognition of an external stimulus by the HK which functions as a dimer, the HK is autophosphorylated in trans on a conserved histidine residue, the phosphoryl group is provided through ATP hydrolysis via the ATPase activity of the HK (Stock et al, 1989). Post

autophosphorylation the phosphate is transferred to a conserved aspartate residue on the receiver domain of the RR, which triggers a conformational change in the protein, usually modulating its DNA binding activity. This mode of action is diagrammatically depicted in Figure 1-5.

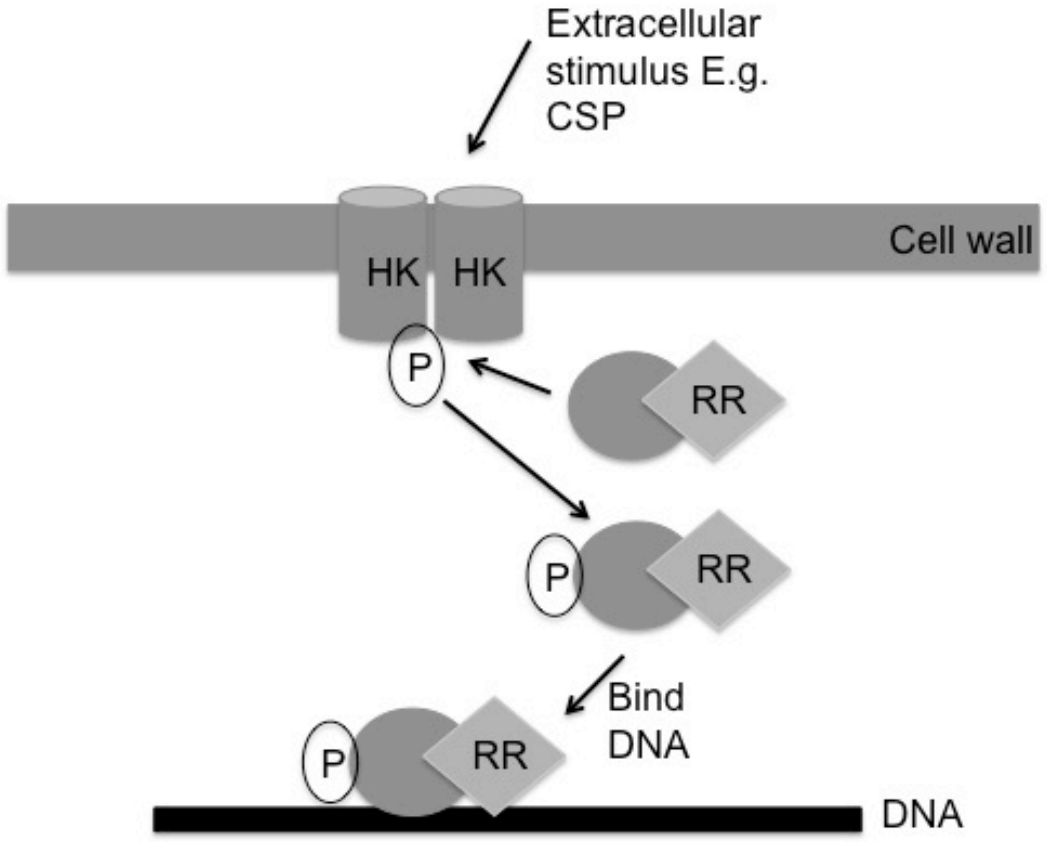


Figure 1-5:Schematic diagram of TCS signal transduction

Diagram modified from Paterson et al., 2006 representing prototypical TCS gene regulation. Upon recognition of specified extracellular stimulus the membrane associated HK is autophosphorylated. The phosphoryl group is subsequently transferred to its cognate RR which undergoes a conformational change enabling it to bind its DNA targets.

HK and RR pairs are usually found in the genome adjacent to each other, in some cases the open reading frames overlap, and they are normally transcribed together (Lange et al., 1999). The numbers of TCS present in a single species varies with none found in Mycoplasma species, 13 in *S.pneumoniae*, roughly 30 in *E.coli* and *B.subtilis*, 63 in *Pseudomonas aeruginosa* and over 100 in *Myxococcus xanthus* (Rodrigue et al., 2000, Jung et al., 2012). Orphan RR have also been identified in a number of species, which have no cognate HK (Wang et al., 2009). The external stimulus recognised by the HK can range from recognition of short peptides, temperature changes, pH changes, recognition of metal ions,

antibiotics and recognition of oxygen etc, in many cases the external stimulus has not been identified (Perry et al., 2011). These TCS have been shown to act as global gene regulators regulating large caches of genes important for survival and often regulate genes required for virulence. More recently TCS have also been shown to regulate a number of small RNA (sRNA) molecules in a number of pathogens, which have been shown to regulate virulence.

The description of the TCS functioning described above is somewhat simplified and other factors have more recently been shown to interact with these systems. This includes phosphorylation of some RR on an alternative threonine residue by a serine/threonine protein kinase, which has been observed in the pneumococcus (this is discussed further below) (Agarwal et al., 2012, Ulijasz et al.,2009). Phosphorylation of orphan response regulators can also occur through a HK of a non cognate TCS pair (Wang et al., 2009). In some bacteria HK have also been shown to form heterodimers with HK of different TCS pairs, preventing signalling (Vincent et al., 2010). However *in vivo* cross talk between HK and RR of different TCS pairs does not occur to enable tight regulation of genes (Boll & Hendrixson, 2011). TCS have also been shown to regulate genes in both their phosphorylated and non phosphorylated forms, with different caches of genes regulated in both forms (Standish et al. , 2007).

1.7.1.1 TCS in *S.pneumoniae*

S.pneumoniae contains 13 TCS pairs and one orphan response regulator (Throup et al., 2000, Lange et al., 1999), summarised in Table 1-1. The RR of each TCS pair have been grouped based on their amino acid sequence identity to each other and RR of different species, the receiver domain of the RR share high homology to each other however the output domain (DNA binding domain) varies considerably due to their different DNA targets (Lange et al., 1999). The first family is classed the OmpR family which includes RR01, RR02, RR04, RR05, RR06, RR08, RR10 and ORR, the second family classed AraC contains RR07 and RR09, the third family is called LuxR and contains RR03 and RR11 and finally the fourth family classed AgrA contains RR12 and RR13 (Lange et al., 1999, Throup et al., 2000).

These large scale studies for initial identification of all TCS in *S.pneumoniae* also showed a large number of these systems play some role in virulence (Throup et al., 2000). Interestingly the initial studies into their role in virulence performed by Lange et al., 1999 showed no difference in virulence of the RR knockouts compared to their parent strain in a mouse model of bacteraemia, except for RR02 and RR13 which could not be deleted and RR07 which could not be deleted in a virulent strain (Lange et al., 1999). This was contrary to the later study conducted by Throup et al., 2000 which showed a role in virulence of RR01, RR04, RR05, RR06, RR07, RR08, RR13 and ORR using a mouse model of pneumonia, with deletion mutants of the RR being attenuated compared to the WT (Throup et al., 2000). In this study RR07 and RR13 could be deleted however this is not the case for RR02 which has been shown to be the only vital RR in *S.pneumoniae*, which shares homology to the indispensible RR of *B.subtilis*, *S.aureus* etc (Fabret & Hoch, 1998, Martin et al., 1999). However HK02 can be deleted in the pneumococcus (Throup et al., 2000). Since these studies a large amount of effort has been put into assessing what genes each of these systems regulate. Some features which have added to the complexity of these studies it that they seem to regulate genes in a strain dependant manner, and therefore their role in virulence is also strain dependant (Lange et al., 1999, Blue & Mitchell, 2003, Hendriksen et al., 2007, Mccluskey et al., 2004). This may be why RR07, RR13 could not be deleted in the initial study (Lange et al., 1999). What is known currently about each of the TCS and genes they regulate will be described below.

TCS	Gene number (TIGR4)	Alternative nomenclature	Role in virulence?	Virulence references
TCS01	HK SP_1632 RR SP_1633	480	Yes	(Throup et al., 2000) (Hava & Camilli, 2002)
TCS02	HK SP_1226 RR SP_1227	YycFG, MicAB, VicRK, WalRK ,492	Yes	(Throup et al., 2000) (Lange et al., 1999)
TCS03	HK SP_0386 RR SP_0387	LiaSR, 474	Yes	(Rosch et al., 2008)
TCS04	HK SP_2083 RR SP_2082	PnpRS, 481	Yes	(Throup et al., 2000) (Mccluskey et al., 2004)
TCS05	HK SP_0799 RR SP_0798	CiaRH, 494	Yes	(Throup et al., 2000), (Hava & Camilli, 2002) (Sebert et al., 2002) (Ibrahim et al., 2004)
TCS06	HK SP_2192 RR SP_2193	CbpSR, 478	Yes	(Throup et al., 2000)
TCS07	HK SP_0157 RR SP_0156	539	Yes	(Throup et al., 2000) (Lange et al., 1999) (Hava & Camilli, 2002) (Lau et al., 2001)
TCS08	HK SP_0082 RR SP_0083	484	Yes	(Throup et al., 2000)

TCS09	HK SP_0662 RR SP_0661	ZmpSR, 488	Yes	(Hava & Camilli, 2002) (Hendriksen et al., 2007) (Blue & Mitchell, 2003)
TCS10	HK SP_0604 RR SP_0603	VncRS, 491	No	
TCS11	HK SP_2001 RR SP_2000	479	No	
TCS12	HK SP_2236 RR SP_2235	ComDE, 498	Yes	(Hava & Camilli, 2002)
TCS13	HK SP_0527 RR SP_0526	BlpRH, SpiR1/2, SpiRH, 486	Yes	(Throup et al., 2000) (Lange et al., 1999) (Dawid et al., 2007)
ORR	RR SP_0376	RitR, 489	Yes	(Throup et al., 2000) (Ulijasz et al., 2004)

Table 1-1: Pneumococcal TCS

Summary of all the current known pneumococcal TCS, information is provided with regards to their role in virulence and alternative names used in the literature.

1.7.1.1.1 TCS01

TCS01 is one of the systems to date that no information is available for with regards to what genes it regulates. However it has clearly been shown to play a role in virulence in the initial large scale screens (Throup et al., 2000), and a later screen has shown this gene is essential for lung infection (Hava & Camilli, 2002).

1.7.1.1.2 TCS02

TCS02 is the only TCS in the pneumococcus which cannot be deleted and is essential (Throup et al., 2000, Lange et al., 1999). Homologues of this system are present in a large number of other gram positive pathogen where it is also essential (Fabret & Hoch, 1998, Liu et al., 2008, Dubrac & Msadek, 2004, Senadheera et al., 2005). Interestingly only the RR is essential in the pneumococcus rather than both the HK and RR seen in other pathogens (Throup et al., 2000, Martin et al., 1999, Fabret & Hoch, 1998). Due to its requirement it has made studies into the genes regulated by TCS02 hard to assess and mechanisms used to do this have included over expressing RR02 or reducing its expression (Ng et al., 2003, Mohedano et al., 2005). The genes regulated by this system were shown to include gene involved in peptidoglycan biosynthesis, fatty acid biosynthesis, cell division and some cell surface virulence factors, including PspA and PcsB which have been shown to be directly regulate by RR02 through binding in its phosphorylated form to their upstream promoter regions (Ng et al., 2003, Ng et l., 2005, Mohedano et al., 2005). PcsB which is positively regulated

by this system is essential and upon its over expression RR02 can be deleted (Ng et al., 2003, Ng et al., 2004). This peptidoglycan hydrolase has been shown to be important for peptidoglycan biosynthesis and localises to the cell septum where it interacts with the cell division proteins (Sham et al., 2011). Mutants constructed altering the expression of *pcsB* or *rr02* show clear defects in their ability to divide properly (Ng et al., 2004). HK02 has been shown to localise throughout the cell periphery (Wayne et al., 2010), contrary to the localisation of WalK to the cell septum in *B.subtilis* (Fukushima et al., 2009). The external stimulus of TCS02 is currently unknown but due to the genes regulated by the system it is thought to respond to change in the cell wall peptidoglycan responding to the presence of certain precursors etc (see review Dubrac et al., 2008).

1.7.1.1.3 TCS03

TCS03 was not shown to play a role in virulence in the initial virulence screens but later studies have implicated it in playing a role in virulence. Deletion of HK03 in a serotype 4 strain (TIGR4) causes a significant increase in survival of infected mice (I.N) compared to the WT (Rosch et al., 2008). This correlated to a significant decrease in the number of bacteria present in the nasopharynx and blood of infected mice 24 hours post infection (Rosch et al., 2008). Within this study one of the genes shown to be regulated by this system was that of the pneumococcal pilus, with RR03 shown to bind directly to the *rlrA* promoter (Rosch et al., 2008). RR03 was also shown to be up regulated in the CSF of infected rabbits, using a meningitis model of infection (Orihuela et al., 2004). More recent studies have further characterised the role of TCS03 with regards to its function in the pneumococcus. This TCS has been shown to be important in helping protect the bacterial cell during autolysis/ fratricide (Eldholm et al., 2010). As described above the pneumococcus is naturally competent, during competence some of the population autolyse to provide DNA for genetic variation etc, which is caused by a number of hydrolytic enzymes (LytA, CbpD, LytC) (Johnsborg, et al., 2008, Steinmoen, et al., 2002). Activity of these enzymes induces cell envelope stress which triggers activation of TCS03. To protect the bacteria from lysis this system regulates a number of genes which are important to prevent this occurring (Eldholm et al., 2010). This phenomenon is backed by earlier studies which show TCS03 is expressed to a higher level in

competent cells rather than non competent cells, protecting this population (Dagkessamanskaia et al., 2004). TCS03 has also been shown to be up regulated when bacterial cells are subjected to vancomycin, which would also cause cell envelope stress, activating this system (Haas et al., 2005).

1.7.1.1.4 TCS04

Initial studies into the role in virulence of RR04 were conflicting, although the Lange study showed no attenuation during a bacteraemia model of infection it did show a clear growth defect *in vitro* (Lange et al., 1999), later studies by Throup et al., 2000 identified this as an important virulence factor required during a pneumonia model of infection (Throup et al., 2000). Later studies looking at the role of this TCS in virulence suggested it plays a strain specific role in virulence, with attenuation during a pneumonia and bacteraemia model of infection only observed in a serotype 4 TIGR4 strain (Not serotype 2/3) (Mccluskey et al., 2004). Gene expression analysis was also performed in this study to characterise the genes regulated by this system, which was shown to also be strain specific. In the serotype 2 background (D39) genes regulated by this system included TCS05 and a serine protease HtrA (Mccluskey et al., 2004). In a serotype 3 background (0100993) genes regulated included RR08 and a number of PTS (phosphotransferase system) genes (Mccluskey et al., 2004). In the serotype 4 strain (TIGR4) some of the genes regulated included that of a manganese ABC transporter made up of PsaA, PsaB and PsaC, which is down regulated upon deletion of RR04 (Mccluskey et al., 2004). This ABC transporter has been shown to play a role in virulence in the pneumococcus (Dintilhac et al., 1997, Berry & Paton, 1996, Ogunniyi et al., 2000, Marra et al., 2002). Homologues of this system in other bacteria have been shown to respond to divalent cations, which is hypothesised to be the external stimulus that activates this system (Groisman, 2001).

1.7.1.1.5 TCS05

TCS05 was the first characterised TCS in the pneumococcus (Guenzi et al., 1994). This system is also called CiaRH (competence induction and altered cefotaxime susceptibility) after some of the phenotypes observed upon its deletion (Guenzi et al., 1994, Guenzi & Hakenbeck, 1995). This TCS has therefore been associated with repressing competence, protecting the cell from

β-lactam antibiotics and protecting the cell for autolysis (Guenzi & Hakenbeck, 1995, Giammarinaro et al., 1999, Lange et al., 1999, Dagkessamanskaia et al., 2004, Zähner et al., 2002, Mascher et al., 2006, Echenique et al., 2000). The genes regulated by this system have been well characterised which include repression of the genes required for competence development and positive regulation of HtrA (High temperature requirement protein A) (Mascher et al., 2003, Sebert et al., 2002). Further the genes directly regulated through binding of RR05 to the upstream promoter have been identified (Halfmann et al., 2007), which has been shown to include a number of sRNA molecules and is the first study in the pneumococcus showing sRNAs regulated by a TCS (Halfmann et al., 2007). Homologues of this system in other streptococci have been shown to also regulate sRNAs suggesting they may play an important role in survival as they are conserved (Marx et al., 2010). One other factor shown to be differentially regulated by this system is the pneumococcal pilus which upon deletion of this TCS is down regulated (Sebert et al., 2002), however this has not been further validated by any other methods and the pilus was not found to be directly regulated via RR05 (Halfmann et al., 2007).

This system has been clearly shown to play a role in virulence through the initial screens and later studies, which show a clear role of the genes regulated by this system in aiding colonisation and systemic infection (Marra et al., 2002, Throup et al., 2000, Sebert et al., 2002). Upon contact of bacterial cells with Detroit 562 cells this system is up regulated (Orihuela et al., 2004). The reduced virulence phenotype observed in a RR05 deletion mutant has been shown to be due to the reduced expression of HtrA, as deletion RR05 and HtrA show similar virulence phenotypes and over expression of HtrA in a RR05 mutant restores virulence (Ibrahim et al., 2004). HtrA has also been shown to post transcriptionally regulate bacteriocin activity, linking this system to that of TCS13 also (Dawid et al., 2009), and is the factor which modulates competence induction (Sebert et al., 2005). Upon deletion of a global gene regulator (serine/ threonine protein kinase) this system is down regulated suggesting this TCS may also be controlled by an alternative kinase (Saskova et al., 2007). This system is also down regulated in a RR04 mutant suggesting this system may regulate TCS05 (Mccluskey et al., 2004).

1.7.1.1.6 TCS06

TCS06 was one of the systems shown to be important for virulence (Throup et al., 2000), further studies have validated this with a decrease adherence observed to Detroit 562 and A549 cells upon deletion of either the HK or RR in a serotype 2 strain (D39) (Standish et al., 2005). Interestingly these strains when assessed in a mouse model of infection (I.N) showed in some instances opposite effects of the HK and RR mutants. In the RR mutant bacteria counts in the lung, blood and nasopharynx were statistically higher than the WT (Standish et al., 2005). TCS06 has also been linked to biofilm formation as a mutant in HK06 shows a reduced biofilm formation. Two virulence factors that are regulated by TCS06 including RlrA/ RrgA (pilus proteins) and CbpA showed a similar reduction in biofilm formation when containing transposon insertions (Muñoz-Elías et al., 2008).

This system has also been designated CbpS/R as the important adhesin CbpA (PspC) is located directly upstream of this TCS and regulates this genes (Standish et al., 2005). RR06 is able to bind directly to the promoter upstream of CbpA, which has been shown to occurs when RR06 is in its non phosphorylated form (Standish et al., 2005, Standish et al., 2007). Interestingly although this TCS contain a cognate HK it is also phosphorylated on a threonine residue by a serine/ threonine protein kinase, which has been shown to modulate its affinity for binding to the CbpA promoter region (Agarwal et al., 2012). Other genes have also been shown to be regulated by this systems this includes the virulence factors PspA which is repressed upon over expression of RR06 in D39. This repression has been shown to occur when RR06 is in its phosphorylated form (Standish et al., 2007). Another virulence factor regulated by this TCS is the pneumococcal pilus which is up regulated upon deletion of HK06, the RR has been shown to bind directly to the *rlrA* promoter region (Rosch et al., 2008).

1.7.1.1.7 TCS07

TCS07 is another system that has not been characterised with regards to the genes it regulates. This TCS however has been shown to play a role in virulence (Throup et al., 2000, Hava & Camilli, 2002, Lau et al., 2001).

1.7.1.1.8 TCS08

TCS08 was also one of the TCS shown to play a role in virulence in the initial screens performed (Throup et al., 2000). Although this has not been further validated in a mouse model of infection deletion of TCS08 has been shown to affect the ability to adhere to A549 cells (Song et al., 2009). TCS08 has also been linked to phase variation with the two variant showing different virulence capabilities as described above, RR08 is down regulated in opaque variant compared to transparent (Trappetti et al., 2011). Studies have also been performed into the genes that are regulated by this TCS with initial studies in a serotype 2 stain (R6) showing this TCS regulates solely a number of genes important for cellobiose metabolism including a PTS (Mckessar & Hakenbeck, 2007). Deletion of TCS08 renders the mutants unable to utilise this as a carbon source. However it is unlikely that the pneumococcus would come across cellobiose in the host and it is thought these systems recognise structural analogues of cellobiose (β-glucosides) which may be derived from the breakdown of host ECM proteins (see review King, 2010). Studies of the role of this TCS in a serotype 4 strain TIGR4 showed a different collection of genes to be regulated by this system, which again may suggest this system acts in a strain dependant manner (Song et al., 2009). Included was that of genes encoded on the pilus islet, which were up regulated upon deletion of RR08 (Song et al., 2009).

1.7.1.1.9 TCS09

In the initial virulence screen TCS09 was not shown to play a role in virulence however further studies suggested RR09 was essential during pneumonia (Throup et al., 2000, Hava & Camilli, 2002). Further characterisation of its role in virulence showed like RR04 it played a strain specific role in virulence (Blue & Mitchell, 2003). Deletion of RR09 in a serotype 2 (D39) background rendered the strain avirulent when administered via the I.P, I.N and Intravenous (I.V) route in a mouse model of infection (Blue & Mitchell, 2003). Conversely deletion of RR09 in a serotype 4 (TIGR4) and serotype 3 (0100993) background strains rendered them attenuated via the I.N route of administration only, this was attributed to the inability of the strains to disseminate into the blood rather than an inability to grow in the lungs (Blue & Mitchell, 2003). Evaluation of the genes regulated by these systems showed this system also regulates genes in a strain dependant manner as only 7 genes were found to be commonly regulated by this TCS in D39 and TIGR4, from the 102 and 80 found to be differentially regulated respectively

(Hendriksen et al., 2007). Genes regulated by RR09 in TIGR4 included that of the pilus islet genes, which were up regulated upon its deletion, PspA was down regulated upon its deletion. In D39 the genes encoding TCS12 were up regulated upon RR09 deletion, the serine protease HtrA was down regulated as well as a number of PTS encoding genes (Hendriksen et al., 2007).

1.7.1.1.10 TCS10

TCS10 has not been shown to play a role in virulence in the large scale screens (Lange et al., 1999, Throup et al., 2000). HK10 was shown to be up regulated upon contact with Detroit 562 cells and in the blood of infected mice (Orihuela et al., 2004). Early studies into the role of this system indicated it played a role in vancomycin tolerance as it lies directly downstream of genes shown to be important for this function, and may also play a role in autolysis (Novak et al., 1999, Novak et al., 2000). However this has been revised and TCS10 was shown to not regulate the upstream genes important for vancomycin tolerance (Haas et al., 2004, Robertson et al., 2002). Upon deletion of RR10 an up regulation of some of the genes present on the pilus islet was observed, however this was not confirmed via any other methods (Haas et al., 2004). HK10 was one of the genes found to be down regulated upon deletion of the ORR and therefore may be regulated by this system (Ulijasz et al., 2004).

1.7.1.1.11 TCS11

TCS11 is one of the least well studied TCS. Currently no genes have been indentified that are regulated by this TCS. This system was also not identified to play a role in virulence in any of the large scale virulence screens so it may not regulate genes important for virulence. However this system has been found to be up regulated when cells are subjected to vancomycin stress (Haas et al., 2005). Upon deletion of ORR this TCS was down regulated which may imply this system is also regulated by the ORR (Ulijasz et al., 2004).

1.7.1.1.12 TCS12

Early studies have suggested TCS12 to play a role in virulence showing HK12 is essential during pneumonia (Hava & Camilli, 2002), further studies have validated its role in virulence where peptide inhibitors able to block TCS12 activation cause an increased survival in infected mice (Zhu & Lau, 2011),

conversely alternative studies have suggested a fitness advantage upon deletion of TCS12 (Kowalko & Sebert, 2008). With regards to the function of this system this is one of the most well characterised. This system is known to function to allow natural competence development, allowing DNA uptake from the environment to allow horizontal gene transfer and genetic variation (Johnsborg, et al., 2008, Steinmoen, et al., 2002), which is discussed in section 1.5.1. Briefly this system has a know activation stimulus which is that of a small peptide named CSP encoded on a gene located upstream of the TCS (Havarstein et al., 1996, Havarstein et al., 1995, Pestova et al., 1996). Genes regulated by this system include those important for DNA uptake and processing to allow recombination as well as positively regulating itself (Peterson et al., 2004). Some of the genes regulated by this system include some virulence factors (cell wall hydrolyses, LytA/ CbpD) which are important for inducing fratricide in a population of cells (Guiral et al., 2005, Kausmally et al., 2005), this phenomenon is also linked to TCS03 as described in section 1.7.1.1.3.

This system has also been linked to the function of TCS05, which is known to repress competence (Dagkessamanskaia et al., 2004, Guenzi et al., 1994, Giammarinaro et al., 1999, Sebert et al., 2005), and plays an important role in autolysis which is important during competence for fratricide, discussed above. This system is also highly up regulated in a serine/ threonine protein kinase mutant, however a reduced expression of TCS05 is also observed in this mutant and therefore this may be due to a knock on effect of this, as TCS05 represses TCS12 expression (Saskova et al., 2007). RR12 and RR13 are the only two members of the AgrA family and both are activated in response to small peptides. This may not be surprising as they have been shown to converge in some instances on the same promoter and regulate the same gene (Knutsen et al., 2004). A number of the genes regulated by TCS13 have also been shown to be induced upon early competence development (Peterson et al., 2004).

1.7.1.1.13 TCS13

TCS13 was initially thought to be essential as knockouts of the system could not be obtained (Lange et al., 1999), although this was found not to be the case this TCS has been shown to play a role in virulence (Throup et al., 2000, Dawid et al., 2007). This TCS is most similar in aa sequence to TCS12 and is similar in that

it is activated by a small peptide encoded by *blpC* located directly downstream of the *blpR*/*blpH* encoded TCS (De Saizieu et al., 2000). Directly downstream of *blpC* there are also two genes *blpA*/ *blpB* which encode an ABC transporter required for BlpC export, which is homologous to the ComA/comB export system required for transport of CSP (De Saizieu et al., 2000). Recent studies of strains that do not contain a functional BlpA are unable to transport BlpC, however the system can still be activated by exogenously produced BlpC if other bacteria in the population are able to export BlpC (Son et al., 2011). Genes regulated by this system include a number of bacteriocin genes and immunity peptides which have been shown to be important for inter and intrastrain competition, important during colonisation where it has to compete to occupy this niche (Dawid et al., 2007, De Saizieu et al., 2000, Lux et al., 2007). The operons containing the genes regulated by this system are highly diverse with regards to the number of genes present which affects their ability to kill off other colonisers, with some strain more equipped to do so (Lux et al., 2007). As described above due to the similarity of TCS12 and TCS13 they are able to converge to regulate the same gene in some instances (Knutsen et al., 2004). This system has also been linked to TCS05 which regulates HtrA a serine protease, this protease has been shown to be able to post transcriptionally regulate bacteriocin activity in phase variants (Dawid et al., 2009). Transparent phase variant have been shown to have no bacteriocin activity yet opaque do, with both showing similar transcript levels, upon deletion of TCS05 or HtrA in transparent variants bacteriocin activity is restored (Dawid et al., 2009).

1.7.1.1.14 ORR

The orphan response regulator is found in the pneumococcal genome without a cognate HK (Throup et al., 2000). This RR was also found to be important for virulence in the large screens, which was further validated in a later study which showed it was important in a lung model of infection (Ulijasz et al., 2004). This study also assessed the genes regulated by this system, which included RR10 and RR11 of other TCS pairs. Other genes regulated by this system included genes required for iron uptake and led to the proposed name of RitR (repressor of iron transport) for this system, as it suppresses a number of genes important for iron transport (Ulijasz et al., 2004). ORR has also been shown to play a role in resistance to oxidative stress through the genes it regulates. The typical

aspartate phosphorylation site in RR is missing in this ORR, which is replaced by an asparagine residue. However ORR has been shown to be phosphorylated on its DNA binding domain (normally occurs on the receiver domain) by a serine/ threonine protein kinase, and this is removed by its cognate phosphatase (Ulijasz et al.,2009). Further to add to the complexity the cognate phosphatase of the kinase can bind directly through a protein-protein interaction with the DNA binding domain of ORR which alters its ability to bind to its promoter regions, the phosphatase does not bind to the DNA or compete with ORR binding (Ulijasz et al.,2009). This protein complex can be broken down via the kinase but how it does this is still unknown (Ulijasz et al.,2009). Other orphan response regulators in other bacteria have been shown to be phosphorylated through HK of a non cognate TCS pair but to date this has not been observed for ORR (Wang et al., 2009).

1.7.2 Serine/ threonine protein kinases

The use of ESTPK (Eukaryotic-like serine threonine protein kinases) as global gene regulator in prokaryotes is currently being elucidated. It was previously thought that the mode of signal transduction in bacteria was distinct from that in eukaryotes with TCS as described above acting to regulate gene expression changes required for adaptation to environmental changes. Whereas in eukaryotes signalling normally occurs via signalling cascades made up of serine/threonine and tyrosine kinases and their cognate phosphatase. The recent discovery of ESTPK in a large number of bacteria has revised this role, which share roughly 35% sequence identity to their eukaryotic homologues (see review Burnside & Rajagopal, 2012). In prokaryotes these kinases typically consist of a N-terminal kinase domain and C-terminal PASTA (Penicillin binding protein and serine/threonine kinase associated domain) domains, which can range in number from 1-5. *S.pneumoniae* StkP contains four PASTA domains, *S.agalactiae* Stk1, *S.pyogenes* SP-STK, *S.mutans* PknB and *S.aureus* Stk1 contain three PASTA domains and *E.faecalis* PrkC contains five PASTA domains (see review Burnside & Rajagopal, 2012). The PASTA domains are extracellular domains and are thought to act to recognise a certain extracellular stimuli. In most instances the stimulus is unknown but PASTA domains function to bind unlinked peptidoglycan in PBPs (penicillin binding proteins) and this is also the case for PrkC in *B.subtilis* (Shah et al., 2010, Gordon et al. 2000, Maurer et al. 2012). The PASTA domains of StkP

in *S.pneumoniae* have been shown to bind to synthetic peptidoglycan which activates the kinase, this is also observed upon incubation with the β-lactam ampicillin which binds to the PASTA domains (Maestro et al. 2010, Beilharz et al. 2012).

ESTPK are often found adjacent to a STP (protein phosphatase) which function in unison with the kinase to dephosphorylate proteins phosphorylated by the kinase (Ulijasz et al. 2009). In most bacterial species only a single ESTPK and STP pair have found, some exceptions to this are *M.tuberculosis* which contains 11 ESTPK yet only PknB contains extracellular PASTA domains (see review Av-gay & Everett, 2000). Some *S.aureus* strains also contain two ESTKP (Didier et al., 2010).

Unlike TCS ESTPK do not contain a DNA binding domain to directly modulate gene expression and alter gene expression in a number of other ways discussed below. These kinases have however been shown to alter the expression of large caches of genes in a number of bacteria this includes *S.pneumoniae, S.aureus*, *S.pyogenes* and *S.mutans* (Banu et al., 2010, Bugrysheva et al., 2011, Donat et al., 2009, Burnside et al., 2010, Saskova et al., 2007).

One mode of action through which ESTPK regulate large numbers of genes is via phosphorylating RR of TCS pairs, which in turn effects their DNA binding to gene targets. Some examples of this include the CovR/S TCS in *S.agalactiae*. CovR RR is normally phosphorylated on an aspartate residue which increased its affinity for its DNA targets (Lin et al., 2011, Lamy et al., 2004, Jiang et al., 2005). However Stk1 is able to phosphorylate CovR on a threonine residue which reduced its affinity for its DNA targets (Lin et al., 2011). In *S.pyogenes* its CovR homologue is also phosphorylated by its ESTPK (ST-STK) on a threonine residue (Agarwal et al., 2011), ST-STK also phosphorylates another RR in *S.pyogenes* that of WalR of the WalR/K TCS (Agarwal et al., 2011). This phenomenon is also observed in the pneumococcus as its ESTPK (StkP) phosphorylates the orphan response regulator known as RitR (Ulijasz et al. 2009), and also RR06 is phosphorylated on a threonine residue which increased its binding affinity to its DNA target (Agarwal et al. 2012). ESTPK also regulate other transcription regulators which modulate gene expression changes. This includes SarA from *S.aureus* which has over 100 known gene targets (Chien et al., 1999, Cheung et

al., 2008), which upon phosphorylation by Stk1 on a threonine residue increases its binding to its DNA targets four fold (Didier et al., 2010). EmbR transcription regulator in *M.tuberculosis* is also a target of PknH (ESTPK) which again enhances its ability to bind to promoter DNA (Sharma et al., 2006b), interestingly EmbR can also be phosphorylated by alternative kinases in *M.tuberculosis* including PknA and PknB (Sharma et al., 2006a, Sharma et al., 2006b).

ESTPK can also act to regulate gene expression through modification of histone proteins. These proteins upon DNA binding can modify the DNA structure and stop other regulatory proteins from binding (see review Dorman & Deighan, 2003). In *E.coli* DNA binding like histone proteins can effect expression of 8% Of the genes in the genome (Oberto et al., 2009). In *S.pyogenes* SP-STK is able to phosphorylate a histone protein, which is thought to contribute to regulation of certain virulence factors (Jin & Pancholi, 2006), this phenomenon is also observed in *S.aureus* (Burnside et al., 2010).

Another hypothesised function of ESTPK with regards to transcription regulation is via phosphorylating RNA or DNA polymerase. In *L.monocytogenes* PrkA (ESTPK) has been shown to interact with the alpha subunit of DNA polymerase and the alpha and beta subunit of RNA polymerase, but how this may alter genes expression in this bacterium is unknown (Lima et al., 2011). Initial studies into the protein targets of StkP in the pneumococcus also indicated it was able to phosphorylate RNA polymerase however further studies were unable to validate this (Novakova et al., 2005, Novakova et al., 2010).

Finally ESTPK can alter translation through phosphorylation of elongation factors which play a vital role in protein biosynthesis. Phosphorylation of elongation factors has been observed in a number of bacteria including *L.monocytogenes*, *B.subtilis* and *M.tuberculosis* (Absalon et al., 2009, Sajid et al., 2011, Lima et al., 2011, Archambaud et al., 2005, Shah & Dworkin, 2010). In *M.tuberculosis* this causes an overall decrease in protein synthesis which can lead to dormancy (Sajid et al., 2011), in *B.subtilis* this phenomenon regulates spore germination (Shah & Dworkin, 2010, Shah et al., 2010).

With regards to their role in virulence ESTPK regulate a number of virulence factors in different bacterial species. In *S.agalactiae* deletion of the kinase and phosphatase lead to a reduction in virulence of 25-100 fold in a neonatal rat sepsis model, perhaps due to Stk1s positive regulation of its toxin β-hemolysin (Rajagopal et al., 2003, Rajagopal et al., 2008). Toxins are also regulated by SP-STK in *S.pyogenes* (Jin & Pancholi, 2006) and by Stk1 in *S.aureus*, which play a role in virulence (Burnside et al., 2010, Débarbouillé et al., 2009, Donat et al., 2009, Tamber et al., 2010). In *S.mutans* deletion of PknB leads to production of weaker biofilms and increased sensitivity to cellular stresses (oxidative/ osmotic/ pH) (Banu et al., 2010, Hussain et al., 2006, Zhu & Kreth, 2011). In the pneumococcus deletion of StkP leads to increased sensitivity to oxidative stress and heat, a reduction in transformation efficiency and reduced virulence in a mouse model of infection (Echenique et al. 2004, Saskova et al. 2007). Further PknB in *M.tuberculosis* is essential (Fernandez et al., 2006, Kang et al., 2005, Sassetti et al., 2003).

1.7.3 Small RNAs

The role of sRNA (small RNAs) in gene regulation in bacteria is currently at the forefront of research. New technology has enabled better identification of these small regulators for instance by tiling array and RNA-seq analysis and a number of internet prediction tools exist, enabling large scale studies to be performed (Rieder et al, 2012, Sharma & Vogel, 2001, Livny & Waldor, 2007). sRNA can function in a number of ways to alter gene expression/ translation, which are described below.

One mode of sRNA regulation is through cis-encoded 5'UTR of mRNA, these sequences found upstream of the ATG start codon can function to prevent translation via blocking the ability of the ribosomes to bind. In a number of pathogens these 5'UTR act as thermometers where at certain temperatures this region is in a closed structure, preventing ribosome binding as it has formed a secondary structure, however upon increased temperature or vice versa the secondary structure is removed and translation initiation occurs (Giuliodori et al., 2010). The first discovered was that of *lcrF* mRNA in *Yersinia pestis* encoding a transcription activator (Hoe & Goguen, 1993, Bohme et al., 2012), further examples include *prfA* mRNA in *L.monocytogenes* which at temperatures below

37°C translation of the genes is blocked yet above these temperature translation can occur, PrfA is a transcription factor and regulates a number of genes involved in virulence (Johansson et al., 2002). These sRNA can also be regulated via binding of small metal ions or change in structure due to altered pH (see review Breaker, 2012). One example includes *gpbA* mRNA in *V.cholerae* which functions as a cell surface adhesin, this gene is regulated via the binding of cyclic diguanosine monophosphate (Kirn et al., 2005, Sudarsan et al., 2008).

A large number of sRNA act through base pairing to the mRNA it regulates. These sRNA are either cis-antisense RNA which means they are encoded on the opposite strand to the gene that is being regulated and is transcribed alongside it. However trans encoded sRNA also exist which are transcribed from a region that is not linked to the gene it regulates. These elements are not likely to be perfect repeats of the mRNA it binds to and therefore form weaker interactions (Thomason & Storz, 2011). Again these sRNA have been linked to roles in virulence, both *M.tuberculosis* and *Salmonella typhimurium* contain cis-antisense RNAs which can modulate virulence (Lee & Groisman, 2011, Padalon-brauch et al., 2008, Gong et al., 2011, Arnvig & Young, 2012, Arnvig & Young, 2009). This is also the case for trans-acting RNA, some examples of these factors modulating virulence can be seen in *S.aureus*, *S.pyogenes*, *L.monocytogenes*, *S.typhimurium* etc (Klenk et al., 2005, Toledo-Arana et al., 2009, Chevalier et al., 2010, Morfeldt et al., 1995, Padalon-brauch et al., 2008, Roberts & Scott, 2007).

Some trans-acting sRNAs require Hfq proteins, which function as sRNA binding proteins and act as a chaperone for the sRNA. These Hfq dependant sRNA have been well characterised in gram negative pathogens and are highly abundant, constituting the largest group of post transcriptional regulators (Sobrero & Valverde, 2012). Hfq proteins have currently not been identified in pathogenic streptococci so will not be discussed further here.

sRNA can also function through direct binding to proteins which modulates their function, some examples of this activity include binding of sRNA to RNAP (RNA polymerase) in *E.coli* which can modulate the cache of genes the RNAP recognises (Cavanagh et al., 2012, Wassarman & Storz, 2000). These also function via modulating the activity of other RNA binding proteins which again

have clearly been shown to modulate virulence in a number of bacterial species (Fortune et al., 2006, Lenz et al., 2004, Rasis & Segal, 2009, Brencic et al., 2010).

As stated above a number of these sRNA are regulated by TCS further adding to the complexity of signalling networks. Conversely sRNA have also been shown to positively regulate TCS, for example translation is prevented of the PhoP/Q TCS in *E.coli* via base paring of the *micA* sRNA to *phoP/Q* mRNA (Coornaert et al., 2011). This type of regulation is also seen in other TCS which form a feedback loop with the sRNA, with the TCS being controlled by and controls the sRNA (Guillier & Gottesman, 2008, Tu et al., 2010).

Until recently in the pneumococcus only a small number of regulatory RNAs had been discovered (Kumar et al., 2010, Halfmann et al., 2007, Tsui et al., 2010), and little work had been done to assess the role of these sRNA in virulence. As described above one of the TCS was shown to regulate five sRNA with two found to be important for autolysis (Halfmann et al., 2007). The most recent studies have identified further sRNA in the pneumococcus with a total of 88-89 identified (Mann et al., 2012, Acebo et al., 2012), the large number would suggest these like in other bacteria may play a key role in virulence gene regulation. Upon deletion of a number of these sRNA a number showed clear alterations in virulence, 26 were identified to be important for colonisation, 18 were important during bacteraemia and 28 were important for lung infection (Mann et al., 2012), this is currently the most comprehensive study into the role of sRNAs in virulence in the pneumococcus and gives an insight into the genes that are regulated by these sRNAs.

1.7.4 Other

Other transcription regulators have been shown to also play key role in gene regulation and virulence in *S.pneumoniae*. A large number were found to be important in the large virulence screens (Hava & Camilli, 2002). Some of the better studied regulators are summarised in Table 1-2:, however due to their large numbers will not be discussed here in further detail.

Transcription regulator	Function	Role in virulence
PsaR	Manganese uptake/ virulence factor regulation	(Hendriksen et al., 2009) (Johnston et al., 2006)
CodY	Amino acid metabolism/ iron uptake/ carbon metabolism.	(Hendriksen et al., 2008a)
GlnR	Glutamate and Glutamine metabolism.	(Hendriksen et al., 2008)
RegR	Competence regulation/ virulence factor regulation	(Chapuy-regaud et al., 2003)
Rgg	Resistance to oxidative stress	(Bortoni et al., 2009)

Table 1-2: pneumococcal transcription regulators

Some example of pneumococcal transcription regulators which are important for virulence or regulate genes that are biologically important.

Other modes of regulation occur which have not been well studied in the pneumococcus, examples include protein to protein interactions such as that of the pilus islet transcription regulation RlrA and adhesin RrgA, which can modulate expression levels of the islet (Basset et al., 2011, Basset et al., 2012). Post transcriptional regulation of bacteriocin activity is also modulated by HtrA in opacity variants perhaps through its protease activity (Dawid et al., 2009). Hydrogen peroxide has also been shown to be able to modulate fatty acid biosynthesis through oxidising FabF (Benisty et al., 2010).

1.8 Aims of this study

The aim of this study was to evaluate some of the key regulatory pathways used in the pneumococcus to modulate gene expression changes to environmental cues.

This includes assessing the role of two-component signal transduction systems in virulence gene regulation. A number of the systems have been shown to regulate each other and their functions are often intertwined, with some systems shown to regulate the same genes. Therefore we wished to evaluate whether these systems are able to compensate for the deletion of another and whether a hierarchy exists between the systems. Analysis of this was performed using microarrays to assess the whole genome expression changes in single and double TCS mutants.

The serine/ threonine protein kinase is another signalling system used in the pneumococcus to modulate gene expression changes. We therefore also wanted to evaluate this system with regards to the genes it regulates. This kinase contain four extracellular sensing domains which are thought to recognise the extracellular stimulus, therefore the function of the kinase when one of these domains was deleted was also evaluated to assess how this affected its ability to modulate gene expression changes.

Often for the above systems the environmental cue is not known. So a final aim was to assess the effect key environmental changes may have on expression of cell surface virulence factors. This includes looking at the role hydrogen peroxide plays in modulating cell surface constituents.

2 Materials and Methods

2.1 Bacterial growth conditions and storage

S.pneumoniae were grown on BAB (Blood agar base, Oxoid, UK) supplemented with 5% Horse blood (E&O laboratories) at 37°C. Throughout BAB will refer to that supplemented with 5% horse blood. Purity of culture was checked by placing an optochin disc (Mast diagnostics, UK) on the area streaked with *S.pneumoniae* prior to incubation. For *S.pneumoniae* strains requiring media supplemented with antibiotics, concentrations used can be found in Table 2-1. For liquid culture and culture storage a single *S.pneumoniae* colony was inoculated into BHI broth (Brain heart infusion broth, Oxoid, UK) and grown statically to mid log (OD_{600nm} 0.6), 15% sterile glycerol was added and 1ml culture aliquotes stored at -80°C (Aaberge et al. 1995). For strains grown anaerobically strains were grown in BHI broth in an anaerobic cabinet. All *S.pneumoniae* strains used in this study are shown in Table 2-2.

E.coli strains were grown from a single colony on LB agar (Luria Bertani agar, Sigma- Aldrich, UK) or in LB (Luria broth, Sigma-Aldrich, UK) (unless otherwise stated) containing the appropriate antibiotic (Table 2-1) at 37°C with shaking 200rpm. For storage cultures were centrifuged at 4000g for 5 minutes (4K15 centrifuge, Sigma-Aldrich, UK) and resuspended in LB supplemented with 10% glycerol. 1ml aliquots were stored at -80°C. *E.coli* strains and plasmids used in this study can be found in Table 2-4 and Table 2-3 respectively.

Antibiotic	**Stock concentration**	**Working concentration** ***E.coli***	**Working concentration** ***S.pneumoniae***
Ampicillin	100mg/ml	100µg/ml	N/A
Kanamycin	100mg/ml	100µg/ml	400µg/ml
Chloramphenicol	10mg/ml	N/A	10µg/ml
Spectinomycin	100mg/ml	N/A	200µg/ml
Erythromycin	10mg/ml	1mg/ml	1µg/ml

Table 2-1: Antibiotic stock and working concentrations used throughout the study.

Strain name	Parent strain	Description	Resistance
T4NO1		Serotype 4 TIGR4. Taken one passage from strains used in Aaberge et al., 1995. T4NO1 genome sequence in Tettelin et al. 2001.	N/A
T4JH		Serotype 4 TIGR4.	N/A
Xen35		Serotype 4 TIGR4 strain containing *luxA-E* in SP_1914. (Francis et al., 2001, Orihuela et al. 2003)	Kan^R
T4Δ*stkP*	T4NO1	Serotype 4 TIGR4 strain containing a transposon insertion in *stkP* (SP_1732).	$Spec^R$
T4Δ*spxB*	T4NO1	Serotype 4 TIGR4 strain containing a transposon insertion in *spxB* (SP_0730).	$Spec^R$
T4Δ*lctO*	T4NO1	Serotype 4 TIGR4 strain with *lctO* (SP_0715) deleted.	Kan^R
T4Δ*spxB*Δ*lctO*	T4NO1	Serotype 4 TIGR4 strain containing a transposon insertion in *spxB* (SP_0730) and *lctO* (SP_0715) deleted.	$Spec^R$/ Kan^R
T4Δ*rr06*	T4JH	Serotype 4 TIGR4 strain with *rr06* (SP_2193) deleted.	$Spec^R$
T4Δ*rr08*	T4JH	Serotype 4 TIGR4 strain with *rr08* (SP_0083) deleted.	$Chlor^R$
T4Δ*rr09*	T4JH	Serotype 4 TIGR4 strain with *rr09* (SP_0661) deleted. (Throup et al. 2000, Hendriksen et al. 2007)	Ery^R
T4Δ*rr086*	T4JH	Serotype 4 TIGR4 strain with *rr08 and rr06* (SP_0083/SP_2193) respectively deleted.	$Chlor^R$/ $Spec^R$
T4Δ*rr096*	T4JH	Serotype 4 TIGR4 strain with *rr08 and rr06* (SP_0083/ SP_2193) respectively deleted.	Ery^R/ $Spec^R$
T4Δ*rr098*	T4JH	Serotype 4 TIGR4 strain with *rr09 and rr08* (SP_0661/ SP_0083) respectively deleted.	Ery^R/ $Chlor^R$
T4Δ*rr0986*	T4JH	Serotype 4 TIGR4 strain with *rr09, rr08 and rr06* (SP_0661/ SP_0083/ SP_2193) respectively deleted.	Ery^R/ $Chlor^R$/ $Spec^R$
T4Δ*stkP*∇*ST*	T4NO1	Serotype 4 TIGR4 strain containing a transposon insertion in *stkP* (SP_1732). WT gene with P2 promoter inserted in SP_1886.	$Spec^R$/ $Chlor^R$
T4Δ*stkP*∇*XST*	T4NO1	Serotype 4 TIGR4 strain containing a transposon insertion in *stkP* (SP_1732). Xen35 *stkP* gene with P2 promoter inserted in SP_1886.	$Spec^R$/ $Chlor^R$
T4P1	T4NO1	Serotype 4 TIGR4 strain with *lux* genes inserted	Kan^R

		in SP_1886 under the control of promoter P1.	
T4P2	T4NO1	Serotype 4 TIGR4 strain with *lux* genes inserted in SP_1886 under the control of promoter P2.	Kan^R
T4P3	T4NO1	Serotype 4 TIGR4 strain with *lux* genes inserted in SP_1886 under the control of promoter P3.	Kan^R
T4P4	T4NO1	Serotype 4 TIGR4 strain with *lux* genes inserted in SP_1886 under the control of promoter P4.	Kan^R
T4P19	T4NO1	Serotype 4 TIGR4 strain with *lux* genes inserted in SP_1886 under the control of promoter P19.	Kan^R
Xen35Δ19	Xen35	Serotype 4 TIGR4 strain containing *luxA-E* in SP_1914. also contains a transposon insertion upstream of SP_1915.	$Spec^R$ /Kan^R
T4Δ*rrgB*	T4NO1	Serotype 4 TIGR4 strain with *rrgB* (SP_0463) deleted.	Kan^R
T4Δ*rlrA*	T4NO1	Serotype 4 TIGR4 strain with *rlrA* (SP_0461) deleted.	Kan^R

Table 2-2: Names of all *S.pneumoniae* strains used in this study.

Table of all *S.pneumoniae* strains used in this study with their antibiotic profiles. All strains unless a reference is given were constructed by the author. T4Δ*rr098* and T4Δ*rr08* were constructed by Kanika Kapoor under the supervision of the author.

Plasmid name	Description	Resistance	Origin
pCEP2	Plasmid containing 2Kb pneumococcal DNA flanking either side (allows integration into genome) of a MIP (Maltose inducible promoter).	*Amp^R / Kan^R	(Guiral et al. 2006)
pCEP2 lux	*lux* genes cloned under the control of a maltose inducible promoter.	*Amp^R/ Kan^R	Generated in TJM lab by author Jenny Herbert
pC2LSD	pCEP2 lux plasmid containing a StuI restriction site upstream of MIP to allow promoter swapping.	*Amp^R/ Kan^R	Generated in TJM lab by author Jenny Herbert
PCP1	pC2LSD plasmid containing promoter P1 in place of the MIP.	*Amp^R/ Kan^R	Generated in TJM lab by author Jenny Herbert
PCP2	pC2LSD plasmid containing promoter P2 in place of the MIP.	*Amp^R/ Kan^R	Generated in TJM lab by author Jenny Herbert
PCP3	pC2LSD plasmid containing promoter P3 in place of the MIP.	*Amp^R/ Kan^R	Generated in TJM lab by author Jenny Herbert
PCP4	pC2LSD plasmid containing promoter P4 in place of the MIP.	*Amp^R/ Kan^R	Generated in TJM lab by author Jenny Herbert
PCP19	pC2LSD plasmid containing promoter	*Amp^R/	Generated in TJM lab by

	P19 in place of the MIP.	Kan^R	author Jenny Herbert
PCP2 ST	PCP2 plasmid containing *stkP* in place of the *lux* genes and $Chlor^R$ in place of the Kan^R.	*Amp^R/ $Chlor^R$	Generated in TJM lab by author Jenny Herbert
PCP2 XST	PCP2 plasmid containing Xen35 *stkP* in place of the *lux* genes and $Chlor^R$ in place of the Kan^R.	*Amp^R /$Chlor^R$	Generated in TJM lab by author Jenny Herbert
pET-33b	Protein expression vector.	*Kan^R	N/A
pET-33bRrgB	*rrgB* gene encoding aa 40-600 cloned into pET-33b.	*Kan^R	Generated in TJM lab by author Jenny Herbert
pR410	Plasmid with Kan^R cassette flanked by IR recognised by MarC9 transposase.	*Amp^R/ Kan^R	(Prudhomme et al. 2007)
pR412	Plasmid with $Spec^R$ cassette flanked by IR recognised by MarC9 transposase.	*Amp^R/ $Spec^R$	(Prudhomme et al. 2007)
pET-29bMarC9	*marC9* hyperactive transposase cloned into pET-29b.	*Kan^R	(Lampe et al. 1999)

Table 2-3: List of plasmids used in this study.

Table of plasmids used in this study and their parent plasmid origins. * Indicates antibiotic added to media for selection in *E.coli*.

Strain	Genotype
XL1-Blue™	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F´ *proAB lacI*qZΔ*M15* Tn*10* (Tetr)].
BL21(DE3)	F^- *ompT gal dcm lon* $hsdS_B(r_B^- m_B^-)$ λ(DE3 [*lacI lacUV5-T7 gene 1 ind1 sam7 nin5*])
Stellar™	*F-, endA1, supE44, thi-1, recA1, relA1, gyrA96, phoA, Φ80d lacZΔ M15, Δ (lacZYA - argF) U169, Δ (mrr - hsdRMS - mcrBC), ΔmcrA, λ-*

Table 2-4: Genotype of *E.coli* strains used in this study.

2.1.1 Viable counting of S.pneumoniae frozen glycerol stocks

At least 24 hours post freezing a vial per strain was thawed at 37°C for 3 minutes. Mixed well and 20µl removed and serial 10 fold dilutions made in 180µl phosphate buffered saline (PBS, Sigma-Aldrich, UK) in a 96 well round bottom plate (Miles & Misra 1931), creating dilutions of 10^{-1} to 10^{-6}. A BAB plate was divided into six sections and for each dilution 3x 20 µl was spotted onto 1 section. Plates were allowed to dry and incubated overnight at 37°C in a candle jar. Colonies from each spot were counted and the dilution factor picked which

contained roughly 20-100 colonies per spot. From this the CFU/ml (colony forming units/ml) can be calculated see Equation 2-1.

Number of colonies spot 1 + spot 2 + spot 3 = N

N / Divide by Three = A (Average of three spots)

A x 50 = A50 (gives number/ml)

A50 x dilution factor = CFU/ml

Equation 2-1: Equation used to calculate the CFU/ml of *S.pneumoniae* glycerol stocks.

2.2 Cloning

2.2.1 Restriction digest

All restriction enzymes were sourced from NEB (New England Biolabs, UK) and used as per manufacturers instructions.

2.2.2 Polymerase chain reaction (PCR)

Throughout the study PCR performed for cloning or for sequencing was performed using high fidelity DNA polymerase Phusion® (NEB, UK), conditions Table 2-6. All other PCRs such as for confirming transformation were performed using GoTaq® DNA polymerase (Promega, UK), conditions Table 2-5. For both a 55°C annealing temperature was kept constant unless otherwise stated. 72°C extension time was calculated using the expected PCR product size. For all PCR 10mM dNTPs and 10mM forward and reverse primers solution were used. All PCRs were run in a Flexigene thermal cycler (Bibby Scientific, UK).

Temperature	Time	Cycle number
95°C	2 minutes	1 cycle
95°C	30 seconds	30 cycles
55°C	30 seconds	
72°C	1 minute/ Kb	
72°C	5 minutes	1 cycle
Hold 4°C		

Table 2-5: Program information used when performing PCR with GoTaq® DNA polymerase.

Temperature	Time	Cycle number
98°C	30 seconds	1 cycle
98°C	10 seconds	30 cycles
55°C	30 seconds	
72°C	20 seconds/ Kb	
72°C	5 minutes	1 cycle
Hold 4°C		

Table 2-6: Program information used when performing PCR with Phusion® DNA polymerase.

2.2.3 Colony PCR

To screen for *S.pneumoniae* transformants single colonies were first streaked onto a BAB plate and grown for 12 hours to obtain more growth. Using a pipette tip a sweep of bacterial growth was resuspended in 10μl of PCR grade water and placed in the microwave (800W microwave) for 2 minutes to lyse the bacterial cells. For PCR 1μl from the 10μl is added to a 50μl reaction replacing the genomic DNA.

2.2.4 Agarose gel electrophoresis

All DNA gels used were at 0.8% (UltraPure™ agarose, Life Technologies, UK) unless otherwise stated, containing SYBR®Safe DNA gel stain (Life Technologies, UK). Agarose was dissolved and run in 1xTAE buffer (Tris-Acetate EDTA) at 100V for 20 minutes. All DNA samples were run alongside 1Kb Plus DNA ladder (Life Technologies, UK) to gauge size. Gels were visualized using the UVPro gel doc system (UVTech).

2.2.5 PCR purification/ gel extraction

PCR purification and gel extraction were performed using the Wizard® SV gel and PCR clean up system (Promega) as per the manufacturers guidelines.

2.2.6 Plasmid purification

For Plasmid purification *E.coli* strains were grown in 5ml LB broth overnight, centrifuged at 4000g for 5 minutes (4K15 centrifuge, Sigma-Aldrich, UK) to pellet

cells and supernatant decanted. The bacterial pellet was processed using the QIAprep spin miniprep kit (QIAGEN) as per manufacturers guidelines.

2.2.7 Ligation

For ligation the plasmid backbone and PCR product was mixed at a ratio of 2:1 (Vector (100ng): insert) calculated using the infusion molar ratio calculator (http://www.clontech.com/US/Support/xxclt_onlineToolsLoad.jsp?citemId=http://bioinfo.clontech.com/infusion/molarRatio.do&section=16260&xxheight=750). 2μl of T4 DNA ligase reaction buffer (10x), 1μl T4 DNA ligase (NEB, UK) were added and the reaction made to 20μl with PCR grade water. The reaction was incubated for 3 hours at room temperature and then stored at -20°C or immediately transformed into XL1-Blue™ chemically competent *E.coli*.

2.2.8 In-Fusion cloning

In-Fusion cloning was used when restriction sites were not available for ligation. For In-Fusion cloning plasmid backbone and PCR products were mixed at a ratio of 2:1 (Vector (100ng): insert) (In-Fusion molar ratio calculator), to this 2μl of 5x In-Fusion HD enzyme Premix (In-Fusion® HD cloning kit, Clontech, USA) was added and reaction made to 10μl with PCR grade water. Reaction mix was incubated for 15 minutes at 50°C and stored at -20°C until required. Transformation was performed into Stellar® competent *E.coli* cells as per the manufacturers guide.

2.2.9 Transformation into E.coli

Transformation into *E.coli* was performed using 50μl of competent cells (Stellar, XL1-Blue and BL21(DE3)) as per the manufacturers guide. Purified plasmids were transformed using 3-5μl (roughly 100ng/μl) and a volume of 5μl-10μl was used for transformation of In-Fusion and ligation reactions.

2.2.10 Transformation into S.pneumoniae

Pneumococcal strains to be transformed were grown in 10ml BHI supplemented with 1mM $CaCl_2$ until OD_{600nm} 0.07-0.12 was reached. 1ml aliquots were removed into sterile 1.5ml eppendorfs and 100ng/ml of competence stimulating peptide

(CSP-2) added. Tubes were incubated at 37°C for 15 minutes and then roughly 1ug of DNA added, samples were then incubated at 37°C for 75 minutes. Samples were plated onto BAB containing the appropriate antibiotic to select for the transformants. Plates were incubated overnight in a candle jar at 37°C.

2.2.11 Sequencing

PCR samples were purified using the Wizard® SV gel and PCR clean up system (Promega) and Plasmids using the QIAprep miniprep kit. Samples were sent to Source Bioscience, Nottingham, UK for sequencing. Sample and primers were supplied at the concentrations required by the company.

2.2.12 gDNA extraction

S.pneumoniae strains were grown in 20ml BHI at 37°C to OD_{600nm} 0.6. The culture was centrifuged at 4000g for 10 minutes to pellet the cells (4K15 centrifuge), the supernatant was removed and pellets froze at -20°C or processed immediately. To begin extraction the pellet was resusended in 1ml lysis buffer (10mM Tris, 100mM EDTA, 0.5% SDS) and incubated for 1 hour at 37°C. Subsequently 20µg/ml proteinase K (Life technologies, UK) was added and incubated for 3 hours at 50°C. 20µg/ml RNase A (Life technologies, UK) was then added and incubated for 30 minutes at 37°C followed by the addition of an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich, UK), and samples inverted to mix. Samples were centrifuged at 13,000g for 3 minutes (113 centrifuge, Sigma-Aldrich, UK), the upper phase was transferred to a fresh tube and to this 0.2 volumes of 10M ammonium acetate (Sigma-Aldrich, UK) followed by 600µl of absolute ethanol were added. Tubes were inverted and DNA precipitation should be observed, the precipitate was then removed using a sterile 200µl pipette tip into a fresh tube. If DNA precipitate cannot be seen tubes were centrifuged for 30 minutes at 13,000g (1K15 centrifuge, Sigma-Aldrich, UK), and the supernatant removed leaving the pellet. DNA pellets were air dried at room temperature for 20 minutes and then resuspended in 200µl PCR grade water, resuspension was aided by heating at 65°C if required.

2.3 Construction of gene knockouts using *in vitro mariner* mutagenesis

A transposon based mutagenesis method was used for construction of T4Δ*stkP* and T4Δ*spxB*. This method was used as it requires only amplification of the PCR product in to which you wish the transposon to insert into. Four plasmidS are available for use with this system containing different antibiotic resistance cassettes, pR408 Erythromycin, pR410 kanamycin, pR412 spectinomycin, pEMCAT chloramphenicol. Each plasmid contains the antibiotic resistance cassette flanked by two inverted repeats that are recognized by the MarC9 transposase (a hyperactive form of the Himar1 transposase) (Lampe et al., 1996, Lampe et al., 1999). After incubation of the chosen plasmid, PCR product and transposase enzyme the antibiotic resistance cassette flanked by inverted repeats is excised from the plasmid and is able to insert into the PCR product at random TA di-nucleotide repeats. Creating PCR products with insertions at different positions along their length, this is transformed into *S.pneumoniae* giving a pool of mutants. Directionality of transposon can be assessed using primer MP128 and position along the PCR product length assessed using primer MP127. See Figure 2-1 and Table 2-9.

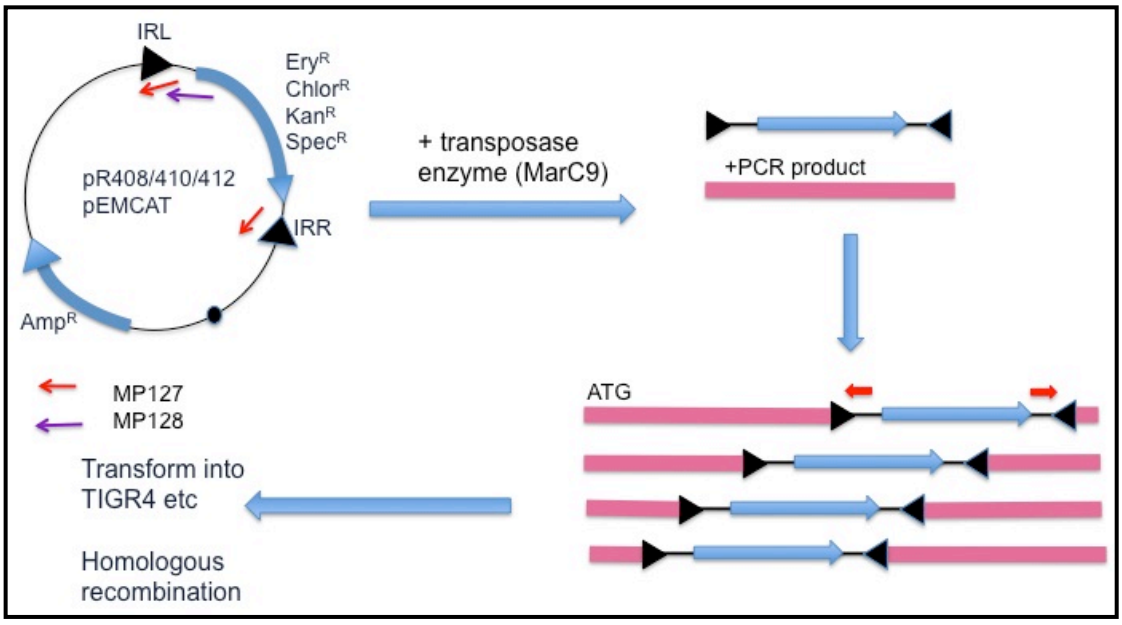


Figure 2-1: Schematic diagram representing the key steps during *in vitro mariner* mutagenesis.

2.3.1 Transposase purification

2.3.1.1 Protein expression

pET-29bMarC9 plasmid was isolated and transformed into BL21(DE3) cells as described in materials and methods 2.2.9. An overnight starter culture was prepared inoculating a single colony into 10ml LB media and grown at 37°C. 5ml of the starter culture was inoculated into 500ml 2xYTmedia (Sigma-Aldrich, UK) and grown to OD_{600nm} 0.6 at 160rpm, 37°C. IPTG (Isopropyl ß-D-thiogalactopyranoside, Melford, UK) was subsequently added to a final concentration of 1mM and culture grown for a further 2 hours under the same conditions. Sample was centrifuged at 5000g at 4°C for 20 minutes (4K15 centrifuge) and resuspended in 5ml resuspension buffer (Table 2-7), 1ml aliquots made and stored at -80°C until purification.

2.3.1.2 Purification

One of the 1ml aliquots was thawed and 10μl of 25mg/ml lysozyme (Fluka, Sigma-Aldrich, UK) solution added, and incubated at room temperature for 5 minutes, followed by 1ml of lysis buffer (Table 2-7) and incubated at room temperature for 15 minutes. 60μl of 1mg/ml DNase 1 from bovine pancreas (Sigma-Aldrich, UK) and 10mM $MgCl_2$ was added and incubated at room temperature for 20 minutes. The sample was centrifuged at 12,000g for 2 minutes at 4°C (1K15 centrifuge) and supernatant discarded. Pellet was washed once in 1ml washing buffer (Table 2-7) and centrifuged at 12,000g for 2 minutes at 4°C (1K15 centrifuge), supernatant discarded. Pellet was subsequently washed twice with 1ml 6M Urea (Fisher Scientific, UK) centrifuged at 12,000g for 2 minutes at 4°C (1K15 centrifuge) and supernatant discarded. Pellet was resuspended in 500μl of column buffer (Table 2-7) centrifuged at 12,000g for 2 minutes at 4°C (1K15 centrifuge) and supernatant collected.

A HiTrap™ DEAE FF 5ml column (GE Healthcare, Fisher Scientific, UK) was equilibrated with column buffer, 500μl of sample supernatant was loaded onto the column at 4°C, followed immediately by 8ml of column buffer. Samples were collected manually in 500μl fractions and run on a SDS gel (Figure 2-2) to confirm protein presence. Fractions 1-3 were pooled, dialysed in dialysis buffer

(Table 2-7) at 4°C while stirring, changing dialysis buffer twice in 8-12 hours. Sample was centrifuged at 12,000g 4°C for 2 minutes (1K15 centrifuge) and supernatant collected (remove any precipitate). Remaining sample was then mixed with an equal volume of 100% glycerol and stored at -80°C.

Buffer	Composition
Resuspension buffer	20mM Tris-HCl pH 7.6, 25% sucrose, 2mM $MgCl_2$, 1mM DTT, 1mM Benzamidine
Lysis Buffer	20mM Tris-HCl pH 7.6, 4mM EDTA, 200mM NaCl, 1% Deoxycholic acid, 1% NP-40, 1mM DTT, 1mM Benzamidine, 0.6mM PMSF (Phenylmethylsulfonyl fluoride)
Washing buffer	0.5% NP-40, 1mM EDTA
Column buffer	20mM Tris-HCl pH 7.6, 4M Guanidine/ HCl, 50mM NaCl, 5mM DTT, 1mM Benzamidine, 1mM PMSF
Dialysis buffer	50mM Tris-HCl pH 7.6, 100mM NaCl, 10mM $MgCl_2$, 1mM DTT

Table 2-7: Buffers used during MarC9 purification.

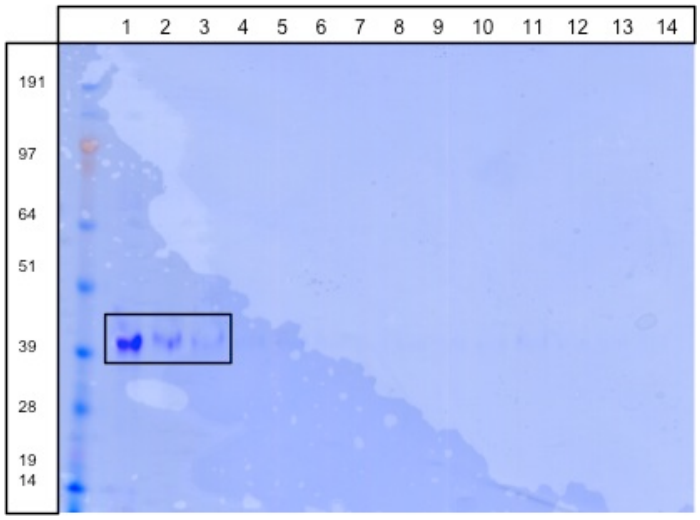


Figure 2-2: Coomassie stained gel of transposase (MarC9) elution fractions.

Boxed are the three fractions containing the 46kDa transposase MarC9, three fractions were pooled and used in later experiments. Left is size in kDa of bands from the SeeBlue® Plus2 pre-stained ladder.

2.3.2 Transposase reaction conditions

Initially the chosen pR plamid was purified using the QIAprep miniprep kit (QIAGEN). The desired PCR product amplified and cleaned using the Wizard® SV gel and PCR clean up system (Promega). The reaction mixture consisted of 1μg

of PCR product, 10μl 2x transposition buffer (Table 2-8), 1μg plasmid DNA, 0.5μl transposase and made to 20μl with PCR grade water.

Buffer	Composition
Transposition buffer	10% glycerol, 2mM DTT, 25mM Hepes pH 7.9, 250μg/ml BSA, 100mM NaCl, 10mM $MgCl_2$ Prepared fresh each time

Table 2-8: Composition of transposition buffer used in transposase reactions.

The reaction mix was incubated for 6 hours at 30°C, cleaned using the Wizard® SV gel and PCR clean up system (Promega) (eluted in 100μl PCR grade water), and heated for 10 minutes at 75°C to inactivate the transposase. To the elute 10μl 10x T4 DNA reaction buffer, 1.5μl 2mM dNTPS, 2μl T4 DNA polymerase (3U/μl) (NEB, UK) and 0.5μl 10mg/ml BSA was added and incubated at 16°C for 30 minutes followed by 75°C for 10 minutes to inactivated the enzyme. Finally 2μl of 1mM NAD+ (β-Nicotinamide adenine dinucleotide, NEB, UK) and 4μl *E.coli* DNA ligase (NEB, UK) were added, incubated at 16°C overnight and then stored at 4°C until transformation.

The whole reaction mix was subsequently transformed into an un-encapsulated TIGR4 strain due to its increased transformation efficiency as described in materials and methods 2.2.10. Transformants were screened using the original primers used for amplification of the PCR product (Table 2-9), which would show a band size or roughly 1.2kb larger due to transposon insertion. MP127 primers were also used in combination with the original primers to show positioning within the PCR fragment and MP128 was used for sequencing to assess directionality of insertion.

Once sequencing was confirmed the original primers were used to amplify the larger fragment from the un-encapsulated TIGR4 strain. This was then transformed into encapsulated T4NO1 creating T4Δ*stkP* and T4Δ*spxB*, for both of which the pR412 plasmid was used giving spectinomycin resistance.

Also included in Table 2-9 are primers 1917F and 1915R. Transposase reaction was also performed on this PCR product and a resulting mutant constructed with

the transposon inserting 20 nucleotides upstream of SP_1915. This insertion was moved from the unencapsulated T4 strain into Xen35 to assess the insertions affects on bioluminescence expression, resulting strain X35Δ19. This mutant was also constructed using plasmid pR412.

Gene	Gene number	Primer name	DNA sequence 5'-3'	size (bp)
stkP	SP_1732	StkPF FL	ATGATCCAAATCGGCAAGA	1980
		StkPR	TTAAGGAGTAGCTGAAGTTG	
spxB	SP_0730	spxB F	ATGACTCAAGGGAAAATTACTG	1776
		spxB R	TTATTTAATTGCGCGTGATTG	
		1917F	GATGCAGGAAATACGTCGCTT	1157
		1915R	TTACCTCATGTTTCTTAGATTTTC	
		MP127	CCGGGGACTTATCAGCCAACC	
		MP128	TACTAGCGACGCCATCTATGTG	

Table 2-9: Primers used for construction and confirmation of transposase mutants.

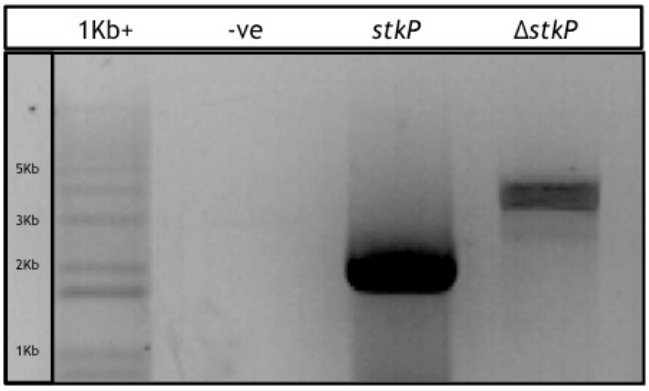


Figure 2-3: Example of PCR confirmation of transposon insertion

In this example a transposon was inserted into *stkP*. PCR was performed using *stkP* primer pair in Table 2-9. *stkP* is roughly 2Kb in size and with transposon insertion ∆*stkP* is roughly 3.2Kb as seen on gel. Numbers on left indicate size of bands in the 1Kb+ ladder.

2.4 Construction of gene knockouts using splice overlap PCR

A number of gene knockouts were also constructed by splice overlap PCR, this included T4Δ*lctO*, T4Δ*rrgB*, T4Δ*rlrA*, T4Δ*rr08*, T4Δ*rr06*. For splice overlap PCR primers were designed containing approx 20bp of gene specific nucleotides and roughly 15bp corresponding to the gene the PCR product is to be joined to. For each gene knockout three primer pairs were required, one to amplify the

upstream region of the gene to be removed, one for the downstream region and one to amplify an antibiotic resistance cassette to replace the gene. For each gene knockout the whole gene was removed unless there was any overlap with another open reading frame, in this instance a small number of nucleotides would remain so as to not perturb the function of surrounding genes. For T4Δ*lctO*, T4Δ*rrgB* and T4Δ*rlrA lctO*/ *rrgB* and *rlrA* were replaced with a kanamycin resistance cassette amplified from the plasmid pR410. For T4Δ*rr08 rr08* was replaced with a chloramphenicol resistance cassette amplified from the strain T4Δ*pspC*. For T4Δ*rr06 rr06* was replaced with a spectinomycin resistance cassette amplified from the plasmid pR412. Some observations upon growth of mutants in BHI indicated the StkP mutants, T4Δ*rr098,* SpxB mutants and Xen35 all seemed to grow slower than the parent strains. Further microscopy indicated StkP mutants had an altered morphology showing increased chain length and altered septation, which was also observed in Xen35. Mutants containing the *rr09* deletion also seemed so show smaller colony morphology.

2.4.1 Construction of single gene knockouts

Each PCR fragment was amplified individually and PCR purified using the Wizard® SV gel and PCR clean up system (Promega). To join the three fragments together 1μl of each fragment was added to a PCR reaction using the outermost primers indicated by a * in Table 2-10, PCR was performed using an extension time that would amplify the correct size band to join all three fragments together. Resulting PCR products were run on a 0.8% agarose gel (UltraPure™ agarose, Life Technologies, UK) and the band of the correct size gel extracted using the Wizard® SV gel and PCR clean up system (Promega). Fragments were then transformed into the desired *S.pneumoniae* strain as described in materials and methods 2.2.10, using the correct antibiotic for selection. Transformants were screened for using PCR and then sequencing.

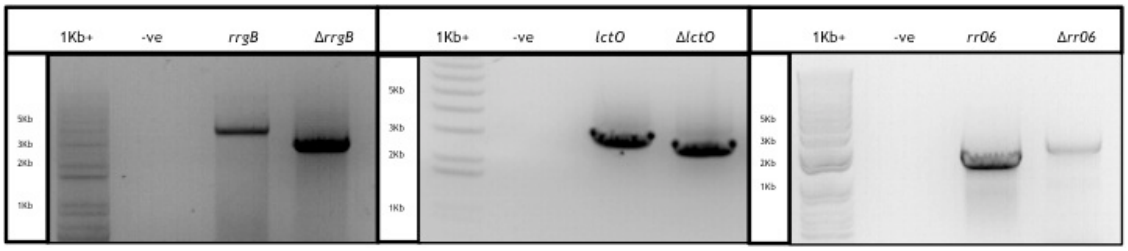


Figure 2-4: Example of PCR confirmation of splice overlap PCR gene knockouts

In these examples from left to right *rrgB*, *lctO* and *rr06,* the whole gene was replaced by an antibiotic resistance cassette. PCR was performed using primer pairs in Table 2-10. Sizes corresponding to the expected WT and ∆ band are shown in Table 2-10. Numbers on left indicate size of bands in the 1Kb+ ladder.

Gene removing	Gene number	Primer name	DNA sequence 5'-3'	size (bp)
lctO	SP_0715	SP_0715 UPF*	TTCGACCAATTCAAGGTTGAGGC	768
WT- 2.7Kb		SP_0715 UPR	ATCCCCAGCTTGAAACTGTCCTCCTCGATTAAG	
Δ*lctO*- 2.3Kb		KAN0715 F	AGGAGGACAGTTTCAAGCTGGGGATCCGTTTGA	795
		KAN0715 R	GGCAATCTGTTTTACTAAAACAATTCATCCAGTAA	
		SP_0715 DWNF	GAATTGTTTTAGTAAAACAGATTGCCTCCACTG	766
		SP_0715 DWNR*	AGCTACGCAGGCACCCAGC	
rrgB	SP_0463	0462F-25C*	CAAGATGGTACTATAACG	1350
WT- 4.1Kb		0462 R	TCCCCAGCTTGGATTTCTCCTTATTCATATC	
Δ*rrgB*- 2.9Kb		KANGB-F	AGGAGAAATCCAAGCTGGGGATCCGTTTG	795
		KANGB-R	CCTTTCTCTCTTACCTAAAACAATTCATCCAGTAA	
		0464F	ATGAATTGTTTTAGGTAAGAGAGAAAGGAGCCATT	807
		0464 R-34R*	TTCTGATTGACAACCGTAATCG	
rlrA	SP_0461	0460F*	ACGTCTGTTATCAAGAATGGTC	834
WT- 3.2Kb		0460R	TGAATTGTTTTAGGTTCATCGTACTGTCTACACA	
Δ*rlrA*- 2.4Kb		KANRL-F	TTCGGTAACTCAAGCTGGGGATCCGTTTGA	795
		KANRL-R	GTACGATGAACCTAAAACAATTCATCCAGT	
		0462F	TCCCCAGCTTGAGTTACCGAATCTTAGTTGC	804
		0462RT-R*	CTTCTGTCAAGGTGTATGTCC	
rr06	SP_2193	36O*	CTTTAGTGGCAAGTTTGGCTG	878
WT – 2.1Kb		2194 R N	GTTCTAGAGCGGTCTCTCCCTTTCTACTACCAG	
Δ*rr06* – 2.6Kb		Spec06F-N	TAGAAAGGGAGAGACCGCTCTAGAACTAGTGGATCC	1150
		Spec06R-N	GGATTTTTTATCATCAATTTTTTTATAATTTTTTTAATC	
		2192 F N	TAAAAAAATTGATGATAAAAAATCCTAAATTAT	546
		36P*	TTGCATTTTACTAGTCACTTC	
rr08	SP_0083	14Y*	ACGATGGCAGGTGAAAACA	356
WT – 1.45Kb		0082R	CAAACAAATTTTTCAGATCTGTCTCTCCTTTGATAAAA	
Δ*rr08* – 1.6Kb		Chlor08F	CAAAGGAGAGACAGATCTGAAAAATTTGTTTGATTTTT	850
		Chlor08R	ACTTTTTAGTTTCATTTATAAAAGCCAGTCATTAGGC	
		0084F	GACTGGCTTTTATAAATGAAACTAAAAAG	385

			TTATATTTTGG	
		14Z*	GCGACAACTGGGCAATCATCAA	
rr09	SP_0661	R9F*	GCGGAGCCGAGTAGGAGATTCTCACC	
WT- 1.5Kb		R9R*	TGTAGAAATGACCTGACCAG	
Δ*rr09* - 1.7Kb				

Table 2-10: Primers used for construction of splice overlap PCR gene knockouts.

Primers and related information used to construct all splice overlap PCR gene knockouts. * Indicates primers used for splice overlap PCR. Blue nucleotides indicate region of the primer specific to the gene being amplified. Δ*rr09* was not constructed in this study and therefore primers stated above are used to amplify and confirm the insertion only.

2.4.2 Construction of double/ triple gene knockouts

Construction of Double and triple mutants were performed via amplification of the Δ PCR products using the splice overlap primers from the parent strain. These were transformed into the desired recipient strain and screened for recombination using PCR.

2.5 Construction of a new bioluminescent strain

To test the potential metabolic burden of high expression of the *lux* genes (required for bioluminescence) *luxA-E* were put under the control of different strength promoters. For this pCEP2 plasmid was used as it allows chromosomal integration of genes cloned into SP_1886 (contained within the plasmid) (Guiral et al. 2006). This plasmid contains a maltose inducible promoter with a multiple cloning site downstream. Flanking either side of this is 2Kb of pneumococcal DNA (SP_1885 and SP_1887) allowing upon transformation integration into the pneumococcal genome, the plasmid is unable to replicate in the pneumococcus and therefore favors recombination. As to not perturb the function of any of the surrounding genes the region containing the maltose inducible promoter recombines out a small part of SP_1886, a IS1167 element containing a frameshift and is therefore already non functional.

2.5.1 Cloning lux genes into pCEP2

Initial cloning steps consisted of cloning the *lux* genes (*luxA-E*) into plasmid pCEP2 (Figure 2-5) at restriction sites NcoI and BamHI. The *lux* genes were amplified from Xen35 using primers in Table 2-11. Plasmid and *lux* genes were

digested with NcoI and BamHI and subsequently ligated together as described in materials and methods 2.2.7. Correct insertion was confirmed by PCR and sequencing resulting in the plasmid pCEP2 lux (Figure 2-6). This construct contains the *lux* genes under the control of a maltose inducible promoter however when transferred into TIGR4 no bioluminescence was visualized (data not shown) implying the promoter was not strong enough to drive expression of *luxA-E*.

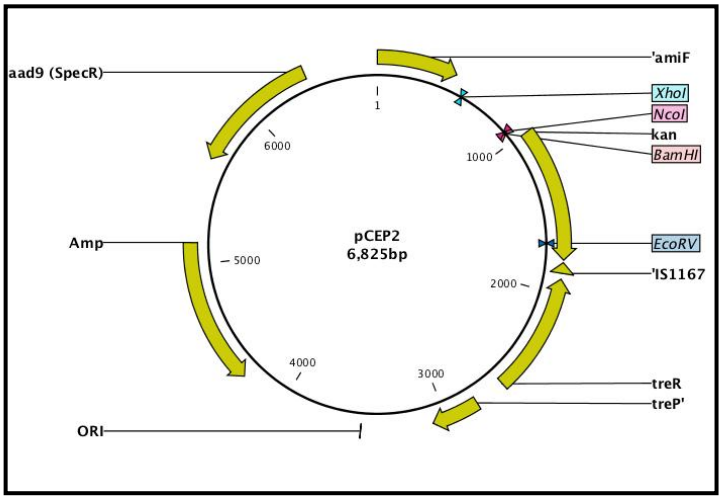


Figure 2-5: Plasmid map of pCEP2

Plasmid map was visulaised in CLC Genomics Workbench 4.5.1.

Primer name	DNA sequence 5'-3'	size (bp)
PcepluxF	TGCTACCATGGAATTTGGAAACTTTTTGC	5606
PcepluxR	CAAACGGATCCTTAACTATCAAACGCTTCGGTT	

Table 2-11: Primers used for amplification of the *lux* genes for cloning into pCEP2.

Primers and related information used for amplification of the *lux* genes from Xen35 for cloning into pCEP2. Light blue and pink region indicates BamHI and NcoI restriction site respectively.

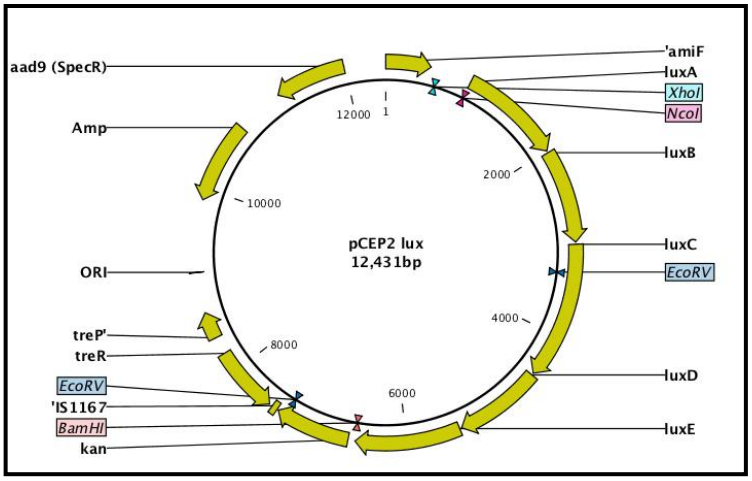


Figure 2-6: Plasmid map of pCEP2lux.

Plasmid map of pCEP2lux constructed from plasmid pCEP2 (Figure 2-5) in CLC Genomics Workbench 4.5.1. pCEP2lux contains the *lux* genes from Xen35 between restriction sites NcoI and BamHI, constructed using primers in Table 2-11.

2.5.2 Creating a StuI site in pCEP2 lux

Due to this we wished to remove the maltose inducible promoter and replace it for a stronger one. Therefore a StuI restriction site was engineered upstream of the maltose inducible promoter allowing complete excision via restriction digest with NcoI and StuI. Site directed mutagenesis was performed to create a StuI site in pCEP2lux using primers in Table 2-13, with PCR conditions in Table 2-12. SDM was performed using 100ng of starting plasmids using high fidelity DNA polymerase Phusion® (NEB, UK). Once run 40μl of the reaction was digested with DpnI enzyme as per the manufacturers guide at 37°C for 3 hours. The reaction was then stored at -20°C until transformed. Transformation was performed into XL1-Blue™ competent *E.coli* and transformants selected for on 100μg/ml ampicillin and confirmed by sequencing and restriction digest. Creating plasmid pC2LSD.

Temperature	Time	Cycle number
98°C	30 seconds	1 cycle
98°C	10 seconds	18 cycles
55°C	30 seconds	
68°C	4.5 minutes	
68°C	10 minutes	1 cycle
Hold 4°C		

Table 2-12: PCR program conditions used for site directed mutagenesis on pCEP2lux.

Primer name	DNA sequence 5'-3'	Size (bp)
Puc18 LSD F	GTTCCGAAAACCAAAGGCCTTGCGCTTGATAAGTTTG	12,431
Puc18 LSD R	CAAGGCTTTTGGTTTCCGGAACGCGAACTATTCAAAC	

Table 2-13: Primers used for Site directed mutagenesis on pCEP2lux.

Primers used for Site directed mutagenesis on pCEP2lux plasmid to create a StuI restriction site. Blue indicates the StuI restriction site, and red the nucleotide that is to be altered in the pCEP2lux plasmid to create the StuI site (A>C/ T>G).

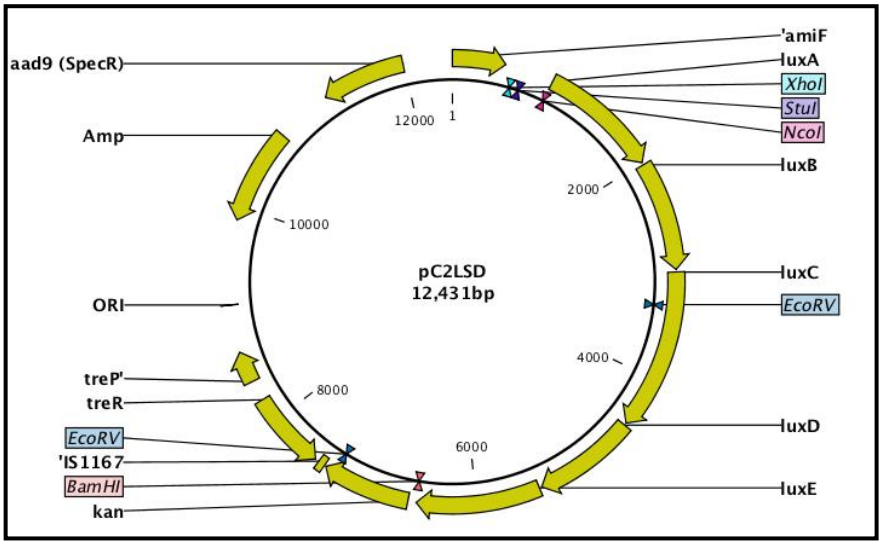


Figure 2-7: Plasmid map of pC2LSD.

Plasmid map of pC2LSD constructed from plasmid pCEP2lux (Figure 2-6) in CLC Genomics Workbench 4.5.1. pC2LSD has had a single nucleotide change creating a StuI restriction site using primers in Table 2-13.

2.5.3 Replacing the maltose inducible promoter

To decide what strong promoters to use RNA-seq data was used to identify genes with high expression (RNA-seq data performed by Dr Jiangtao Ma and Dr Andrea Mitchell). Genes with the highest expression (RPKM) values were identified and the upstream region assessed for the likelihood of a promoter being present. Expression of surrounding genes was also taken into consideration as they may be expressed as part of an operon. Finally sequence regions upstream of chosen genes were further assessed for the presence of promoter sequences using BPROM-softberry program that detects potential sigma70 promoter recognition sites: (http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb). All regions selected contained a potential sigma70 binding site and as a control the program was run using the region containing the maltose inducible promoter from pCEP2, which also contained a binding site.

For promoter cloning pC2LSD was digested using restriction enzymes StuI and NcoI removing the maltose inducible promoter. Promoters were amplified from TIGR4 using primers in Table 2-14, and products PCR purified, digested with restriction enzymes StuI and NcoI and then PCR purified again for cloning. Vector and insert were ligated together as described in materials and methods 2.2.7, and then transformed into XL1-Blue cells. Transformants were selected for on 100μg/ml ampicillin and confirmed by PCR and sequencing. Plasmids constructed were pC2LSD P1/P2/P3/ P4 and P19.

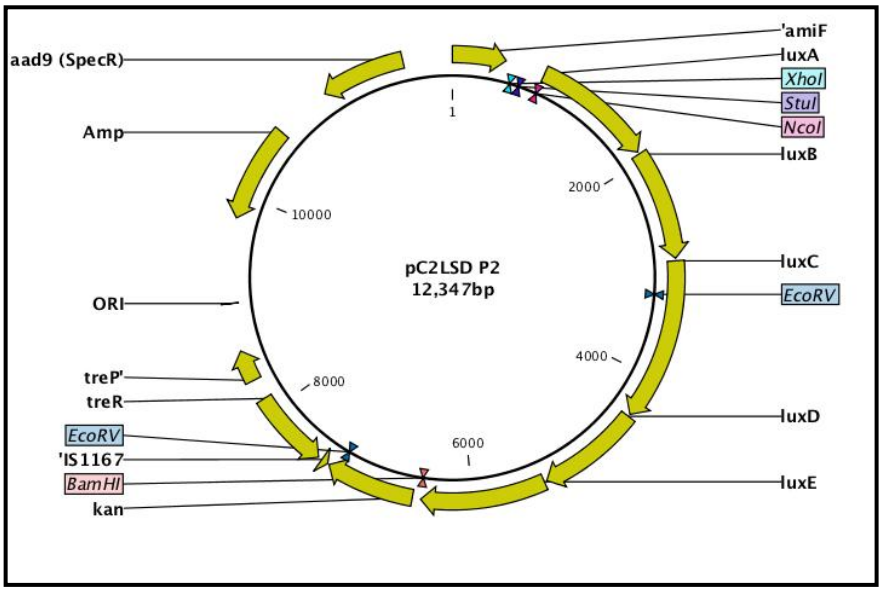


Figure 2-8: Plasmid map of pC2LSD P2

Plasmid map of pC2LSD P2 constructed from plasmid pC2LSD (Figure 2-7) in CLC Genomics Workbench 4.5.1. pC2LSD contains the P2 promoter amplified from T4 and cloned between restriction sites StuI and NcoI constructed using primers in Table 2-14. Other promoter plasmids are identical to pC2LSD P2 with variations only between StuI and NcoI restriction sites.

All plasmids were isolated and transformed into T4NO1, selected for on 100μg/ml kanamycin, and integration into the genome confirmed by PCR using primer amiFF T4G (Table 2-15) and the corresponding promoter reverse primer.

Gene number	Primer name	DNA sequence 5'-3'	size (bp)
5' SP_1489	P1 F	GCTAGGCCTGTGGATGGATAATGCTGA	206
	P1 R	TAGCCATGGTTGAGTAAAAGCCTCCAAT	
5' SP_2012	P2 F	CTAGGCCTTAAGGGATTCCTTGGTTTAC	176

	P2 R	TAGCCATGGTAGTGATTTCCTCCTTATG	
5’ SP_1128	P3 F	GCTAGGCCTGAAAACAGTATATCATAAA	163
	P3 R	TAGCCATGGTTTTTACTCTCCTTATGAG	
5’ SP_0232	P4 F	TAGGCCTAGCGTTTTTCACACTTGC	116
	P4 R	GCCATGGGCAAAAGCACCTCCATAA	
5’ SP_1915	P19 F	TAGGCCTCATGTAAGTTAAGCTAGTC	140
	P19 R	GCCATGGATCTCCTTTCTGACTCTA	

Table 2-14: Primers used for cloning different *S.pneumoniae* promoters into pC2LSD.

Primers and related information used for cloning different *S.pneumoniae* promoters into pC2LSD. Blue nucleotides indicate region of the primer specific to the gene being amplified.

2.6 Complementation of gene knockouts

Complementation of the *stkP* gene in T4Δ*stkP* was performed to assess the functionality of the Xen35 *stkP* allele, which contains a deletion of one of the extracelleular sensing domins (PASTA domains) in the C-terminal of the protein. To do this *stkP* was amplified from T4NO1 and Xen35 and cloned into the plasmid pC2LSD P2. This put the different *stkP* alleles under the control of the strong P2 promoter. The kanamycin resistance cassette was also removed from the pC2LSD P2 plasmid and replaced with a chloramphenicol resistance cassette to allow transformation into Xen35 (Kan^R) and T4Δ*stkP*.

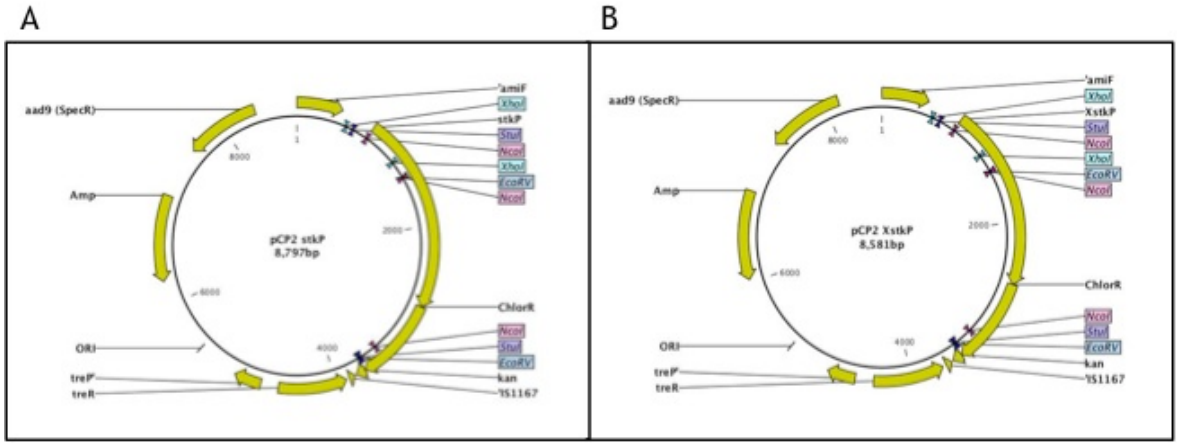


Figure 2-9: Plasmid map of pCP2 ST and pCP2 XST.

Plasmid map of pCP2 ST (A) and pCP2 XST (B) created in CLC Genomics workbench 4.5.1. Constructed from cloning T4 *stkP* and Xen35 *stkP* into plasmid pC2LSD P2 respectively along with a chloramphenicol resistance cassette using primers in Table 2-15.

pC2LSD P2 plasmid was isolated using the Wizard® SV gel and PCR clean up system (Promega) and then restriction digested with NcoI and EcoRV. The digest was run on an agarose gel and the required band gel extracted. PCR was performed to amplify the two *stkP* alleles from T4NO1 and Xen35 and the chloramphenicol resistant cassette was amplified from T4Δ*rr08*. Initially the *stkP* alleles were joined via splice overlap PCR to the chloramphenicol resistance cassette (Primers Table 2-15). The correct size band was gel extracted and this used for cloning into pC2LSD P2. For cloning In-Fusion cloning technology was used (In-Fusion® HD cloning kit, Clontech, USA), see materials and methods 2.2.8. Transformants were selected for on 100μg/ml ampicillin and confirmed by PCR and sequencing. From this plasmids pCP2 ST and pCP2 XST were isolated using the QIAprep spin miniprep kit (QIAGEN). Plasmids were subsequently transformed into T4Δ*stkP* and transformants selected for on 10μg/ml chloramphenicol and confirmed by PCR, using primer amiFF T4G to confirm integration into the genome (primer binds outside of the plasmids). Creating strains T4Δ*stkP*∇*ST* and T4Δ*stkP*∇*XST* containing the T4 *stkP* allele and Xen35 *stkP* allele respectively between genes SP_1885/SP_1887. No positive transformants were obtained when transforming these plasmids into Xen35.

Gene	Gene number	Primer name	DNA sequence 5'-3'	size (bp)
stkP	SP_1732	STKP COMP F*	CACTACCATGGATGATCCAAATCGGCAAG	1980 (T4) 1764 (X35)
		STKP COMP R	TTTCAGGGATCCTTAAGGAGTAGCTGAAGTT G	
		CHLOR ST C F	CCTTAAGGATCCCTGAAAAATTTGTTTGATTT	850
		CHLOR COMP R*	CTTCCCCGATATCTTATAAAAGCCAGTCATTA G	
amiF	SP_1886	amiFF T4G	AGGATGAAGAAGAACGTAAAG	

Table 2-15: Primers used for cloning of two *stkP* alleles into plasmid pC2LSD P2.

Primers and other related information used for the cloning of two *stkP* alleles into plasmid pC2LSD P2 for T4Δ*stkP* complementation. * indicates primers used for splice overlap PCR. Blue nucleotides indicate region of the primer specific to the gene being amplified.

No complemented strains were constructed for any of the other gene knockouts due to time limitiations therefore phenotypic observations seen in the knockout strains would need to be further confirmed to ensure this is not due to accumulation of mutations at other regions within the genome.

2.7 Whole genome sequencing (WGS)

Whole genome sequencing was performed on strains Xen35, T4NO1, T4JH and T4P2. Genomic DNA extraction was performed by Dr Andrea Mitchell.

Pre sequencing DNA was fragmented using a Bioruptor sonicator and processed using a TruSeq DNA library preparation kit (Illumina, USA). Quality and quantity was checked using the bioanalyser and Kapa library quantification kits (KAPA Biosystems, UK) respectively. Samples were loaded onto the flow cells at 12pM on the Illumina Cluster station and sequenced using the IlluminaGAII (Xen35) or IlluminaGAIIx (T4NO1, T4JH and T4P2). Pre sequencing preparation was performed by Mrs Julie Galbraith at the Glasgow Polyomics Facility.

Post sequencing processing was performed by Dr Pawel Herzyk.

Data analysis was performed in CLC Genomics Workbench 4.5.1 (CLC bio). Xen35 genome sequence was reference assembled to T4 genome sequence available at NCBI (NC_003028), followed by SNP and Indel analysis. A De Novo assembly was also performed assembling the reads against themselves to give an indication if regions of low coverage are too divergent from the reference to assemble or are deletions. Preliminary assemblies, SNP and Indel testing were performed by Dr Andrea Mitchell.

Only prelimnary genome sequence data is available for T4NO1, T4JH and T4P2. Where genome sequence data was reference assembled to T4 genome sequence available at NCBI (NC_003028), followed by SNP and Indel analysis. Analysis was performed by Dr Andrea Mitchell. None of the genome changes have been further validated.

A number of changes detected in Xen35 were confirmed via PCR followed by sequencing using primers in Table 2-16.

Gene number	Primer name	DNA sequence 5'-3'	size (bp)	Changes confirmed
SP_1732	StkPF	AAGAAGGAACGAAAATCAAT	716	40/ 41
	StkPR	TTAAGGAGTAGCTGAAGTTG		
SP_1915	1915F	ATGAAGTTACGAATTGAGATTG	447	125-142

	1915R	TTACCTCATGTTTCTTAGATTTTC		
SP_201	201F	GAGGAGACAAATACGGAAG	302	2
	201R	CAAGATATTGATTTGGTTATTT		
SP_1908	1908F	ATGTATAATAAAGTTATCTTGATTGG	396	47-59
	1908R	TTAAAATGGCAATTCTTCCTC		
SP_1909	1909F	ATGGCAAAAAATGTTGTGAT	762	62-67
	1909R	TTATTTTTTATGAACCATGAAACC		
SP_1910	1910F	AAAAGGAAACAATGATTTTTACAT	639	68-106
	1910R	TTAGGCAGTCCAGTGTTTCGC		
SP_1911	1911F	ATGATACAGCCAGCAAGTTT	336	107-116
	1911R	CATTGTTTCCTTTTTCTCCTAT		
SP_1927	1927F	AGTAAAAACTGGACTATAACTTATT	459	189-195
	1927R	ACCAGAATCATTCGCTAAT		
SP_1928	1928F	ATGAAGGCTCAAGCGATT	473	196-208
	1928R	GTTGTGAATGGATAAATATATCAT		
SP_1935	1935F	GTGAAGAAGAGTTCAGTCAG	396	236-240
	1935R	GTTATTTTCTAAAAGTTGTTCAG		
0885-0886	0885-0886F	CTATCGGATTAAGGAAATTAAC	505	16
	0885-0886R	ATATCTTCCCTTTTCTTTAGTT		
1029-1030	1029-1030F	CATTTTTATATTTAAAGGAGCG	557	19
	1029-1030R	TTCATTCAATCTTACATTGAT		
1199-1200	1199-1200F	GCTATAAGTATAACACTATATGAAA	426	26-29
	1199-1200R	AGTTATCAGAAATGCCTTTC		
1717-1718	1717-1718F	TTTCACCTCCGAAATTTCT	391	36-39
	1717-1718R	AACAATTCAGGATTAAAAATAG		
1777-1778	1777-1778F	GAGAAAATTGACAACCGCTA	546	44
	1777-1778R	AATTGAAACTCAAAAAGCC		
1851-1852	1851-1852F	AGATAACCACCAAACCACT	404	45
	1851-1852R	ACGGATAAAGAAAAAGCCC		
2105-2106	2105-2106F	TAAGGCTGAGGACAGTAAAAAC	396	243
	2105-2106R	GCTTGCTGACTACGAAGAC		
SP_0927	0927F	ATGAACATTCAACAATTACGC	880	18
	0927R	CTTGCATGACTTCTACGAA		
0482-0483	0482F	GAATTGCCAATGGTGTATCTGT	515	6/7
	0483R	GTGTTTCATACCGGAAAGAGA		
1912-1913	1913R	AGATGCTTCGCAATACCTAC	946	117-120
	1911R	As above		
1928-1929	1928F	As above	838	209-214
	1929R	AAGCAGAAGTGTACTATTCTAG		
	1918F	CCTGACCTTTGAAGTTTTCA	1601	152-167
	1920R	TAGATGGGTTTTAATTTAAC		

Table 2-16: Primers used for confirmation of genome sequence changes seen in Xen35.

Primers and other related information used for confirmation of genome sequence changes seen in Xen35. Changes confirmed refer to the table of Xen35 whole genome changes seen in appendix III.

2.8 RNA WORK

2.8.1 RNA extraction

For RNA extraction 10ml *S.pneumoniae* cultures were grown to mid log (OD_{600nm} 0.6) in BHI, centrifuged at 4000g for 5 minutes (4K15 centrifuge, Sigma-Aldrich, UK), supernatant decanted and pellets froze in liquid nitrogen and stored at -80°C until extraction. For each strain three cultures were grown at the same time in the same broth to reduce variability. Therefore three RNA extractions were performed per strain giving three biological replicas.

To begin extraction the bacterial pellet was resuspended in 200μl lysis buffer (lysozyme from chicken egg white 15mg/ml (Sigma-Aldrich, UK), 1mM EDTA (0.5M EDTA pH8.0), 10mM Tris (1M Tris HCl, Ambion, Life Technologies, UK)) made in nuclease free water (Ambion, Life Technologies, UK), and incubated at room temperature for 15 minutes, vortexing (rotamixer, Hook and Tucker instruments, UK) every 2 minutes. All buffers used throughout are from the RNeasy mini RNA purification kit (Qiagen, UK) unless otherwise stated. Samples were transferred to 1.5ml eppendorfs and 700μl RLT buffer added to each and vortexed for 10 seconds. Samples were then transferred into a 1.5ml eppendorf containing 25-50mg of 100μm glass beads (Sigma-Aldrich, UK) and cells lysed using a Hybaid Ribolyser (Hybaid, UK) at speed 4 for 4x 20 seconds. Samples were centrifuged briefly for 10 seconds at 13,000g (1K15 centrifuge, Sigma-Aldrich, UK) and supernatant removed into a 1.5ml eppendorf. 500μl of 100% ethanol (Fisher Scientific, UK) was added and mixed without vortexing. 700μl of sample was added to an RNeasy column and centrifuged for 30 seconds at 13,000g, flow through discarded and the remaining volume of sample applied to the column and centrifuged for 30 seconds at 13,000g, flow through discarded. 350μl of RW1 buffer was added to the column and centrifuged for 5 minutes at 13,000g. 80μl of RNase-free DNase 1 (27.3 Kunitz units) (Qiagen, UK) was pippetted directly onto the column membrane and incubated at room

temperature for 15 minutes. 700µl of RW1 buffer was added to the column and centrifuged for 30 seconds at 13,000g discarding flow through. 500µl of RPE buffer was added to the column and centrifuged for 30 seconds at 13,000g, flow through discarded, a further 500µl of RPE buffer was added and centrifuged for 2 minutes at 13,000g to remove any residual ethanol. The column was transferred to a new 1.5ml eppendorf and 50µl of nuclease free water pippetted directly onto the membrane and left to stand for 2 minutes at room temperature. Samples were centrifuged at 13,000g for 1 minute to elute RNA. For all RNA samples a second DNase step was performed at this stage to ensure removal of any contaminating DNA. The TURBO DNA-free™ kit (Ambion, Life Technologies, UK) was use as per the manufacturers guide. RNA was aliquoted into 5µl samples and stored at -80°C until required.

2.8.2 RNA quantity and quality

RNA concentration was measure on the nanodrop ND 1000 (Thermo Scientific, UK), and quality assessed using the Bioanalyser 2100 (Agilent, UK). PCR was also performed on RNA samples to confirm absence of DNA contamination.

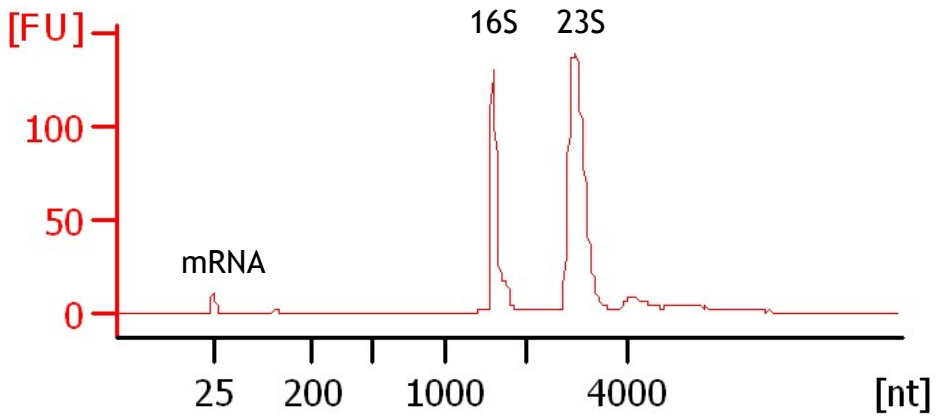


Figure 2-10: Bioanalyser 2100 total RNA readout.

Bioanalyser 2100 total RNA readout showing two prominent peaks (16S and 23S RNA), which are used as an indicator of RNA isolation and purity. If RNA is degraded these peaks decrease in size and shift to the left.

2.8.3 RNA vs. RNA Microarray

Microarray technology was used to look at gene expression differences in mutant strains. Microarrays used were designed and printed at the Bacterial Microarray

group at St. Georges Hospital, London (Bμg@S). Probes printed onto the microarray slide were designed based on the genome sequenced strain TIGR4 (Tettelin et al., 2001), representing all 2236 open reading frames, a further 117 probes were added to represent unique genes seen in the R6 genome sequence (Hoskins et al., 2001), array version SPv1.1.0.

On each microarray slide was a Cy3 labelled control sample (always T4 RNA) used as a baseline for expression and a Cy5 labelled sample (RNA of strain tested). Microarrays were done in pairs each slide containing the Cy5 labelled samples to be compared e.g. T4 vs. Xen35 both Cy5 labelled on separate slides (also contains a Cy3 labelled T4 sample). Arrays were performed in triplicate (6 slides per experiment, 3 per strain) with RNA on each slide being from a different biological replica.

2.8.3.1 Microarray slide preparation

Microarray slide preparation was performed alongside microarray sample preparation. Microarray slides were placed in 0.2μm filtered pre-hybridised solution (8.75ml 20x SSC buffer, 250μl 20% SDS, 5ml 100mg/ml BSA, made to 50ml with sterile distilled water) pre-heated to 65°C. Slides were incubated at 65°C for 30 minutes before washing in sterile distilled water for 1 minute and then in propan-2-ol for a further minute. Slides were centrifuged to remove any residual liquid for 5 minutes at 1500g (4K15 centrifuge, Sigma-Aldrich, UK) and stored in a dust free box until required.

2.8.3.2 cDNA synthesis

All incubations were performed using a Techgene thermal cycler (Bibby Scientific, UK). 2μg of total RNA was mixed with 1μl of random primers (Life Technologies, UK) and made to 11μl with nuclease free water. Samples were incubated at 70°C for 10 minutes and allowed to snap cool on ice. To each sample 5μl 5x first strand buffer*, 2.5μl DTT* (100mM), 2.3μl dNTP mix (5mM dGTP/ dATP/ dTTP and 2mM dCTP, Life Technologies, UK), 1.7μl Cy3-dCTP/ Cy5-dCTP (GE Healthcare, Fisher Scientific, UK) and 2.5μl Superscript 11 (200U/μl) were added. Samples were incubated at 25°C for 10 minutes followed by 45°C for 90 minutes.

* Came as a kit with Superscript 11 from Life Technologies, UK.

Post incubation a Cy3 labelled sample (control) was combined with a Cy5 labelled sample (test strain) and cDNA sample purified using the MinElute PCR purification kit (Qiagen, UK) as per manufacturers guide. Final elution was performed in 15.9μl nuclease free water. 14.9μl of sample was mixed with 4.6μl of filtered 20x SSC buffer (Ambion, Life Technologies, UK) and 3.5μl of 0.2μm filtered 2% SDS (20% sodium dodecyl sulphate, Ambion, Life Technologies, UK). Samples were incubated at 95°C for 2 minutes, briefly allowed to cool and then pippetted under the raised lifter slip (Erie Scientific Company, USA) covering the microarray. Samples were pippetted from the bottom left hand corner of the lifter slip and liquid drawn up over the array area via capillary flow action. Microarray slides were sealed in hybridization cassettes, submerged in water pre-warmed to 65°C and incubated over night in the dark for 16-20 hours in a Techne Hybridiser HB-1D (Techne, USA).

After incubation microarray slides were washed in 65°C pre-warmed wash A (20ml 20xSSC, 1ml 20% SDS made to 400ml with sterile distilled water) for 2 minutes with agitation, allowing the lifter slips to slide off the array slide. Slides were subsequently washed in wash B (1.2ml 20xSSC made to 400ml with sterile distilled water) for 4 minutes with agitation. Slides were centrifuged to remove any residual liquid at 1500g for 5 minutes, and stored in a dust free box until scanning.

2.8.3.3 Microarray analysis

Microarray slides were scanned using ScanArray Express™ (Packard biosciences, Biochip Technologies, Perkin Elmer) and the resulting TIFF (tagged image file format) images created used for analysis. TIFF images were imported into Bluefuse for microarrays 3.5© (BlueGnome LTD, UK) with control data (Cy3 labelled) in channel 1 and test strain data (Cy5 labelled) in channel 2. The files containing the microarray gridmap were created and provided by BμG@S, this (SPv1_1_0_CGH_Gridmap.bcf) automatically removes control spots from the analysis in Bluefuse. Further preliminary post processing analysis involved initial exclusion of unreliable data due to poor hybridization with a confidence estimate of below 0.1. To account for spatial, intensity and dye related effects a

"Global Lowess excluding all with text." normalization step was performed with confidence flags set at default. Replicas of each dye swap were combined by fusion.

Further data analysis was performed in Genespring GX 7.3.1 (Agilent Technologies, USA). Files created in Bluefuse (Output_fused.xls) for each of the three biological replicas were imported into Genespring 7.3.1. Initial normalization consisted of a "dye swap" and a "per gene" step. Statistical analysis of RNA expression was performed in Genespring using the statistical analysis (ANOVA) tool, performing a 1-way parametric test without assuming variances are equal. False discovery rate was set to 0.05 (5% gene false discovery rate), and a Benjamini and Hochberg false discovery rate multiple testing correction applied. This analysis was used to create lists of genes that were statistically differentially regulated between the parent strain and the mutant strains. Although in the literature the cutoff for a true change is classed a 2 fold or above however all the data was included in the analysis as small changes such as a 1.1 fold change would still equate to a 10% change in transcript level which may be biologically relevant. This was validated by the fact some genes showed low expression changes but were true changes confirmed by RT-PCR and were of important cell surface virulence factors.

Validation of microarray experiments was performed using real time PCR (RT-PCR). RT-PCR was performed to confirm some of the gene expression changes seen from the microarray experiments. The same starting RNA was use as for the microarrays so a direct comparison can be made between techniques. For each condition RNA was extracted from three biological replicas as for microarrays, cDNA synthesis was performed on each replica and each ran as an individual sample. Analysis was then performed on the three replicas.

2.8.4 cDNA synthesis

For real time PCR cDNA synthesis was performed as described for the microarrays however all DNTPs concentrations were 5mM, and the Cy dye was replaced with 1.7μl of nuclease free water. Alongside cDNA synthesis a No-RT (no reverse transcriptase) control was performed for each cDNA sample, where the Superscript 11 was replaced with 2.5μl nuclease free water. All cDNA

samples and their No-RT control were diluted 1/50 in nuclease free water and stored at -20°C until required.

2.8.5 RT-PCR

Real-time PCR was performed using FastStart Universal SYBR green master mix (ROX) (Roche, UK). Each sample consisted of 12.5μl of master mix, 1μl of forwards and reverse primer mix (10mM working solution), 0.5μl of sample (cDNA 1/50 dilution or No-RT control etc) and made to 25μl with nuclease free water. Samples were run in 0.2ml white 96 well PCR plates (ABgene, Fisher Scientific, UK) or 0.2ml white 8-well strips (ABgene, Fisher Scientific, UK), with optically clear 8- strip caps (ABgene, Fisher Scientific, UK). All samples were run under the same conditions (Table 2-17) unless otherwise stated on a Chromo4™ system CFB-3240 (Bio-Rad, USA).

Temperature	Time	Cycle number
50°C	2 minutes	1 cycle
95°C	10 minutes	1 cycle
95°C	15 seconds	40 cycles
55°C	30 seconds	
72°C	30 seconds	
72°C	5 minutes	1 cycle
Hold 4°C		
72°C- 95°C		Melting curve

Table 2-17: RT-PCR program information.

2.8.5.1 RT-PCR primers

gyrA was used as an internal control to normalize for cDNA synthesis variations as its expression shouldn't vary under different conditions. For each primer pair along with the cDNA samples and their corresponding No-RT control a gDNA sample was run to ensure the primers amplify and a no-DNA control to ensure the master mix was not contaminated with DNA. Primers were designed manually against the TIGR4 whole genome sequence (NC_003028). Primers were confirmed to not form primer dimers and to form no secondary structure using the online primer design tool provided by Sigma Aldrich (http://www.sigmaaldrich.com/configurator/servlet/DesignTool), as this may give false positives results during RT-PCR by forming DS-DNA that the SYBR green

dye would bind to. This would also be corrected for in the No-RT control. All primers used for RT-PCR analysis can be seen in Table 2-18.

Gene	Gene number	Primer name	DNA sequence 5'-3'	size (bp)
rr01	SP_1633	RR01 RT-F	GGGAAAATGCTCTCTGAAT	120
		RR01 RT-R	ACAAGGGCAAACCAATATC	
rr02	SP_1227	RR02 RT-F	GGTCGTGAAGCGCTAGAG	106
		RR02 RT-R	TACGAATGGTCTTAGCAACTT	
rr03	SP_0387	RR03 RT-F	GACCTCCAAGACGATGTAGA	143
		RR03 RT-R	ATTGCTAAGGTCGCGTCA	
rr04	SP_2082	RR04 RT-F	GTGACAAATGGACGGAAG	166
		RR04 RT-R	ATTCATCACTTTTCGCAGA	
rr05	SP_0798	RR05 RT-F	GATTTTGCTGGATTTGATGTTGCC	146
		RR05 RT-R	CCGCTCCCAGTTCAAATCCAT	
rr06	SP_2193	RR06 RT-F	TGAGTGCTCTGGGAGATGAAACT	148
		RR01 RT-F	CGCCAAAGATCCTCTATGACG	
rr07	SP_0156	RR07 RT-F	GTGGGATATGGAGGTCGTCG	132
		RR07 RT-R	TCCCGAATCATATCAAGCCCT	
rr08	SP_0083	RR08 RT-F	GGCAGGTTATCAGGTCTTGG	157
		RR08 RT-R	AGGCTGCTCTGGTGATAAGTA	
rr09	SP_0661	RR09 RT-F	CAGGTGCCAGATATCATTT	147
		RR09 RT-R	GACAGCAGACAAGGCATAA	
rr10	SP_0603	RR10 RT-F	GTGGCTATGAAACTATTGAGG	120
		RR10 RT-R	GGACTTCTAAGCCGTTGAG	
rr11	SP_2000	RR11 RT-F	GCTTCAACCGGATGTAGAG	151
		RR11 RT-R	TTCTGCTCGTATCCACTCC	
rr12	SP_2235	RR12 RT-F	CCAATATCATACAAGACAACG	130
		RR12 RT-R	GAGCCACTTCAAATCCCT	
rr13	SP_0526	RR13 RT-F	AAAGCACATCATATCATTCC	137
		RR13 RT-R	AGTCCCTTCATCTCTTCATT	
orr	SP_0376	ORR RT-F	CCAGAAAGAGCAGTATCGG	145
		ORR RT-R	TCGGCTCAATTTTTCTGC	
psrP	SP_1772	PsrP RT-F	AATGAGTCAGCAGTACTTG	100
		PsrP RT-R	TCGCTGAATTACTTGTAG	
rlrA	SP_0461	0461 RT-F	CCATCGCAACAGGCTACC	185
		0461 RT-R	TGTGACCCAATCCATACTTCC	
rrgA	SP_0462	0462 RT-F	AACCAGTCCAGCGATAGG	185
		0462 RT-R	CTTCTGTCAAGGTGTATGTCC	
rrgB	SP_0463	0463 RT-F	ATACACCTGTGAACCACCAAG	104

		0463 RT-R	CATTCTATCGCTCCAGTTTGC	
rrgC	SP_0464	0464 RT-F	GTATCTTCTTTGTTATGGCTCTG	185
		0464 RT-R	ATCATCATAGGAATACGAATCATC	
srtB	SP_0466	0466 RT-F	GGTGTCTCGCTTGTATTATCG	86
		0466 RT-R	TGTCAGCCTCATCCAACG	
srtC	SP_0467	0467 RT-F	GTGTCTCGTTATTATTATCGTATTG	91
		0467 RT-R	CCTCAAGTTCTGCCTTATCC	
srtD	SP_0468	0468 RT-F	TCTCGCCTACAATCAACGC	169
		0468 RT-R	ATAATCTGCTCCCAAATAAACCG	
hyp	SP_1914	1914 RT-F	TGGCGTGTAGATTTGAAAGTAG	127
		1914 RT-R	CGTAGACAATTTCCAACAACC	
hyp	SP_1915	RT-1915F*N	TCAAGCTCTATGAGTTGGAGTC	118
		1915 RT-R	TTAGGAGGGATTGGTAATGCCG	
Ser/thr phosphatase	SP_1201	SP_1201 RT-F	GCTGGGATGCTGGAAGAC	150
		SP_1201 RT-R	GGACAAACAGATAGCCCCTT	
gyrA	SP_1219	gyrA RT-F	GCGCGAGCTCTTCCTGATGT	100
		gyrA RT-R	TATGGGGTTTGTCTGGGGTC	
luxA	Xen35_	LuxA RT-F	GGAGCATCATTTCACGGAGTTTG	114
		LuxA RT-R	GTGGGAAGAACAATAGCGGCAGT	
luxB	Xen35_	LuxB RT-F	CAGATAATGGTGTTGTCGGCG	120
		LuxB RT-R N	CTATGCGGACAGGATGATGAGTTG	
luxC	Xen35_	LuxC RT-F	GTGTTTACCTGCCAATATTGAATGAC	166
		LuxC RT-R	TTTAAGTCACGAATGTATGTCCTGCG	
luxD	Xen35_	LuxD RT-F	GCCAGAAGAAAACAGCCCAAAGAG	88
		LuxD RT-R	CGCCAGACCAGCAAAATGAT	
luxE	Xen35_	LuxE RT-F	TGATGATTTGATTTTTTCGAGCG	161
		LuxE RT-R	CCGTAATATTGTCATCTACTTTGTGTGC	

Table 2-18: Primers used for RT-PCR.

2.8.5.2 Analysis RT PCR

Analysis was performed in Opticom Monitor™ version 3.1. Background was subtracted in the software and replicas grouped together with at least two replicas used for analysis. Data was analysed using the $2^{-\Delta\Delta C}{}_T$ method (Livak & Schmittgen 2001), graphical data represent was performed in Prism version 4.0b (GraphPad Software), each bar representing the sample replica means ±

standard deviation error bars. Statistical analysis was performed using a 1-way ANOVA with a Tukeys testing correction comparing the dCT values (Ct of test gene minus Ct of control genes (*gyrA*)) of the control strain (normally TIGR4) against that of the test strain.

2.9 RNA-seq

2.9.1 mRNA purification for transcriptomics

RNA-seq analysis was performed on T4JH and Xen35. Starting total RNA used was from the same batch as that used for microarray and RT-PCR, allowing direct comparison of the techniques. Starting RNA for each sample was 20μg, ribosomal RNA was depleted using the MICROB*Express*™ Kit (Ambion, Fisher Scientific, UK) as per manufacturers instructions with the following exceptions. Volume used of everything was doubled, as the recommended starting RNA concentration is 2-10μg. Volume of Oligo MagBeads used per sample was 200μl to increase efficiency of rRNA binding. And final resuspension volume was decreased to 16.5μl to obtain a more concentrated mRNA preparation. To confirm rRNA depletion samples were run on the Bioanalyser 2100 (Agilent, UK) and concentration measures using the nanodrop ND 1000 (Thermo Scientific, UK).

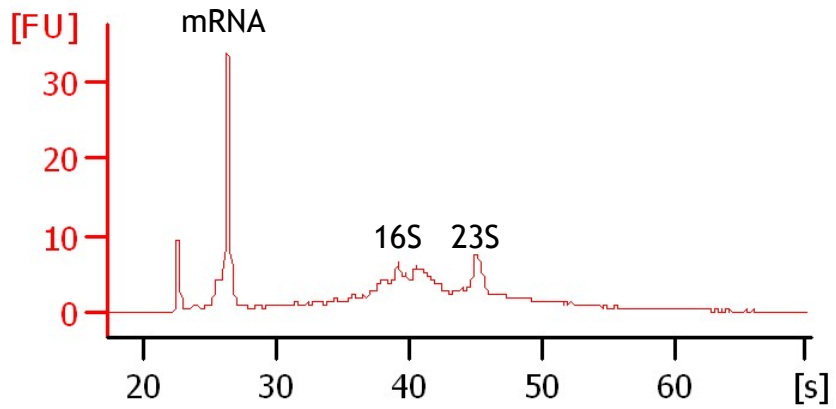


Figure 2-11: Bioanalyser 2100 readout of rRNA depleted total RNA.

Bioanalyser 2100 readout of rRNA depleted total RNA showing small 16S and 23S RNA peaks due to rRNA depletion. mRNA labelled is represented by the prominent peak now the most abundant RNA due to rRNA depletion.

2.9.2 cDNA synthesis from mRNA

cDNA synthesis was performed on a starting volume of 2μg of mRNA. Added to 2μg of mRNA was 1μl (3μg) Random hexamers (Life Technologies, UK) this was made to 23μl with RNase free water and incubated at 70°C for 10 minutes. 8μl of 5x first strand buffer, 4μl 0.1M DTT and 2μl 10mM dNTPs was added and incubated at 25°C for 2 minutes. A further 3μl of superscript III was added and incubated for 25°C for 10 minutes, 42°C for 90 minutes and 70°C for 15 minutes. Added to this reaction on ice was 186μl DNase free water, 60μl 5x second strand reaction buffer, 6μl 10mM dNTP mix, 2μl *E.coli* DNA ligase (10U/μl), 4μl *E.coli* DNA polymerase (10U/μl), 2μl *E.coli* RNase H (2U/μl) giving a total volume of 300μl.This was incubated at 16°C for 3 hours.

300μl of phenol:chlorophorm:isomayl (25:24:1) was added, vortexed for 30 seconds and centrifuged for 1 minute at 12,000g. The aqueous phase removed to a fresh tube and 300μl of chloroform added, vortexed for 30 seconds and centrifuged for 1 minute at 12,000g. The aqueous phase was removed into a fresh tube and 3μl of Glycogene (10mg/ml, Gene Link, USA) added. 300μl 4M ammonium acetate and 300μl of isopropanol were added, and samples mixed via inverting 4 times and incubated at 4°C overnight to aid precipitation of cDNA. Samples were then centrifuged at 4°C, 12,000g for 30 minutes (1K15 centrifuge, Sigma-Aldrich, UK), supernatant carefully removed and pellet rinsed with 500μl 70% ethanol twice centrifuging in between at 14,000g for 2 minutes and discarding supernatant. The pellet was dried at 37°C for 10 minutes and finally resuspended in 10μl DNase free water. cDNA concentration and quality was measure on the nanodrop ND 1000 and Bioanalyser 2100 respectively. 2μg of cDNA was sent from each sample for sequencing.

2.9.3 Downstream cDNA processing and sequencing

Pre sequence processing was performed as described in materials and methods 2.7, by Mrs Julie Galbraith. Paired end RNA-seq was performed using the IlluminaGAIIx at the Glasgow Polyomics Facility.

Data analysis was performed in CLC Genomics Workbench 4.5.1 (CLC bio, USA) T4JH RNA-seq data was assembled to the genome sequence TIGR4 data (NCBI) and Xen35 RNA-seq data was assembled to the Xen35 genome sequence (Assembled from WGS data by author and Dr Andrea Mitchell). RPKM (reads per kilobase of exon model per million mapped reads) values given were used for comparison between expression analysis performed by microarray.

2.10 Western blotting

2.10.1 Sample preparation

For all western blots *S.pneumoniae* strains were grown to OD_{600m} 0.6 in 15ml BHI, strains were centrifuged at 4000g for 10 minutes (4K15 centrifuge) and the supernatant discarded. The bacterial pellets were then resuspended in 750μl PBS and sonicated on ice at 10 microns for four repeats of 30 seconds with 30 seconds break using a Soniprep 150 sonicator (MSE, UK). Protein concentration in each sample was measured using the Qubit® fluorometer (Life Technologies, UK) as per the manufacturers manual. Protein samples being compared were normalised to that of the sample with the lowest concentration, samples were diluted if required with PBS and NuPAGE® LDS sample buffer (4X) (Novex®, Life Technologies, UK) added in the appropriate volume. Samples were boiled at 70°C for 10 minutes.

2.10.2 SDS-PAGE gels and western blotting

Samples prepared as above were run on NuPAGE® Novex 4-12% Bis-Tris gels, 15 well (Life Technologies, UK). Gels were run in XCell SureLock® Mini-Cell tank (Life Technologies, UK) for 50-60 minutes at 200V. 5-10μl of sample were run in each well along with 5μl of SeeBlue® Plus2 pre-stained standard marker (Life Technologies, UK). Gels were transferred using the iBlot® module and iBlot® transfer stack, mini (nitrocellulose) (Life Technologies, UK), transfer was run on program 2 as per the manufacturers guide. The membrane was blocked in 3% skimmed milk (Marvel, UK) in PBS overnight at 4°C or shaking at 37°C for 1 hour. The membrane was then incubated with a 1/4000 dilution of the primary antibody* in 3% skimmed milk for 3 hours shaking at 37°C, washed 6x 5 minutes in PBS with 0.1% Tween 20 (Sigma-Aldrich, UK). A 1/20,000 dilution of the HRP

labelled secondary antibody (Goat Anti-Rabbit IgG HRP linked $F(ab)_2$, GE Healthcare, Fisher Scientific, UK) was added and incubated shaking at 37°C for 1 hour, washed 6x5 minutes in PBS with 0.1% Tween 20 (Sigma-Aldrich, UK). Membranes were developed using Immobilon Western Chemiluminescent HRP substrate (Millipore, UK) with 18x24cm X-ray film (Kodak, USA).

* Anti-Rabbit RrgB – Kindly provided by Novartis Vaccines (De Angelis et al. 2011)

* Anti-Rabbit GroEL (*E.coli*) pAb (Enzo Life Sciences, UK)

2.11 Tissue culture

2.11.1 Maintenance of cells

HBMEC (Human brain microvascular endothelia cells) were grown in advanced RPMI 1640 media (Life technologies, UK) supplemented with 20% FBS (Fetal bovine serum (EU approved), Biosera, UK), 2mM L-Glutamine (Sigma Aldrich, UK), 1% 100X Penicillin streptomycin solution (Sigma Aldrich, UK), and 1% Fungizone® Antimycotic (Gibco®, Life Technologies. UK).

A549 (Human lung epithelial carcinoma cell line, ATCC-CCL-185) cells were grown in Hams F12K (Kaighns modification) media (Life Technologies, UK) supplemented with 10% FBS, Penicillin streptomycin and Fungizone® as above.

Detroit 562 (Human Pharyngeal epithelial carcinoma cell line, ATCC-CCL-138) cells were grown in Minimal Essential Media Eagle (Sigma-Aldrich, UK) supplemented with 10% FBS, 2mM L-glutamine, Penicillin streptomycin and Fungizone® as above.

2.11.2 Growing cells from liquid nitrogen

When resuscitating cells from liquid nitrogen 1ml aliquots were thawed quickly, cells were made up to 10ml with pre-warmed media in a falcon and centrifuged at 1000g for 2 minutes (4K15 centrifuge, Sigma-Aldrich, UK). The supernatant was then removed and the cell pellet resuspended in 15ml media placed into a 75cm^2 vented flask and grown at 37°C in 5% CO_2. The exception to this is the

HBMEC cells which once the 1ml vial was thawed were placed immediately into 15ml pre-warmed media in a 75cm^2 vented flask without centrifugation.

2.11.3 Splitting cells

Cells were split when they reached 70-80% confluence. The tissue culture media was decanted and roughly 15ml of sterile PBS washed over the cells to remove any residual FBS. To digest the cells off the flask 4ml 0.25% Trypsin-EDTA (Sigma-Aldrich, UK) was added and incubated for 5 minutes at 37°C. When cells were visually detached they were removed into a flacon and made up to 10ml with media, centrifuged at 1000g for 2 minutes, supernatant removed and the pellet resuspended in 10ml media. The cell suspension was diluted 1/10 in a 75cm^2 vented flask containing fresh media and grown at 37°C in 5% CO_2.

2.11.4 Adherence assay

For adherence assay all cell lines were grown in the same media as stated in section **Error! Reference source not found.**, however the penicillin streptomycin solution and fungizone® was removed. All adherence assays were performed in 24 well microtitre plates. For adherence assays the protocol for splitting cells was followed however the bacterial suspension produced was used to seed the correct number of tissue culture cells into the 24 well plates. Cell numbers from the bacterial suspension were first counted using a haemocytometer and the volume containing 2x10^5 viable cells seeded into each well and made to 1ml with media. The plates were incubated at 37°C in 5% CO_2 for 48 hours (cells confluent) and could then be used for the adherence assay.

Prior to seeding the HBMEC cells into the 24 wells plates the plates were incubated overnight with a fibronectin solution to allow the cells to adhere. 150μl of 1mg/ml fibronectin from bovine plasma (Sigma-Aldrich, UK) was added to 10ml of sterile PBS, 200μl of this solution was added to each well of the 24 well plate and incubated overnight at 37°C in 5% CO_2. The fibronectin solution was then removed and stored at 4°C, the solution can be used twice.

To begin adherence assay 1ml of *S.pneumoniae* at 10^7 CFU/ml are seeded in to a single well containing tissue culture cells. The volume of glycerol stock

containing the required number of bacteria was calculated using Equation 2-1. The required volume was centrifuged at 13,000g for 3 minutes (113 centrifuge) the supernatant discarded and the bacterial pellet resuspended in 1ml tissue culture media. The assay was incubated at 37°C in 5% CO_2 for 2 hours. Each strain per assay was performed in duplicate and each assay run in triplicate. After two hours 100μl of media was removed from each well, samples from wells containing the same strain (two wells per assay) are pooled and serial dilutions made and viable count performed as stated in materials and methods 2.1.1. The CFU/ml is calculated using Equation 2-1 giving the number of non-adherent cells. The remaining media is removed and the cells washed 3 times with 1ml PBS, 200μl of 0.25% Trypsin-EDTA added to each well to detach the cells and the plate incubated at 37°C for 10 minutes. 800μl of 0.0125% Triton-X-100 (Sigma-Aldrich, UK) in PBS was then added to each well to lyse the tissue culture cells. 200μl was removed from each well into a 96 well plate from each individual well. Serial dilutions of these were created as described previously and neat to 10^{-5} dilutions plated onto BAB plates. The CFU/ml is then calculated from this giving the number of adherent bacteria, from this an average of the two duplicate wells is calculated. Percentage adherence is then calculated by adding the non-adherent and adherent CFU/ml values together and from this calculating the percentage of adherent from the total. Graphical presentation and statistical analysis was performed in Prism version 4.0b (GraphPad Software). Data presented was normalized to that of the wild type strain (100%), so percentage adherence was calculated relative to this. Error bars on graphs represent the standard error of the mean from three replicas. Statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparison test.

2.12 Electron microscopy

2.12.1 Sample preparation

Samples for electron microscopy were taken from 1ml frozen *S.pneumoniae* glycerol stock. Stocks were first defrosted and 500μl centrifuged at 4000g for 5 minutes (113 centrifuge, Sigma-Aldrich, UK), supernatant removed and the pellet washed once in an equal volume of 0.2μm filtered 1x PBS, supernatant was removed and the pellet resuspended in 100μl 0.2μm filtered 1x PBS.

20μl of the prepared bacterial suspension were pippetted onto glow discharge poly-lysine coated nickel grid and incubated at room temperature for 15 minutes to allow bacterial attachment. Grids were then placed sample side down onto 20μl droplets of 1% PFA (paraformaldehyde, Sigma-Aldrich, UK) in 1X PBS on Nescofilm (VWR Jencons) to fix cells and left for 30 minutes at room temperature. All further washed mentioned were performed via placing the grids sample side down on 20μl droplets of the solution on Nescofilm. Grids were then washed 5x for 2 minutes in 0.2μm filtered 1xPBS followed by 3x 5 minute washes in 0.2% BSA (Albumin from bovine sera, Sigma-Aldrich, UK) in 1xPBS. Any excess liquid was removed using filter paper. The grid was then placed sample side down onto 20μl droplets of a 1/10 dilution of the primary antibody (Anti-Mouse RrgB, see section 2.18) in 0.2% BSA PBS, and incubated at room temperature for 1 hour. Grids were then washed 6x 5 minutes in 0.2% BSA PBS, and any excess liquid removed. Grids were then placed onto 20μl of a 1/20 dilution of the secondary antibody (Goat-anti-Mouse IgG (H&L) gold conjugate (10nm particles), Aurion, Holland) in 0.2% BSA PBS and incubated at room temperature for 1 hour. Grids were subsequently washed 6x 5 minutes in 0.2% BSA PBS, then 5x 2 minutes in 0.2μm filtered 1xPBS. Cells were then fixed again in 1% PFA for 30 minutes at room temperature. Grids were washed 6x 2 minute washes in 0.2μm filtered 1xPBS followed by 6x 5 minute washes in 0.2μm filtered distilled water. Any residual liquid was removed using filter paper and finally grids stained for 20 seconds with 0.5% aqueous uranyl acetate (Sigma-Aldrich, UK) (made in distilled water). Residual uranyl acetate was removed using filter paper and grids allowed to dry at room temperature for 10 minutes.

2.12.2 Viewing samples

Grids were viewed between x5000 and x16,000 magnification on a LEO 912 Transmission Electron Microscope.

2.13 Fluorescence activated cell sorting (FACS)

2.13.1 Sample preparation

S.pneumoniae strains were grown to OD_{600nm} 0.6 in 5ml BHI. Cultures were centrifuged at 4000g for 5 minutes (4K15 centrifuge), and bacterial pellet

resuspended in 1ml 2% paraformaldehyde (Sigma-Aldrich, UK) and incubated at room temperature for 1 hour to fix the cells. Cells were centrifuged at 4000g for 5 minutes (113 centrifuge) and resuspended in 1ml buffer 1 (0.2μm filtered 1x PBS, 1% BSA) (Sigma-Aldrich, UK), and blocked at 4°C over night.

For antibody staining 100μl of fixed bacterial suspension was centrifuged at 4000g for 5 minutes (113 centrifuge, Sigma-Aldrich, UK) and resuspended in 500μl buffer 2 (0.2μm filtered 1x PBS, 1% BSA, 0.05% Tween 20) (Sigma-Aldrich, UK), 1μl of each primary antibody* (1/500 dilution) was added and incubated at room temperature for 1 hour. Cells were washed once (buffer 2) and then resuspended in 500μl buffer 2. 1μl of each secondary antibody** was added and incubated for 1 hour in the dark at room temperature. Cells were washed three times in 500μl buffer 2 and then resuspended in 500μl buffer 1.

* Anti-Mouse RrgB polyclonal (Made in house, see section 2.18)

* Anti-Rabbit Type Serum 4 (Statens Serum Institute, Denmark)

** Goat Anti-Rabbit IgG (H&L chain specific) Allophycocyanin (APC) conjugate (Southern Biotech, USA).

** Goat Anti-Mouse IgG (γ chain specific) Fluorescein (FITC) Conjugate (Southern Biotech, USA).

2.13.2 Running fluorescence activated cell sorting

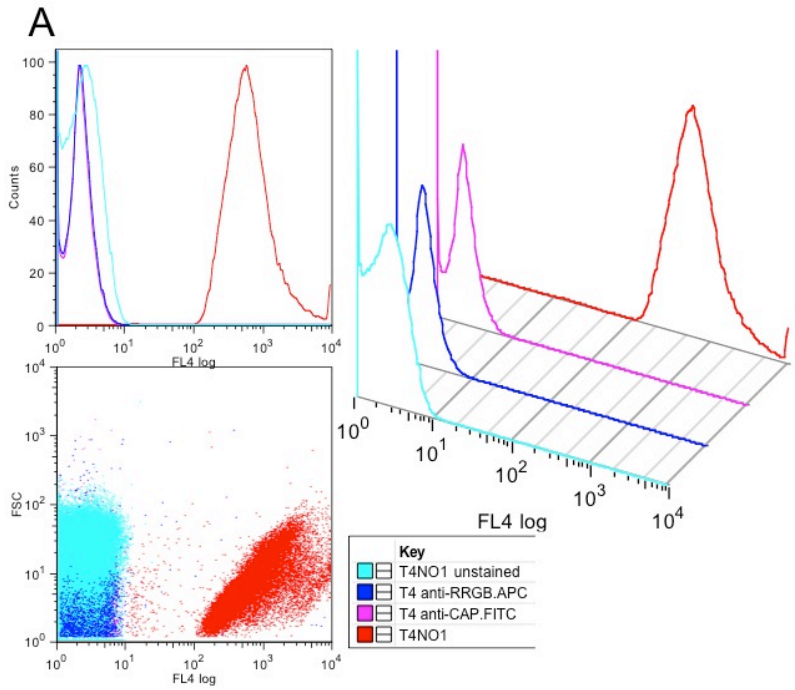
Samples were diluted 1:1 in buffer 1 and run on a FACScalibur flow cytometer (BD biosciences, USA), samples were acquired in CellQuest-Pro software (BD biosciences, USA). FACS settings were as follows: the primary parameter was set as the forwards scatter (FSC), voltage set at E00 on a log scale, side scatter (SSC) voltage was set at 587 on a linear scale, FL1 voltage was set at 624 on a log scale and FL4 voltage was set at 682 on a log scale. The threshold was set at 0 and no compensation was required. FACS analysis was performed in FlowJo 9.4.10 for Macintosh (Tree Star, USA). Cells were gated initially on being capsule positive (APC+) and then this population gated on selecting RrgB positive (FITC+) and RrgB negative populations. Percentage RrgB positive and negative

populations were compared between parent and mutant strains. Cell populations throughout this study refer to these gates.

2.13.3 Fluorescence activated cell sorting controls

FACS controls were performed to confirm no cross reactivity between the two antibodies. This was performed by using the Anti-rabbit capsule antibody with the Anti-mouse FITC secondary and the Anti-mouse RrgB antibody with the Anti-rabbit APC secondary. This was also confirmed by staining a T4NO1 bacterial population with the single antibody pairs alone and confirmed they gave the same percentage positive and negative populations. FACS analysis was also confirmed in all cases by visually assessing the samples using fluorescence microscopy.

Based on the unstained T4NO1 population data the values for the capsule positive (FL4) and RrgB positive (FL1) gates were set. For capsule positive cells anything above 10^1 log FL4 were classed as capsule positive, during fluorescence microscopy no events were observed to be APC positive and not be a bacterial cell. For RrgB positive cells anything above 12^1 log FL1 were classed as RrgB positive and anything below this negative for RrgB. All values referring to different cell population throughout this study refer to these gates.



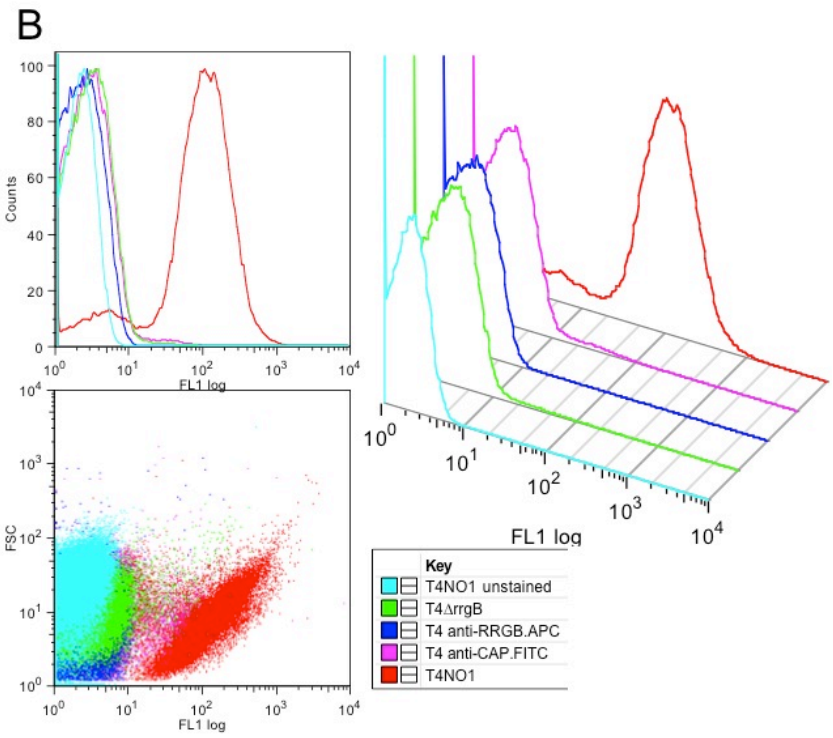


Figure 2-12: FACS controls swapping the secondary antibodies.

FACS was performed on T4NO1 and T4Δ*rrgB*. T4NO1 was run as an unstained sample, stained with a anti-mouse RrgB/ anti-rabbit APC, stained with a anti-rabbit capsule/ anti-mouse FITC or stained with a anti-mouse RrgB/ anti-mouse FITC and anti-rabbit capsule/ anti-rabbit APC. T4Δ*rrgB* was stained with anti-mouse RrgB/ anti-mouse FITC and anti-rabbit capsule/ anti-rabbit APC antibody combinations. (A) Shows histograms of the capsule positive population of T4NO1 and the negative controls showing no APC positive cells. Histograms/ polychromatic plot show negative (left) and positive (right) capsule populations. (B) Shows the capsule stained populations being gated on for being RrgB positive in T4NO1, and the negative controls being negative for FITC (RrgB). Histograms/ polychromatic plot show negative (left) and positive (right) RrgB populations in each strain.

A

Strain	FL4- (%)	FL4+ (%)
T4NO1	0	100
T4 anti-CAP FITC	100	0.01
T4 anti-RRGB APC	99.9	0.07
T4NO1 unstained	99.9	0.1

B

Strain	FL1- (%)	FL1+ (%)
T4NO1	12.1	87.9
T4 anti-CAP FITC	98	2
T4 anti-RRGB APC	100	0.04
T4Δ*rrgB*	98.4	1.6
T4NO1 unstained	100	0.006

Table 2-19: Percentage APC and FITC positive cells in FACS control samples.

(A) Shows the percentage APC (FL4) positive and negative cells in T4NO1 samples unstained or stained with different antibody combinations. (B) Shows the percentage FITC (FL1) positive and negative cells in T4NO1 samples unstained or stained with different antibody combinations and T4Δ*rrgB*. FACS analysis was performed in FlowJo.

From the control FACS data the secondary antibody was swapped for one that should not bind to the primary antibody (Figure 2-12). This confirmed in most instances that both the antibodies were unable to cross react with another antibody or cellular components (Table 2-19). Based on the gates however there may be a small amount of cross reactivity between the capsule antibody and the FITC antibody with the cells stained with this antibody combination showing 2% of events FITC positive. Alternatively the FITC antibody may bind weakly to some other cellular component irrespective of the capsule antibody binding. The pilus knockout strain T4Δ*rrgB* also showed 1.6% of cells positive for FITC when stained with both antibody pairs, however fluorescence microscopy confirmed T4Δ*rrgB* cells to be negative for FITC and therefore could potentially be very weak FITC positive cells undetectable by fluorescence microscopy. This finding must be kept in mind when looking at FACS data analysis.

FACS controls were also performed with the single antibody pairs to confirm that using the double antibody staining simultaneously didn't alter the results (Figure 2-13). The capsule stained population of T4NO1 from the single stained and double stained cells gave the same number of cells positive for the capule (Table 2-20). The RrgB populations in T4NO1 with the single and double stained populations were roughly 2% different, this can be seen from the histogram as an increase in the unstained RrgB population (left hand side) in the single stained T4NO1 sample and can likely be put down to the fact this is where the events would be that weren't cells, as with this sample there is no way of selecting the cells from the debris as APC staining was not performed and this is likely the cause of this difference (Table 2-20). Corroborated by the fact the T4NO1 double stained population showed roughly 2% of event were unstained for the capsule and were therefore not cells.

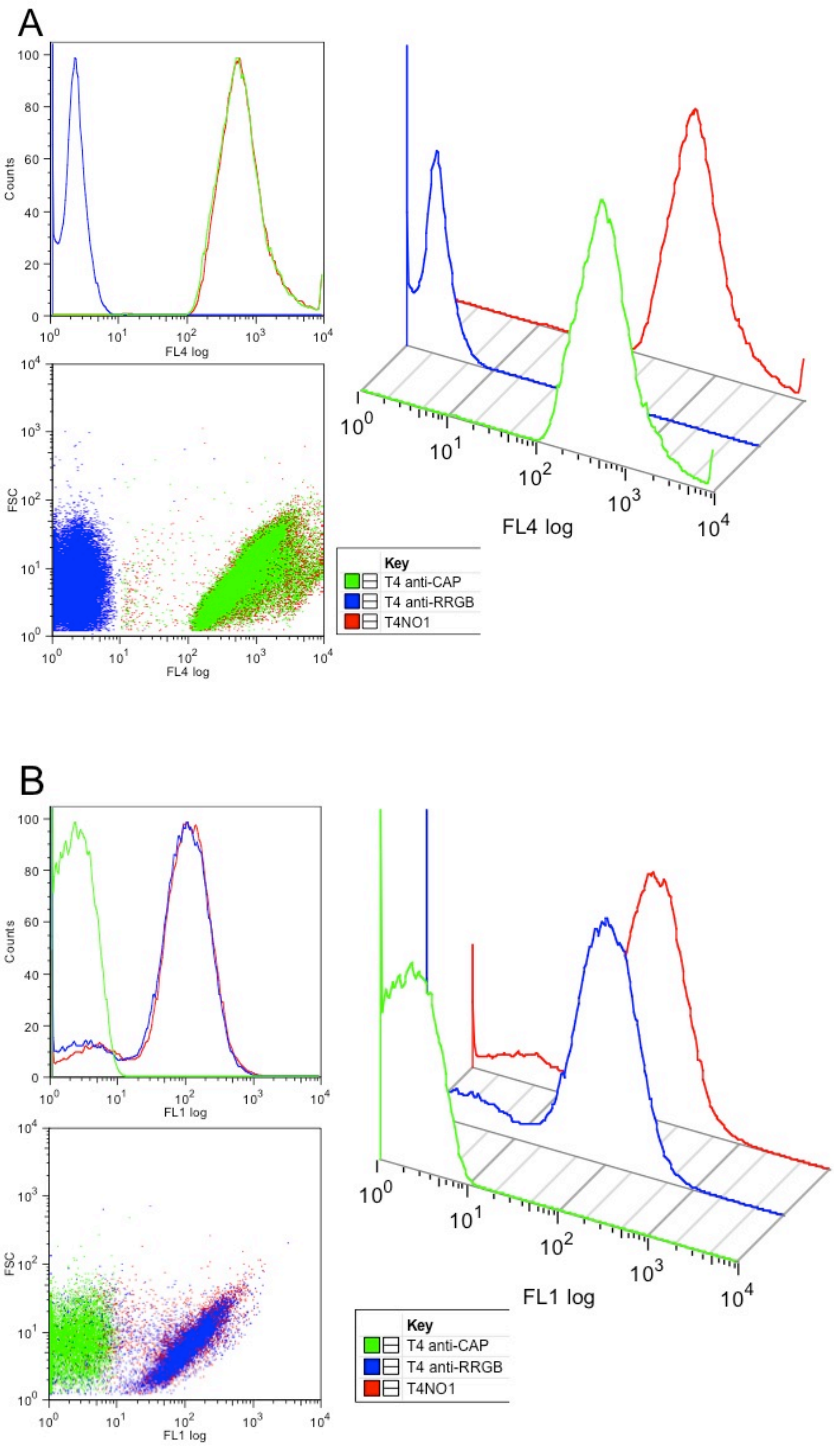


Figure 2-13: FACS controls staining T4NO1 with single antibody pairs

FACS was performed on T4NO1. T4NO1 was run as a sample stained with the two antibody pairs anti-mouse RrgB/ anti-mouse FITC and anti-rabbit capsule/ anti-rabbit APC or with each antibody pair singly. (A) Shows histograms of the capsule positive (APC) population of T4NO1 with the double and single antibody staining and the single RrgB stained population negative for APC positive cells. Histograms/ polychromatic plot show negative (left) and positive (right) capsule populations. (B) Shows the capsule stained populations being gated on for being RrgB positive in T4NO1, and the single RrgB and capsule antibody stained T4NO1 sample gated on also. Histograms/ polychromatic plot show negative (left) and positive (right) RrgB populations in each strain.

A

Strain	FL4+ (%)	FL4- (%)
T4NO1	98.3	1.7
T4 anti-CAP	98	2

B

Strain	FL1- (%)	FL1+ (%)
T4NO1	12.1	87.9
T4 anti-RrgB	14.9	85.1

Table 2-20: Percentage APC and FITC positive cells in FACS single antibody pair control samples.

(A) Shows the percentage APC (FL4) positive and negative cells in T4NO1 samples stained with both or a single antibody pair. (B) Shows the percentage FITC (FL1) positive and negative cells in T4NO1 samples stained with both or a single antibody pair. FACS analysis was performed in FlowJo.

2.13.4 Fluorescence microscopy

10μl of antibody stained bacteria preparation was pippetted onto a 76x22mm glass slide (Menzel Gläser, Germany) and a 22x22mm glass cover slip (Menzel Gläser, Germany) placed over the sample. Samples were heated for 60 minutes at 50°C to fix the sample. All fluorescence microscopy images were taken at X40 and X100 magnification using a Zeiss AxioscopeM1 fluorescence microscope with the correct filters. Images were analysed in Volocity software 6.0.1 (Perkin Elmer). Images were arranged in Adobe Photoshop elements 10.

2.14 Measuring bioluminescence over time.

To follow bioluminescence over time, $1x10^6$ CFU of *S.pneumoniae* were added in 20μl volumes to a black F96 MicroWell™ plate (Nunc, Fisher Scientific, UK) containing 180μl BHI, each strain was tested in triplicate. Luminescence was measured on a FLUOstar OPTIMA (BMG) taking readings every 20 to 30 minutes for roughly 10 hours. Graphical presentation was performed in Prism version 4.0b (GraphPad Software), each data point representing the mean of the triplicate luminescence reading.

2.15 Preparation of T4Δ*spxB* samples with addition of hydrogen peroxide

The volume of culture grown will vary depending on the downstream technique being used e.g. FACS/ western blotting. Cultures of T4Δ*spxB* were grown in BHI to OD_{600nm} 0.3 and then 10μl/ml of Hydrogen peroxide 30% w/v AnalaR NORMAPUR® (VWR, UK) added from 1/10 (contains 9mM), 1/25 (contains 3.6mM) and 1/50 (contains 1.8mM) dilutions. Higher concentrations were used at roughly 20mM but all T4Δ*spxB* bacteria were killed by this concentration. Samples were grown to OD_{600nm} 0.6 and processed according to the technique being used.

2.16 Hydrogen peroxide assay

Hydrogen peroxide assay was performed on some strains to look at their ability to produce hydrogen peroxide. *S.pneumoniae* cultures were grown to OD_{600nm} 0.6 in BHI, 180μ of culture were added in triplicate to a 96 well flat bottom plate. To this 20μl of 3mg/ml ABTS (2,2 azinobis (3-ethylbenzthiazolinesulfonic acid) diammonium salt, Sigma-Aldrich, UK), 0.2mg/ml HRP (Peroxidase from horseradish Type II, Sigma-Aldrich, UK) in a 0.1M sodium phosphate buffer pH7.0 was added. The assay was left to develop for 30 minutes at 37°C followed by centrifugation at 1500g for 3 minutes (4K15 centrifuge). 100μl from each well was removed and placed into a fresh well. Absorbance was measured using a spectrophotometer at 540nm (FLUOstar OPTIMA, BMG). Hydrogen peroxide standards were used as a positive control by making serial dilutions of Hydrogen peroxide 30% w/v AnalaR NORMAPUR® (VWR, UK) in BHI. From this a standard curve was generated in Microsoft Excel plotting known hydrogen peroxide concentrations against the absorbance (Figure 2-14). From this the concentration of Hydrogen peroxide produced from pneumococcal strains was obtained. Graphical presentation of hydrogen peroxide assay data was performed in Prism version 4.0b (GraphPad Software). Data presented was the hydrogen peroxide (mM) produced from each strain tested, obtained from averaging a triplicate absorbance value and from this value using the standard curve below to give the amount of H_2O_2 produced in mM. No statistical analysis was performed.

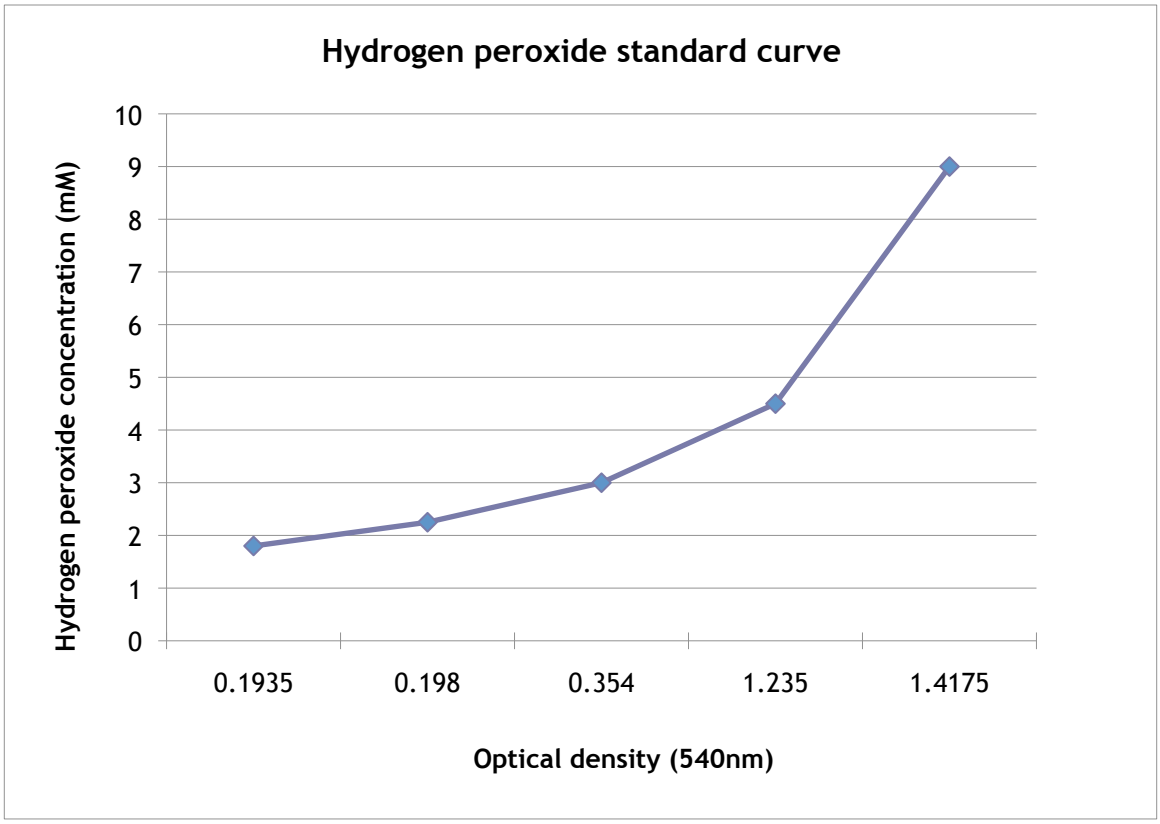


Figure 2-14: Hydrogen peroxide assay standard curve

Standard curve of known hydrogen peroxide concentrations and absorbance values obtained when usied in a hydrogen peroxide assay. Graph used throughout the thesis to calculate hydrogen peroxide concentration produced by pneumococcal strains.

2.17 *In Vivo* experiments

All animal experiments were performed in 6-8 week old MF1 outbred mice (Harlan, UK).

2.17.1 Animal passage

Prior to challenge *S,pneumoniae* strains were intraperitoneally passaged through MF1 outbred mice (Harlan, UK). To ensure the correct dosing glycerol stocks were viable counted giving the CFU/ml (materials and methods 2.1.1). From the CFU/ml a calculation was performed (Equation 2-2) giving the volume containing $2.5x10^6$ CFU/dose ($1x10^7$ CFU/ml). The dosage volume is 200μl/ mouse, the amount of inoculum prepared will vary depending on the number of mice infecting.

$0.5^{*} \times 1x10^{7**} = N$

N divide by CFU/ml of glycerol stock = dose in ml***

Equation 2-2: Equation used to calculate the volume required from glycerol stocks for intraperitoneal passage of *S.pneumoniae* strains through MF1 mice.

***0.5 is the amount in ml of inoculum you wish to prepare (in this case 0.5ml).**

****1x10^7 as stated above is the concentration /ml you wish the inoculum to be.**

*****Dose in ml containing the number of bacteria required to give 1x10^7 CFU/ml in 0.5ml, the volume is made up to the required volume (0.5ml) with 1x PBS.**

2.17.1.1 Standard inoculum preparation

Glycerol stocks were thawed and centrifuged at 13,000g for 3 minutes (113 centrifuge, Sigma-Aldrich, UK) to pellet the bacteria, supernatant removed and the pellet resuspended in an equal volume of sterile PBS. From this the calculated volume of glycerol stock required was removed into a new sterile eppendorf and made to the correct volume with PBS.

2.17.1.2 Challenge

MF1 mice were infected via the I.P route with 2.5x10^6 CFU in 200µl, at 6 hours post infection mice were culled via terminal exsanguination under general anesthetic and death confirmed via cervical dislocation. Blood obtained was placed into 20ml BHI and incubated overnight at 37°C. 1ml of overnight culture was then place in 50ml BHI plus 20% FBS (Fetal bovine serum, Life Technologies, UK) and grown at 37°C to OD_{600nm} 0.6, 1ml aliquots were made and stored at -80°C.

2.17.1.3 Viable counting of *S.pneumoniae* frozen standard inoculum

For counting of bacterial SI (standard inoculum) at least 24 hours post freezing three x 1ml vials were thawed per strain at 37°C for 3 minutes. SI were centrifuged at 13,000g for 3mins (113 centrifuge, Sigma-Aldrich, UK), supernatant removed and the pellet resuspended in 1ml PBS. Each vial was then viable counted (materials and methods 2.1.1), and from the three vials an

average CFU/ml was calculated. This value was used to calculate the dosage for challenge experiments.

2.17.2 In vivo challenge intranasal (I.N)

For intranasal challenge mice were infected with $5x10^6$ CFU in 50µl, 25µl given into each nostril. To calculate the dosage **Error! Reference source not found.** was used as for the IP passage, 1.5ml of inoculum was made per strain as 20 mice were infected per strain.

2.17.2.1 Preparation of inoculum

A vial of SI per strain was thawed at 37°C for 3 minutes. SI was centrifuged at 13,000g for 3mins (113 centrifuge, Sigma-Aldrich, UK), supernatant removed and the pellet resuspended in 1ml PBS. The volume required containing the correct number of bacteria as calculated from **Error! Reference source not found.** was moved to a new sterile 5ml bijoux. This volume was then made up to 1.5ml with PBS giving a final concentration of $1x10^8$ CFU/ml. Prior to inoculation the inoculum was viable counted (materials and methods 2.1.1) to confirm the correct dosing this is also performed post animal inoculation to account for any death/growth of the bacteria in this period of time.

2.17.2.2 T4 vs. Xen35 vs. T4P2

20 MF1 mice were infected I.N per strain (T4NO1/ Xen35/ T4P2). Mice were split into four groups of 5, 24 hour group, 48 hour group, 72 hour group and a survival group. 24 hours post inoculation the 24 hour group mice were sacrificed and lungs, brain, nasal wash and blood removed for bacterial enumeration. The same was done at 48 hours for the 48 hour group and at 72 hours for the 72 hour group. If mice became sick before this period of time they were culled and time of death noted. The survival group was left until the mice became sick or culled at the end of the experiment 100 hours post inoculation.

For enumeration of bacterial counts from blood and nasal wash viable counting was performed as previously described, for blood dilutions of 10^{-1} to 10^{-6} were created and plated onto BAB, as described in section 2.1.1. For nasal wash

dilutions of 10^{-1} to 10^{-5} were created and plated onto BAB along with the neat wash, CFU/ml for both was calculated using Equation 2-1.

Enumeration of bacterial counts from organs varied slightly. Upon removal from mice the lungs and brain were placed into 3ml PBS. The weight of the PBS was measured prior to and post addition of the organ to obtain the exact weight of the organ, allowing us to work out the CFU/ gram of tissue. The organs in PBS were homogenized followed by viable counting of the homogenate, dilutions 10^{-1} to 10^{-5} were created and plated onto BAB along with the neat homogenate. The CFU/ml was calculated for each organ using Equation 2-1 and then this used to calculated the CFU/gram of tissue as seen in Equation 2-3.

CFU/ml x 3* = T (total CFU/organ)

T x O** = CFU/gram of tissue

Equation 2-3: Equation used for calculating the bacterial burden in mouse organs from animal challenge experiments.

***3 indicated volume of PBS organ placed in.**

****** **O indicated organ weight, calculated from subtracting the PBS weight from that of the organ and PBS together.**

2.17.2.3 Analysis

Graphical presentation and statistical analysis was performed in Prism version 4.0b (GraphPad Software). CFU/ml data for nasal wash and blood were used to compare colonization and bacteremia between different strains. CFU/gram of tissue was used for lungs and brain to compare the organ bacterial load between strains. Comparisons were made at time points 24/ 48 and 72 hours. Statistical analysis was performed using a non-parametric Mann-Whitney two sample rank test, significance $P<0.05$. This statistical test was also used for comarison of the percentage weight loss of the mice over time. Survival of mice infected with different strains was compared using a Kaplain meier survival curve and analysed using a Log-rank Test ($P<0.05$).

T4NO1 and Xen35 animal inoculations were performed by Mr Ryan Ritchie and Dr Kirsty ross. Processing was performed by Mr Ryan Ritchie, Dr Kirsty Ross, Dr Carol McInally and author. For T4P2 all inoculations and processing was performed by author.

2.17.3 IVIS imaging

Mice infected with Xen35 or T4P2 were imaged for 5 minutes while under general anesthetic using the IVIS spectrum CCD camera (Xenogen corporation, USA). All images were displayed using the same settings in Living image 4.0 software (Xenogen corporation, USA), to allow comparison between images and strains. Images allowed visualization of progression of pneumococcal disease *in vivo*.

Help was supplied throughout by Mr Ryan Ritchie for animal imaging.

2.18 RrgB antibody production

2.18.1 Cloning rrgB into pET-33b

A partial fragment of the *rrgB* gene encoding amino acid 40-600 (to remove cross reactivity with other pilins) was cloned into pET-33b at restriction sites BamHI and HindIII. pET-33b was initially isolated using the QIAprep miniprep kit (QIAGEN), digested with the stated restriction enzymes for 3 hours at 37°C and then purified using the Wizard® SV gel and PCR clean up system (Promega). *rrgB* was amplified using primers in Table 2-21, with the primers incorporating the required restriction sites at the end of the PCR product, the PCR product was purified using the Wizard® SV gel and PCR clean up system (Promega) followed by restriction digest with the required enzymes as above, followed by a further purification and ligation into plasmid (materials and methods 2.2.7).

Transformation was performed as described into XL1-Blue cells and sample plated onto LB agar containing 100μg/ml kanamycin. Positive colonies were screened for by PCR using T7 and RrgBR-Ab primers and confirmed by sequencing (Table 2-21).

pET-33bRrgB plasmid was then purified using the QIAprep miniprep kit (QIAGEN) and transformed into BL21(DE3) chemically competent cells, and transformants screened for as above. This strain was used for protein expression.

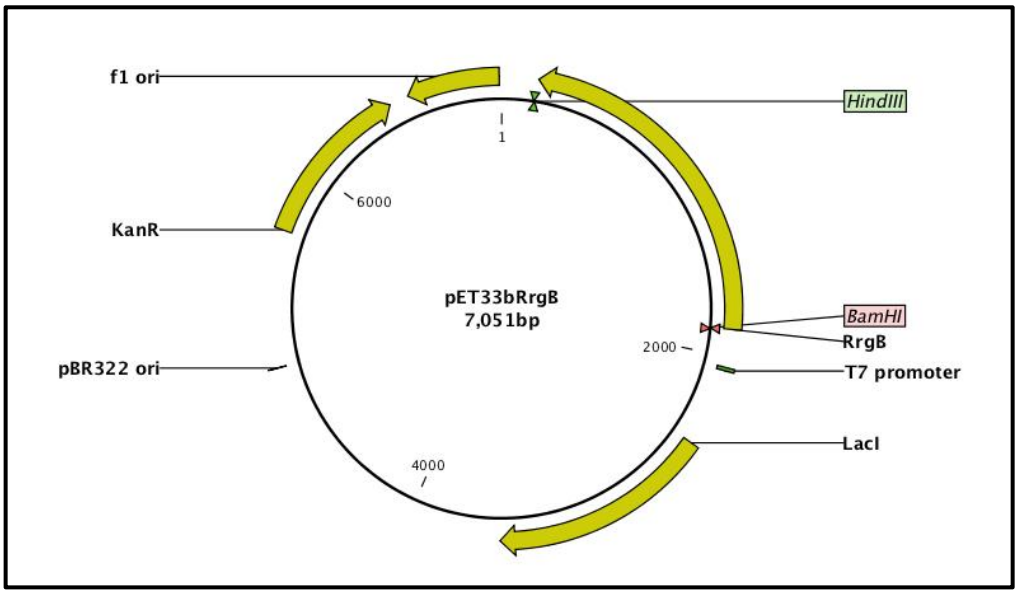


Figure 2-15: Plasmid map of pET-33bRrgB.

Plasmid map of pET-33bRrgB from CLC Genomics Workbench 4.5.1. Constructed via cloning part of *rrgB* encoding amino acid 40-600 at restriction sites BamHI and HindIII using primers in Table 2-21.

Gene	Gene number	Primer name	DNA sequence 5'-3'	Size (bp)
rrgB	SP_0463	RrgBF	AATGGGTCGGGATCCGCATAAACTATTGGCAACAGAT	1731
		RrgBR-Ab	AGTGCGGCCGCAAGCTTTTAATAAGAAGTTGCAGTGACTTC	
		Pet T7	TAATACGACTCACTATAGGG	

Table 2-21: Primers used for cloning of rrgB into pET33b.

Primers and related information used for cloning and confirmation of insertion of rrgB into pET33b for protein expression and purification. Dark Blue nucleotides indicate region of the primer specific to the gene being amplified. Red indicates the stop codon incorporated at the end of the *rrgB* gene sequence. Light blue and green represent BamHI and HindIII restriction site respectively.

2.18.2 RrgB protein purification

2.18.2.1 Expression

BL21(DE3) pET33bRrgB was grown in 10ml LB overnight containing 100μg/ml kanamycin. The starting culture in the morning was inoculated into 1 liter of LB

containing 100μg/ml kanamycin and cells were grown at 37°C, 220rpm until OD_{600nm} 0.8 was reached, IPTG (Melford, UK) was added to a final concentration of 1mM and cells grown at 30°C, 220rpm for a further 4 hours. Culture was then centrifuged at 4000g for 40 minutes (Beckman® J-6B centrifuge) supernatant decanted and pellets stored at -20°C until purification.

2.18.2.2 Purification

Cell pellet was resuspended in 50ml PBS, Benzamidine and DNase 1 added (Sigma-Aldrich, UK) and sonicated on ice with rotation for 4x 30 seconds with 1 minute in between each at 84% amplitude. Samples were further centrifuged for 45 minutes at 4°C at 17,000g (4K15 centrifuge), pellet discarded and supernatant filtered using a 0.2μm filter and placed in a fresh falcon tube.

Due to the addition of 6 Histidines at the C-terminal of RrgB in pET33b the Initial purification step consisted of Nickel affinity chromatography. A 1ml HisTrap HP (GE Healthcare, Fisher Scientific, UK) nickel affinity column was charged by running through 5ml of 0.2M nickel solution (benchtop peristaltic pump, masterflex C/L). Sample was then looped through the column for 30 minutes using a benchtop peristaltic pump, leaving the protein bound to the column. To elute the protein off the column 15ml of elution buffer was run through the column using a peristaltic pump and fractions collected on ice manually. Elution buffer consisted of dilutions made of a 0.5M NaCl and 0.5M imidazole pH7.4 solution made with PBS, giving final solution concentrations of 0.03125M, 0.0625M, 0.125M, 0.175M, 0.25M and 0.5M. Buffers were run through the column as above starting with the lowest concentration. As the buffer concentration increases imidazole displaced the bound protein, all collected fractions were run on a SDS gel (Figure 2-16). Fractions 0.175M, 0.125M and 0.0625 were pooled and purified further by anion exchange chromatography. Samples were dialysed overnight at 4°C and buffer exchanged into in 20mM Tris-HCl pH7.4.

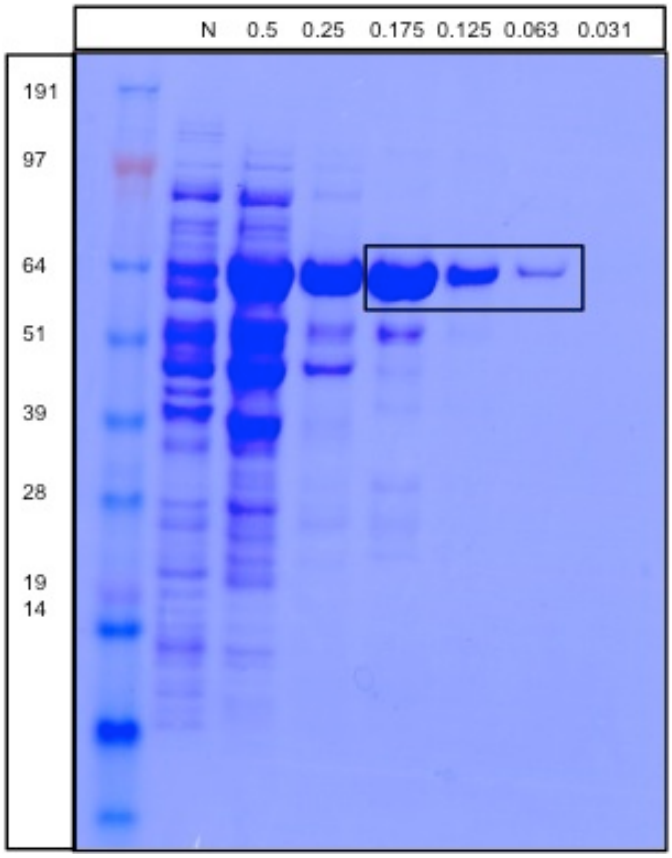


Figure 2-16: Coomassie stained gel of nickel affinity chromatography elution fractions during RrgB purification.

Boxed are the three fractions containing the 64kDa RrgB protein, three fractions were pooled and taken forward to anion exchange. Left is size in kDa of bands from the SeeBlue® Plus2 pre-stained ladder.

To begin anion exchange 20ml anion exchange starter buffer (20mM Tris-Hcl pH8.0) was run through a HiTraP™ Capto™Q column followed by the pooled dialysed protein fractions for 30 minutes to allow protein to bind. Column was transferred to the ÄKTAprimer plus (GE Healthcare, Fisher Scientific, UK) and an anion exchange protocol performed eluting protein in fractions using a gradient of anion exchange elution buffer ranging from 0- 1M NaCl (20mM Tris-HCl pH8.0). Following anion exchange fractions were run on a SDS gel (Figure 2-17) and fractions 14-19 pooled, dialysed overnight at 4°C in PBS. Purified protein was used for antibody production (2.18.3).

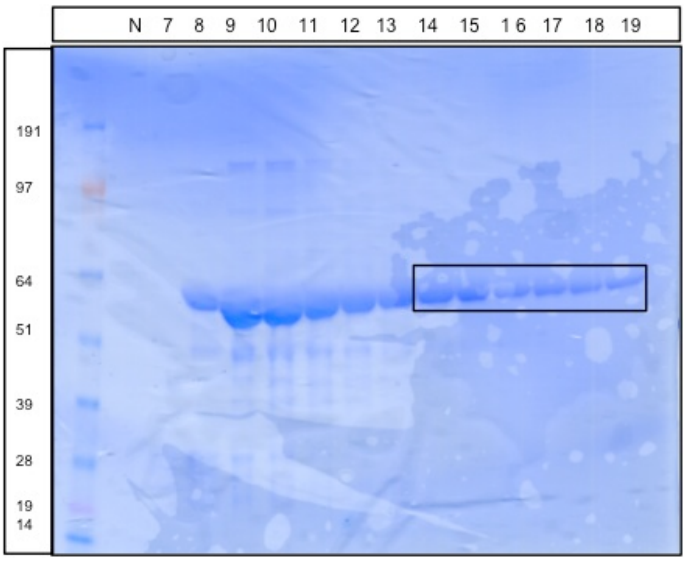


Figure 2-17: Coomassie stained gel of anion exchange chromatography elution fractions during RrgB purification.

Boxed are the six fractions containing the 64kDa RrgB protein, these fractions were pooled and used for later experiment. Left is size in kDa of bands from the SeeBlue® Plus2 pre-stained ladder.

2.18.3 Antibody production

Anti-RrgB antibody was made via vaccinating five MF1 mice with purified RrgB protein. Mice were vaccinated subcutaneously with 10μg of purified RrgB protein emulsified in 50μl of Freunds adjuvant incomplete (Sigma-Aldrich, UK) made to 100μl with PBS, on three occasions, with two week intervals between each vaccination. Two weeks after the final vaccination mice were culled via terminal exsanguination under general anesthetic and death confirmed by cervical dislocation. The blood was transferred to sterile 1.5ml eppendorfs and stored at 4°C overnight for the blood to clot and then spun at 13,000g for 10 minutes at 4°C (1K15 centrifuge, Sigma-Aldrich, UK), the clear sera was removed into fresh 1.5ml eppendorfs and pooled from all 5 mice. The antibody specificity for RrgB was tested by western blotting and subsequently used for all FACS experiments.

Vaccination and bleeds were performed by Dr Kirsty Ross.

3 Virulence gene regulation by two-component signal transduction systems

3.1 Aims of this chapter

In *S.pneumoniae* there are 13 TCS and one orphan response regulator (ORR) (Lange et al., 1999), described in chapter 1.7.1.1. The aim of the work described in this chapter is to assess virulence gene regulation by TCS (two-component signal transduction systems), determined via microarray analysis of single TCS mutants. As well as looking at virulence factors regulated by these systems in these single knockouts the expression levels of all the other RR of TCS pairs were evaluated elucidating whether there is potential cross regulation between the systems and whether a hierarchy exists. Initial experiments were performed on a *rr08* and *rr09* knockout constructed in TIGR4 assessing the global expression changes seen upon their deletion. Further analysis was performed looking at what effects removal of both systems has to global expression changes.

Interestingly deletion of six TCS (TCS03, TCS05, TCS06, TCS08, TCS09 and TCS10) in the pneumococcus have shown to cause changes in the expression of the pneumococcal pilus. To further assess the potential interactions between TCS a knockout was constructed in *rr06*, and from this construction of a number of double mutants (T4Δ*rr086* and T4Δ*rr096*) and a triple mutant (T4Δ*rr0986*) performed. As all single mutants have been shown to regulate the pilus, in these mutants the expression levels of the whole pilus islet was evaluated by RT-PCR, to illuminate further any potential cross regulation between the systems. FACS analysis was also performed on all strains to assess potential changes in expression of the pilus at the population level. Due to time limitations no mutants were constructed in TCS10, TCS05 and TCS03. Adherence capabilities of these TCS mutants were also assessed to different cell lines to try and pinpoint where these TCS may be important *in vivo,* during pneumonia, meningitis or colonisation.

Studies performed on TCS are often performed in different parental strains and under different laboratory conditions, which can make it hard to compare between their functions reported in the literature. TCS have been shown to regulate different genes in different strains and under different growth conditions, therefore this study enables clear comparisons to be made with

regards to what genes are commonly regulated by RR09 and RR08 under the same conditions and how these systems may interact with one another.

3.2 Genes regulated by RR08

Initial analysis was performed on T4Δ*rr08* to assess gene regulation by this TCS and its regulation of other transcription regulators including other TCS. A total of 57 genes were differentially regulated in T4Δ*rr08* compared to T4JH, (Table 3-1). When increasing the P value to P<0.1 a total of 131 genes were differentially regulated (data not shown).

Gene	Description	Abbrev	Fold change
SP_0043	Competence factor transport protein	*comB*	1.8
SP_0044	Phosphoribosylaminoimidazole-succinocarboxamide	*purM*	15.5
SP_0048	Glycinamide ribonucleotide transformylase	*purN*	17.0
SP_0049	vanZ protein	*vanZ*	15.6
SP_0050	phosphoribosylamine	*purH*	14.5
SP_0051	Hypothetical protein		14.0
SP_0053	Phosphoribosylaminoimidazole carboxylase	*purE*	14.4
SP_0055	Hypothetical protein		4.3
SP_0271	Ribosomal protein S12	*rpsL*	1.2
SP_0288	Xanthine/ uracil permease protein		6.9
SP_0409	Hypothetical protein		4.6
SP_0461	Transcriptional regulator	*rlrA*	2.1
SP_0462	Cell surface anchor family protein	*rrgA*	1.9
SP_0626	Branched chain amino acid transport system		1.4
SP_0703	Hypothetical protein		2.1
SP_0785	Hypothetical protein		2.1
SP_0786	ABC transporter		2.0
SP_0802	ATP dependant DNA helicase		1.1
SP_0820	ATP dependant Clp protease	*clpE*	1.2
SP_0825	5,10-methylene tetrahydrofolate dehydrogenase	*folD*	1.1
SP_0918	Spermidine synthase	*speE*	1.4
SP_1225	VicX protein	*vicX*	1.7
SP_1226	Histidine kinase 02	*hk02*	1.7
SP_1227	Response regulator 02	*rr02*	1.6
SP_1228	A/G specific adenine glycosylase		2.6
SP_1269	Choline kinase		1.3
SP_1357	ABC transporter		1.4
SP_1368	PSR protein	*psr*	1.8
SP_1369	Prephenate dehydratase	*pheA*	2.0
SP_1370	Shikimate kinase	*aroK*	1.9
SP_1371	3-phosphoshikimate 1- carboxyvinyltransferase	*aroA*	1.8
SP_1501	ABC transporter		1.5
SP_1527	ABC transporter	*aliB*	3.3
SP_1552	Cation efflux pump	*mntE*	1.2
SP_1587	Oxalate: formate antiporter		6.6
SP_1626	Ribosomal protein S15	*rspO*	1.5
SP_1697	ATP dependant DNA helicase	*recG*	1.4
SP_1699	Phosphopantetheinyl transferase	*acpS*	1.4
SP_1701	Phospho-2-dehydro-3-deoxyheptonate aldolase	*aroG*	1.5
SP_1709	GTP binding protein	*engA*	1.3
SP_1802	Hypothetical protein		2.3
SP_1804	General stress protein 24		2.4
SP_1814	Tryptophan biosynthesis	*trpC*	2.1
SP_1862	Hypothetical protein		1.5
SP_1871	Iron ABC transporter	*fecE*	3.6

SP_1978	Diaminopimelate decarboxylase	*lysA*	1.7
SP_1986	Hypothetical protein		1.8
SP_1987	ABC transporter, ATP binding protein		1.7
SP_1988	Immunity protein, putative		1.9
SP_2072	Glutamate amidotransferase	*guaA*	4.1
SP_2077	Transcriptional repressor, putative	*argR*	1.2
SP_2078	Aminoacyl-tRNA synthetase	*argS*	1.4
SP_2095	5-formyltetrahydrofolate cyclo-ligase family		1.4
SP_2105	Hypothetical protein		1.9
SP_2167	L-fuculose kinase	*fucK*	5.3
SP_2194	ATP dependant Clp protease		1.4
SP_2201	Choline binding protein D	*cpbD*	1.9

Table 3-1 : Table of genes differentially regulated in T4Δ*rr08*

Table shows genes differentially regulated in T4Δ*rr08* compared to its parent T4JH (P<0.05). Fold change represents that seen in T4Δ*rr08* compared to T4JH, red showing genes up regulated in T4Δ*rr08* and blue are those that are down regulated.

Genes that are regulated by RR08 include a number of the genes present on the pilus islet, which are up regulated in T4Δ*rr08* including the *rlrA* regulator. A number of genes involved in purine biosynthesis (SP_0044/ SP_0048/ SP_0050/ SP_0053) and the virulence factor *cbpD* are also up regulated upon RR08 deletion. The transcriptional repressor *argR* is down regulated in T4Δ*rr08*, which may be responsible for some of the genes that are differentially regulated in T4Δ*rr08*, discussed later. A number of ABC transporter including one for iron, a PTS system, and a manganese efflux pump are also all differentially regulated in the knockout. Interestingly both *rr02* and *hk02* are up regulated in T4Δ*rr08,* which suggest RR08 is a repressor of this TCS. Alternatively TCS02 expression could be modulated indirectly by RR08 as deletion of *rr08* could cause a stress on the cell which induces expression of this gene.

RR02 is part of TCS02 which is also referred to as *vicRK*, *micAB*, *yycFG* and *walRK*, this system was shown to be differentially regulated by microarray analysis of T4Δ*rr08*. So RT-PCR analysis was performed to confirm this change and the expression levels of all the other RR were also evaluated (Figure 3-1), as subtle changes may not have been identified by microarray analysis. RT-PCR analysis confirmed there is a trend towards an increase in expression of *rr02* in T4Δ*rr08* however this was not statistically significant, perhaps due to the small change, and would need further validation. Interestingly a statistically significant down regulation of *rr*01 was observed by RT-PCR, which was not observed during microarray analysis suggesting RR08 positively regulates this

TCS. Although some of the other RR including *rr*07, *rr09* and *rr*12 look to show a small down regulation these were not statistically significant. With regards to the other genes shown to regulate the pilus there seems to be no expression change in the TCS known to regulate the pilus (*rr03*, *rr05*, *rr06*, *rr10*). RT-PCR analysis and microarray analysis also did not show any other genes shown to regulate the pilus to be differentially regulated by RR08, other than that of *mntE*. However MntE is unlikely to modulate pilus expression through its direct binding to the islet, so RR08 may act through direct binding to the pilus islet. A summary of the major genes regulated by RR08 can be seen in Figure 3-2, including virulence factors, transcription regulators and groups of genes showing a big expression difference in T4Δ*rr08*.

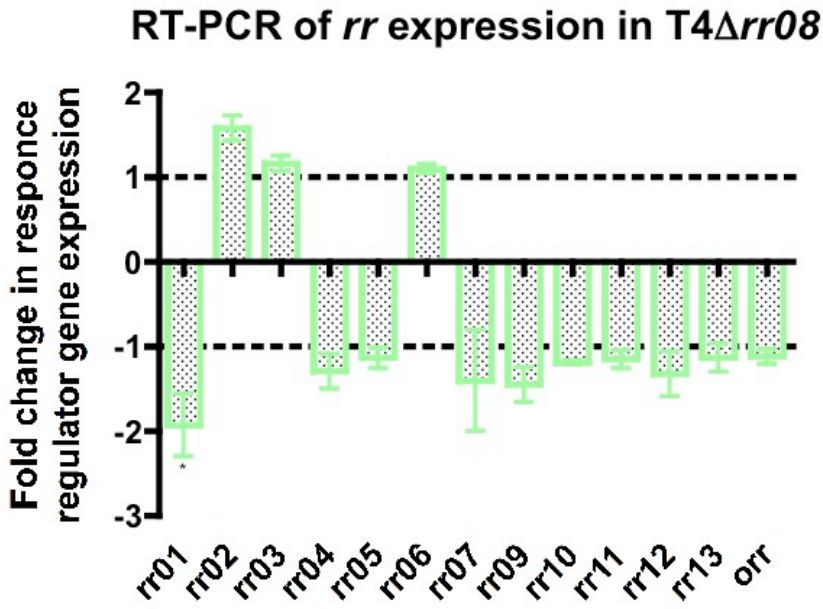


Figure 3-1: RT-PCR graph of response regulator expression in T4Δ*rr08.*

Graph shows RT-PCR of all the pneumococcal response regulators in T4Δ*rr08.* Fold change represents that T4Δ*rr08* compared to T4JH. Each bar represents the average of three replicas (three biological replicas) and errors bars the standard deviation. Statistical analysis was performed by a 1-way ANOVA with a Tukeys testing correction comparing the dCt values of the control strain (TIGR4) to the test strain (T4Δ*rr08*), * P<0.05.

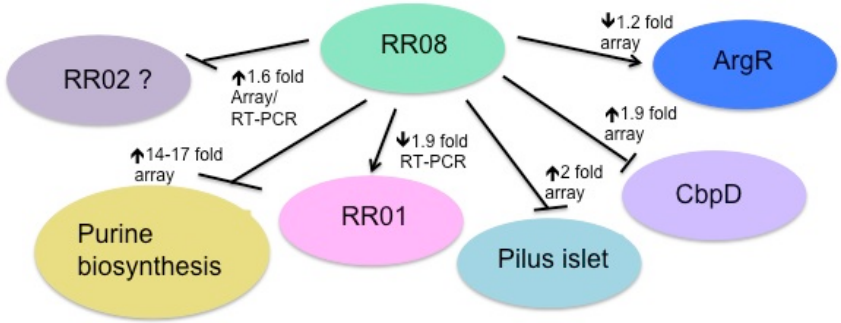


Figure 3-2: Summary of genes regulated by RR08

Summary of genes regulated by RR08 has been compiled from microarray and RT-PCR data. Arrow represents RR08 positively regulating the gene and a bar represent RR08 repression of the gene. Values indicate the fold change of the gene observed during microarray analysis or RT-PCR in T4Δ*rr08*.

3.3 Genes regulated by RR09

Further microarray analysis was performed on T4Δ*rr09*, which has also previously been shown to regulate the pneumococcal pilus. A total of 43 genes were shown to be differentially regulated between T4Δ*rr09* and its parent strain T4JH, shown in Table 3-2. When increasing the P value to P<0.1 a total of 87 genes were shown to be differentially regulated between the two (data not shown).

Gene	Description	Abbrev	Fold change
SP_0047	Phosphoribosylaminoimidazole synthetase	*purM*	11.8
SP_0048	Glycinamide ribonucleotide transformylase	*purN*	11.5
SP_0055	Hypothetical protein		2.2
SP_0117	Pneumococcal surface protein A	*pspA*	2.3
SP_0287	Xanthine/ uracil permease protein		4.2
SP_0290	Dihydrofolate synthetase	*folC*	2.4
SP_0409	Hypothetical protein		3.6
SP_0415	Enoyl-CoA hydratase	*phaB*	1.4
SP_0461	Transcriptional regulator	*rlrA*	4.6
SP_0463	Cell surface anchor family protein	*rrgB*	5.1
SP_0464	Cell surface anchor family protein	*rrgC*	6.4
SP_0648	Beta-galactosidase	*bgaA*	3.8
SP_0715	Lactate oxidase	*lctO*	2.2
SP_0862	Ribosomal protein S1	*rpsA*	1.2
SP_0868	Hypothetical protein		1.7
SP_0875	Lactose phosohotransferase system repressor	*lacR*	15.2
SP_0876	Phosphofruktokinase	*fruB*	14.0
SP_0877	PTS system, fructose specific	*fruA*	13.4
SP_1225	VicX protein	*vicX*	1.5
SP_1501	ABC transporter		1.3
SP_1578	Hypothetical protein		1.6
SP_1675	ROK family protein	*glk*	2.1
SP_1676	N-acetyl neuraminate lyase	*npl*	2.5
SP_1677	Hypothetical protein		2.6
SP_1682	ABC transporter		3.2

SP_1708	Hypothetical protein		3.0
SP_1724	Sucrose-6-phosphate hydrolase	*scrB*	12.7
SP_1801	Hypothetical protein		2.6
SP_1803	Hypothetical protein		2.7
SP_1804	General stress protein 24		2.8
SP_1853	Galactokinase	*galK*	6.1
SP_1884	Trehalosse PTS system	*treP*	3.5
SP_1895	Sugar ABC transporter	*msmG*	10.8
SP_1896	Sugar ABC transporter	*msmF*	10.5
SP_1897	Sugar ABC transporter	*msmE*	10.4
SP_1898	Alpha-galactosidase	*aga*	12.5
SP_1922	Hypothetical protein		2.0
SP_1924	Hypothetical protein		2.3
SP_1925	Hypothetical protein		2.3
SP_1967	Hypothetical protein		1.2
SP_2063	lysM domain containing protein		1.6
SP_2107	4-alpha-gluconotransferase	*malM*	1.7
SP_2150	Ornithine carbamoyltransferase	*argF*	3.8

Table 3-2 : Table of genes differentially regulated in T4Δ*rr09*

Table shows genes differentially regulated in T4Δ*rr09* compared to its parent T4JH (P<0.05). Fold change represents that seen in T4Δ*rr09* compared to T4JH, red showing genes up regulated in T4Δ*rr09* and blue are those that are down regulated.

No TCS were differentially regulated in a *rr*09 knockout strain as determined by microarray analysis. However RT-PCR was performed to assess the expression levels of all the other RR incase as seen in T4Δ*rr08* some were differentially regulated yet not detected by microarray analysis. RT-PCR analysis of *rr* expression in T4Δ*rr09* showed a potential down regulation of *rr*01, *rr*07 and *rr08* suggesting RR09 acts as a positive regulator of these TCS (Figure 3-3). However none were found to be statistically significant.

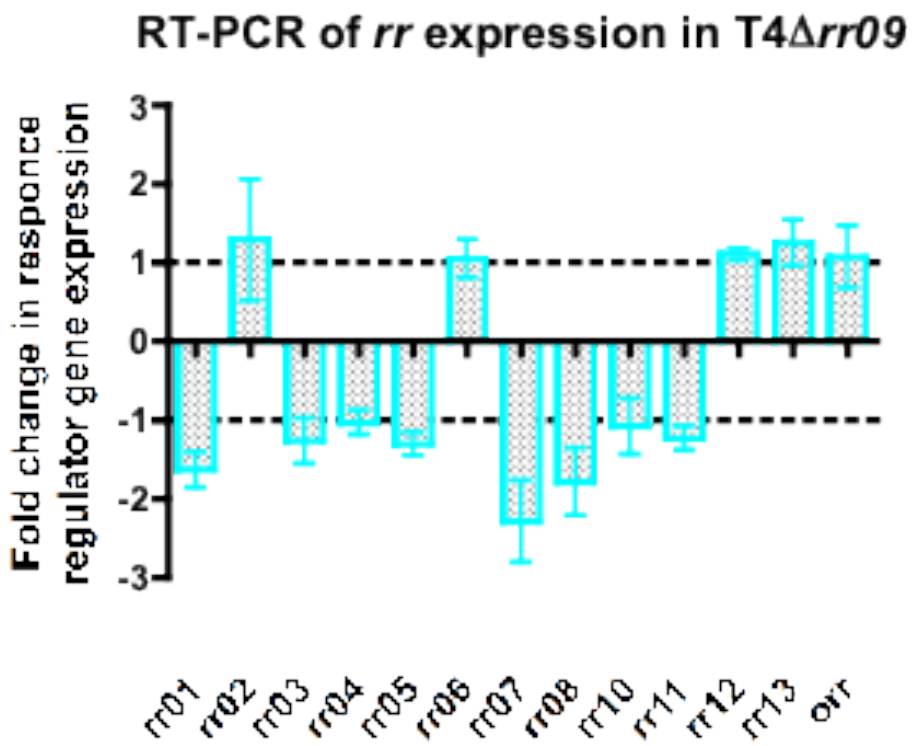


Figure 3-3: RT-PCR graph of response regulator expression in T4Δ*rr09*.

Graph shows RT-PCR of all the pneumococcal response regulators in T4Δ*rr09*. Fold change represents that T4Δ*rr09* compared to T4JH. Each bar represents the average of three replicas (three biological replicas) and errors bars the standard deviation. No statistical difference was observed.

A summary of the genes regulated by RR09 can be seen below in Figure 3-4. Encompassing virulence factors, transcription regulators and groups of genes showing a big expression difference in T4Δ*rr09*. When comparing this to what genes are regulated by RR08 we see a number of similarities, both repress the expression of the pilus islet and genes involved in purine biosynthesis. They potentially both regulate RR01 to a similar level (not significant difference in T4Δ*rr09*) and RR09 may positively regulate RR08, however again this difference was not seen as significant.

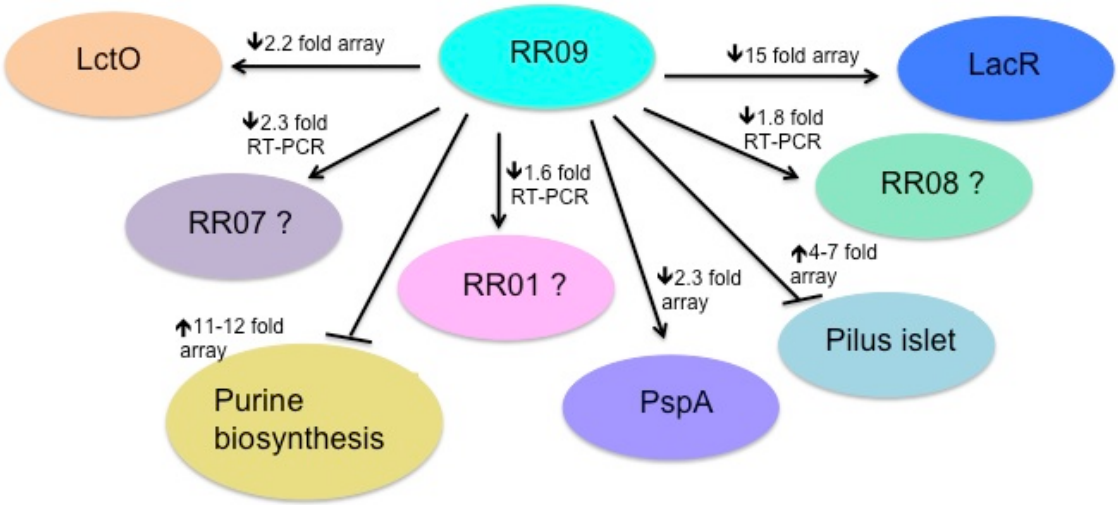


Figure 3-4: Summary of genes regulated by RR09

Summary of genes regulated by RR09 has been compiled from microarray and RT-PCR data. Arrow represents RR08 positively regulating the gene and a bar represent RR09 repression of the gene. Values indicate the fold change of the gene observed during microarray analysis or RT-PCR in T4Δ*rr09*.

3.4 Genes differentially regulated in T4Δ*rr098*

To try and understand further the potential cross regulation of these TCS a double knockout was constructed and microarray analysis performed on this strain. A total of 122 genes were differentially regulated in the double knockout compared to the parent strain T4JH, shown in Table 3-3. When increasing the P value to P<0.1 a total of 327 genes are differentially regulated (data not shown).

Gene	Description	Abbrev	Fold change
SP_0004	GTP dependant nucleic acid binding protein	*engD*	1.3
SP_0044	Phosphoribosylaminoimidazole-succinocarboxamide	*purM*	13.1
SP_0048	Glycinamide ribonucleotide transformylase	*purN*	12.3
SP_0049	vanZ protein	*vanZ*	12.3
SP_0050	phosphoribosylamine	*purH*	9.3
SP_0051	Hypothetical protein		13.1
SP_0053	Phosphoribosylaminoimidazole carboxylase	*purE*	9.8
SP_0054	Phosphoribosylaminoimidazole carboxylase	*purK*	11.1
SP_0055	Hypothetical protein		3.1
SP_0056	Adenylosuccinate lyase	*purB*	2.2
SP_0063	PTS system IID component		2.5
SP_0064	PTS system IIA component		2.0
SP_0066	Aldolase-1 epimerase		2.0
SP_0099	Hypothetical protein		1.5
SP_0107	LysM domain containing protein		2.3
SP_0199	Cardiolipin synthetase	*cls*	1.5
SP_0201	Hypothetical protein		1.6
SP_0258	Hypothetical protein		1.9
SP_0259	Hypothetical protein	*ruvB*	1.2
SP_0288	Hypothetical protein		6.5
SP_0290	Dihydrofolate synthetase	*folC*	2.1
SP_0341	Hypothetical protein		1.3
SP_0409	Hypothetical protein		4.0
SP_0419	Enoyl-(acyl-carrier protein) reductase	*fabK*	1.4
SP_0422	3-oxacyl-(acyl-carrier protein) synthase II	*fabF*	1.3
SP_0461	Transcriptional regulator	*rlrA*	4.4
SP_0462	Cell surface anchor family protein	*rrgA*	3.6
SP_0464	Cell surface anchor family protein	*rrgC*	2.9
SP_0467	Sortase	*srtC*	4.1
SP_0468	sortase	*srtD*	2.8
SP_0487	Hypothetical protein		1.4
SP_0626	Branched chain amino acid transport system		1.6
SP_0627	Hypothetical protein		1.3
SP_0657	Ribonuclease BN, putative		1.2
SP_0688	UDP-N-acetly muramoylalanine- D-glutamate ligase	*murD*	1.2
SP_0689	Undecaprenyldiphospho-muramoylpentapeptide beta-N-acetylglucosaminyltransferase	*murG*	1.1
SP_0703	Hypothetical protein		1.6
SP_0720	ABC transporter ATP binding protein		1.3
SP_0742	Hypothetical protein		2.2
SP_0746	ATP dependant clp protease	*clpP*	1.3
SP_0784	Glutathione reductase		1.4
SP_0785	Hypothetical protein		2.3
SP_0786	Hypothetical protein		2.2
SP_0787	Hypothetical protein		2.0

SP_0790	Hypothetical protein		1.2
SP_0811	Transposase family protein		1.2
SP_0820	ATP dependant Clp protease	*clpE*	1.4
SP_0835	Purine nucleoside phosphorylase	*deoD*	1.3
SP_0898	Transcriptional repressor, degenerate		1.2
SP_0966	Adherence and virulence protein A	*pavA*	1.1
SP_1018	Thymidine kinase	*tdk*	1.2
SP_1023	acetyltransferase		1.3
SP_1026	Hypothetical protein		1.8
SP_1070	Hypothetical protein		1.4
SP_1103	Hypothetical protein		1.4
SP_1107	Ribosomal protein L27	*rpmA*	1.3
SP_1114	ABC transporter, ATP binding protein		1.2
SP_1119	Glyceraldehyde-3-phosphate dehydrogenase	*gapdh*	1.6
SP_1160	Lipoate protein ligase		1.7
SP_1161	Acetoin dehydrogenase, E3 component		1.5
SP_1162	Dihydrolipoamide acetyltransferase		1.5
SP_1163	Acetoin dehydrogenase, E1 component		1.5
SP_1225	VicX protein	*vicX*	1.7
SP_1226	Histidine kinase 02		1.6
SP_1227	Response regulator 02		1.8
SP_1228	A/G specific adenine glycosylase		2.4
SP_1231	Phoshpopantothenoylcystein decarboxylase		1.6
SP_1232	Hypothetical protein		1.7
SP_1249	Guanosine 5'-monophosphate oxido reductase		2.3
SP_1299	Ribosomal protein L31 type B	*rpmE2*	1.3
SP_1356	chlorohydrolase	*Atz*	2.0
SP_1357	ABC transporter, ATP binding/permease protein.		1.7
SP_1369	Prephenate dehydratase		1.9
SP_1371	3-phosphoshikimate 1-carboxyvinyltransferase		1.8
SP_1393	Putative membrane protein	*pstA*	1.6
SP_1396	Phosphate transporter, ATP binding protein	*pst*	1.3
SP_1415	Glucosamine-6-phosphate isomerase	*nagB*	1.9
SP_1427	U32 family peptidase		1.6
SP_1428	Hypothetical protein		1.5
SP_1501	Amino acid ABC transporter, ATP binding protein		1.6
SP_1509	F0F1 ATP synthase gamma subunit	*alpG*	1.1
SP_1526	ABC transporter authentic frameshift		3.5
SP_1527	Oligopeptide ABC transporter	*aliB*	3.7
SP_1532	Authentic frameshift		1.5
SP_1535	Hypothetical protein		1.3
SP_1554	tRNA CCA-pyrophosphorylase		1.5
SP_1572	Starved cell/ iron storage peroxide induced protein	*dpr*	1.4
SP_1584	Transcriptional repressor	*codY*	1.3
SP_1587	Oxalate formate antiporter		2.1
SP_1626	Ribosomal protein S15	*rpsO*	1.3
SP_1697	ATP dependant DNA helicase	*recG*	1.4
SP_1701	Phospho-2-dehydro-3-deoxyheptonate aldolase		1.5
SP_1708	Hypothetical protein		2.9
SP_1749	GTP binding protein	*yqeH*	1.1
SP_1772	Cell wall surface anchor family protein	*psrP*	1.3
SP_1802	Hypothetical protein		3.1
SP_1804	General stress protein 24		3.4
SP_1941	Competence damage inducible protein A	*cinA*	1.3
SP_1978	Diaminopimelate decarboxylase		1.7
SP_1983	Ribulose-3-phosphate epimerase		1.4
SP_1985	Dimethyladenosine transferase	*ksgA*	1.5
SP_1987	ABC transporter, ATP binding protein		1.9
SP_1988	Immunity protein, putative		2.0
SP_2039	Hypothetical protein	*sapR*	1.6

SP_2065	MATE efflux family protein		1.6
SP_2066	Threonine synthase		1.5
SP_2071	Hypothetical protein		2.0
SP_2072	Glutamine amidotransferase		3.3
SP_2073	ABC transporter, ATP binding/permease protein		1.4
SP_2075	ABC transporter, ATP binding/permease protein		1.6
SP_2091	NADH dependant glyceraldehyde-3-phosphate dehydrogenase	*gpsA*	1.2
SP_2095	5-formyltetrahydrofolate cyclo-ligase family		1.3
SP_2105	Hypothetical protein		1.8
SP_2148	Arginine deiminase		3.3
SP_2167	L-fuculose kinase	*fucK*	3.0
SP_2193	Response regulator 06	*cbpS*	1.4
SP_2194	ATP dependant Clp protease		1.7
SP_2196	ABC transporter, ATP binding protein		1.4
SP_2198	ABC transporter, permease protein		1.5
SP_2199	Hypothetical protein		2.1
SP_2218	Rod shape determining protein	*mreC*	1.2
SP_2238	rRNA large subunit transferase		1.9

Table 3-3 : Table of genes differentially regulated in T4Δ*rr098*

Table shows genes differentially regulated in T4Δ*rr098* compared to its parent T4JH (P<0.05). Fold change represents that seen in T4Δ*rr098* compared to T4JH, red showing genes up regulated in T4Δ*rr098* and blue are those that are down regulated.

RT-PCR analysis was performed to assess the expression levels of the other RR after deletion of both *rr08* and *rr09* (Figure 3-5). Not surprisingly a down regulation of *rr*01 is observed in T4Δ*rr098* as this has already been seen in T4Δ*rr08,* however this down regulation is more than the fold change observed in T4Δ*rr08,* which may indicate the change in *rr01* expression observed in T4Δ*rr09* although not significant may be a true change and in the double knockout the increased difference is due to the effect of both of these systems. In the double knockout a similar down regulation of *rr07* to that observed in T4Δ*rr09* alone is seen, although again in T4Δ*rr09* this was not significant it may indicate this is a true change and suggests RR09 alone acts to regulate RR07. In the double knockout *rr02* expression was increased to a level similar to that observed in T4Δ*rr08* alone as measured by microarray, but in T4Δ*rr098* the increased expression of *rr02* was not validated by RT-PCR. *rr06* was shown to be up regulated by microarray analysis in T4Δ*rr098* however again this was not validated by RT-PCR. Interestingly in T4Δ*rr098* statistically significant expression changes are observed in a number of the other RR that were not seen in the single mutants alone, including down regulation of *rr04, rr05* and *rr13*. A direct comparison between *rr* expression differences via RT-PCR in T4Δ*rr08,* T4Δ*rr09* and T4Δ*rr098* can be seen in appendix II.

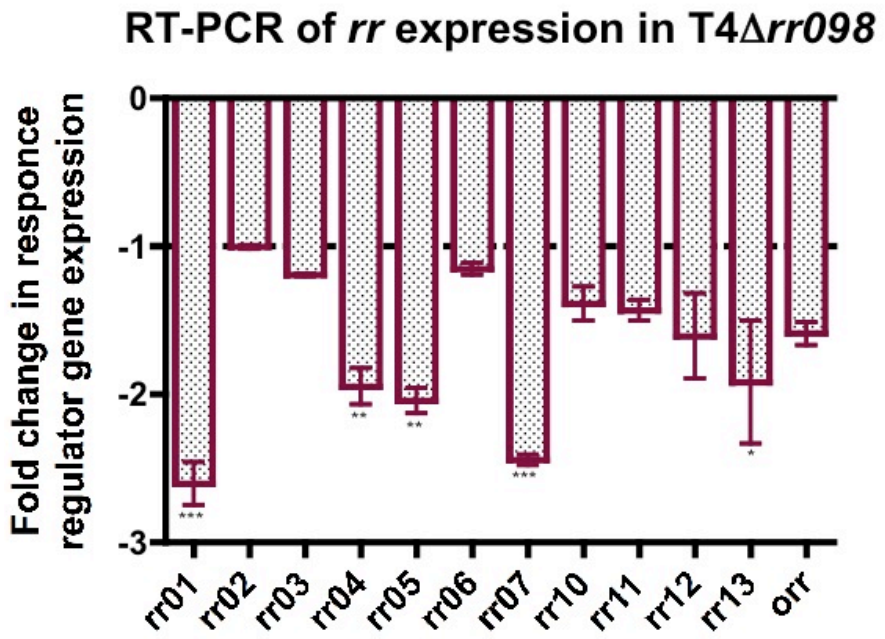


Figure 3-5: RT-PCR graph of response regulator expression in T4Δ*rr098.*

Graph shows RT-PCR of all the pneumococcal response regulators in T4Δ*rr098.* Fold change represents that of T4Δ*rr098* compared to T4JH. Each bar represents the average of three replicas (three biological replicas) and errors bars the standard deviation. Statistical analysis was performed by a 1-way ANOVA with a Tukeys testing correction comparing the dCt values of the control strain (TIGR4) to the test strain (T4Δ*rr098*), * P<0.05, ** P<0.01, *** P<0.001.

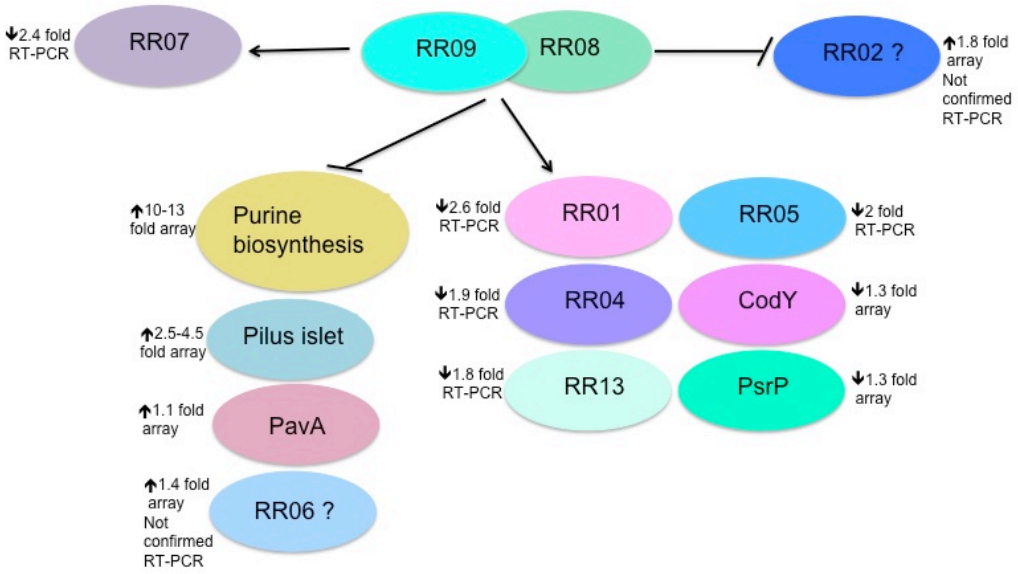


Figure 3-6: Summary of genes regulated by RR09 and RR08 alone or together.

Summary of genes regulated by RR09 and RR08 has been compiled from microarray and RT-PCR data. Arrow represents positively regulated genes and bars represent genes repressed. Values indicate the fold change of the gene observed during microarray analysis or RT-PCR in T4Δ*rr098.* Genes present to either side represent genes regulated by RR09 and RR08 alone, genes below represent genes regulated by both RR09 and RR08 shown from differential regulation upon deletion of both.

A summary of the genes regulated by RR08 or RR09 alone and genes regulated by both is shown in Figure 3-6. The genes regulated encode virulence factors, transcription regulators and includes groups of genes showing a big expression difference in T4Δ*rr098*. A large number of these changes include genes that are not differentially regulated in the single knockout mutants alone. This suggests that single knockout mutants can be compensated for by the other systems, in the double knockout this cannot happen.

Direct comparison of the genes regulated by each single mutant and double mutant by microarray analysis can be seen in Figure 3-7. Comparisons were performed on both the $P<0.05$ (Figure 3-7) and $P<0.1$ (appendix V) gene lists from each experiment. From this it is clear only a small proportion of genes are commonly differentially regulated in all three strains, 7 genes $P<0.05$ and 22 genes $P<0.1$ (appendix V). The majority of these genes show similar expression levels in all three knockout mutants suggesting RR08 and RR09 (not significant) both regulate these genes equally, perhaps through a common gene regulated by both. For instance RR01, which is controlled by RR08 and RR09 and may therefore control a common set of genes. However the pilus expression in the single RR08 mutant is lower than that of the single RR09 and double mutant. Alternatively these systems may respond to a common stress which cannot occur when either is deleted leading to differential expression of a common set of genes.

In most instances it is clear that both RR act independently of each other and regulate their own set of genes. Only a quarter to half of the genes differentially regulated in the single RR09 mutant were seen to also be differentially regulated in the double mutant. This was slightly higher in the RR08 single mutant with two thirds of the genes regulated in the single mutant seen to also be differentially regulated in the double mutant. The fact a large number of genes are differentially regulated in the double mutant that are not observed in either of the single mutant alone would however suggest they both may converge on some of the same pathways and perhaps compensate for the deletion of the other. Deletion of both leads to a large collection of genes being differentially regulated, with roughly two thirds of all the genes differentially regulated in T4Δ*rr098* not seen to be differentially regulated in either of the single mutants.

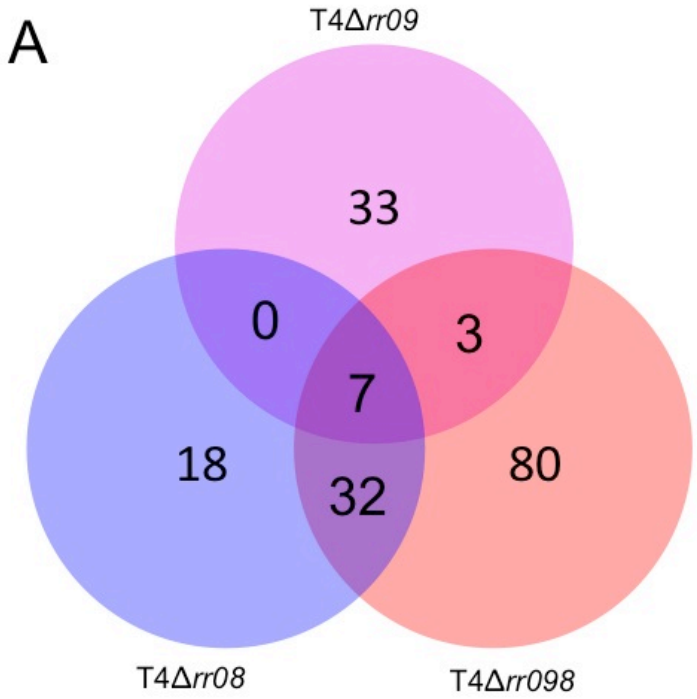


Figure 3-7: Venn diagram of gene commonly regulated by *rr08* and *rr09* in T4JH.

Venn diagrams were compiled from our gene expression data of T4Δ*rr08,* T4Δ*rr09 and* T4Δ*rr098*. (A) Compares the gene list In Table 3-1, Table 3-2 and Table 3-3 (P<0.05).

3.5 TCS regulation of the pneumococcal pilus

To further assess how TCS may interact with one another, a further set of RR mutants were constructed. A deletion mutant of *rr06* in T4JH was constructed and subsequently transformed into the other RR mutants creating a total of seven mutants, the three described previously and T4Δ*rr06,* T4Δ*rr096,* T4Δ*rr086* and T4Δ*rr0986*. All of the systems chosen have previously been shown to regulate the pneumococcal pilus. These mutants were used to assess potential cross regulation of these systems via evaluating their effect on pilus islet expression.

Analysis was performed on all mutants comparing the expression level of the pilus islet genes by RT-PCR, including the transcription regulator (*rlrA*), the three pilins (*rrgA*, *rrgB*, *rrgC*) and the three sortases (*srtB*, *srtC*, *srtD*). FACS analysis was performed to assess the expression of the pilus at the population level. To allow systematic evaluation of the effect of each deletion to the double and triple mutants the analysis has been split into three sections, comparing T4Δ*rr06,* T4Δ*rr08,* T4Δ*rr086* and T4Δ*rr0986,* then T4Δ*rr06,* T4Δ*rr09,* T4Δ*rr096,* T4Δ*rr0986,* and finally T4Δ*rr08,* T4Δ*rr09,* T4Δ*rr098* and T4Δ*rr0986*.

3.5.1 Regulation of the pilus in T4Δrr06, T4Δrr08, T4Δrr086 and T4Δrr0986

RT-PCR analysis of pilus expression in T4Δ*rr06* showed a decrease in pilus expression compared to T4JH (Figure 3-8), this is contrary to its role as a repressor stated in the literature. Possible reasons for this will be discussed later. In this study, RR06 acts as a positive regulator of the pilus islet. T4Δ*rr08* pilus expression shows a small increase in pilus expression compared to T4JH, validating what is observed via microarray analysis. Interestingly in the double mutant (T4Δ*rr086*) there is a large increase in pilus expression, which would suggest that RR06 acts as a repressor in this instance. Further to this deletion of RR09 in T4Δ*rr086* (T4Δ*rr0986*) led to a drop in pilus expression to that of levels similar to T4Δ*rr08*, suggesting in this case RR09 acts as a positive regulator of the pilus.

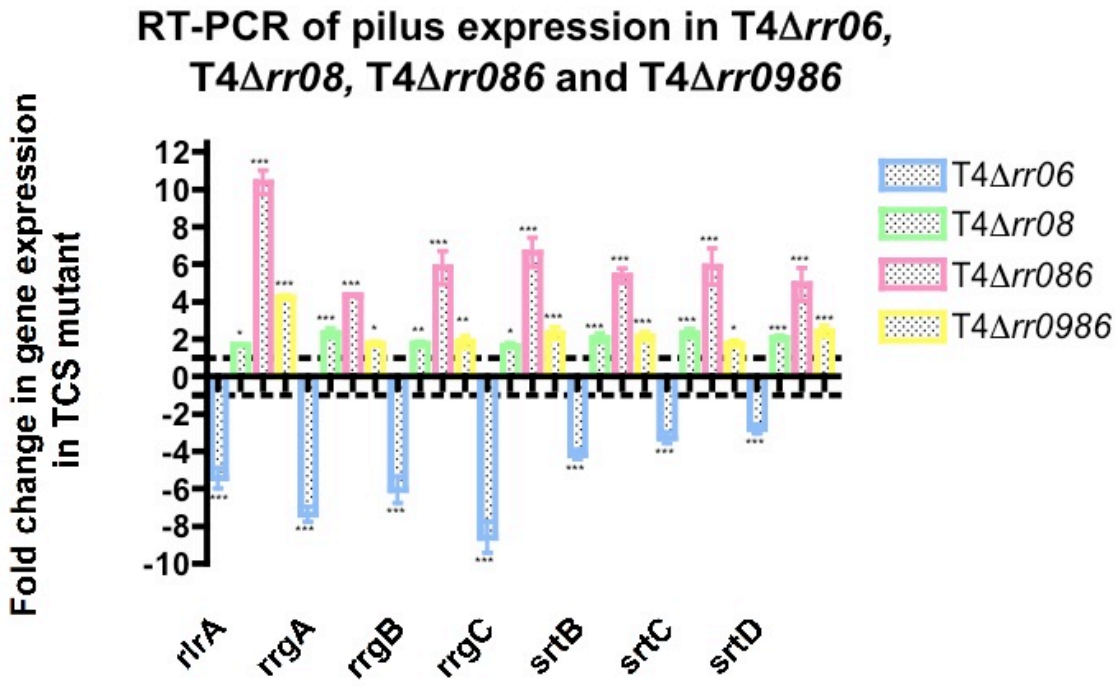


Figure 3-8: RT-PCR graph of pilus expression in T4Δ*rr06*, T4Δ*rr08*, T4Δ*rr086 and* T4Δ*rr0986*. Graph shows RT-PCR of the whole pilus islet (*rlrA, rrgA, B, C, srtB, C, D*) in T4Δ*rr06*, T4Δ*rr08*, T4Δ*rr086* and T4Δ*rr0986*. Fold change represents that of T4Δ*rr06*, T4Δ*rr08*, T4Δ*rr086* and T4Δ*rr0986* compared to T4JH. Each bar represents the average of three replicas (three biological replicas) and errors bars the standard deviation.

FACS analysis performed on these mutants showed variation in the level of pilus positive cells in a growing population rather than increased levels on a single cell (Figure 3-9, Table 3-4). Analysis confirmed the decrease in pilus expression in T4Δ*rr06* with only 4% of cells positive for RrgB on the cell surface compared to 20.5% of that of the parent T4JH, this was also seen via fluorescence microscopy (Figure 3-14).

T4Δ*rr08* had an increase in 17% of RrgB positive cells compared to T4JH. There is likely no difference in the amount of pili on single cells with the RrgB positive peak lying directly above that of T4JH suggesting a similar fluorescence intensity of RrgB positive cells (similar levels of pili), fluorescence microscopy confirmed the increase in pili positive cells shown inFigure 3-14.

T4Δ*rr086* had 57% of RrgB positive cells within a growing population confirmed in Figure 3-14, this is 37% more than in T4JH, 20% higher than T4Δ*rr08* and 53% higher than T4Δ*rr06*. Interestingly by FACS analysis there is only one large peak

compared to that of the normal double peak showing clear RrgB positive and negative populations. This larger peak is also shifted to the left suggesting a decrease in fluorescence intensity of the RrgB positive cells compared to T4JH and the other mutants. This suggests deletion of both RR06 and RR08 causes the cells to lose the ability to regulate the amount of pili on the cell surface, as well as that at the population level.

Further deletion of RR09 in T4Δ*rr086* caused the pilus expression to drop. With T4Δ*rr0986* showing only 27% of cells to be RrgB positive shown in Figure 3-14, this is only 7% higher than that of T4JH. In this mutant we again see two distinct peaks representing that of RrgB positive and negative populations, with the positive population of a similar fluorescence intensity to T4JH and T4Δ*rr08*.

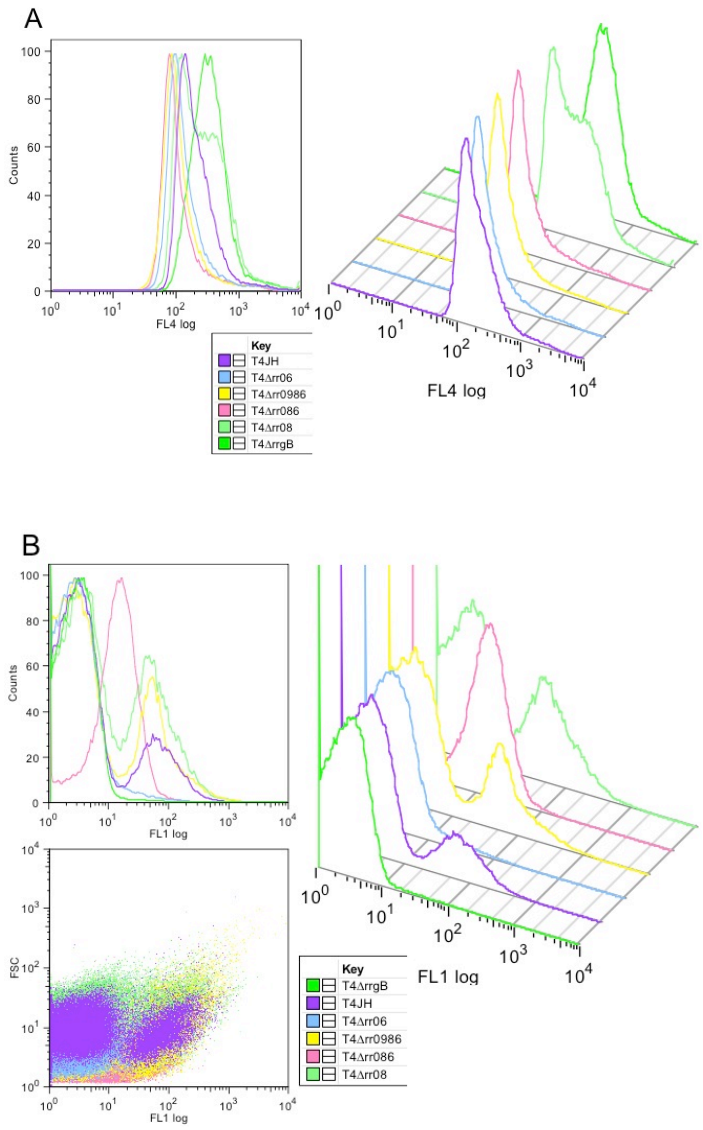


Figure 3-9: FACS analysis of RrgB surface expression in T4Δ*rr06*, T4Δ*rr08*, T4Δ*rr086* and T4Δ*rr0986*.

FACS was performed on T4JH, T4Δ*rr06*, T4Δ*rr08*, T4Δ*rr086*, T4Δ*rr0986* and T4Δ*rrgB*. (A) Shows histograms of the capsule positive populations selected for from samples stained with a capsule antibody, population used for further analysis. (B) Shows the capsule stained populations being gated on for being RrgB positive, histograms/ polychromatic plot show negative (left) and positive (right) RrgB populations in each strain.

Strain	RrgB- (%)	RrgB+ (%)
T4JH	79.5	20.5
T4Δ*rr06*	95.9	4.1
T4Δ*rr08*	63.1	36.9
T4Δ*rr086*	42.8	57.2
T4Δ*rr0986*	73.1	26.9
T4Δ*rrgB*	98.4	1.6

Table 3-4: Percentage RrgB positive cells in a T4Δ*rr06*, T4Δ*rr08*, T4Δ*rr086* or T4Δ*rr0986* population from FACS analysis.

Table shows the percentage RrgB positive and negative cells in a growing bacterial population. FACS analysis was performed in FlowJo.

3.5.2 Regulation of the pilus in T4Δrr06, T4Δrr09, T4Δrr096 and T4Δrr0986

The RT-PCR data for T4Δ*rr06* and T4Δ*rr0986* is that already discussed in section 3.5.1, with an increase in pilus expression observed in T4Δ*rr0986* and a decrease seen in T4Δ*rr06* (Figure 3-10). This data confirms the findings observed by microarray analysis for T4Δ*rr09*, as all the pilus islet genes are statistically up regulated in this strain. Deletion of RR09 in T4Δ*rr06* creating T4Δ*rr096* shows a drop in expression compared to T4Δ*rr09* but an increase compared to T4Δ*rr06*, with expression levels of the pilus islet now only marginally higher than that of T4JH. Further deletion of RR08 in T4Δ*rr096* causes little difference in the expression levels of the pilus islet compared to T4Δ*rr096*.

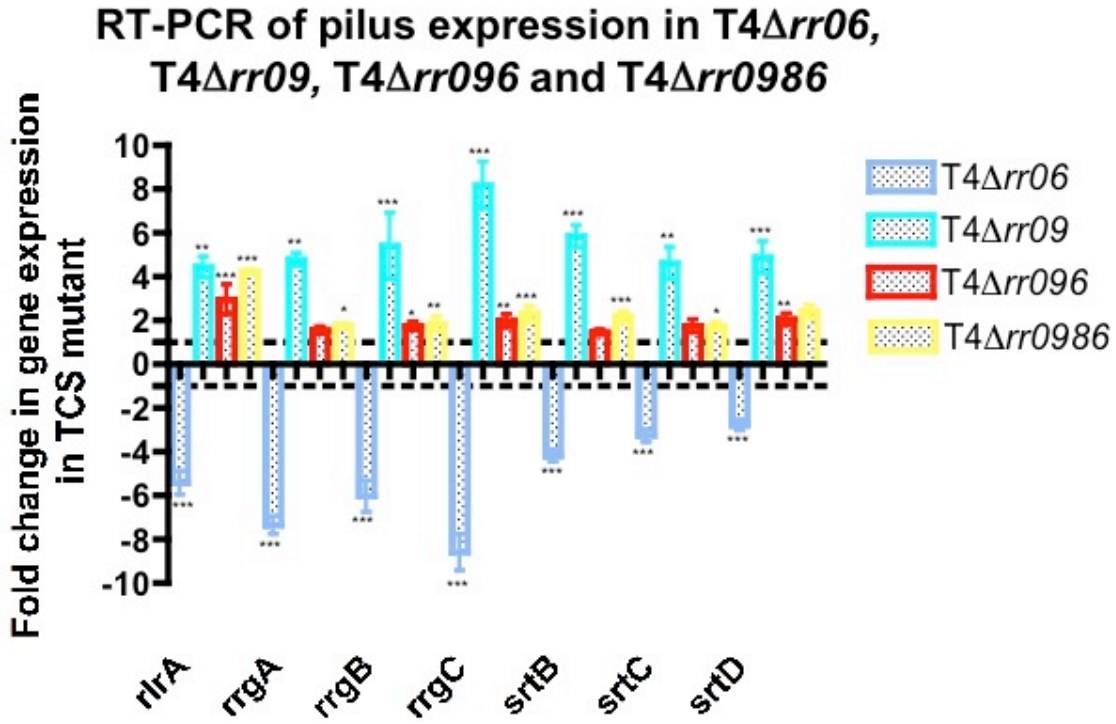


Figure 3-10: RT-PCR graph of pilus expression in T4Δ*rr06*, T4Δ*rr09*, T4Δ*rr096* and T4Δ*rr0986*.

Graph shows RT-PCR of the whole pilus islet (*rlrA, rrgA, B, C, srtB, C, D*) in T4Δ*rr06*, T4Δ*rr09*, T4Δ*rr096* and T4Δ*rr0986*. Fold change represents that of T4Δ*rr06*, T4Δ*rr09*, T4Δ*rr096* and T4Δ*rr0986* compared to T4JH. Each bar represents the average of three replicas (three biological replicas) and errors bars the standard deviation.

FACS analysis was performed comparing the RrgB surface exposure of these TCS mutants (Figure 3-11, Table 3-5). As already discussed a T4Δ*rr06* knockout shows

only 4% of cells positive for RrgB on the cell surface. Upon deletion of RR09 we know from earlier that we see an increase in pilus expression and this corresponds to an increase in the number of cells expressing RrgB on the cells surface compared to T4JH. 91% of cells upon RR09 deletion contain pili on the surface (confirmed by fluorescence microscopy,Figure 3-14), this is 70% more than that of T4JH. Interestingly there is one large peak similar to T4Δ*rr086,* suggesting an inability to regulate the cell surface constituents. However the RrgB positive peak lies above that of T4JH, T4Δ*rr096* and T4Δ*rr0986* suggesting the amount of pili on the single cells is similar, and there is not large shift to the left as observed with T4Δ*rr086*.

Deletion of RR06 in T4Δ*rr09* causes a large drop in the number of pili positive cells with only 28% of cells pili positive in T4Δ*rr096* (Figure 3-14). Again there are two peaks representing RrgB positive and negative populations. Further deletion of RR08 in T4Δ*rr096* has little effect on the number of pili positive cells with 27% of cells positive for RrgB on the cell surface. The fact there is only 1% difference between T4Δ*rr096* and T4Δ*rr0986* may suggest that without the presence of RR06 and RR09 RR08 is unable to perform its role in regulating pilus expression.

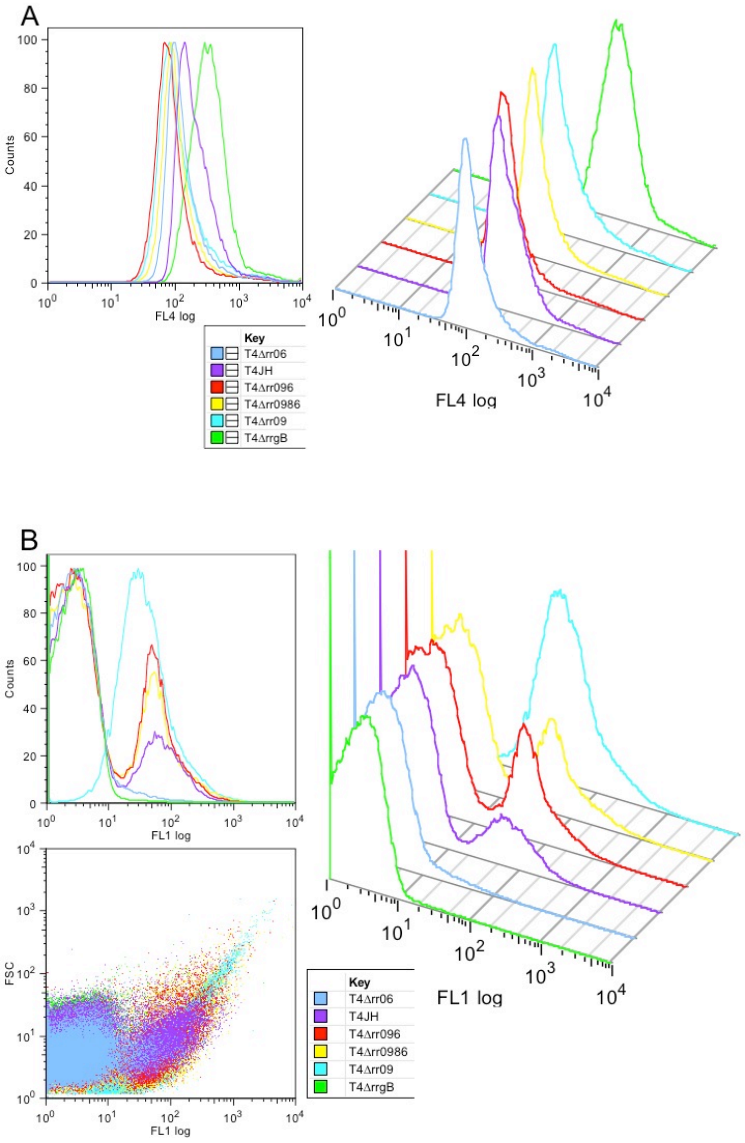


Figure 3-11: FACS analysis of RrgB surface expression in T4Δ*rr06*, T4Δ*rr09*, T4Δ*rr096* and T4Δ*rr0986*.

FACS was performed on T4JH, T4Δ*rr06*, T4Δ*rr09*, T4Δ*rr096*, T4Δ*rr0986* and T4Δ*rrgB*. (A) Shows histograms of the capsule positive populations selected for from samples stained with a capsule antibody, population used for further analysis. (B) Shows the capsule stained populations being gated on for being RrgB positive, histograms/ polychromatic plot show negative (left) and positive (right) RrgB populations in each strain.

Strain	RrgB- (%)	RrgB+ (%)
T4JH	79.5	20.5
T4Δ*rr06*	95.9	4.1
T4Δ*rr09*	9.3	90.7
T4Δ*rr096*	71.8	28.2
T4Δ*rr0986*	73.1	26.9
T4Δ*rrgB*	98.4	1.6

Table 3-5: Percentage RrgB positive cells in a T4Δ*rr06*, T4Δ*rr09*, T4Δ*rr096* or T4Δ*rr0986* population from FACS analysis.

Table shows the percentage RrgB positive and negative cells in a growing bacterial population. FACS analysis was performed in FlowJo.

3.5.3 Regulation of the pilus in T4Δrr08, T4Δrr09, T4Δrr098 and T4Δrr0986

The RT-PCR data has already been described above in sections 3.5.1 and 3.5.2 for pilus islet expression of T4Δ*rr08,* T4Δ*rr09* and T4Δ*rr098*6, this data compares the pilus expression in these strains and T4Δ*rr098* (Figure 3-12). Interestingly pilus expression in T4Δ*rr098* is lower than that of T4Δ*rr09,* which was not clear from microarray analysis, and higher than that of T4Δrr08. Therefore deletion of *rr08* in T4Δ*rr09* causes a decrease in pilus expression. Deletion of RR06 in T4Δ*rr098* causes a further drop in pilus expression, which is at a similar level to that of T4Δ*rr08*.

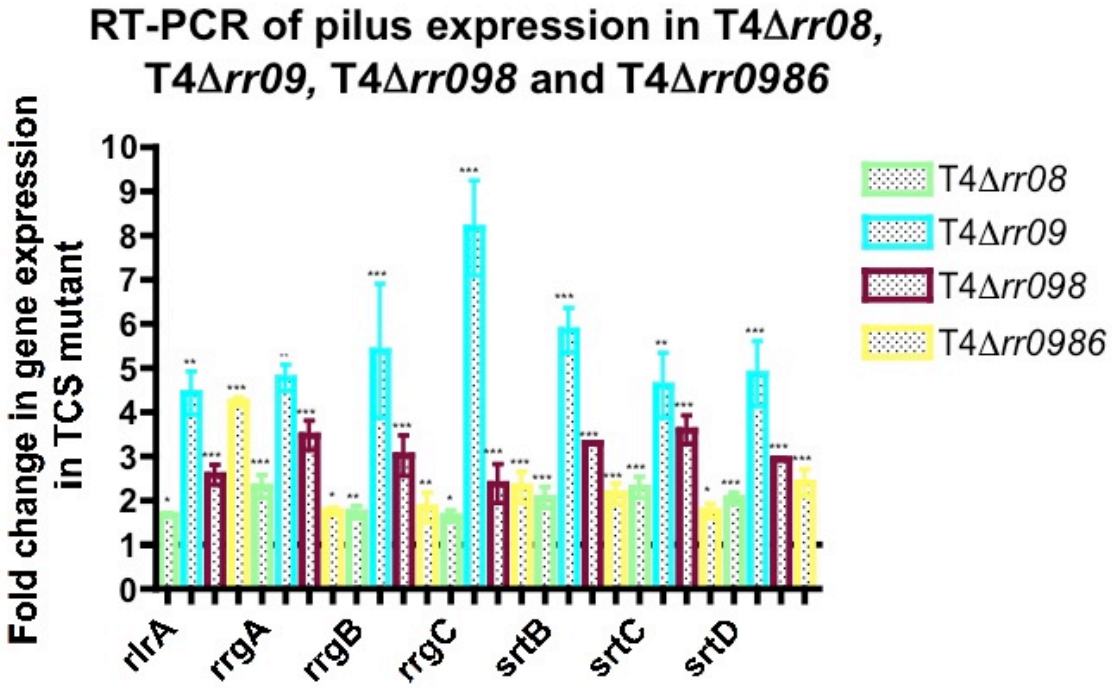


Figure 3-12: RT-PCR graph of pilus expression in T4Δ*rr08,* T4Δ*rr09,* T4Δ*rr098 and* T4Δ*rr0986.*

Graph shows RT-PCR of the whole pilus islet (*rlrA, rrgA, B, C, srtB, C, D*) in T4Δ*rr08,* T4Δ*rr09,* T4Δ*rr098* and T4Δ*rr0986.* Fold change represents that of T4Δ*rr08,* T4Δ*rr09,* T4Δ*rr098* and T4Δ*rr0986* compared to T4JH. Each bar represents the average of three replicas (three biological replicas) and errors bars the standard deviation.

FACS analysis on T4Δ*rr08,* T4Δ*rr09* and T4Δ*rr0986* has already been described above in sections 3.5.1 and 3.5.2. T4Δ*rr098* pilus expression level as already shown by RT-PCR is in between that of T4Δ*rr08* and T4Δ*rr09*. FACS analysis confirmed this with 56% of cells positive for RrgB on the cell surface in T4Δ*rr098*

compared to T4Δ*rr08* (37%) and T4Δ*rr09* (91%) (Figure 3-13, Table 3-6). Deletion of RR08 in T4Δ*rr09* restored the two clear populations (RrgB positive and negative). Similar pili positive cell numbers were observed in T4Δ*rr086* (57%). However only one large peak was observed during FACS analysis, which was not seen in T4Δ*rr098*. This can also be observed via fluorescence microscopy shown in Figure 3-14.

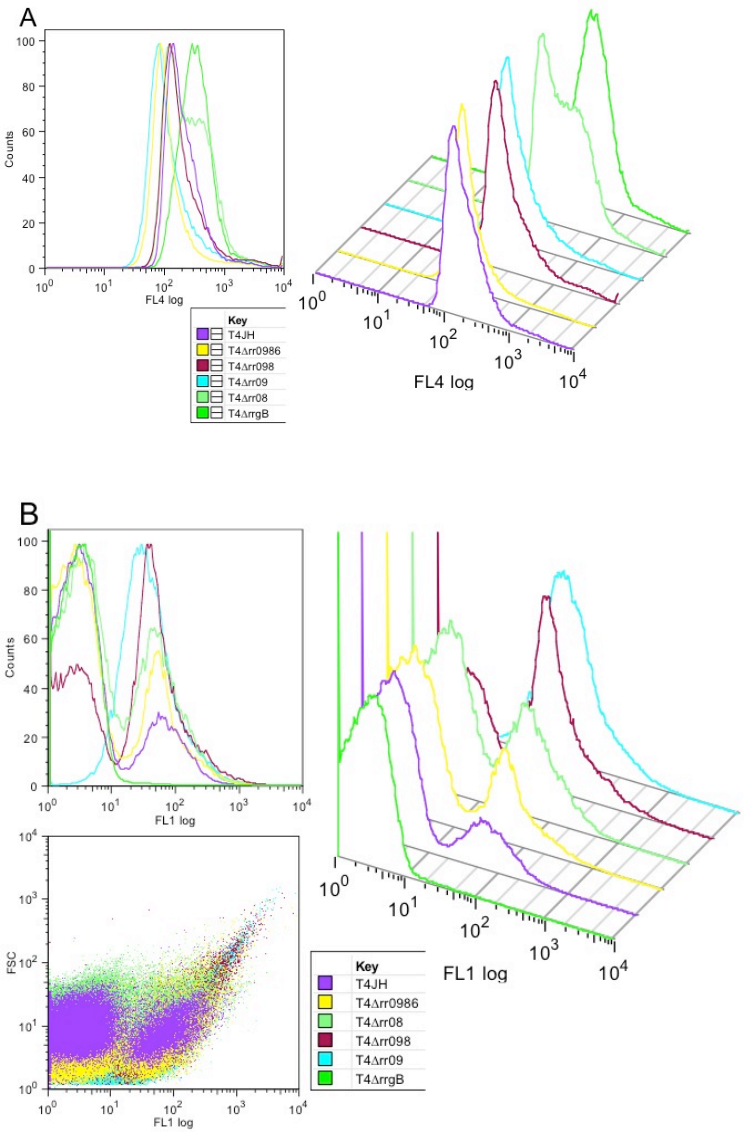


Figure 3-13: FACS analysis of RrgB surface expression in T4Δ*rr08,* T4Δ*rr09,* T4Δ*rr098 and* T4Δ*rr0986.*

FACS was performed on T4JH, T4Δ*rr08,* T4Δ*rr09,* T4Δ*rr098,* T4Δ*rr0986* and T4Δ*rrgB*. (A) Shows histograms of the capsule positive populations selected for from samples stained with a capsule antibody, population used for further analysis. (B) Shows the capsule stained populations being gated on for being RrgB positive, histograms/ polychromatic plot show negative (left) and positive (right) RrgB populations in each strain.

Strain	RrgB- (%)	RrgB+ (%)
T4JH	79.5	20.5
T4Δ*rr08*	63.1	36.9
T4Δ*rr09*	9.3	90.7
T4Δ*rr098*	44	56
T4Δ*rr0986*	73.1	26.9
T4Δ*rrgB*	98.4	1.6

Table 3-6: Percentage RrgB positive cells in a T4Δ*rr08,* T4Δ*rr09,* T4Δ*rr098* or T4Δ*rr0986* population from FACS analysis.

Table shows the percentage RrgB positive and negative cells in a growing bacterial population. FACS analysis was performed in FlowJo.

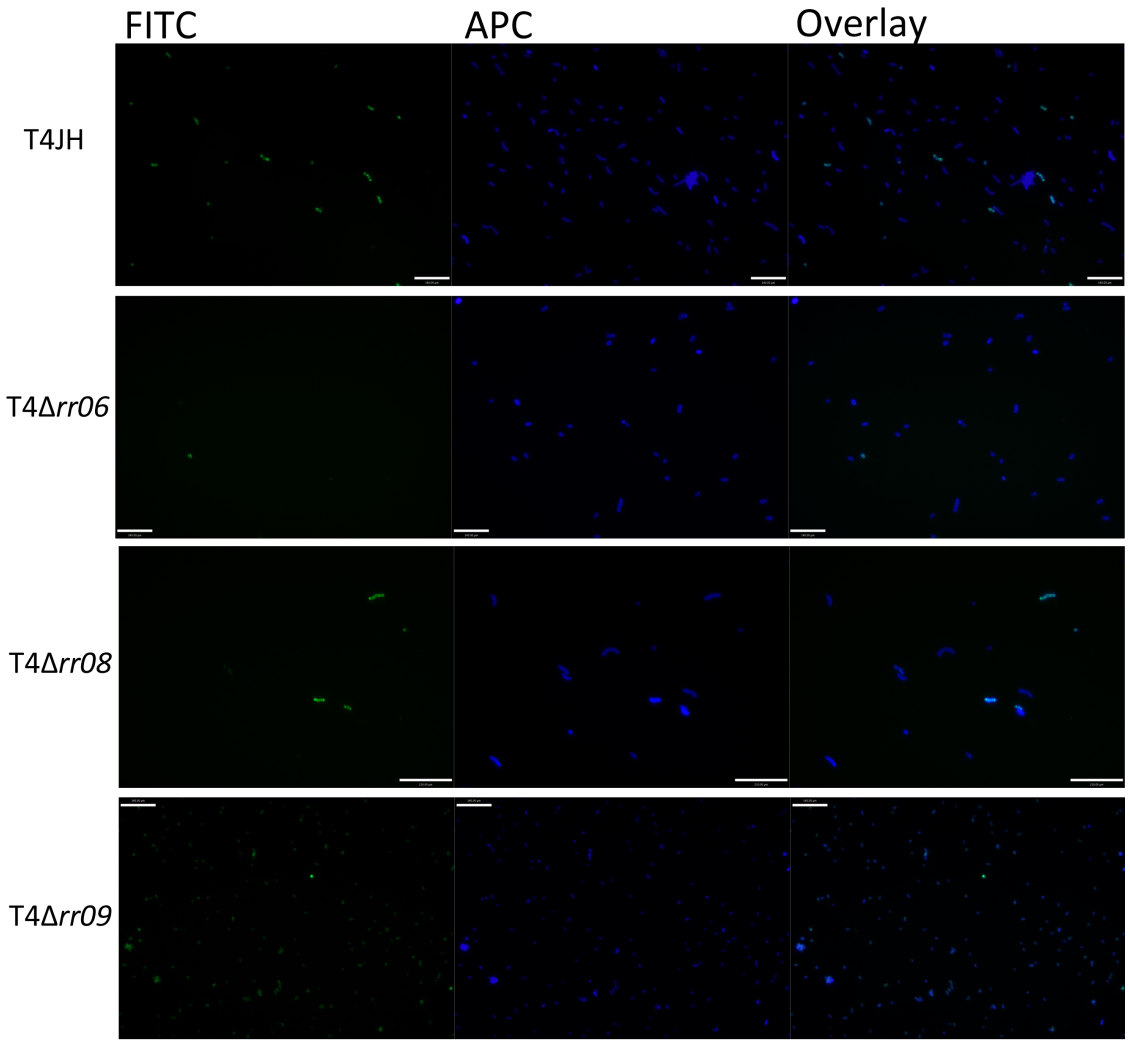


Figure 3-14: Fluorescence microscopy of TCS mutants

One representative images of fluorescently labelled TCS mutants used for FACS analysis. Cells were stained for the presence of RrgB (FITC) and the capsule (APC). Scale bars above represent, T4JH-140μm, T4Δ*rr06* - 140μm, T4Δ*rr08* - 210μm and T4Δ*rr09* - 140μm. The same representative image will be used throughout this study for each strain, more images with can be seen in appendix I.

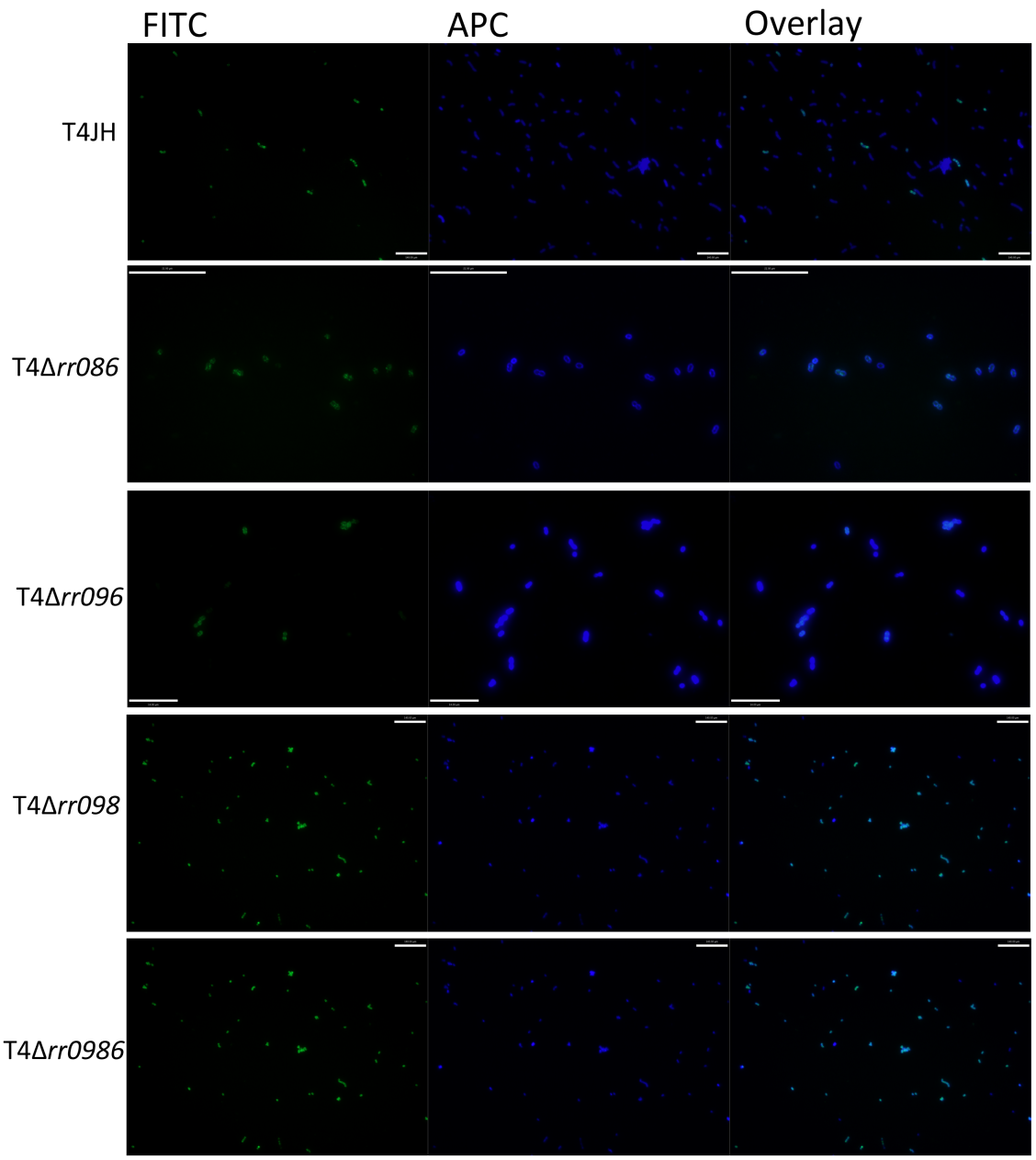


Figure 3-15: Fluorescence microscopy of TCS mutants

One representative images of fluorescently labelled TCS mutants used for FACS analysis. Cells were stained for the presence of RrgB (FITC) and the capsule (APC). Scale bars above represent, T4JH-140μm, T4Δ*rr086* - 22μm, T4Δ*rr096* - 14μm, T4Δ*rr098* - 140μm and T4Δ*rr0986* - 140μm. The same representative image will be used throughout this study for each strain, more images with can be seen in appendix I.

With regards to the pneumococcal pilus being regulated at the population level, currently only one gene has been shown to modulate this function, which is RrgA. RrgA has been shown to bind directly to RlrA through a protein-protein interaction and modulate RlrAs ability to positively regulate the pilus islet. Upon deletion of *rrgA* almost all cells within a growing population are shown to contain pili on the cell surface (Basset et al., 2011, Basset et al., 2012). All of

the TCS have been shown to modulate the pilus at the population level, therefore direct comparisons were made between the expression levels of *rrgA* and *rlrA* of all TCS mutants, shown in Figure 3-16. This RT-PCR data has already been shown previously in sections 3.5.1, 3.5.2 and 3.5.3 along with the expression of the other genes present on the islet. This graph provides a direct comparison between the *rrgA* and *rlrA* expression levels in all of the TCS mutants. This data may indicate if the regulation at the population level of the pilus by TCS is through modulating the expression levels of *rlrA* and *rrgA*. However it must be kept in mind that RNA for RT-PCR was isolated from all the bacterial cells present in a growing population, and therefore this analysis gives the average expression of these genes in the whole population, which must vary from cell to cell as FACS analysis has shown in all mutants different numbers of pili positive and negative cells.

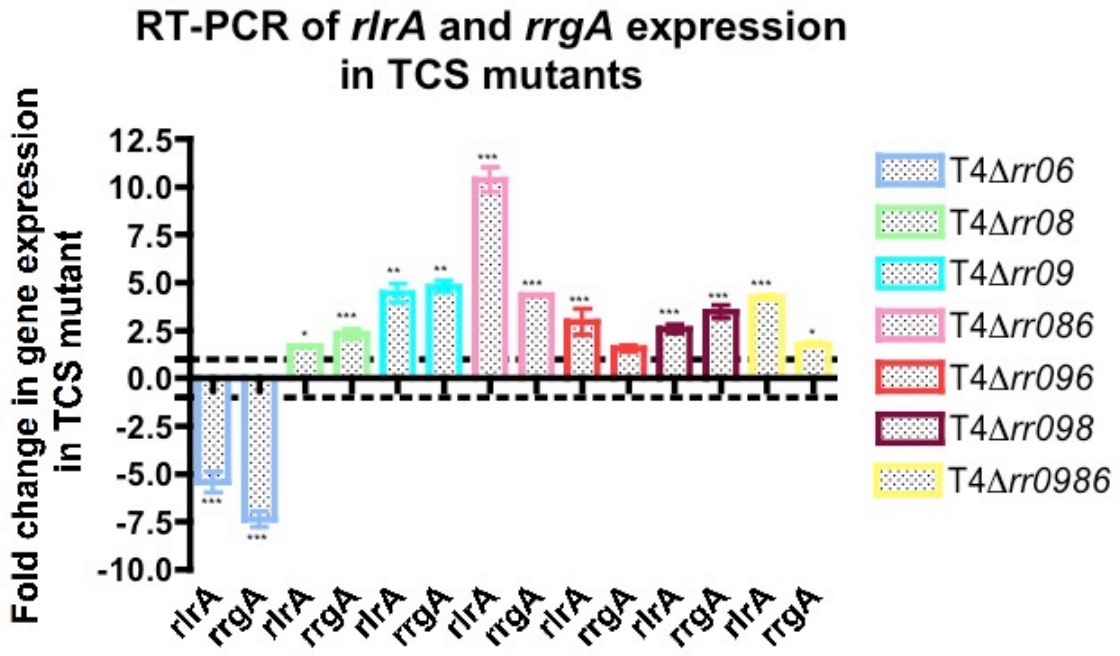


Figure 3-16: RT-PCR graph of *rlrA* and *rrgA* expression in TCS mutants

Graph shows RT-PCR of *rlrA* and *rrgA* in all the TCS mutants. The data represented here is that already seen in previous graphs, this graph provides a direct comparison of *rlrA* and *rrgA* levels in all TCS mutants. Fold change represents that of T4Δ*rr08,* T4Δ*rr09,* T4Δ*rr098* and T4Δ*rr0986* compared to T4JH. Each bar represents the average of three replicas (three biological replicas) and errors bars the standard deviation. Statistical analysis was performed by a 1-way ANOVA with a Tukeys testing correction comparing the dCt values of the control strain (TIGR4) to the test strain (TCS mutant), * P<0.05, **P<0.01, ***P<0.001.

With regards to the TCS modulating the *rlrA* and *rrgA* levels this seems to be the case for T4Δ*rr086,* which shows a much higher level of *rlrA* relative to *rrgA*. This may be why there is a large increase in pili positive cells in this strain. Other strains show a similar pattern such as T4Δ*rr096* and T4Δ*rr0986* which show moderately higher expression levels of *rlrA* relative to *rrgA*. This is not the case for T4Δ*rr08* and T4Δ*rr098* however as the difference in the expression of both genes is small. T4Δ*rr06* has a clear drop in both *rlrA* and *rrgA* expression levels, the decrease in *rrgA* expression is higher than *rlrA* so is likely not modulated via the repression of the islet through binding of RrgA to RlrA. Interestingly T4Δ*rr09* shows very similar expression levels of *rlrA* and *rrgA* yet shows the biggest increase in pili positive cells, with 91% of cells pili positive in T4Δ*rr09*. This suggests RR09 modulates its activity by an alternative means than through changing the expression levels of *rrgA* and *rlrA*. The phenotype of almost all cells being pili positive in T4Δ*rr09* is similar to that of the *rrgA* knockout noted in the literature. Therefore RR09 may act to modulate in some way the protein-protein interaction of RlrA and RrgA.

3.5.4 Expression of other RR in TCS mutants

RT-PCR analysis was also performed on TCS mutants to assess whether deletion of any RR altered the expression levels of any of the others, focusing on *rr06*, *rr08* and *rr09*. This may give an indication if one of the studied RR regulates another, modulating pilus expression through regulation of another.

RT-PCR analysis showed no variation in *rr06* expression in T4Δ*rr08,* T4Δ*rr09* and T4Δ*rr098* (Figure 3-17), this data has already been seen for T4Δ*rr08,* T4Δ*rr09* and T4Δ*rr098,* but below directly compares this on the same graph. *rr08* expression was slightly lower in T4Δ*rr09* and T4Δ*rr096* however neither were statistically significant perhaps due to the small change, but as this is seen in both mutants this could be a true change and RR09 may positively regulate *rr08* expression. *rr09* expression is slightly lower in T4Δ*rr08* suggesting RR08 may regulate *rr09* expression, however this was not seen in T4Δ*rr086,* and neither were noted to be statistically different.

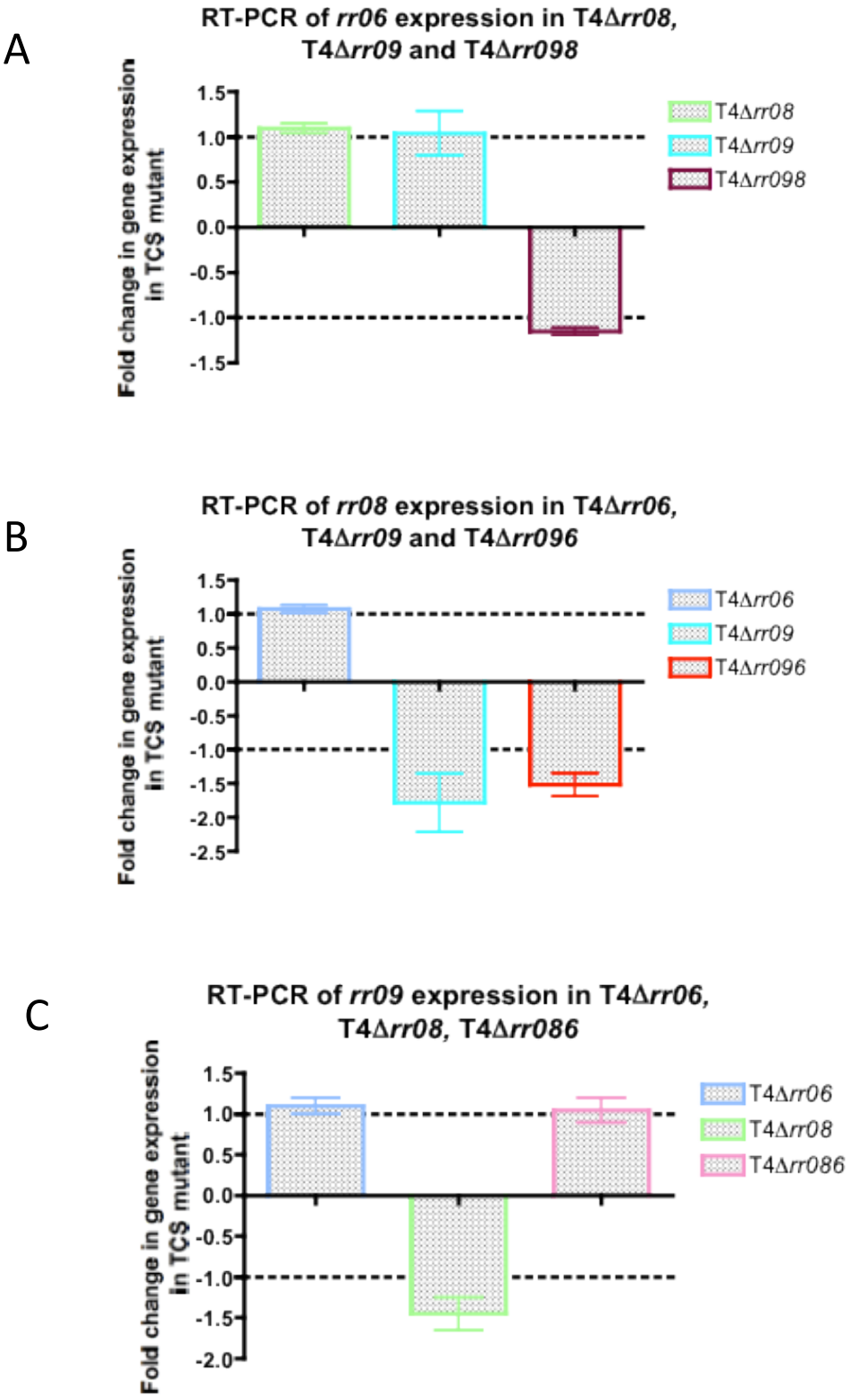


Figure 3-17: RT-PCR graph of RR expression in TCS mutants.

Graph shows (A) RT-PCR of *rr06* expression in T4Δ*rr08,* T4Δ*rr09* and T4Δ*rr098*, (B) RT-PCR of *rr08* expression in T4Δ*rr06,* T4Δ*rr09* and T4Δ*rr096*, (C) RT-PCR of *rr09* expression in T4Δ*rr06,* T4Δ*rr08* and T4Δ*rr086*. Fold change represents that of the TCS mutant compared to T4JH. Each bar represents the average of three replicas (three biological replicas) and errors bars the standard deviation. No statistical difference was observed.

3.6 Adherence assay analysis of TCS mutants

All RR mutants were tested for their ability to adhere to different cell lines. A number of the TCS have been shown to regulate pneumococcal virulence factors and adhesins, which affect adherence. None of the genes required for capsule biosynthesis were shown to be differentially regulated in T4Δ*rr08,* T4Δ*rr09* and T4Δ*rr098* ascertained through microarray analysis. Therefore it is assumed variation in capsule levels would not be the cause of any changes in adherence in these strains, which has been shown to alter the cell surface exposure of adhesins (Sanchez et al., 2011). The capsule level was not evaluated in T4Δ*rr06,* T4Δ*rr086,* T4Δ*rr096* and T4Δ*rr0986.* In the double and triple mutants there is also no data which may indicate if surface adhesins other than the pilus are differentially regulated in these strains which may alter adhesion. RR06 is known to also regulate CbpA and PspA therefore we assume these factors may also modulate changes in adhesion in T4Δ*rr06* (Standish et al., 2005, Standish et al., 2007). In T4Δ*rr08* no known cell surface adhesins were shown to be differentially regulated other than the pilus. In T4Δ*rr09* PsaA was shown to be down regulated which may alter adherence. Finally In T4Δ*rr098* the adhesin PsrP was down regulated 1.3 fold and PavA was up regulated 1.1 fold.

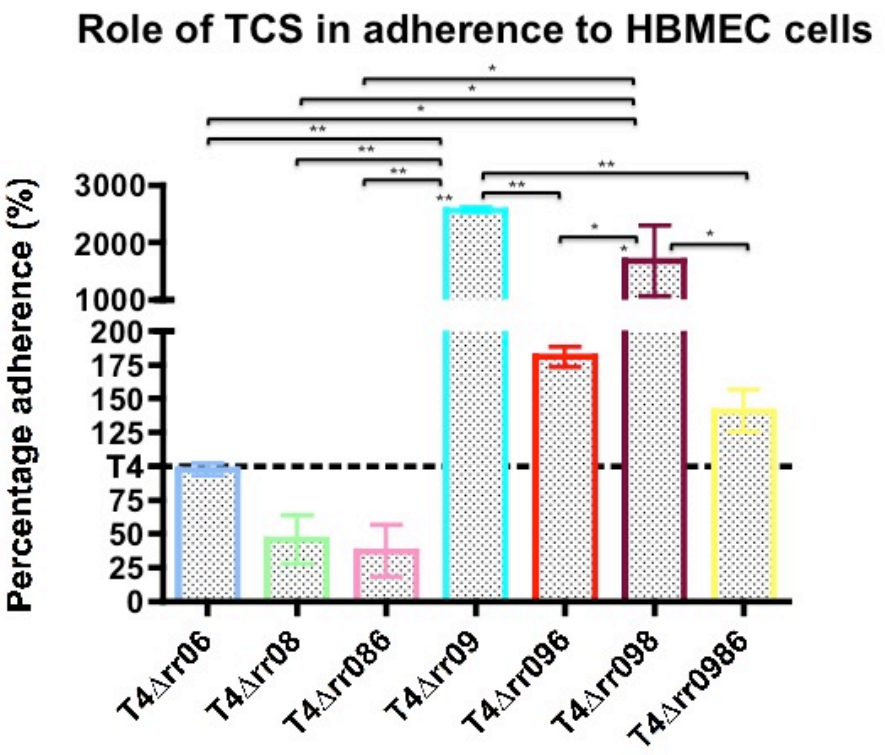
Adherence assay analysis showed clear difference in adherence capabilities upon deletion of a number of the TCS (Figure 3-18). Strains with a deletion of *rr09* showed the biggest increase in adherence compared to T4JH to all cell lines, with the biggest increase seen in T4Δ*rr09* and T4Δ*rr098.* In this instance this would correspond to the strains producing the highest amount of pili shown the highest adherence. It would perhaps in this instance be expected that T4Δ*rr086* would also show an increased adherence. However, this was not the case and this strain showed a trend towards a reduced adherence to HBMEC and A549 cells and a trend towards an increase in adherence to D562 cells, however these differences were not significant. Deletion of RR08 alone showed a trend towards a small reduction in adherence to all cell lines, however again this was not significant compared to T4JH.

Deletion of RR06 showed no difference in adherence to HBMEC cells, however roughly a 5 fold increase in adherence to A549 and D562 cells was observed

compared to T4JH, but this is not significant. As we see a reduced level of pili in T4Δ*rr06* this is clearly not causing the altered adherence and is likely due to another surface protein differentially expressed in T4Δ*rr06,* for instance CbpA.

We see no significant difference in adherence between T4Δ*rr09* and T4Δ*rr098,* suggesting RR08 deletion in T4Δ*rr09* does not significantly alter adherence. However there is a significant difference in adherence of T4Δ*rr096* and T4Δ*rr0986* compared to T4Δ*rr09 and* T4Δ*rr098,* suggesting deletion of RR06 in these strains causes the decrease in adherence. Similar adherence capabilities are observed between T4Δ*rr0986* and T4Δ*rr096*, which show similar levels of pili expressed on the cell surface.

A



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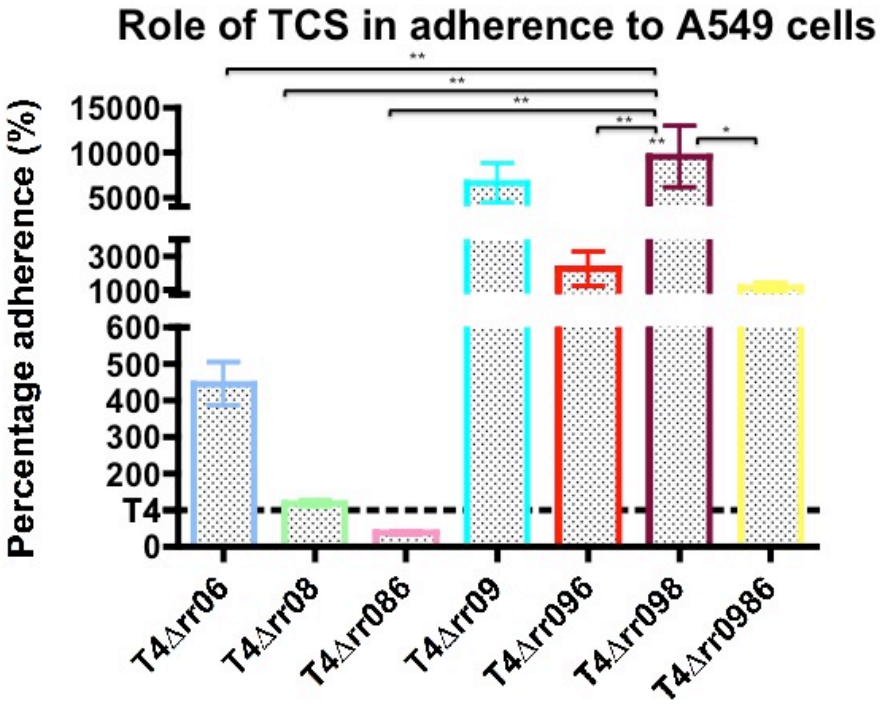

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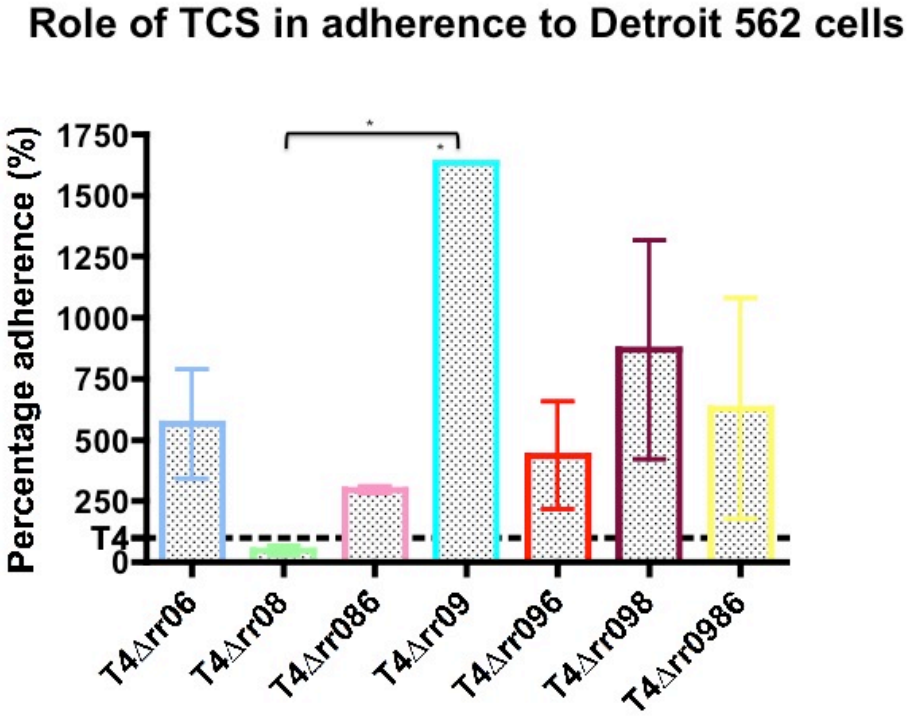

Figure 3-18: Adherence of TCS mutants to different cell lines.

Adherence of TCS mutant strains was assessed to HBMEC (A), A549 (B) and (C) Detroit 562 cell lines. Data is represented as percentage adherence relative to that of T4JH (100%, dashed line), each bar is an average of three replicas except for all HBMEC data and T4Δ*rr09,* T4Δ*rr086,* T4Δ*rr098* Detroit 562 data which is an average of two repeats, the error bars represent the standard error of the mean. Statistical analysis was performed using a 1-way ANOVA with a Tukeys testing correction, for (A) and (C) * P<0.05, ** P<0.001, for (B) * P<0.05, ** P<0.01. * above the bar represent statistical significance compared to T4JH (not represented as a bar on the graphs).

3.7 Discussion

It must initially be noted that the mutants evaluated within this chapter have not been complemented to confirm the phenotype observed in these mutants is not due to accumulation of genetic changes at an alternative point within the genome. However with regards to the alterations in pilus expression all three RR evaluated have been linked in the literature to regulation of this virulence factor and therefore we assume this is due to true regulation by the TCS. Further the gene expression data for RR09 mutant matches that observed in the literature, indicating these changes are due to the RR09 deletion. To fully confirm all changes complementation of all mutants needs to be performed.

A number of the genes regulated by RR08 in our microarray experiments confirmed what was already known from the literature about genes regulated by this system. This system causes repression of the pneumococcal pilus as a deletion mutant showed increased expression of the pilus islet genes (Song et al., 2009). Our microarray data showed no similarities to that of genes differentially regulated in a HK08 mutant constructed in R6, which suggested a role of RR08 in cellobiose metabolism (Mckessar & Hakenbeck, 2007). This may be due to strain specific regulation of genes via this system, as observed in other systems. However in the McKessar study genes classed to be differentially regulated had to show a change in expression of 3 fold or above. This cut off has not been applied to our data sets and therefore genes differentially regulated in this strain below the threshold may show similarities to our data. As this study evaluated a HK kinase mutant rather than a RR mutant this may also be why there are few similarities.

One of the genes differentially regulated in T4Δ*rr08* is that of *argR* (SP_2077) which in D39 has been shown to regulate a number of genes involved in arginine metabolism (Kloosterman & Kuipers, 2011). In the Kloosterman & Kuipers study a single knockout of *argR* showed no similarities between genes regulated by *argR* and RR08. However deletion of another ArgR type regulator *ahrC* (SP_1203) creating D39Δ*argR*Δ*ahrC* shared a number of similarities to that of genes regulated by RR08 in this study, including *purM*, *purN*, *vanZ*, *purH* and *aliB*. In D39Δ*argR*Δ*ahrC bgaA* is differentially regulated which has previously been shown to be regulated by RR08 (Song et al., 2009). Although *ahrC* was not differentially

regulated in our study it may be that in TIGR4 ArgR regulates these genes alone. However, this would need further study.

RR08 in TIGR4 positively regulates *rr01*. Microarray analysis also indicated RR08 regulates RR02 which is repressed by RR08. However this was not shown to be significant via RT-PCR. With regards to RR01 no studies have been performed to assess what genes are regulated alone by this system and so we cannot attribute any of the expression changes observed in T4Δ*rr08* to that of differential expression of this gene.

RR02 was repressed by deletion of RR08 in TIGR4. When comparing the genes regulated by RR02 to that seen in T4Δ*rr08* there were very few similarities perhaps due to experiment being performed in different parent strains (R6 and JNR7/87) which may again suggest RR02 acts in a strain dependant manner (Ng et al., 2005, Mohedano et al., 2005). Both of these studies show RR02 plays a clear role in fatty acid metabolism of which none of the genes required for this are differentially regulated in T4Δ*rr08*. This may indicate that the change in *rr02* expression in T4Δ*rr08* is not a true change. Alternatively perhaps the fact RR02 is the only system which is indispensible means that its regulation is controlled tightly and small changes in its expression are compensated for.

Other than the pilus islet RR08 has been shown to negatively regulate *cbpD* a putative murein hydrolase shown to play a role in competence induced cell lysis (Kausmally et al., 2005). Deletion of *cbpD* in TIGR4 causes a reduced ability to colonise the nasopharynx in an infant rat model and showed a slightly reduced adherence to Detroit 562 cells (Gosink et al., 2000). A RR08 knockout in TIGR4 has previously been shown to be attenuated upon intranasal infection (Throup et al., 2000). However no other studies have analysed the *in vivo* role of RR08. Adherence to D562 cells and HBMEC cells showed a trend towards a reduced adherence in T4Δ*rr08,* however again this was not statistically significant.

One of the other genes shown to be up regulated in T4Δ*rr08* is *mntE* a manganese efflux pump, which has been linked to altered pilus expression, with a T4Δ*mntE* strain when grown in high manganese concentrations showing increased expression of the pilus islet genes (Rosch et al., 2010). There are a

number of other similarities between the expression changes in T4Δ*mntE* and T4Δ*rr08* including in both *cbpD,* SP_1501 and SP_2095 are up regulated and *fucK* is down regulated. From this it could be hypothesised that HK08 responds to external manganese levels and modulates its cellular content by regulating *mntE*. Deletion of RR08 causes an increase in expression of *mntE,* which would correspond with RR08 acting as a repressor of *mntE*. Variations in magnesium concentrations *in vivo* could correspond to being present in a certain niche where lower levels of pili are required and this islet is repressed alongside *mntE* by RR08.

Genes regulated by RR09 in this study again confirmed what was already known about genes regulated by RR09. This includes an up regulation of the pilus islet genes, genes involved in purine biosynthesis and down regulation of a general stress protein, beta-glalctosidase, *pspA* (adhesin) and a ABC transporter (SP_1895-1897) (Hendriksen et al., 2007).

One transcription regulator showing a large down regulation of 15 fold in a RR09 mutant is that of *lacR* (lactose phosphotransferase system repressor). Interestingly this was opposite to that seen in the Hendriksen study, which showed an up regulation of this gene in a RR09 deletion mutant (Hendriksen et al., 2007). *lacR* is known to act as a repressor of genes required for lactose uptake and catabolism. However little data is available in the pneumococcus as to what genes may be differentially regulated when *lacR* is deleted and therefore its contribution to the gene expression changes observed in T4Δ*rr09* cannot be evaluated (Zeng et al., 2010). Other than *lacR* there is no differential regulation of genes in T4Δ*rr09* that are linked to lactose metabolism. The reason for this may be due to the fact there are two *lacR* genes in *S.pneumoniae,* that of SP_0875 differentially regulated in this study and that of SP_1182, which is located next to a large number of genes important for lactose metabolism. This is not the case for SP_0875 and perhaps this *lacR* gene is redundant, or its reduced expression is compensated for by the other *lacR* gene (SP_1182).

In T4Δ*rr09* no other TCS were shown to be statistically differentially regulated. However *rr01*, *rr07* and *rr08* looked to be marginally down regulated. Indication these may be true changes have come from the fact *rr01* and *rr07* are shown to

be statistically differentially regulated in T4Δ*rr098,* which cannot be fully accounted for due to the *rr08* mutant. Further *rr08* expression levels are also down regulated in T4Δ*rr096* to a similar level as in T4Δ*rr09,* which is not due to the *rr06* deletion, and may indicate RR09 positively regulate RR08 at the expression level. This may account for why some of the genes are commonly regulated by RR09 and RR08. As mentioned earlier little is known about RR01 and what genes are regulated by this system, however if this is a true change it seems to be positively regulated by both RR08 and RR09 to a similar level. *rr*07 may also be positively regulated by RR09, this system is also one of the other systems that has not been evaluated with regards to what genes it regulates and would likely contribute to regulating some of the large number of genes differentially regulated in T4Δ*rr09,* but which ones are unknown.

RR09 has also been shown to positively regulate the choline binding protein PspA, as in T4Δ*rr09 pspA* expression is reduced. This virulence factor is highly immunogenic which is why it is a candidate protein to include in a protein based pneumococcal vaccine and has been shown to interfere with complement activation and can bind lactoferrin (Talkington et al., 1991, Hammerschmidt et al., 1999, Tu et al., 1999, Briles et al., 1996). Currently none of the genes shown to be regulated by RR09 have been implicated in regulating *pspA* and therefore this TCS may regulate this gene through binding directly to its promoter region. There is a large increase in the adherence capability of T4Δ*rr09* to all cell lines compared to its parent. The only virulence factor that is up regulated in this strain is that of the pneumococcal pilus so it is assumed this is the reason for the increased adherence. Looking at the cell surface adhesins expression in T4Δ*rr08,* T4Δ*rr09* and T4Δ*rr098* there seems to be no pattern of reduced expression of one cell surface adhesin while there is an increase in expression of the pilus.

Once the genes regulated by the single systems alone were evaluated it allowed estimation of how these systems may interact with regards to the gene they regulate and the potential for each to compensate for the deletion of the other through the use of a double mutant. Comparison of the gene regulated by each system and the double mutant via microarray analysis were done using a venn diagram. From this it seemed that the systems when deleted alone generally regulated their own set of genes as only a small number of genes were shown to

be commonly differentially regulated in both the single and double mutants. This includes *rr07*, which in the double knockout shows similar expression levels to that of the single RR09 mutant, which would indicate this decreased expression is due to regulation by RR09 alone.

22 genes (P<0.1) were shown to be commonly regulated in all three microarray experiments. In most instances the expression differences of the genes in this group compared to T4JH were similar in all three knockout strains. This would suggest that in terms of regulating these genes both systems play an equal role. With perhaps both systems regulating the same gene, which regulates these common genes, for example RR01. Although if this was the case it would be expected in the double mutant that the change in expression of these genes would be equal to the sum of both of the expression changes seen in each of the single mutants. Alternatively there is evidence upon deletion of *rr09* that *rr08* is down regulated so genes found to be similar between the two mutants may be due to the fact RR09 deletion has a knock on effect on *rr08* expression levels. Regulation in this way is summarised in Figure 3-19 using the repression of the genes involved in purine biosynthesis as an example.

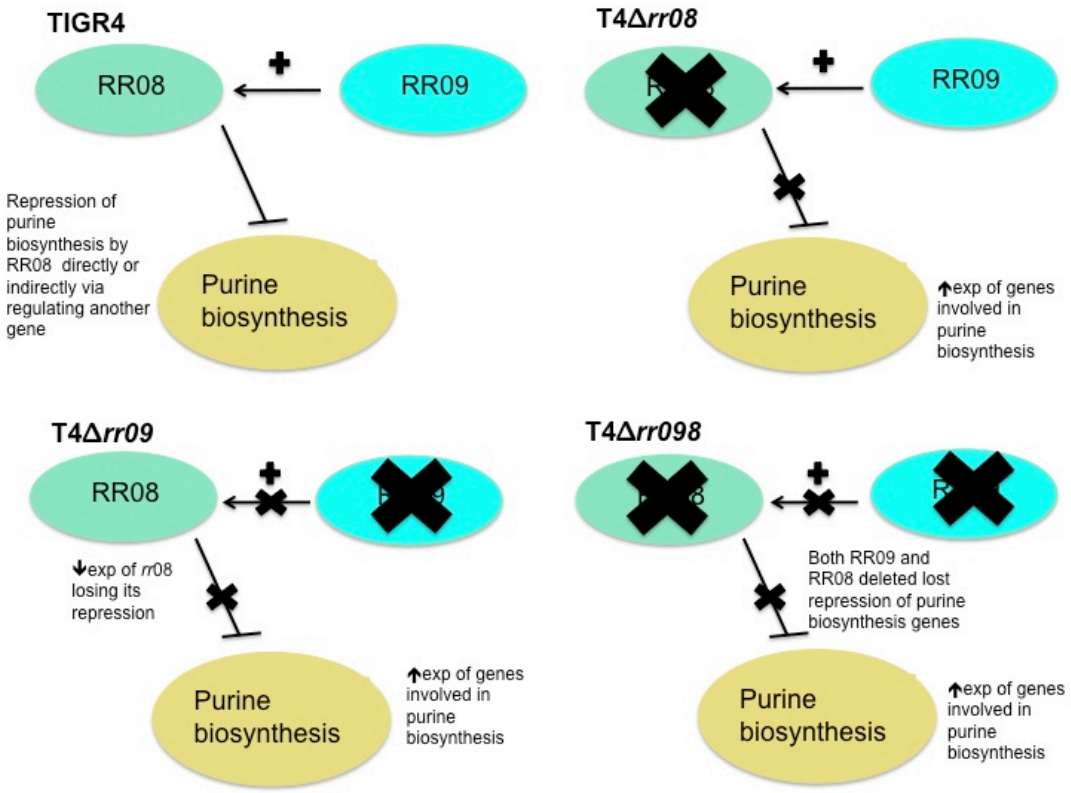

Diagram of gene regulation by RR08 and RR09 when RR09 positively regulates RR08

Figure 3-19: Hypothetical mode of regulation of common genes by RR08 and RR09.

Diagram represents a potential mode of how RR09 and RR08 regulate the same gene., using the regulation of purine biosynthesis genes as an example. Arrows represents positively regulated genes and bars represent genes repressed. Hypothesis is based on RR09 positively regulating *rr08* and RR08 positively regulating *rr09*.

If this was the case it would be expected that genes regulated by RR09 would include all the genes regulated by RR09 alone and all the genes shown to be regulated by RR08. Yet only a small number of genes are shown to be commonly regulated by the two systems. Further the expression of the pilus islet showed the regulation is more complex as deletion of both *rr09* and *rr08* results in a phenotype which shows expression levels between that of the single mutants.

These systems must to some extent interact with one another as deletion of both TCS leads to a large number of genes being differentially regulated that are not seen in the single mutants alone. In the double mutant a total of 210 genes ($P<0.1$) were not differentially regulated in either of the single mutants. The fact deletion of both leads to a totally new collection of genes being differentially regulated would suggest that deletion of both singly is compensated for by the other systems. Whether this would occur upon deletion of two different RR would give an indication if this is common or RR09 and RR08 are both important TCS.

However another explanation for this observation may be that deletion of both systems puts a stress on the cell, or alters in some way the surrounding environment, which leads to the differential expression of the genes which are not shown to be differentially regulated by the single systems alone. The large number of genes shown to be differentially regulated in the double mutant are likely due to the differential expression of *rr04*, *rr05* and *rr13* in the double mutant which are not seen to be differentially regulated in the single mutants.

codY is one of the genes differentially regulated in the double mutant but not in the single mutants. *codY* is a global regulator of genes that are important for adaptation to different nutritional environments. In T4Δ*rr098* there is only one gene shown to be regulated by *codY* in D39 that is also regulated in the double RR mutant, this is that of *dpr* (SP_1572) a starvation induced protein (Hendriksen et al., 2008). These two genes are both down regulated in the double RR mutant

to similar levels. However it has been shown that *codY* cannot be deleted and in the above study genome changes had accumulated in the strain to counteract this harmful deletion, shown via whole genome sequence (Caymaris et al., 2010). Due to this some of the genes shown to be regulated by *codY* in this study may not fully represent its true function. This latter paper links *codY* function with potentially playing a role in altering competence therefore suggesting some regulation of RR12 and potentially having an interaction with *ritR* (ORR). However there was not statistical differential regulation of these systems in T4Δ*rr098* (Caymaris et al., 2010).

Microarray analysis in the double mutant showed an increase in the expression of *rr02* however this was not confirmed by RT-PCR. Yet in T4Δ*rr098* a number of genes involved in fatty acid biosynthesis (P<0.1) were differential regulated shown via microarray analysis, which are known to be regulated by RR02 (Mohedano et al., 2005). However due to the number of regulators that are changed in the double mutant its not unlikely that this may have come about due to one of these. For this reason the genes regulated by each of these systems will not be discussed further with regard to their likely contribution to the genes differentially regulated in T4Δ*rr098*. In regards to *rr06* this like *rr02* was shown to be up regulated by microarray however this was not confirmed by RT-PCR.

In the double mutant along with some RR there are also virulence factors that are differentially regulated that are not observed in the single mutants alone, including *psrP* and *pavA*. *pspA* was not observed to be differentially regulated in the double mutant at all yet *cbpD* which was differentially regulated in T4Δ*rr08* is observed in the P<0.1 gene list of the double mutant. *psrP* (pneumococcal serine rich repeat protein) is found in the genome downstream of a large number of glycosyltransferase which function to glycosylate this protein, none of these were differentially regulated in the double mutant bar *psrP*s adjacent gene SP_1771, and therefore whether this is a true change would require further validation.

psrP has been shown to be important for biofilm formation and lung infection, with it able to bind to keratin 10 on lung epithelial cells (Sanchez et al., 2010,

Shivshankar et al., 2009). PsrP was down regulated in T4Δ*rr098* so wouldn't contribute to the increased adherence to all cell lines observed in this strain, with the highest to that of A549 cells. Although not significant there is a trend towards a lower adherence in T4Δ*rr098* compared to T4Δ*rr09* to HBMEC and D562 cells, this correlates well with the lower number of pili expressing cells in the double mutant compared to the single.

With regards to expression of *pavA* in the double mutant there is only a small 1.1 fold increase in the expression of this gene, this was not further validated by RT-PCR and may not be a true change. This fibronectin binding protein has been shown to be essential for virulence in D39 in a mouse model of bacteraemia (Holmes et al., 2001). Upon deletion of PavA in a serotype 35A and R800 strain a large reduction in adherence and invasion of A549 and HBMEC cells is observed, this may in part account for the increased adherence of the double mutant to these cell lines as *pavA* is marginally up regulated in this strain. However this would not account for the increased adherence in the single *rr09* mutant, which shows similar adherence to the double mutant.

We can clearly see that these systems interact with one another from the data obtained in this study, yet this is just looking at one aspect (gene expression changes). For a clearer picture on how they interact more information is required about the genes that are directly regulated by each RR, which has only been assessed for RR05 on a large scale (Halfman et al., 2007), which evaluated the promoters that RR05 directly bound to through its DNA binding domain. Another key feature elucidated in this paper is that of the role of RR in regulation of small non-coding RNAs, which would not be picked up by our microarrays. Information about whether there is potential for the RR to interact with each other at the protein level may further add to the complexity, but more likely is the potential of one HK to phosphorylate multiple RR. Another key to understanding these systems is what the extracellular stimulus to which they respond. This is known to be CSP for HK12 and BLP for HK13 yet this is still unknown for all the other systems, (de Saizieu et al., 2000, Pestova et al., 1996). This is vital to understand during infection where these systems might be activated.

Further studies to try and understand potential communication between these systems was using the expression of the pilus genes to give an indication of their interactions, as the systems chosen were all able to alter pilus expression. This was performed by assessing the expression level of all seven genes on the pilus islet in the mutant strains relative to T4JH. FACS analysis was used to determine the RrgB expression within a population of these mutants, as recent studies have indicated within a growing population pili negative and positive cells exist (Basset et al., 2011, De Angelis et al., 2011). To date only RrgA has been shown to modulate this function through forming a protein-protein interaction with RlrA, preventing it from positively regulating the islet, upon deletion of *rrgA* almost all bacterial cells are pili positive (Basset et al., 2011, Basset et al., 2012). This analysis will show in these TCS mutants whether the altered pili expression is due to increases in pili on a single cells or increased numbers containing the pilus, or perhaps both.

Contrary to the published role of TCS06 in pilus regulation our data showed RR06 to act as a positive regulator of the operon rather than that of a repressor (Rosch et al., 2008). The most likely reason for this is that studies performed by Rosch were performed in a HK06 mutant rather than a RR06 mutant which is used in this study (Rosch et al., 2008). In an earlier study when assessings TCS06 role in virulence deletions of HK06 or RR06 showed opposite effect with regards to virulence, it is therefore possible different genes are regulated upon deletion of RR06 or HK06, which may be linked to the fact RR06 can be phosphorylated by an alternative kinase (StkP) (Standish et al., 2005, Agarwal et al., 2012). Alternatively this could also be due to variations in the growth conditions under which both experiments were performed, with TCS06 acting as a repressor of the pilus islet upon growth in C+Y media when grown to mid log (Rosch et al., 2008), and TCS06 acting as a positive regulator of the operon when grown in BHI to OD_{600nm} 0.6 (this study). Variations in growth conditions of a T4Δ*rr09* strain have been shown to alter whether RR09 acts as a repressor or activator of the pilus islet (Hendriksen et al., 2007). In our study all three RR have also shown to act both as repressors and positive regulators of the pilus islet depending on the presence of the other RR and this may be why we see variations in the RR function when grown under different conditions as variations in the activity of

other interacting TCS are occurring. The role of the three RR in the presence of others is summarised in Table 3-7.

Strain	RR remaining	RrgB positive cells	Repressor/ activator
T4JH	RR06, RR08, RR09	20%	N/A
T4Δ*rr06*	RR08, RR09	4%	RR06 activator (dec exp comp T4JH)
T4Δ*rr08*	RR06, RR09	37%	RR08 repressor (inc exp comp T4JH)
T4Δ*rr09*	RR06, RR08	91%	RR09 repressor (inc exp comp T4JH)
T4Δ*rr086*	RR09	57%	RR08 repressor (inc exp comp T4Δ*rr06*) RR06 repressor (inc exp comp T4Δ*rr08*)
T4Δ*rr096*	RR08	28%	RR09 repressor (inc exp comp T4Δ*rr06*) RR06 activator (dec exp comp T4Δ*rr09*)
T4Δ*rr098*	RR06	56%	RR09 repressor (inc exp comp T4Δ*rr08*) RR08 activator (dec exp comp T4Δ*rr09*)
T4Δ*rr0986*	NONE	27%	RR06 activator (dec exp comp T4Δ*rr098*) RR08 neither (no diff comp T4Δ*rr096*) RR09 activator (dec exp comp T4Δ*rr086*)

Table 3-7: Summary table of RR function

Summary table of findings in this study. Showing strain, which RR are still present after deletion, the amount of RrgB positive cells within a growing population and whether in each strain the RR acts as a repressor or activator of the pilus operon. Dec = decrease, inc= increase, exp= expression, comp = compared to.

One initial hypothesis taken from this data is that RR08 does not modulate the altered pilus expression observed upon its deletion in T4JH via direct binding to the *rlrA* promoter. Indication this is the case is that deletion of RR08 in T4Δ*rr096* (T4Δ*rr0986*), causes no difference in pilus expression and no change in the number of pili positive cells within a population compared to T4Δ*rr096*. However upon deletion of RR08 in T4Δ*rr09* or T4Δ*rr06* mutants alone there is a decrease and increase in pilus expression and pili positive cells observed respectively. This may suggest RR08 regulates alterations in pili via both RR09 and RR06, however this is likely not at the expression level as there is no observed difference in *rr06* and *rr09* expression in T4Δ*rr08*.

Even in the triple RR mutant there is still an increased pilus expression with an increase of roughly 6% more pili positive cells compared to T4JH, this confirms there are other factors than these three RR interacting to alter pilus expression under the conditions of our experiment. This may suggest that factors competatively bind to the pilus islet promoters, and deletion of RR08, RR09 and RR06 allows binding of other factor, however whether RR09 and RR08 bind directly to the islet is still unknown.

The single RR09 mutant alone causes a large increase in the number of pili positive cells within a population with 91% of cells pili positive upon its deletion. In this strain the ability to distinguish between positive and negative cells is lost with one large peak seen via FACS rather than the two clear positive and negative populaitons seen in T4JH.

As already noted RR06 in our studies acts as a positive regulator of the pilus islet as upon its deletion there is a decrease in pilus expression as well as a decrease in the number of pili positive cells (4%). However when *rr06* is deleted in T4Δ*rr08* there is an increase in pili expression. Interstingly FACS analysis of T4Δ*rr086* again showed one single peak rather than the two positive and negative populations. This could either indicate that RR09 is non-functional in T4Δ*rr086* or in the *rr09* knockout the alterations in pilus expression is due to RR08 and RR06 being non-functional in T4Δ*rr09*. Another interesting finding is that upon deletion of *rr06* or *rr09* in T4Δ*rr08* the same phenotype is observed with 56% and 57% of cells being pili positive in T4Δ*rr098* and T4Δ*rr086* respectively.

It has been shown that modulation of the number of pili positive cells at the population levels occurs through a protein-protein interaction of RlrA and RrgA (Basset et al., 2011, Basset et al., 2012). RrgA once bound to RlrA prevents it binding to the promoters of the pilus islet and positively regulating its expression. All the TCS tested in this study have been shown to alter pilus expression at the population level and this may be through modulating the expression levels of *rrgA* realtive to *rlrA*. T4Δ*rr086* shows a large increase in expression of *rlrA* relative to *rrgA*, which may account for why over double the number of T4Δ*rr086* cells contain pili on the surface relative to T4JH. Both T4Δ*rr0986* and T4Δ*rr096* also show marginally higher expression levels of *rlrA* relative to *rrgA*. However this is not the case for T4Δ*rr098* and T4Δ*rr08* which have slighlty lower *rlrA* levels relative to *rrgA*. Most surprisingly *rrgA* and *rlrA* expression levels are almost identical in T4Δ*rr09* yet this strain showed the largest increase in the number of pili positive cells within the population. Therefore RR09 must act to modulate pilus expression at the population level by another means.

From this data a model of how these systems regulate the pilus and therfore interact has not been constructed as no data it available yet as to whether RR08 and RR09 bind directly to the *rlrA* promoter. A further limiting factors is in this study only three of the genes known to affect pilus expression are being assessed, yet data is available to show RR03, MgrA and MerR bind directly to the *rlrA* promoter modulating its activity(Rosch et al., 2008, Hemsley et al., 2003). Further RR10 and RR05 alter pilus expression upon their deletion either directly or indirectly through another factor (Haas et al., 2004, Sebert et al., 2002).

Regulation at the population level of pili has also been observed in *S.pyogenes* but how this is modulated is yet to be discovered. It may be that TCS in this species are also important in this function (Nakata et al., 2009). In this instance however the link has been made to the conditions under which this phenomenon is modulated, with varying pili levels observed when grown at different temperatures. It is likely in the pneumococcus that some form of environmental cue is also modulating this regulation and this function as shown in this study is through the regulation by TCS.

It perhaps seems unnecessary for these RR to all act on the pilus islet, however *in vivo* this is likely not the case. Our experiments are performed upon growth in BHI media, which contains all the nutrients required for growth. *In vivo* in different niches (lungs, nasopharynx, brain, blood) different nutrients will be limited and it is likely under these conditions there would be variations in the regulation of the pilus by these RR. It being the case that one TCS is able to recognise presence in the nasopharynx, and alter pilus expression accordingly, one may sense being in the lungs and modulate pilus expression etc. Currently the external stimulus that activates these TCS is unknown and requires further study.

More recent data has indicated that response regulators are not always phosphorylated by their cognate HK on an aspartate residue. Two pneumococcal response regulators RitR (ORR) and RR06 are also phosphorylated by StkP (serine/ threonine protein kinase). Although RitR has no cognate HK, RR06 does. RR06 phosphorylation by StkP is on a threonine residue and has been shown to alter its binding capacity for target genes, when aspartate is not phosphorylated (Agarwal et al., 2012), this has also been seen in other bacterial species (Lin et

al., 2011). StkP in the pneumococcus contains four extracellular PASTA domains which in penicillin binding proteins have been shown to bind to unlinked peptidoglycan (Gordon et al. 2000, Maurer et al. 2012). The PASTA domains in StkP have been shown to bind to synthetic peptidoglycan, which activates the kinase which then functions to phosphorylate protein targets (Maestro et al. 2010, Beilharz et al. 2012). The fact StkP phosphorylates RR06 may suggest this kinase is also able to modulate the cell surface constituents as this RR has been shown to regulate three key pneumococcal adhesins (pilus, PspA, CbpA) (Rosch et al. 2008, Standish et al., 2005, Standish et al., 2007).

This may indicate a more complex scenario of how RR modulate gene expression by being able to modulate genes in more that two forms. RR could be found in a non phosphorylated state, phosphorylated on an aspartate residue or phosphorylated on a threonine residue, with in each form a different set of genes can be regulated due to the conformational change of the DNA binding domain. It could perhaps be envisaged that phosphorylation on both sites at the same time could create another conformation, regulating another set of genes. There is currently no data available which directly compares phosphorylation on different sites and how this may alter gene regulation.

We can summarise from this study that all three RR do interact with each other, whether that be competing for the same binding site or forming some protein-protein interaction with one another etc, currently it is not know which and would require further study. Binding of two response regulators to each other has not been observed, but HK from two different TCS pairs can act in this way to impede autophosphorylation (Vincent et al., 2010). RR08 seems to require either RR06 or RR09 to function properly, as RR08 shows no functionality when both are deleted. Upon deletion of RR06 or RR09 in a T4Δ*rr08* mutant we see the same resulting phenotype, suggesting RR08 is required in some way to modulate their function. Deletion of RR09 alone causes a phenotype where the amount of pili positive cells is deregulated, we also see this upon deletion of RR08 and RR06, this again may suggest RR09 requires RR06 or RR08 to modulate its function and in T4Δ*rr086* RR09 has a reduced function.

Genes regulated by these systems are often important for virulence confirmed by our adherence assay data, this data has also given an indication on potential

niche specific regulatory functions of these RR. The highest increase in adherence to all cell lines was observed upon deletion of RR09. With the highest increase seen in T4Δ*rr09* and T4Δ*rr098,* with no statistical difference between the percentage adherences of the two strains to all cell lines. The highest increase in the adherence of the RR09 mutant strains was observed to A549 cell lines suggesting a role of this RR in pneumonia, perhaps modulating the levels of adhesins required in certain environments.

T4Δ*rr09* has been shown to have a reduced virulence upon intranasal infection, with a reduced ability to disseminate to the blood (Hendriksen et al., 2007). Perhaps an inability to regulate the pilus hinders translocation to the blood where you would perhaps require fewer surface adhesins. Other adhesins were also differentially regulated in T4Δ*rr09* from our microarray data including *pspA* in T4Δ*rr09* and *psrP* in T4Δ*rr098*, however both were down regulated and therefore wouldn't contribute to the increased adherence.

In T4Δ*rr096* and T4Δ*rr0986* there is a drop in adherence to all cell lines compared to T4Δ*rr09* and T4Δ*rr098* however there is a trend towards a higher adherence than that of T4JH, yet not significant. The drop correlates with that of decreased pilus expression with both strains showing 26-27% pili positive cells within a population, both show similar adherence capabilities to all cell lines with no significant difference between the two.

Deletion of RR08 has shown an increase in adherence to A549 cells after incubation for 3 hours and a reduced adherence to D562 cells post 3 hours incubation (Song et al., 2009), no difference was observed post 1 hour incubation. In the adherence assays reported here bacteria were incubated with cells for 2 hours and no statistical difference in adherence of T4Δ*rr08* compared to T4JH to all cell lines was seen. There is a trend towards a reduced adherence to HBMEC cell lines however this was not significant. There is an increase in expression of the pilus in T4Δ*rr08* so you would perhaps expect to observe an increased adherence in this strain however this is not the case.

Adherence assay data of T4Δ*rr086* showed no statistical difference in adherence to all cell lines compared to T4JH, with its adherence capabilities similar to that

of T4Δ*rr08*. There is a trend towards an increase in adherence to D562 cells however this is not significant. The large increase in pili positive cells in T4Δ*rr086* would be expected to cause a marked increase in adherence, but although more cells contain pili on the surface there are less pili per cell as indicated by a shift to the left of the FITC peak via FACS analysis. This could indicate the pili are shorter, or perhaps just fewer pili per cell and therefore are unable to bind as efficiently. T4Δ*rr08* has been shown to be attenuated using intranasal infection models (Throup et al., 2000), however no other studied have analysed the *in vivo* role of RR08.

The decreased expression of the pneumococcal pilus in T4Δ*rr06* was not associated with reduced adherence to all cell lines. A reduced adherence was observed to D562 and A549 cells in a RR06 mutant constructed in D39 and R6 background (Standish et al., 2005, Ma & Zhang, 2007). Whole genome expression analysis was not performed on our T4Δ*rr06* mutant, however that of published data showed another important virulence factor *cbpA* is regulated by RR06 and may be affecting adherence (Standish et al., 2005, Ma & Zhang, 2007). Deletion of RR06 in D39 caused a increase colonisation phenotype in a *in vivo* mouse model and showed an increased ability to cause lung infection, this was shown not to be due to CbpA and D39 does not contain the pilus and is therefore not due to this, which could be due to other virulence factors also regulated by RR06, such as PspA (Standish et al., 2005, Standish et al., 2007). Upon deletion of HK06 in TIGR4 no virulence defect was observed in a pneumonia mouse model (Rosch et al., 2008).

The facts in all RR mutants we see variations at the population level in pilus expression rather than at the single cell level (bar in T4Δ*rr086* where we see variations in both) would suggest these systems may act as quorum sensing systems. With the set environmental stimulus e.g. Mn^{2+} concentration upon reaching a certain level causes activation of the TCS. Cells in the proximity of the set external stimulus will therefore have activated TCS and for example up regulate cellular pili levels and the cells further away will contain no pili. During *in vitro* growth you would perhaps not expect much variation in the external environment conditions with regards to metabolite proximity to certain cells, however metabolites produced by some bacterial cells and not others may

provide this external stimulus e.g. exogenous H_2O_2 levels. A number of other TCS in the pneumococcus are regulated by quorum sensing (ComDE, BlpHR), which recognise levels of an external peptide, which upon reaching a critical level activates the TCS (de Saizieu et al., 2000, Pestova et al., 1996). Further study into the external stimulus activating the TCS would help to confirm or refute this hypothesis.

4 Xen35

4.1 Aim of this chapter

Xen35 is a bioluminescent serotype 4 *S.pneumoniae* strain, used for studying disease progression *in vivo*. This strain enables users to visually track disease progression in a mouse model of pneumonia, septicaemia and meningitis. However this strain has been genetically manipulated to enable this trait yet has not been fully characterised to show no other genotype and phenotype changes have occurred upon genetic manipulation. Preliminary experiments using Xen35 showed some clear differences in phenotypic traits compared to TIGR4 therefore in this chapter Xen35 is fully evaluated in regards to genotype and phenotype changes compared to is parent TIGR4.

Preliminary experiments indicated Xen35 was less virulent than TIGR4. Initial whole genome sequencing was performed on Xen35 to deduce any genetic changes in this strain lying outwith the known *lux* insertion site, and confirmed positioning of this insertion. Gene expression changes were also analysed using microarray, RT-PCR and RNA-seq analysis. There were noticeable amount of genomic and expression changes, including those of some key virulence factors in the pneumococcus.

To further evaluate changes in one of these virulence factors, the pneumococcal pilus, western blotting and FACS analysis was performed to see if differences seen by microarray correspond to that of changes at the protein level. Finally to further assess the potential impact of all these changes on virulence MF1 mice were infected with Xen35 and TIGR4 and both of their abilities to cause disease determined.

4.2 Background

Bioluminescence is produced by a chemical reaction that occurs in living organism resulting in the emission of energy in the form of light (see reviews Engebrecht & Silverman., 1984, Meighen., 1991). A wide variety of organism in nature are able to do this, including fungi, fish, squid and most importantly here bacteria. Five genes encode the required proteins needed for the bioluminescence reaction. LuxA and LuxB form the luciferase enzyme heterodimer required for catalysing the bioluminescence reaction. LuxC, LuxD and LuxE form a multienzyme fatty acid reductase complex, composed of a reductase, transferase and a synthetase respectively. These enzymes are required for the synthesis of the aldehyde substrate required during the bioluminescence reaction. These five genes are common to all species that bioluminesce however the substrate of the reactions can vary (see reviews Engebrecht & Silverman., 1984, Meighen., 1991). A common feature of all bioluminescence reactions is that of the requirement of oxygen for the reaction to occur. Other regulatory genes are also often found in a number of species however these are not vital for the reaction. The bioluminescence reaction itself consists of oxidisation of reduced flavin mononucleotide ($FMNH_2$) and a long chain aldehyde (RCHO) producing light, flavin mononucleotide (FMN), fatty acids (RCOOH) and water shown below in Equation 4-1.

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + H_2O + RCOOH + Light\ (490nm)$$

Equation 4-1: Equation of bioluminescence reaction.

This chemistry can be used as a scientific tool by using the *lux* genes as reporter genes *in vitro* and *in vivo* (Engebrecht et al., 1985, Contag et al., 1995, Jenkins et al., 2003). Xen35 the bioluminescent version of *S.pneumoniae* strain TIGR4 (Tettelin et al., 2001). Xen35 is one of a number of *S.pneumoniae* Xen strain constructed by Xenogen Corporation. These Xen strains have been used widely in the pneumococcal field to assess virulence and disease progression of different strains, strains tissue tropisms and *in vivo* gene expression (Orihuela et al., 2003, Orihuela et al., 2004).

The first Xen strain constructed was that of a serotype 2 strain D39 (Xen7) (Francis et al., 2001). This strain was constructed via placing the *lux* genes (*luxA-E*) amplified from *Photorhabdus luminescens* (formerly known as *Xenorhabdus luminescens*) upstream of a kanamycin resistance cassette into a plasmid flanked by two *S.aureus* transposon (Tn4001) inverted repeats, this plasmid (pAUL-A Tn4001 luxABCDE Km^R) also contained the transposon gene required for excision of this region (Francis et al., 2001). The *lux* gene configuration *luxABCDE* was previously altered from that of *luxCDABE* naturally seen in *P.luminescens,* and the ribosome binding site altered for use in gram positive pathogens (Francis et al., 2000). Upon transformation of pAUL-A Tn4001 luxABCDE Km^R Into the naturally competent *S.pneumoniae* strain D39 the transposon flanked *lux* genes were excised from the plasmid and randomly integrated into the D39 genome. Transformants were selected using kanamycin. As no promoter was placed upstream of the *lux* genes in the plasmid transformants were screened using an ICCD camera for high bioluminescence indicating the insertion of the *lux* genes downstream of a promoter, creating Xen7. Xen7 genomic DNA was then isolated and transformed into a number of other *S.pneumoniae* strains constructing a collection of *S.pneumoniae* bioluminescent strains that can be used for *in vivo* analysis of disease progression (Francis et al., 2001, Orihuela et al., 2003). This technology allowed integration into the genome of the *lux* genes whereas previous studies expressed the *lux* genes on a plasmid. Using chromosomal integration increases the length bioluminescence can be followed as no antibiotic selection is required unlike with the plasmid based method (Francis et al., 2000).

4.3 Xen35 whole genome sequence

4.3.1 Genome sequence

Preliminary virulence analysis showed Xen35 was less virulent that TIGR4. Therefore whole genome sequencing was performed on Xen35 to assess any genome changes other than the *lux* insertion found in this strain compared to TIGR4, which may alter virulence. Sequence reads were reference assembled to the TIGR4 genome sequence data available at NCBI (NC_003028). A de novo assembly, assembling the sequence reads against themselves was also performed. Upon reference assembly of the sequence reads there were a total of 18 low coverage regions, where the sequence data was either of too low quality, sequence reads did not assemble due to variation within the region between the two strains, or this region was not present in Xen35. All of these low coverage regions were analysed further by Sanger sequencing to confirm changes in these regions. The de novo assembly created 271 contigs of which contig 48 contained the *lux* genes (unable to align to the TIGR4 sequence). The de novo assembly enabled further assessment of the low coverage regions, as divergent regions in Xen35 would assemble. SNP and indel analysis was also performed on the reference assembly. A total of 243 changes (SNPs/insertions/ deletions) were seen in Xen35 when compared to TIGR4, all of which can be seen in appendix III. This includes 8 deletions (Table 4-1), 15 insertions (Table 4-2) and 220 SNPs. Of theses SNPs 139 are located in intergenic regions or synonymous, but 81 lead to 73 amino acid changes in 34 genes (Table 4-3).

Gene	Function	Position TIGR4	Position Xen35	NT	Outcome
SP_0200 / 0201	SP_0200 Competence-induced protein Ccs4. SP_0201 Hypothetical protein	188293	188292-188293	C	SP_0200 removal of stop codon. SP_0201 frame shift. Premature stop codon.
INT SP_0296 -0297		273379	273378-273379	G	
SP_0491	Hypothetical protein	469288	469286-469287	C	Frameshift. Removal of stop codon
INT SP_1029		972510	972509-972510	T	

-1030					
SP_1732	Serine/Threonine protein kinase.	1634948 - 1635163	1634951 - 1634952		216bp deletion between amino acid repeats TQIVLTVAKKA/ TQIVLTVAKKV, removing PASTA domain 3.
INT SP_1851 -1852		1759031	1758820 - 1758821	A	
SP_1920	MarR family transcriptional regulator.	1829756	1837438 - 1837439	T	Frameshift. Premature stop codon.
SP_1920	As above.	1829840	1837521 - 1837522	A	Frameshift. Premature stop codon.

Table 4-1: Deletions in Xen35 compared to TIGR4.

INT refers to changes observed in intergenic regions.

Gene	Function	Position TIGR4	Position Xen35	NT	Outcome
SP_0206	Hypothetical protein	192436-192437	192435	C	Frameshift, removal of stop codon.
INT SP_0630-0631		597325-597326	597324	G	
INT SP_0885-0886		834922-834923	834923	C	
INT SP_1053-1054		991106-991107	991106	C	
INT SP_1199-1200		1132344-1132345	1132346	G	
INT SP_1199-1200		1132391-1132392	1132394	G	
SP_1715	Hypothetical protein	1618515-1618516	1618519	G	Frameshift. Premature stop codon.
INT SP_1775-1776		1113285-1113286	1113286	C	
INT SP_1776-1777		1695199-1695200	1694988	T	
INT SP_1777-1778		1696085-1696086	1695875	C	
INT SP_1928-1929		1835757-1835758	1843439-1843440	AT	
INT SP_1928-1929		1835782-1835783	1843466-1843489	24b	

				p	
INT SP_1928-1929		1835840-1835841	1843548	A	
SP_2076	Authentic frameshift	1985218-1985219	1992927	G	Frameshift, removal of frameshift.
INT 2105-2106		2016331-2016332	2024041	G	

Table 4-2: Insertions in Xen35 compared to TIGR4

INT refers to changes observed in intergenic regions.

Gene	Function	Position TIGR4	Position Xen35	Amino acid TIGR4	Amino acid Xen35	Origin (D39/ TIGR4)
SP_0148	ABC transporter, substrate binding protein.	146055	146055	Leu (TTG)	Phe (TTC)	D39
SP_0272	30S ribosomal protein S7.	247805	247805	Lys (AAA)	Glu (GAA)	D39
SP_0715	Lactate oxidase	681177	681176	Gly (GGA)	Stop (TGA)	Neither
SP_0730	Pyruvate oxidase	695462	695461	Glu (GAG)	Stop (TAG)	Neither
SP_0784	Glutathione reductase.	737108	737107	Cys (TGC)	Arg (CGC)	D39
SP_0807	Septation ring formation regulator EzrA.	762843	762842	Gln (CAA)	Lys (AAA)	Neither
SP_0904	Hypothetical protein.	857447	857447	Asn (AAC)	Thr (ACC)	Neither
SP_0927	LysR family transcriptional regulator.	880600	880600	Ala (GCT)	Val (GTT)	Neither
SP_1166	MATE efflux family protein.	1101585/ 1101586	1101585 /1101586	Arg (CGT)	Ala (GCT)	D39
SP_1190	Tagatose 1,6-diphosphate aldolase.	1127020	1127022	Val (GTT)	Leu (CTT)	D39
SP_1190	As above.	1127021	1127023	Cys (TGC)	Trp (TGG)	D39
SP_1343	Prolyl oligopeptide family protein.	1267219/ 1267220	1267222 / 1267223	Thr (ACG)	Ser (AGC)	D39
SP_1715	Hypothetical protein	1618223	1618226	Glu (GAA)	Gly (GGA)	D39
SP_1733	Phosphatase, putative.	1636745	1636533	Ala (GCT)	Gly (GGT)	Neither
SP_1891	ABC transporter, oligopeptide binding	1796273	1796062	His (CAT)	Tyr (TAT)	Neither

	protein AmiA.					
SP_1908	Single stranded DNA binding protein.	1822699	1822488	Ala (GCT)	Val (GTT)	Neither
SP_1908	As above.	1822817	1822606	Leu (TTG)	Met (ATG)	D39
SP_1909	Short chain dehydrogenase/ reductase family oxidoreductase.	1823433/ 1823434	1823222 / 1823223	Gln (CAG)	Arg (AGG)	D39
SP_1910	Hypothetical protein.	1823880	1823669	Leu (CTC)	Phe (TTC)	D39
SP_1910	As above.	1823968/ 1823969	1823757 / 1823758	Thr (ACT)	Ile (ATA)	D39
SP_1910	As above.	1824141	1823930	Thr (ACA)	Ser (TCA)	D39
SP_1910	As above.	1824220	1824009	Asp (GAT)	Glu (GAA)	D39
SP_1910	As above.	1824264	1824053	Asp (GAT)	Asn (AAT)	D39
SP_1911	Putative thioredoxin	1824391/ 1824393	1824180 / 1824182	Glu (CAA)	Asp (GAC)	Neither
SP_1911	As above.	1824588	1824377	Ser (AGC)	Gly (GGC)	D39
SP_1911	As above.	1824609	1824398	Ile (ATT)	Leu (CTT)	D39
SP_1913	Authentic frameshift.	1825256	1825045	Ser (AGT)	Gly (GGT)	D39
SP_1914	Hypothetical protein	1825712	1825501	Ile (CTA)	Thr (ACT)	D39
SP_1914	As above.	1826142	1825931	Leu (CTC)	Ile (ATC)	D39
SP_1915	Hypothetical protein	1826478/ 1826479	1834161 / 1834162	Ala (GCA)	Val (GTT)	D39
SP_1915	As above.	1826503	1834186	Asn (AAT)	Ser (AGT)	D39
SP_1915	As above.	1826525	1834208	Ser (TCT)	Leu (GCT)	D39
SP_1916	PAP2 family protein.	1827142	1834825	Asp (GAT)	Asn (AAT)	D39
SP_1916	As above.	1827352	1835035	Cys (TGT)	Arg (CGT)	D39
SP_1916	As above.	1827368	1835051	Ser (AGC)	Arg (AGA)	D39
SP_1918	ABC transporter, ATP binding protein.	1828094	1835777	Pro (CCG)	Ser (TCG)	D39
SP_1918	As above.	1828115	1835798	Arg (CGT)	Cys (TGT)	D39
SP_1918	As above.	1828142	1835825	Arg (AGA)	Gly (GGA)	D39
SP_1919	ABC transporter, permease protein.	1828907	1836590	Leu (CTC)	Phe (TTC)	D39
SP_1919	As above.	1829040/ 1829041	1836723 /1829024	Leu (TTG)	Stp (TAA)	D39
SP_1919	As above.	1829231	1836914	Ile (ATC)	Val (GTC)	D39
SP_1919	As above.	1829312	1836995	Ser (TCA)	Pro (CCA)	D39
SP_1919	As above.	1829318	1837001	Arg (CGT)	Cys (TGT)	D39

SP_1919	As above.	1829335	1837018	Gly (GGC)	Asp (GAC)	D39
SP_1919	As above.	1829383	1837066	Thr (ACG)	Met (ATG)	D39
SP_1919	As above.	1829537	1837220	Ser (TCC)	Pro (CCC)	D39
SP_1919	As above.	1829561	1837244	Ser (TCG)	Pro (CCG)	D39
SP_1919	As above.	1829587	1837270	Glu (GAA)	Gly (GGA)	D39
SP_1920	MarR family transcriptional regulator.	1829980	1837661	Pro (CCT)	Ser (TCT)	D39
SP_1920	As above.	1830130	1837811	His (CAC)	Tyr (TAC)	D39
SP_1923	Pneumolysin.	1832174	1839855	Asn (AAT)	Asp (GAT)	D39
SP_1924	Hypothetical protein.	1833393	1841074	Tyr (TAC)	His (CAC)	D39
SP_1927	IS1381, transposase OrfA	1834935	1842616	Tyr (TAT)	Asp (GAT)	D39
SP_1927	As above.	1834962	1842643	Ala (GCA)	Thr (ACA)	Neither
SP_1927	As above.	1835012	1842693	Ile (ATA)	Met (ATG)	D39
SP_1928	IS1381, transposase OrfB	1835482/ 1835483	1843163 / 1843164	Gly (GGT)	Asp (AAT)	Neither
SP_1928	As above.	1835507	1843188	Cys (TGT)	Tyr (TAT)	D39
SP_1928	As above.	1835558	1843239	Ala (GCT)	Val (GTT)	Neither
SP_1928	As above.	1835629	1843310	Glu (GAA)	Lys (AAA)	D39
SP_1928	As above.	1835657	1843338	Tyr (TAT)	Cys (TGT)	Neither
SP_1928	As above.	1835699	1843380	Ile (ATT)	Ser (AGT)	D39
SP_1930	Hypothetical protein	1836482	1844190	Leu (CTC)	Phe (TTC)	D39
SP_1931	Hypothetical protein	1836923	1844631	Glu (GAA)	Val (GTA)	D39
SP_1931	As above.	1836993	1844701	His (CAT)	Tyr (TAT)	D39
SP_1931	As above.	1837074	1844782	Pro (CCT)	Ser (TCT)	D39
SP_1933	Hypothetical protein.	1837534	1845242	Stp (TGA)	Gly (GGA)	Neither
SP_1933	As above.	1837543	1845251	Val (GTT)	Ile (ATT)	Neither
SP_1933	As above.	1837602	1845310	Ile (ATT)	Thr (ACT)	Neither
SP_1934	Hypothetical protein.	1838778	1846486	Tyr (TAT)	His (CAT)	D39
SP_1934	As above.	1838873	1846581	Gly (GGG)	Ala (GCG)	D39
SP_1934	As above.	1839074	1846782	Leu (TTG)	Ser (TCG)	D39
SP_1935	Hypothetical protein	1839292	1847000	Gly (GGA)	Val (GTA)	Neither
SP_1935	As above.	1839298	1847006	Ala (GCA)	Val (GTA)	D39

Table 4-3: SNPs causing amino acid changes in Xen35 genes compared to TIGR4

Using the table above the major genome changes have been compiled into Table 4-4, consisting of genes that contain frameshifts due to insertions or deletions or contain amino acid changes leading to introduction of a premature stop codon.

Or includes genes that contain large deletions which may alter the functionality of the resulting protein. Although SNPs leading to single amino acid changes in the resulting protein may have a severe effect on the proteins function these have not been further validated with regards to their effect in Xen35 due to their shear number.

Gene	Function	Change	Outcome
SP_0200/ 0201	SP_0200 -Competence-induced protein Ccs4. SP_0201 Hypothetical protein	C deletion	SP_0200 removal of stop codon. SP_0201 frame shift. Premature stop codon.
SP_0206	Hypothetical protein	C insertion	Frameshift, removal of stop codon.
SP_0491	Hypothetical protein	C deletion	Frameshift. Removal of stop codon
SP_0715	Lactate oxidase (LctO)	SNP (G>T)	Amino acid change, Glycine > stop.
SP_0730	Pyruvate oxidase (SpxB)	SNP (G>T)	Amino acid change, Glutamine > stop.
SP_1715	Hypothetical protein	G Insertion	Frameshift. Premature stop codon.
SP_1732	Serine/Threonine protein kinase. (StkP)	216bp deletion	Removal of PASTA domain 3 between amino acid repeats TQIVLTVAKKA/ TQIVLTVAKKV,
SP_1914	Hypothetical protein	*luxA-E* insertion	Gene loss of function
SP_1919	ABC transporter, permease protein.	SNP (TG>AA)	Amino acid change, Leucine > stop
SP_1920	MarR family transcriptional regulator.	T and A deletion	Frameshift. Premature stop codon.
SP_2076	Authentic frameshift	G Insertion	Frameshift, removal of frameshift.

Table 4-4: Genome changes in Xen35 resulting in loss of function of the protein encoded or proteins containing large in frame deletions.

Table compiling the changes in Xen35 that cause the gene encoded protein to become non-functional or to likely cause a reduced function.

Included in Table 4-4 is the gene encoding the eukaryotic like serine/ threonine protein kinase, a global regulator of gene expression in *S.pneumoniae*, which contains a deletion of 216bp (72 amino acid) (Saskova et al., 2007). This deletion still leaves the protein in frame so whether this affects the functionality of StkP is unknown. It appears that the deletion has occurred between two identical 10 amino acid repeats (TQIVLTVAKK) present at the end of the second and third PASTA (penicillin binding protein and serine threonine kinase associated) domains respectively. Which have been shown to play a role in sensing the external environment and relaying this information to the N-terminal kinase

domain (Biol et al., 2002). SP_1914 is non-functional as this is the gene into which the *lux* genes were inserted (Figure 4-1). Interestingly both the pyruvate oxidase and lactate oxidase which function under aerobic growth conditions contain amino acid changes creating a premature stop codon in both genes (Taniai et al., 2008). Gene alignments of *spxB* and *lctO* from Xen35 to other genome sequenced *S.pneumoniae* strains available at NCBI showed this amino acid change is not common, not being seen in any other strains (data not shown). SpxB has been shown to be an important virulence factor in *S.pneumoniae* producing hydrogen peroxide as a by-product of its enzymatic reaction, and is important for intraspecies competition in the nasopharynx (Pericone et al., 2000).

Of the changes seen in Xen35 a large proportion were located around the *lux* gene insertion, summarised in Table 4-5. Due to this each change was mapped as to whether it corresponded to that seen in the corresponding gene in D39 (NC_008533). Xen35 was constructed via transformation of Xen7 (D39) genomic DNA into TIGR4. Therefore it was hypothesised that along with the *lux* gene insertion a large proportion of the genes surrounding the *lux* insertion in Xen7 were also recombined into TIGR4 to make Xen35. This seemed to be the case as 170 out of 194 of the gene changes seen surrounding the *lux* insertion corresponded to the changes of a D39 gene rather than a TIGR4 gene. There were a few exceptions to this, which may be due to some variation of the TIGR4 strain Xen35 was constructed in compared to the genome sequenced TIGR4 strain (Tettelin et al., 2001). Or potential variations in the D39 strain Xen7 was constructed in compared to that of the genome sequenced strain (Lanie et al., 2007). Figure 4-2 shows the region surrounding the *lux* genes (17kb), which was also recombined into TIGR4 creating Xen35.

Change	Surrounding *lux* insertion	Rest of the Genome
SNPS	189	31
Insertions	3	12
Deletions	2	6

Table 4-5: Location of genome changes seen in Xen35

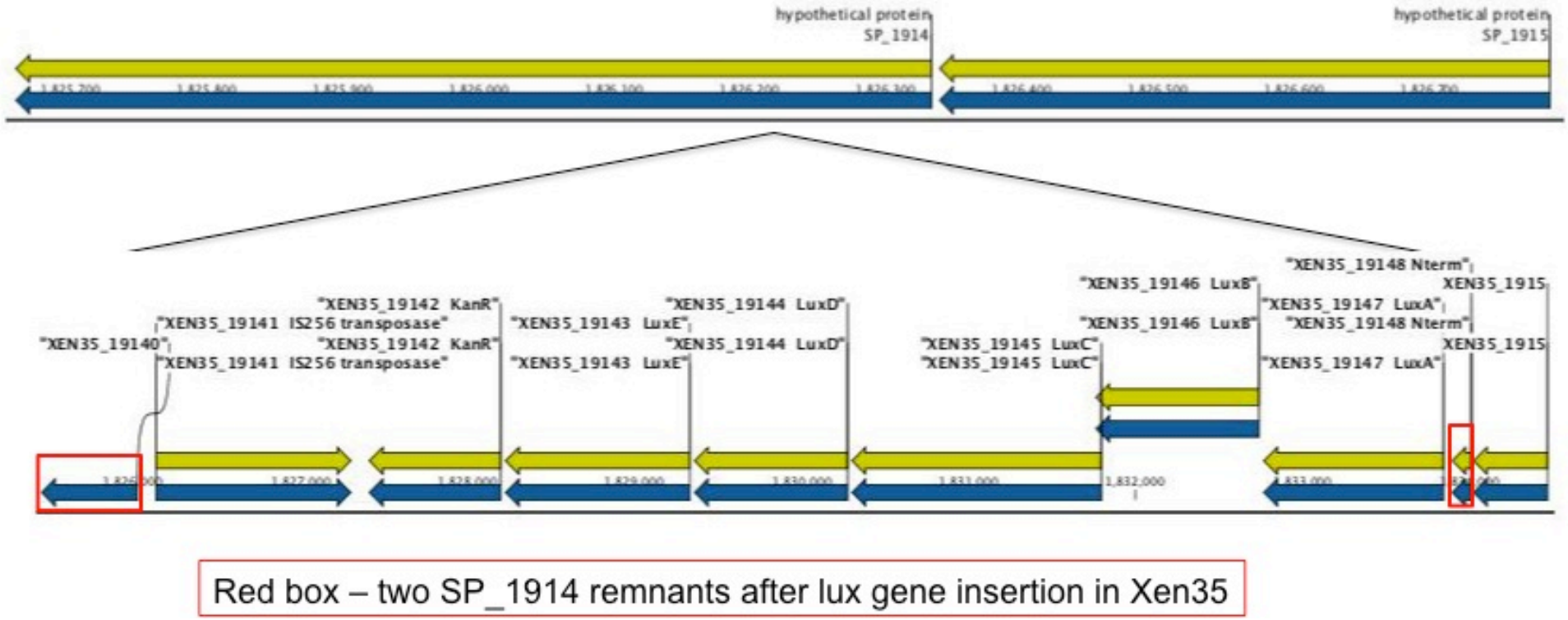

Figure 4-1: Schematic diagram of the *lux* gene insertion site in Xen35

Diagram showing the *lux* insertion site in Xen35, constructed in CLC genomics workbench. LuxA-E are the five genes required for the bioluminescence reaction which are inserted into SP_1914 in Xen35.

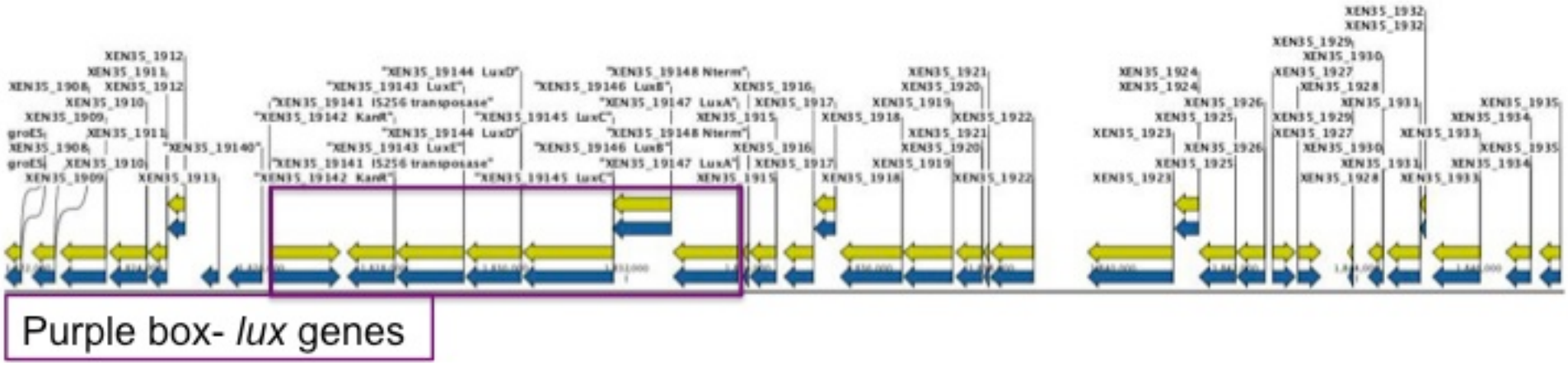

Figure 4-2: Schematic diagram showing region recombined into Xen35 from Xen7 (D39)

Diagram shows whole region SP_1908-1935 in Xen35 that now contains Xen7 (D39) alleles rather than TIGR4, which spans roughly 17kb not including the *lux* genes. Boxed in purple is the *lux* genes. Diagram was constructed in CLC genomics workbench.

The rest of the genome changes lying outwith the *lux* insertion site were also mapped to the D39 genome sequence. A number of these changes were also shown to be in D39, which could indicate multiple recombination events occurring during a single transformation, which has been shown can occur (Croucher et al., 2012). However it could also be from genome variations in the Xen35 TIGR4 parent to that of the genome sequenced strain, which is likely the case for the in frame deletion in StkP. PCR was performed and this deletion was shown not to be present in Xen7, which contains the full length StkP(data not shown). The only other explanation would be this deletion has occurred in Xen35 over time in the laboratory. The SNP causing the loss of function of SpxB is also not present in Xen7, as Xen7 produced similar levels of hydrogen peroxide to TIGR4 (Figure 4-3).

4.3.2 Hydrogen peroxide production of Xen35

Due to the noted SNP in *spxB* in Xen35, a hydrogen peroxide assay was performed on this strain to confirm the resulting SpxB protein was non-functional. SpxB produces over 90% of the cells H_2O_2 as a by-product of its enzymatic reaction therefore it would be expected to see severely reduced levels of H_2O_2 in Xen35 compared to T4JH (Pericone et al., 2000). The presence of hydrogen peroxide leads to a colour change to purple visualised in the assay. This assay was also performed on all the other Xen strains created. This included Xen7 (serotype 2, D39), Xen9 (serotype 19, HUS-TMBIG), Xen10 (serotype 3, A66), Xen11 (serotype 19, Ef3030), Xen12 (serotype 14, 140301) and Xen34 (serotype 23, 230401). This assay will assess if reduced H_2O_2 production is a common feature of all the Xen strains, which is brought about due to the insertion of the *lux* genes or expression of the *lux* genes. These strains were compared to T4JH as we did not have their parent strains available for comparison.

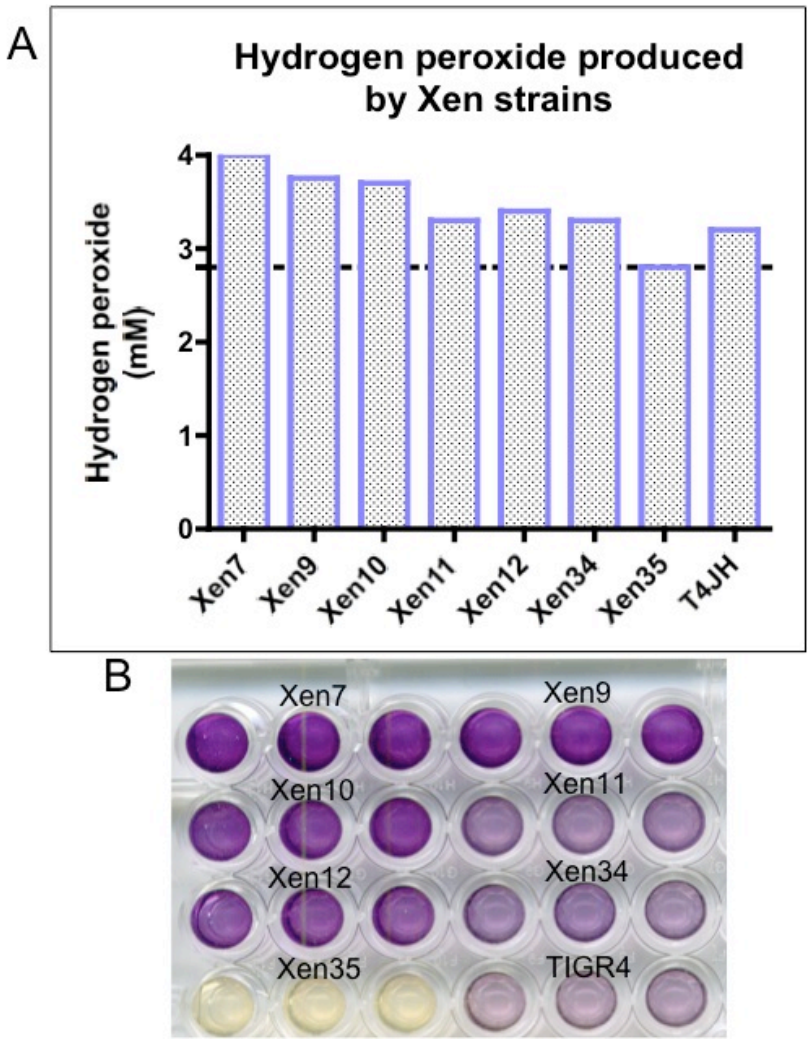

Figure 4-3: Hydrogen peroxide production of Xen strains

Each strain was represented in triplicate in the Hydrogen peroxide assay. (A) The graph gives the hydrogen peroxide production in mM of each strain calculated using a standard curve of known hydrogen peroxide concentrations (See Figure 2-14), the dotted line represent the limit of detection. (B) Shows visually the assay performed in a 96 well plate with each strain represented in triplicate.

Hydrogen peroxide assay confirmed the lack of H_2O_2 production in Xen35 and therefore the fact that SpxB is non-functional in this strain. Although there is only a small decrease in Xen35 H_2O_2 production compared to T4JH, visually it is clear there is no H_2O_2 produced by Xen35 (Figure 4-3). The other Xen strains all produce H_2O_2 and therefore this SNP in Xen35 *spxB* is clearly not a common feature in all Xen strains. The fact this is not the case in Xen7 would also indicate this change did not come from this strain during production of Xen35. This SNP in Xen35 likely arose from the original TIGR4 strain Xen35 was constructed in.

4.4 Expression changes in Xen35

4.4.1 Microarray analysis of Xen35

Due to the large number of genome changes seen in Xen35 gene expression changes were assessed to deduce the downstream effect these genome changes may have in Xen35. For this microarray analysis was performed on Xen35 compared to T4JH.

A total of 33 genes were differentially regulated between Xen35 and T4JH ($P<0.05$) shown in Table 4-6. When increasing the P value to $P<0.1$ a total of 65 genes were differentially regulated (data shown in appendix IIII). Although not within our designated $P<0.05$ cut off this list is referred to as it may contain genes that show true expression changes, for instance it may include genes that are contained within the same operon of genes in the $P<0.05$ list.

Included in the genes that were differentially regulated is that encoding the pilus backbone protein (*rrgB*), and a number of glycosyltransferases, which function to glycosylate a large surface protein know as the pneumococcal serine rich repeat protein (PsrP). *psrP* was shown to be differentially regulate in the $P<0.1$ gene list. Both are important for virulence in *S.pneumoniae* contributing to adherence and biofilm formation respectively (Hemsley et al., 2003, Sanchez et al., 2010). *rlrA, rrgA* and *rrgC* were also shown to be differentially regulated in the $P<0.1$ list. Also Included were a number of transcriptional regulators and a HK (*hk06*) of a TCS pair, the RR (*rr06*) was also differentially regulated in the increased P value list ($P<0.1$). The lactate oxidase (*lctO*) was shown to be down regulated in Xen35, however from the genome sequence data we know this gene encodes a truncated protein.

Interestingly the genes surrounding the *lux* insertion were both highly up regulated in Xen35. SP_1914 a hypothetical protein is non-functional due to the *lux* insertion, however the adjacent gene SP_1915 also encoding a hypothetical protein still remains functional (contains a number of SNPs). SP_1915 located downstream to SP_1914 is up regulated over 100 fold in Xen35. The surrounding genes were not differentially regulated and therefore we hypothesise the promoter driving the expression of the *lux* genes lies downstream of SP_1915.

Due to their huge up regulation it is likely the *lux* genes interruption of SP_1914 altered some regulatory unit causing the huge up regulation of both SP_1914 and SP_1915. Both protein are of unknown function and therefore the effect of this to virulence of Xen35 is unknown.

Gene	Description	Abbrev	Fold change
SP_0095	Hypothetical protein		2.9
SP_0107	LysM domain containing protein		3.3
SP_0202	Anaerobic ribonucleoside triphosphate reductase	*nrdD*	1.5
SP_0463	Cell wall surface anchor family protein	*rrgB*	13.7
SP_0663	Hypothetical protein		1.8
SP_0715	Lactate dehydrogenase	*lctO*	2.3
SP_0726	Phosphomethylpyrimidine kinase	*thiD*	2.8
SP_1159	Tyrosine recombinase (chromosome seg)	*xerS*	1.4
SP_1427	U32 family peptidase		1.7
SP_1464	Acetyltransferase		1.3
SP_1468	Pyridoxal biosynthesis lyase	*pdxS*	2.4
SP_1546	Hypothetical protein		2.1
SP_1572	Non-heme containing ferritin		1.9
SP_1587	Oxalate formate antiporter		4.6
SP_1757	Hypothetical protein		5.4
SP_1764	Glycosyltransferase family protein		5.9
SP_1765	Glycosyltransferase family protein		2.5
SP_1766	Glycosyltransferase family protein		4
SP_1767	Glycosyltransferase family protein		3.8
SP_1769	Glycosyltransferase family protein, authentic frameshift.		4.2
SP_1861	Choline transporter	*proV*	1.7
SP_1862	Hypothetical protein		2.1
SP_1863	MarR family transcriptional regulator	*marR*	2.2
SP_1895	Sugar ABC transporter, permease protein		5
SP_1898	Alpha-galactosidase	*aga*	5
SP_1914	Hypothetical protein		66.1
SP_1915	Hypothetical protein		185.6
SP_2010	Penicillin binding protein 2A	PBP 2A	1.1
SP_2057	Hypothetical protein		1.4
SP_2106	Glycogen phosphorylase family protein		1.7
SP_2185	Hypothetical protein		1.7
SP_2192	Sensor Histidine kinase	*hk06*	1.6
SP_2195	Transcriptional regulator	*ctsR*	1.5

Table 4-6: Genes differentially regulated in Xen35 compared to T4JH

Table shows genes differentially regulated in Xen35 compared to T4JH (P<0.05). Fold change represents that seen in Xen35 compared to T4JH, red showing genes up regulated in Xen35 and blue are those that are down regulated.

4.4.2 Real time PCR validation of microarray analysis

RT-PCR (Real-time PCR) was performed on a number of genes shown to be differentially regulated in Xen35 to confirm changes seen by microarray analysis. Of the genes included were the whole pneumococcal pilus islet (SP_0461-64/

SP_0466-68), PsrP (SP_1772) and the two hypothetical proteins surrounding the *lux* insertions (SP_1914/ SP_1915).

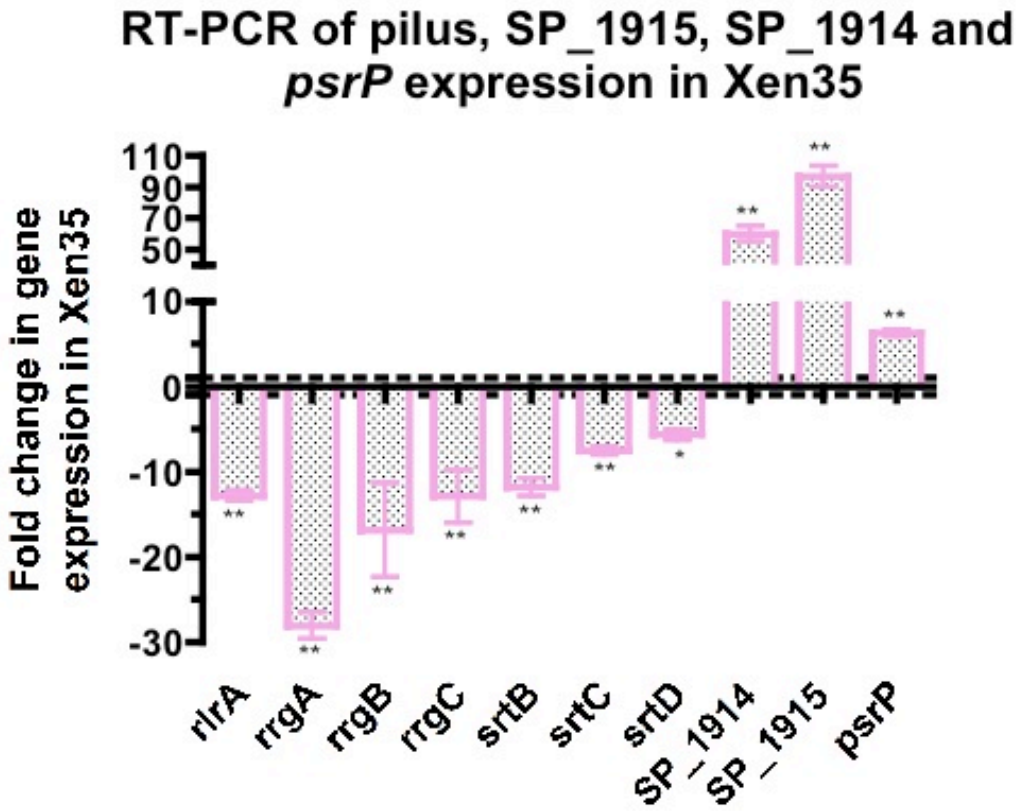

Figure 4-4: RT-PCR graph of pilus islet genes, *psrP* and SP_1914/15 expression in Xen35

Graph shows RT-PCR of the whole pilus islet (*rlrA*, *rrgA*, *rrgB, rrgC*, *srtB*, *srtC, srtD*), *psrP*, SP_1914 and SP_1915 in Xen35. Fold change represents that of Xen35 compared to TIGR4. Each bar represents the average of three replicas (three biological replicas) and errors bars the standard deviation. Statistical analysis was performed by a 1-way ANOVA with a Tukeys testing correction comparing the dCt values of the control strain (T4JH) to the test strain (Xen35), * P<0.01, **P<0.001.

Gene	Microarray	RT-PCR
SP_0461 (*rlrA*)	6.3	12.8
SP_0462 (*rrgA*)	13.3	28.1
SP_0463 (*rrgB*)	13.7	16.2
SP_0464 (*rrgC*)	7.1	12.6
SP_0466 (*srtB*)	2.4	11.8
SP_0467 (*srtC*)	2.0	7.5
SP_0468 (*srtD*)	1.8	5.6
SP_1914	66.1	59.7
SP_1915	185.6	96.3
SP_1772 (*psrP*)	4.3	6.3

Table 4-7: Comparison of expression changes of the pilus islet genes, *psrP,* SP_1914 and SP_1915 in Xen35 from microarray (P<0.05/ P<0.1) and RT-PCR analysis.

Table shows fold changes in gene expression of the whole pilus islet, *psrP*, SP_1914 and SP_1915 in Xen35 compared to T4JH. Fold changes show that from microarray data and those from RT-PCR analysis.

RT-PCR for the selected genes confirmed the changes seen by microarray analysis. RT-PCR was performed on the same RNA preparations that microarray analysis was performed on so variations between the two experimental techniques could also be assessed. Microarray seemed to slightly underestimate the down regulation of the pneumococcal pilus in Xen35, showing a decrease in 28-5.5 fold in the different genes required for pilus assembly. RT-PCR also showed the huge difference in the genes surrounding the *lux* insertion is a true change. The up regulation of *psrP* was also confirmed.

Due to their being such a large change in the expression of the pneumococcal pilus RT-PCR was performed on some of the genes known to regulate the pneumococcal pilus. This includes TCS shown to all be repress expression of the pilus genes (Hendriksen et al., 2006, Rosch et al., 2008, Song et al., 2009). *rr06* was also shown to be differentially expressed in Xen35 by microarray ($P<0.1$). This will assess whether they may be causing this decrease in pilus expression seen in Xen35.

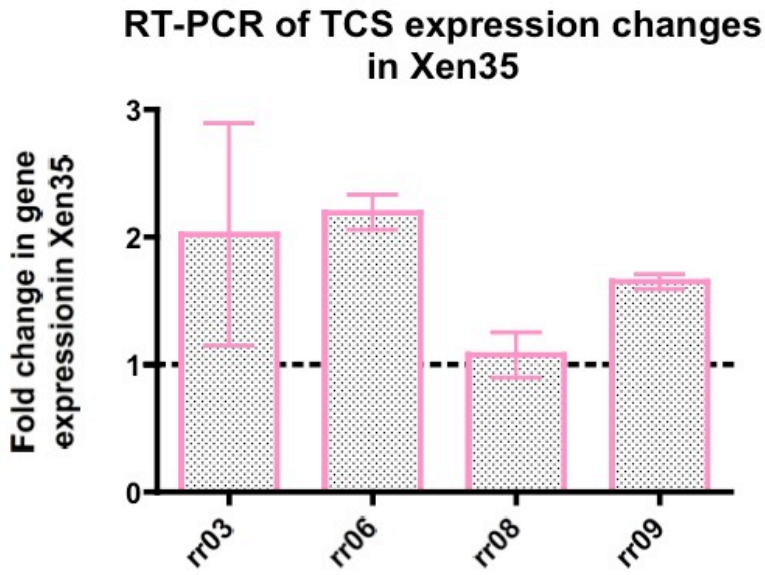

Figure 4-5: RT-PCR graph of TCS expression in Xen35

Graph shows RT-PCR of *rr03*, *rr06*, *rr08* and *rr09* in Xen35. Fold change represents that of Xen35 compared to TIGR4. Each bar represents the average of three replicas (three biological replicas) and errors bars the standard deviation. No statistical difference was observed.

Gene	Microarray	RT-PCR
SP_0387 (*rr03*)	1.2	1.9
SP_2193 (*rr06*)	1.6	2.2
SP_0083 (*rr08*)	1.4	1.1
SP_0661 (*rr09*)	1.8	1.6

Table 4-8: Comparison of expression changes of TCS in Xen35 from microarray and RT-PCR analysis.

Table shows fold changes in gene expression of *rr03*, *rr06*, *rr08* and *rr09* in Xen35 compared to TIGR4. Fold changes show that from microarray data and those from RT-PCR.

RT-PCR performed on the four TCS known to regulate the pneumococcal pilus showed three of them were up regulated in Xen35, however none were classed as statistically significant (Table 4-8, Figure 4-5). *rr06* was shown to be up regulated by RT-PCR (not significant) and by microarray analysis. Whether this may contribute to the altered pilus expression would require further study.

4.4.3 RNA-seq analysis of expression changes in Xen35

RNA-seq analysis was also performed on Xen35 compared to T4JH. mRNA purification was performed on the same total RNA preparation used for microarray analysis and RT-PCR analysis and therefore is directly comparable. No statistical analysis has been performed on this data set to date so comparison is performed using the RPKM (reads per kilobase of exon model per million mapped reads) expression values assigned in CLC genomics workbench. Only a few chosen genes are shown below in Table 4-9. This gives an indication of the expression levels of both genes in the two strains and from this the fold change is calculated.

Gene	RPKM Xen35	RPKM TIGR4	Fold change RNA-seq	Fold change Microarray	Fold change RT-PCR
SP_0461 (*rlrA*)	3.33	38.6	11.6	6.3	12.8
SP_0462 (*rrgA*)	11.17	249.34	22.3	13.3	28.1
SP_0463 (*rrgB*)	41.22	612.78	14.9	13.7	16.2
SP_0464 (*rrgC*)	41.34	671.84	16.3	7.1	12.6
SP_0466 (*srtB*)	4.58	40.56	8.9	2.4	11.8
SP_0467 (*srtC*)	10.32	57.22	5.5	2.0	7.5
SP_0468 (*srtD*)	11.35	60.25	5.3	1.8	5.6
SP_1772 (*psrP*)	21.07	4.63	4.6	4.3	6.3
SP_0387 (*rr03*)	92.64	99.76	1.1	1.2	1.9
SP_2193 (*rr06*)	304.3	239.65	1.3	1.6	2.2
SP_0083 (*rr08*)	53.1	102.04	1.9	1.4	1.1
SP_0661 (*rr09*)	56.36	129.27	2.3	1.8	1.6

Table 4-9: Comparison of expression changes of the pilus islet, *psrP* and TCS in Xen35 from microarray, RT-PCR and RNA-seq analysis.

Table shows fold changes in gene expression of the pilus islet (*rlrA*, *rrgA*, *rrgB, rrgC*, *srtB*, *srtC, srtD*), *psrP, rr03*, *rr06*, *rr08* and *rr09* in Xen35 compared to TIGR4. Fold changes show that in Xen35 from microarray, RT-PCR and RNA-seq analysis.

Initial comparisons were performed looking at the pilus islet genes expression which showed as for the microarray and RT-PCR a decrease in pilus islet expression in Xen35, with the fold change values being similar to both RT-PCR and microarray analysis. This was also the case with *psrP,* which also showed an up regulation in the RNA-seq data corresponding to that shown by microarray and RT-PCR analysis. Analysis of the four TCS shown to play a role in pilus regulation by RNA-seq showed some variations to the data obtained by microarray and RT-PCR analysis. *rr03* and *rr09* expression shown by RNA-seq analysis was opposite to that shown by RT-PCR and microarray, perhaps confirming that these are not true changes and would need further validation. *rr08* showed a down regulation in Xen35 by RNA-seq analysis however RT-PCR showed no difference. *rr06* still showed a small up regulation in Xen35 during RNA-seq analysis. Due to the small fold changes in the TCS it is hard to validate if this is a true change due to variation within the techniques used.

4.5 Changes in pilus expression in Xen35

To further confirm this decrease in pilus expression in Xen35 western blotting was performed to assess if this correlates to a decrease in protein levels. And FACS analysis was performed to assess the spread of the pilus in the population level.

4.5.1 Western blot analysis of Xen35

Western blot analysis was used to determine the amount of pilus backbone protein (RrgB) in Xen35 and T4JH. Western blot analysis was performed on samples prepared at varying ODs (OD_{600nm} 0.2/ 0.6 and 1.0) to assess any variation in the pilus at different ODs. Analysis showed a clear decrease in the amount of RrgB in Xen35 compared to T4JH at all ODs (Figure 4-6). There does seem to be some variation at the different ODs with the biggest difference in RrgB expression seen at OD_{600nm} 0.6. There is likely some variation in the fold

changes stated due to variations in the technique, however at all ODs it is clear there is a large decrease in RrgB expression in Xen35 compared to T4JH.

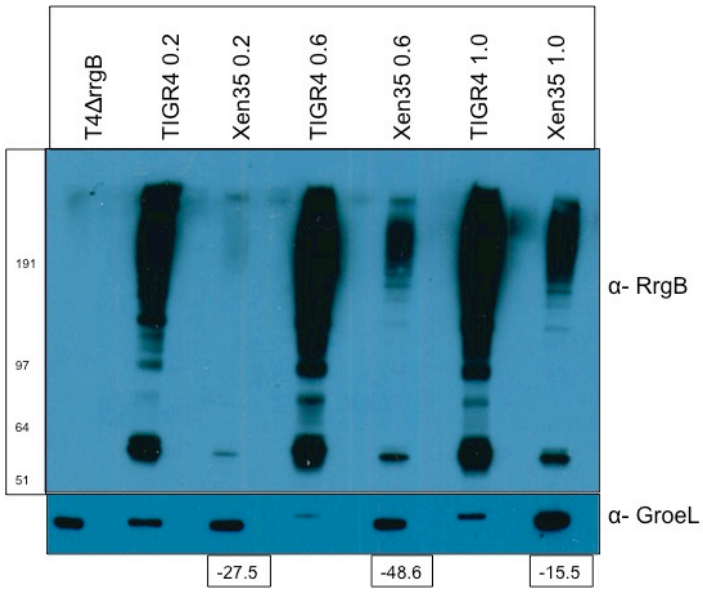

Figure 4-6: Western blot of Xen35 RrgB expression compared to TIGR4.

Western blotting analysis was performed on TIGR4, Xen35 at varying ODs (OD_{600nm} 0.2/ 0.6/ 1.0) and T4Δ*rrgB* looking for RrgB protein levels in all (α-RrgB antibody). Equal protein loading was confirmed by equal expression of GroeL (α-GroeL antibody), normalised against in analysis. Western blotting quantification was performed using ImageJ with fold changes in RrgB expression in Xen35 compared to TIGR4 boxed below the strain, analysis was performed comparing the Xen35 OD sample to that of its corresponding TIGR4 same OD sample. Left hand side numbers represent the size in kDa of proteins run to the same point.

It has recently been shown that the pneumococcal pilus in a population is not distributed evenly between cells. Within a growing population a number of cells are positive for the pilus and some negative (Basset et al., 2011, De Angelis et al., 2011). It was therefore important to evaluate in Xen35 if the decrease in pilus expression correlated with a decrease in pili on single cells or if the number of cells expressing the pilus were fewer than T4JH.

4.5.2 FACS and fluorescence microscopy analysis of Xen35

FACS analysis was performed initially by staining a fixed bacterial population grown in BHI (OD_{600nm} 0.6) with a anti-rabbit capsule antibody specific for the serotype 4 capsule and co-staining with a anti-mouse RrgB antibody. Samples were then stained with a anti-rabbit APC conjugate and a anti-mouse FITC conjugate antibody. This left the bacterial cells stained positive for the capsule

(APC+), which were gated on to confirm events acquired were that of the bacterial population. This capsule stained population was then gated on for being RrgB positive (FITC+) or RrgB negative (FITC-).

FACS performed to look at the change in pilus expression at the population level was performed on Xen35 and two TIGR4 strains, T4JH used for all experiments to date and T4NO1 a strain taken only a single passage from the original genome sequenced strain (Tettelin et al., 2001, Aaberge et al., 1995). Both strains were used as we do not have access to the parent strain of Xen35 (Figure 4-7). We know from the Xen35 genome sequence data there is a degree of variability between different strains and likely strains of the same serotype. We thought it important then to confirm the same pilus expression in the two TIGR4 strains with T4JH being used for microarray, RT-PCR, RNA-seq, western blotting experiments and T4NO1 being used as the reference for the genome sequence data.

FACS analysis performed on Xen35 showed that only 5.5% of the population were positive for the pneumococcal pilus (Table 4-10). This showed that rather than a decrease in pili present on a single cell there are fewer cells that contain the pilus on their cell surface.

With regards to the TIGR4 strains, interestingly there is a big difference in the number of cells expressing the pilus on the cell surface. T4NO1 showed 88% of cells positive for the pilus however T4JH only showed 20.5% of the cells expressing the pilus (Table 4-10), which is similar to the 30% seen in TIGR4 in published data (Basset et al., 2011). The reasons for this difference in these two strains will be discussed in section 4.8. Due to this it was decided that all future experiments would be performed using T4NO1 (the strain the original genome sequence was performed on) (Tettelin et al., 2001, Aaberge et al., 1995).

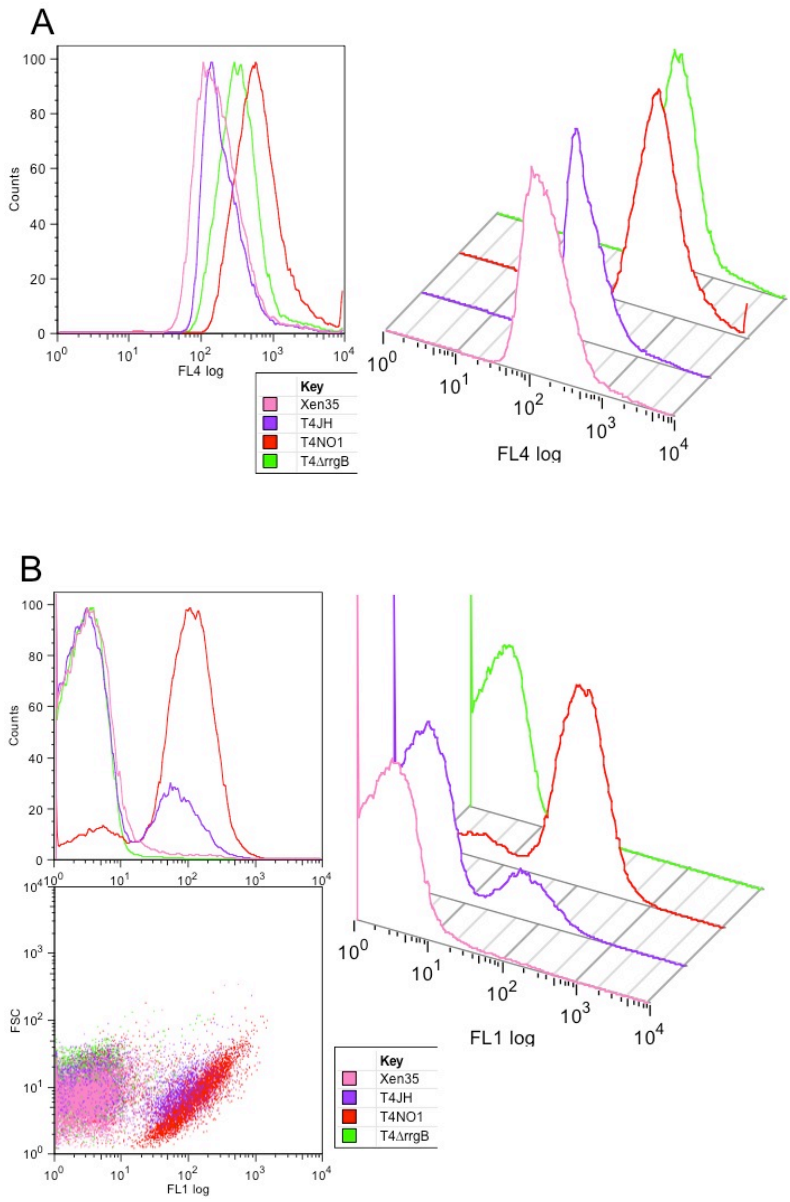

Figure 4-7: FACS analysis of RrgB surface expression in Xen35

FACS was performed on Xen35, T4NO1, T4JH and T4Δ*rrgB*. (A) Shows histograms of the capsule positive populations selected for from samples stained with a capsule antibody, population used for further analysis. (B) Shows the capsule stained populations being gated on for being RrgB positive, histograms/ polychromatic plot show negative (left) and positive (right) RrgB populations in each strain.

Strain	RrgB- (%)	RrgB+ (%)
T4NO1	12.1	87.9
T4JH	79.5	20.5
Xen35	94.7	5.35
T4Δ*rrgB*	98.4	1.6

Table 4-10: Percentage RrgB positive cells in a Xen35 population from FACS analysis.

Table shows the percentage RrgB positive and negative cells in a growing bacterial population. FACS analysis was performed in FlowJo.

Fluorescence microscopy confirmed all changes seen during FACS analysis as seen in Figure 4-8. T4NO1 showed the majority of cells contain the pilus and this is present over the whole cell, this is also the case for T4JH however a smaller number of cells have the pili. Xen35 showed a number of cells with pili present all over the cell surface but some also contained just a small region on the cells where pili were present. No T4Δ*rrgB* cells were observed to be RrgB positive by fluorescence microscopy shown in Figure 4-8. Further representative fluorescent microscopy images can be seen in appendix I.

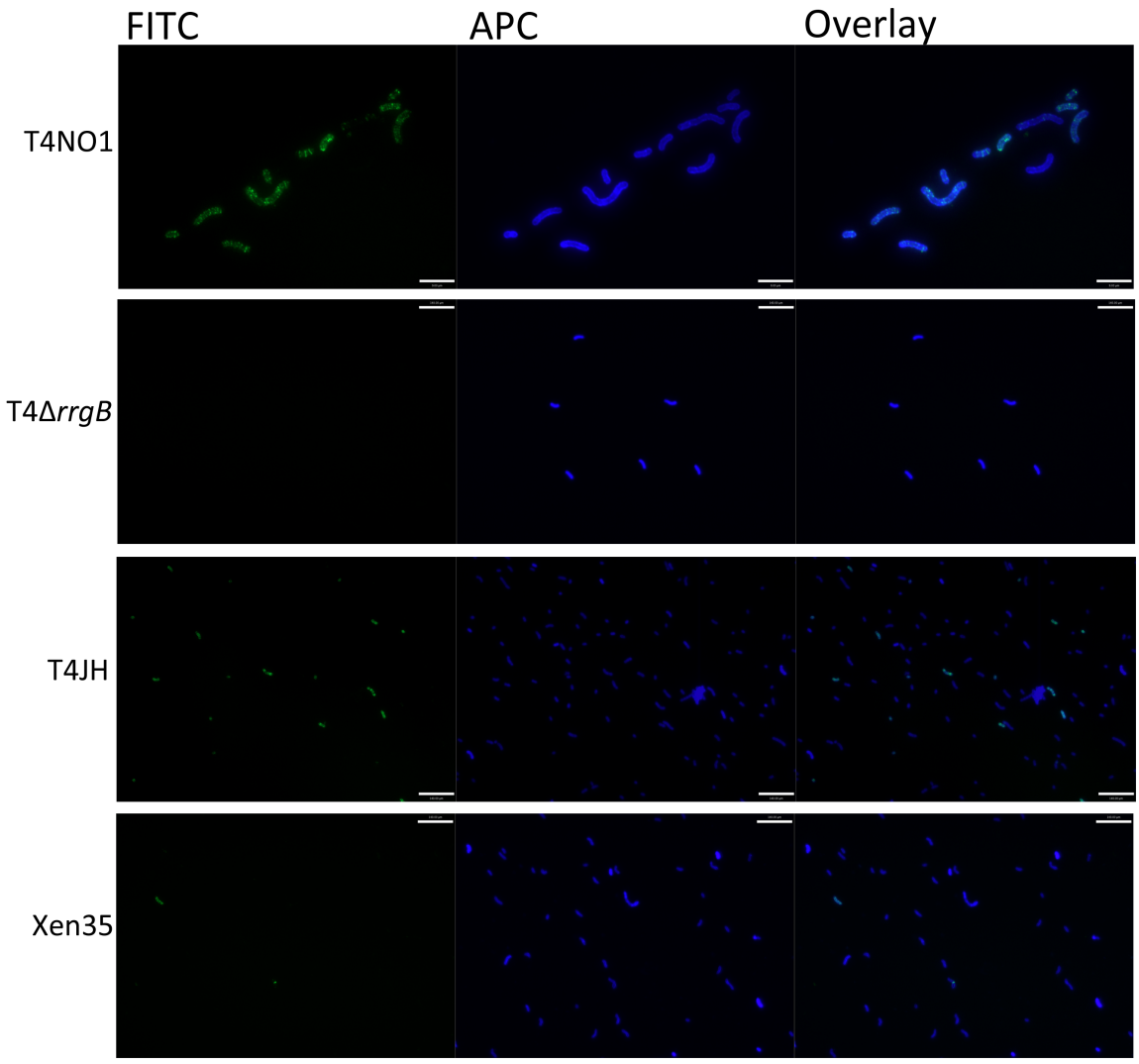

Figure 4-8: Fluorescence microscopy of Xen35.

One representative image of fluorescently labelled T4NO1, T4JH, T4Δ*rrgB* and Xen35 used for FACS analysis. Cells were stained for the presence of RrgB (FITC) and the capsule (APC). Scale bars above represent, T4NO1-9μm, T4Δ*rrgB* - 140μm, T4JH - 140μm and Xen35 - 140μm. The same representative image will be used throughout this study for each strain, more images can be seen in appendix I.

4.6 Electron microscopy analysis of Xen35

Electron microscopy was performed on Xen35 to evaluate mainly any obvious structural changes in the pilus, but also any changes in cell morphology, which may give a hint as to whether StkP in Xen35 is non-functional. StkP mutants have been shown to affect cell division forming longer cells with multiple septa (Giefing et al., 2008). Microscopy was performed using a α-RrgB primary antibody with a 10nm gold particle conjugate secondary antibody. This labelled the length of the pilus shaft with gold particles. Microscopy was also performed on T4NO1. T4Δ*rrgB* was used to confirm absence of the pilus and no cross reactivity of the antibody to any other surface proteins. Images were taken between 4000x and 16000x magnification.

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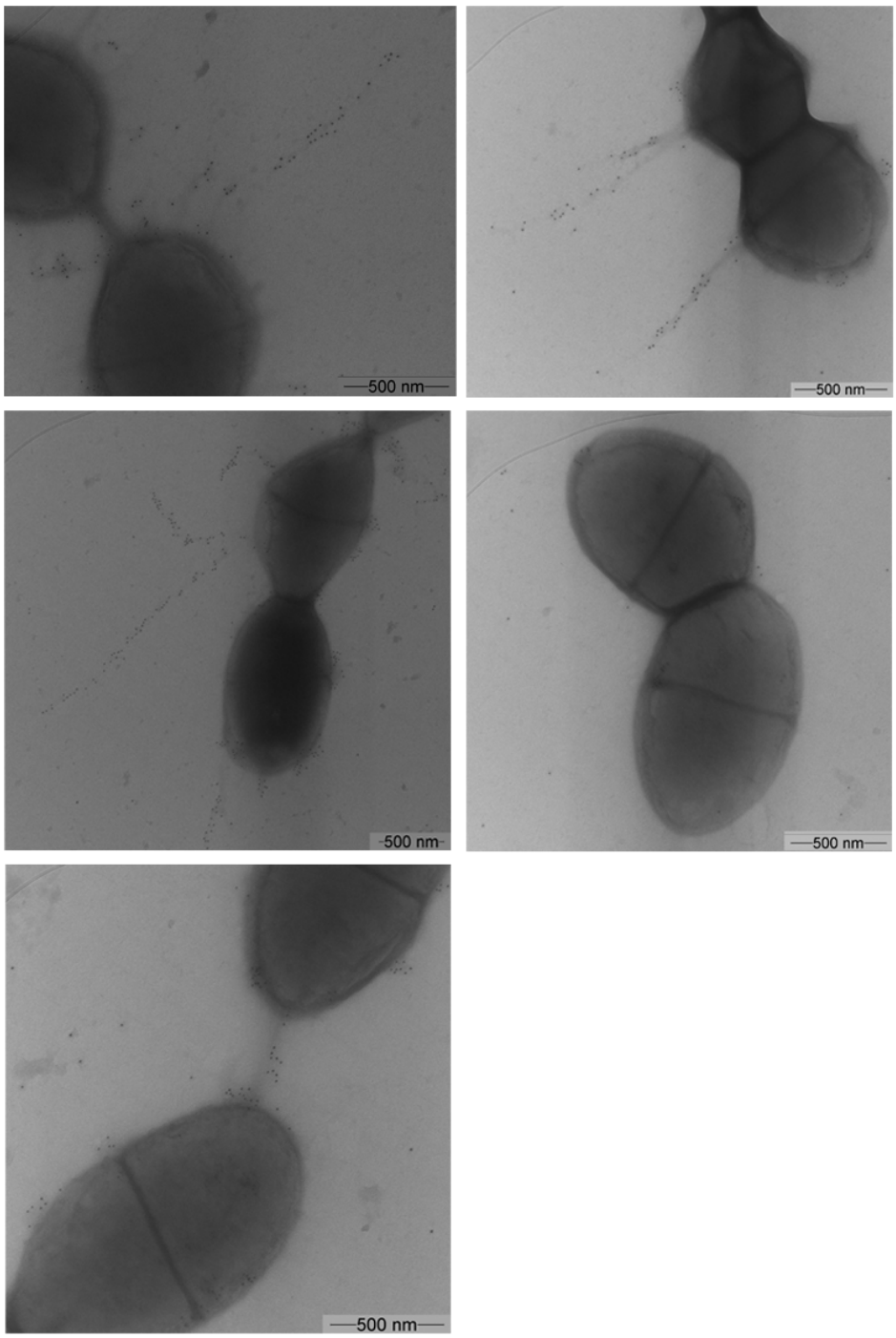


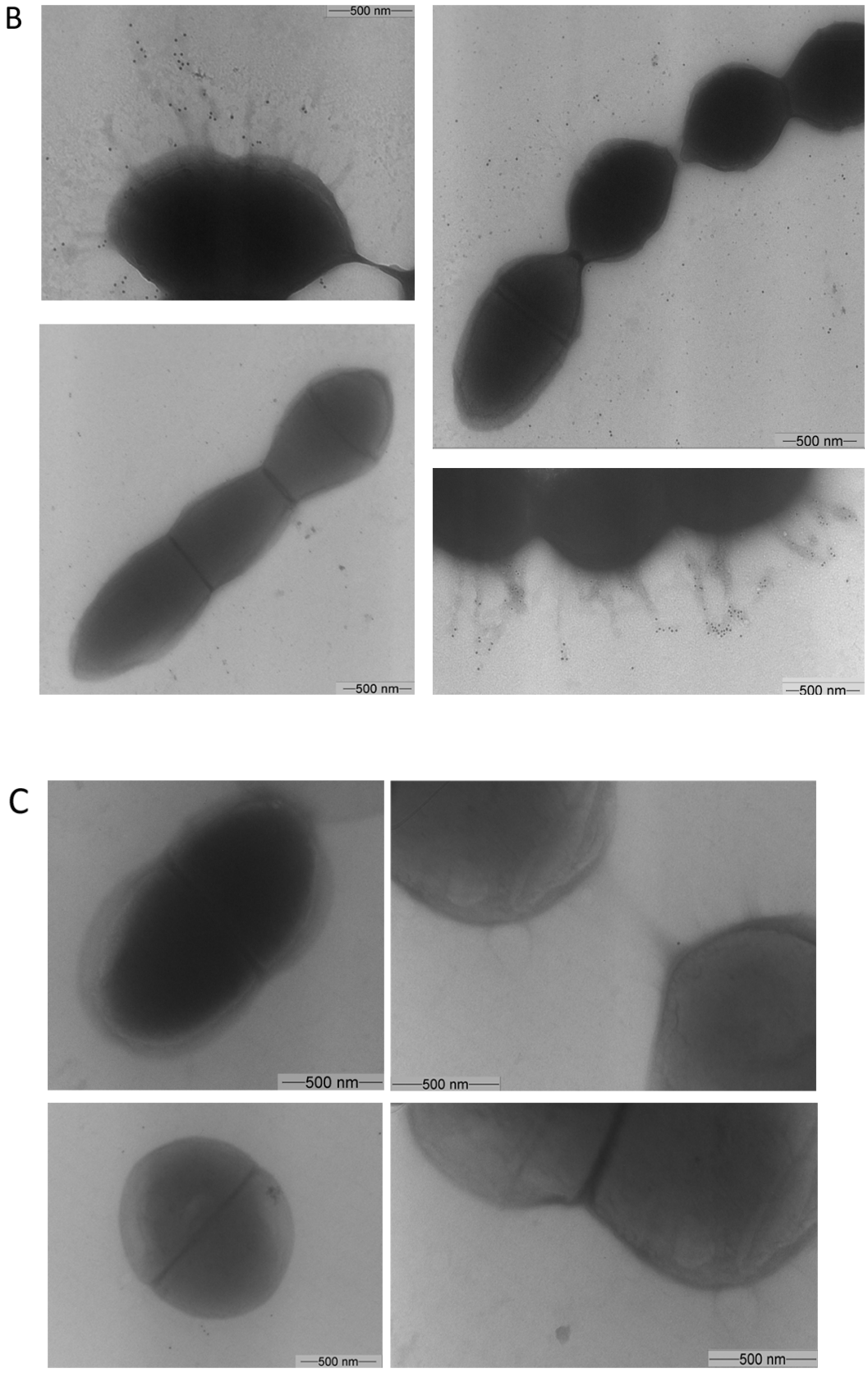


Figure 4-9: Electron microscopy of T4NO1, Xen35 and T4Δ*rrgB*.

Electron microscopy images of T4NO1 (A), Xen35 (B) and T4Δ*rrgB* (C). Cells were stained using a α-RrgB antibody and a secondary 10nm gold conjugate antibody. Cells were visualised at 4000-16000x magnifications.

Electron microscopy further confirmed that T4Δ*rrgB* contains no pili on the surface, and the gold conjugate antibody did not cross react with any other bacterial surface component (Figure 4-9). Images of Xen35 cells showed that some cells did express the pneumococcal pilus on the surface as shown by FACS however a large majority did not. One of the Xen35 cells visualised showed an elongated morphology with multiple septa along the length suggesting this cell is unable to divide properly. Which may indicate StkP in this strain is non-functional or has a reduced functionality (Figure 4-9). The majority of T4NO1 cells contained pili on the surface with often more than one pilus coming from the same cell. However some had no pili. These structures reach a long way from the cell surface in T4NO1. In Xen35 the pili appear shorter, however when performing electron microscopy fewer Xen35 cells were observed on the nickel grid and therefore these cells imaged may not be representative of a normal Xen35 population. More experiments would have to be performed to assess the length of pili in Xen35 cells compared to T4NO1.

4.7 Analysis of virulence of Xen35

Due to the huge genome changes, expression changes and changes in the surface expression of the pilus we wanted to assess difference in virulence of Xen35 compared to T4NO1.

4.7.1 Adherence assay analysis of Xen35

Initial experiments were performed assessing the ability of Xen35 to adhere to different cell lines. Chosen cell lines included HBMEC cells (human brain microvascular endothelial cells), A549 cells (Human lung epithelial carcinoma cells line) and D562 cells (Detroit 562, Human pharyngeal carcinoma cell line), used to mimic meningitis, pneumonia and colonisation respectively. The pneumococcal pilus has been shown to aid adherence to A549 cells (Hemsley et al., 2003). *psrP* has also been shown to be important for pneumonia and aids adherence to A549 cells (Shivshankar et al., 2009). SpxB has been shown to be important for colonisation and is non-functional in Xen35 (Pericone et al., 2000). All these factors likely affect the virulence of Xen35.

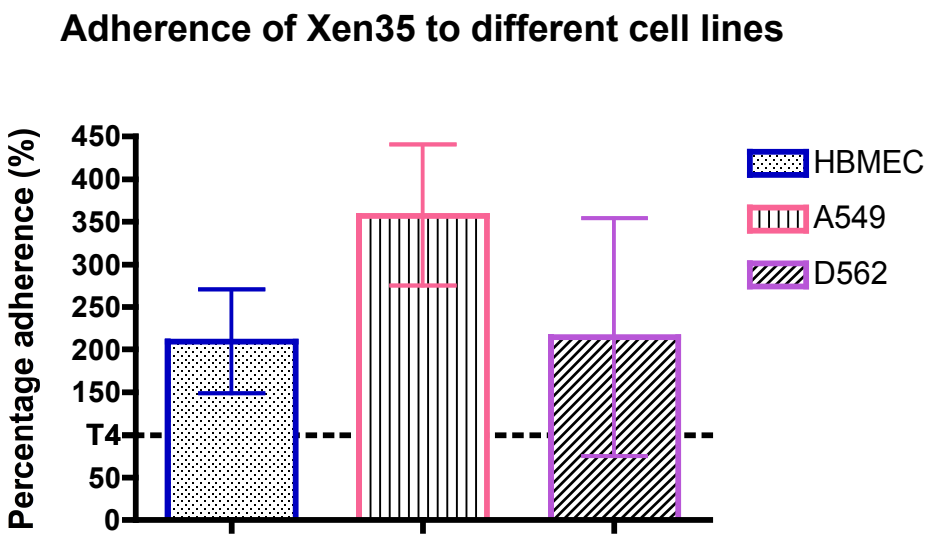


Figure 4-10: Adherence of Xen35 to different cell lines.

Adherence of Xen35 was assessed to HBMEC, A549 and Detroit 562 cell lines. Data is represented as percentage adherence relative to that of T4NO1 (100%, dashed line), each bar is an average of three replicas and the error bars represent the standard error of the mean. Statistical analysis was performed using a 1-way ANOVA with a Tukeys testing correction, no statistical difference was observed.

Adherence of Xen35 to all cells lines showed a trend towards an increase compared to T4NO1, however none were significant. With the highest trend towards an increase seen to the A549 cells, this is likely due to the increased expression of *psrP*. It is possible *psrP* is also able to aid adherence to other cells, and may also contribute to the trend towards an increase in adherence observed to HBMEC and D562 cells also.

4.7.2 In vivo analysis of Xen35

Virulence was also assessed *in vivo* using a mouse model of infection. For this MF1 mice were infected intranasally with $5x10^6$ cfu/50μl with either Xen35 or T4NO1 (already animal passaged). 20 mice were infected per bacterial strain and mice split into four groups, 24hour time point, 48hour time point, 72hour time point and survival. At each time point the mice in each group were culled and nasal wash, blood, lung and brain bacterial counts enumerated. If mice got sick before their time point they were culled and time of death noted. The survival group were culled if sick or culled at the end of the experiment at 96 hours post inoculation.

Initial analysis was performed comparing the survival of mice infected with both strains using a Kaplan-meier survival curve. This analysis was performed only on the 5 mice of the survival group of each strain. From this it is clear T4NO1 is more virulent than Xen35 showing a statistically significant increase in survival time with mice infected with Xen35 (Figure 4-11). The average survival time of mice infected with T4NO1 was 43 hours whereas the mice infected with Xen35 had an average survival time of 76 hours.

Over the course of the experiment the weight of the mice was noted as this can be used to assess disease progression. Percentage weight loss of mice infected with T4NO1 or Xen35 was compared at different time points over the course of the experiment. Data showed at all time points mice infected with T4NO1 had a statistically significant higher percentage weight loss than mice infected with Xen35 (Figure 4-11). This suggests disease progression in T4NO1 is quicker than in Xen35. To assess at what point during infection Xen35 is attenuated bacterial count from the brain, lung, blood and nasal wash of each mouse were compared to that of T4NO1 at varying time points.

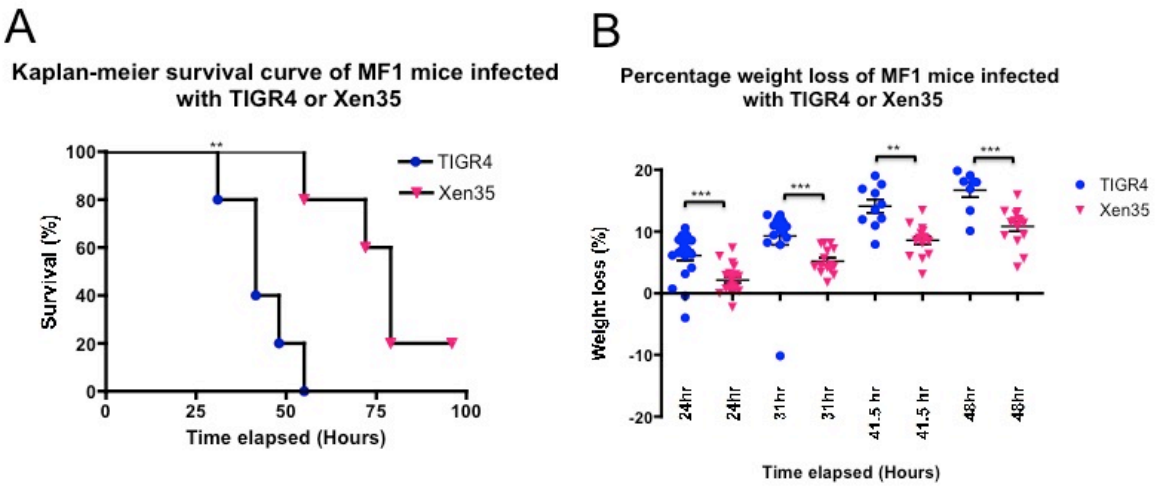


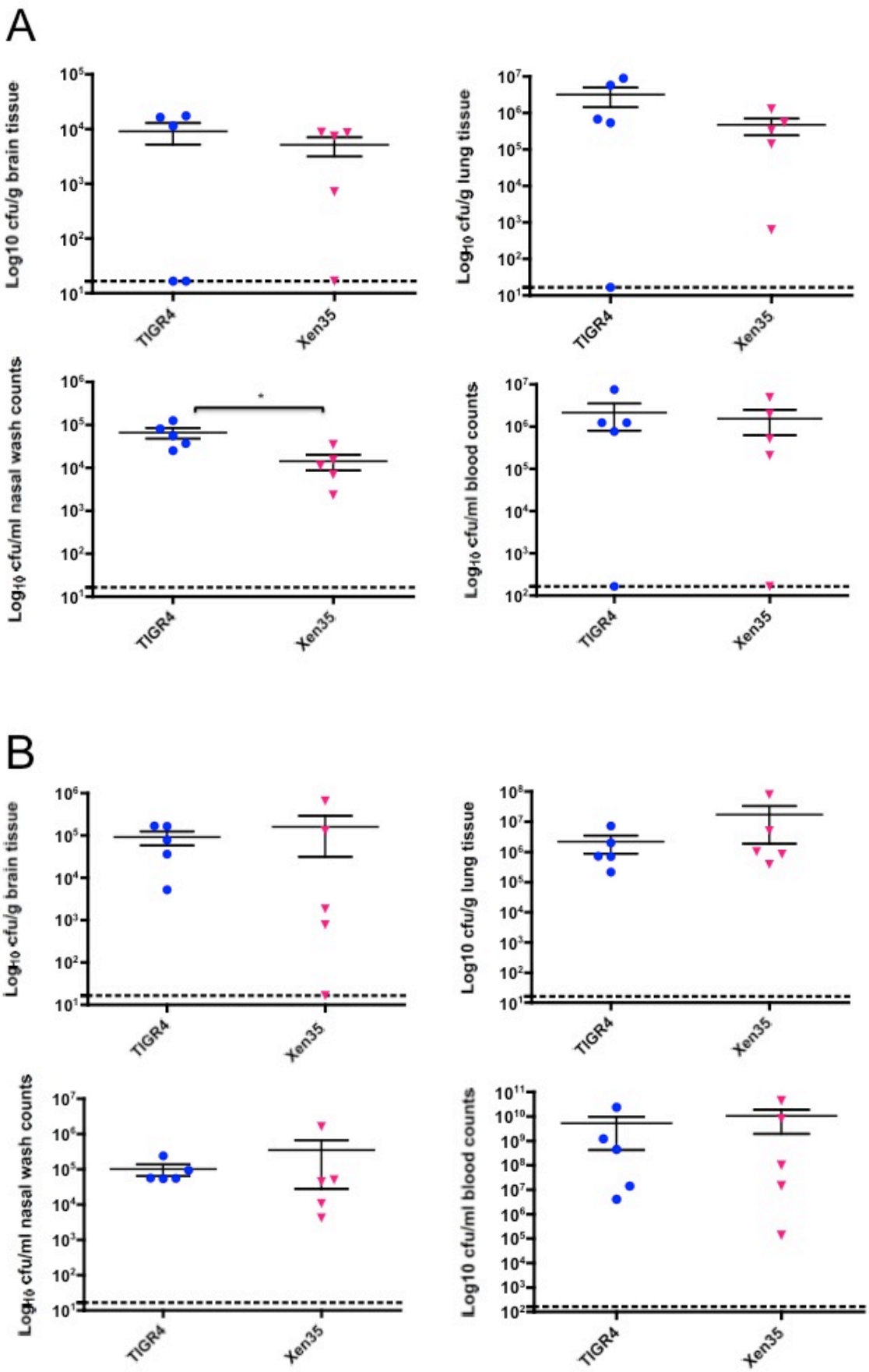
Figure 4-11: Survival and weight loss of mice infected with Xen35

(A) Shows percentage survival of mice infected with T4NO1 or Xen35 over time, statistical analysis was performed using a logrank Test, ** P<0.01. ** above the strain indicated a statistical difference compared to Xen35. (B) Shows percentage weight loss of mice infected with either Xen35 or T4NO1. Statistical analysis was performed using a non-parametric Mann-Whitney two sample rank test, **P< 0.01 and ***P<0.001.

Comparison of bacterial counts in body organs and fluids were performed comparing the four groups stated above against each other. Dotted lines on the

graphs represent the limit of detection, if no counts were observed in the organs/ bodily fluids the limit of detection value was ascribed. It must be taken into account when analysing this data that the mice in each group did not necessarily die at the same time, for instance for the 72 hour group all T4NO1 infected mice were culled between 31-66 hours post inoculation (mice were too sick) and these counts are represented in the 72 hour graph, whereas the Xen35 72 hour group were culled between 55- 72 hours. This is less so the case at earlier time points, at 24 hours all the mice of both groups were culled at the same time, and therefore this data is a good direct comparison of disease progression. For the 48 hour group all Xen35 infected mice were culled at this time point however T4NO1 infected mice were culled between 31-48 hours.

Comparison of bacterial counts at different time points showed a statistically significant difference in nasal wash bacterial counts at 24 hour and survival time points (Figure 4-12). This suggests perhaps Xen35 is unable to colonise as well as T4NO1 in mice. This could be due to the decrease in pilus expression (although this has not been validated *in vivo*) or due to the non-functional SpxB. There is a trend towards lower bacterial counts in the lungs at 24 hours in Xen35 infected mice, however not significant (Figure 4-12). At later time points there is a trend towards higher numbers of bacteria counts in Xen35 infected mice in the lungs, perhaps due to up regulation of PsrP which is known to play a role in biofilm formation and pneumonia (although this has not been validated *in vivo*). Bacterial counts in the brain of mice infected with either strain seem to be fairly similar at all time points with no statistical difference between them. However analysis of tail vein bleeds at the 24 hours time point showed a statistical significant decrease in the bacterial number in the blood of Xen35 infected mice when comparing all 20 mice infected per strain (data not shown). This again is perhaps due to the decreased number of bacteria able to colonise in Xen35 infected mice causing a lag in the time taken for invasive disease to be initiated. Xen35 infected mice were imaged at each time point to visually assess disease progression shown in (Figure 4-13).


A
Log10 cfu/g brain tissue
Log10 cfu/g lung tissue
Log10 cfu/ml nasal wash counts
Log10 cfu/ml blood counts
TIGR4
Xen35
*
B
Log10 cfu/g brain tissue
Log10 cfu/g lung tissue
Log10 cfu/ml nasal wash counts
Log10 cfu/ml blood counts
TIGR4
Xen35

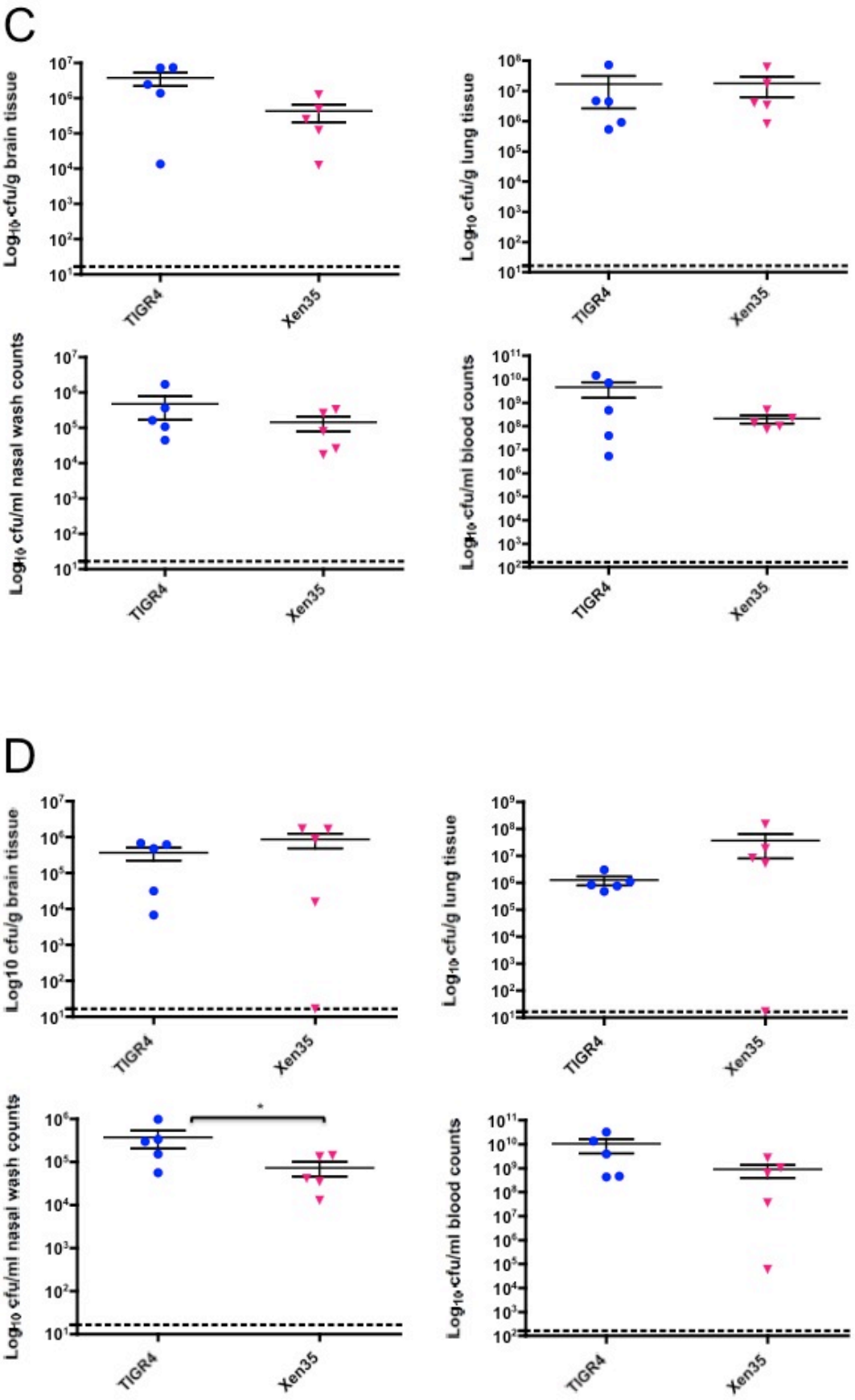


Figure 4-12: Bacterial counts in organs and bodily fluids of MF1 mice infected with Xen35 or T4NO1.

Bacterial counts were enumerated from brain, lungs, nasal wash and blood of mice infected with either Xen35 or T4NO1. Counts were enumerated from 5 mice for each strain over varying time points, graph (A) represent the 24 hour post infection time point, (B) 48 hours, (C) 72 hours and (D) the survival time point. Statistical analysis was performed using a non-parametric Mann-Whitney two sample rank test, *P< 0.05.

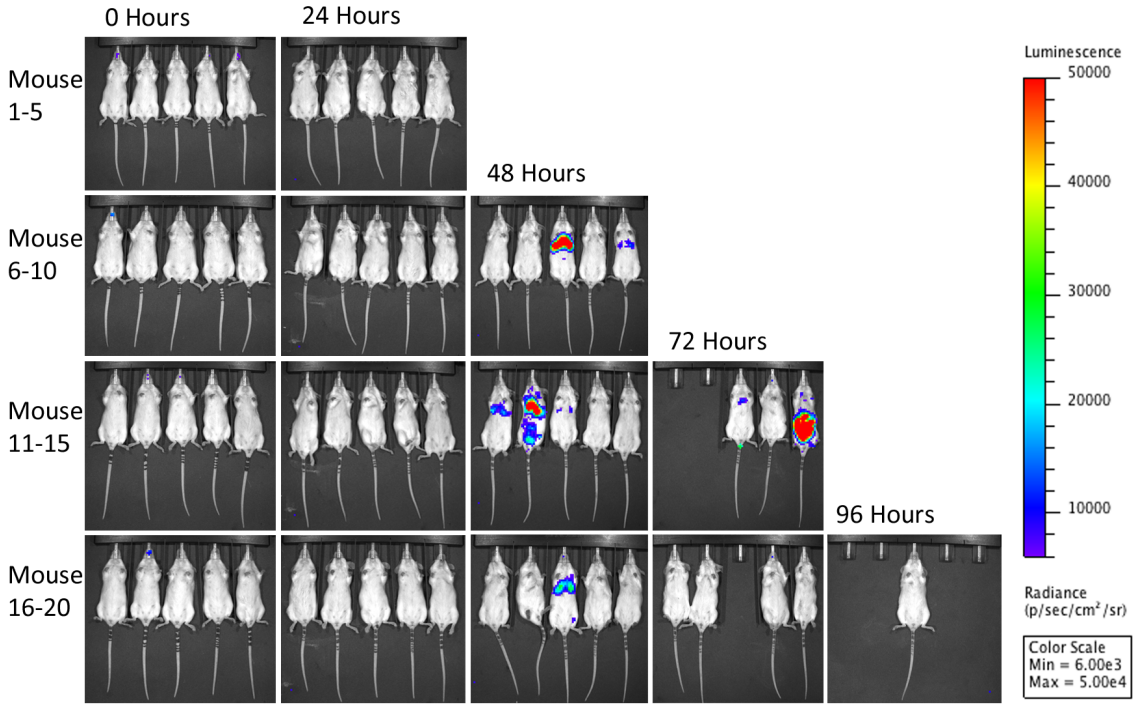


Figure 4-13: Images of mice infected with Xen35

Images of mice infected with Xen35 over time showing *in vivo* infection in the lungs and abdomen of some mice. Images were acquired using the IVIS spectrum *in vivo* imaging system, imaging for 5 minutes for each image. Hours above the images indicate that of the time elapsed since inoculation. Numbers to the side indicate the mice numbers in each group. If mice are not present in the image they have been culled.

4.8 T4NO1 and T4JH genome sequence

Whole genome sequence analysis was also performed on T4NO1 and T4JH to assess any genome changes between these strains and that of the genome sequenced strain (NC_003028). One clear phenotypic change noticed between T4NO1 and T4JH is that in a growing population 88% of T4NO1 cells contain pili (RrgB) on the cells surface whereas only 20.5% contain pili on the cell surface in T4JH. This difference may be due to a genetic difference between the strains. Only preliminary data is currently available for the genome sequence data of these strains, which includes reference assembling the sequence reads of both strains to the available genome sequence (NC_003028), followed by SNP and

indel testing and assessing if there are any low coverage regions. This analysis was performed by Dr Andrea Mitchell. The changes observed have currently not been confirmed by other methods.

4.8.1 T4NO1

Preliminary whole genome sequence changes in T4NO1 compared to the genome sequence data available at NCBI (NC_003028) can be seen in Table 4-11. T4NO1 was taken only a single passage from that of the genome sequence strain (NC_003028) and therefore few genome changes would be expected. Only three changes were observed, one indel, one SNP and one region of low coverage. Only one change was located in a gene, which was a SNP in *comF*. However this did not result in an amino acid change and probably does not alter the genes function. The region of low coverage was located in the intergenic region between SP_1199 and SP_1200. This region was also found to contain a number of changes in Xen35. It may be that this region is a mutational hotspot and readily accumulates changes, as an indel in this region is also seen in T4JH (Table 4-12). Alternatively there may have been mistakes in the assembly of the original genome sequence and these changes are common to all TIGR4 strains. The final indel was located in an intergenic region located between SP_1045 and SP_1046, again the function of this change is not known.

Gene	Information	Position TIGR4	Variant		In Xen35?
INT SP_1045-1046		985056	Indel	T	No
INT SP_1199-1200			Low coverage		Likely, Xen35 contains 4 Indel/ SNPS in this region
SP_2207	ComF	2127997	SNP	G>T (no aa change)	No

Table 4-11: Preliminary whole genome sequence changes in T4NO1.

4.8.2 T4JH

Preliminary whole genome sequence changes of T4JH compared to the genome sequence data available at NCBI (NC_003028) can be seen in Table 4-12. A total of five indels and three SNPs were observed in this strain. Interestingly six of these changes are also observed in Xen35, which may indicate these are

mutational hotspots. The majority of these changes are found in intergenic regions however two are located in genes. This includes the indel in SP_0206, which results in removal of the stop codon, this is also seen in Xen35. This gene is a hypothetical protein and its function is currently unknown. There are no genome changes in any of the genes known to regulate the pilus islet, which may account for the differences in expression of the pilus at the population level in T4NO1 and T4JH. However there is a SNP in the pilus anchor protein (*rrgC*) which has been shown to be present at the base of the pilus (El Mortaji et al., 2012). Currently no structure is available for RrgC so if this amino acid change (His>Tyr) would alter its function is unknown. The change occurs at aa 20 and is present in the N-terminal of the protein so would not effect the cell wall sorting signal or the LPXTG variant (VPDTG) motif required for sortase recognition. This data may suggest that like RrgA, RrgC plays a role in modulating expression of the pilus islet. Perhaps through a protein- protein interaction with one of the other pilus proteins (Basset et al., 2011, Basset et al., 2012).

Gene	Information	Position TIGR4	Variant		Amino acid change	In Xen35?
SP_0206	Hypothetical protein	192436	Indel	C		Yes
SP_0464	RrgC- pilus anchor protein	443333	SNP	C>T	His>Tyr (aa 20)	No
INT SP_0482-0483		463630	SNP	G>A		Yes
INT SP_0496-0497		476406	SNP	G>T		Yes
INT SP_1199-1200		1132344	Indel	G		Yes
INT SP_1359-1360		1282011	Indel	G		No
INT SP_1777-1778		1696086	Indel	C		Yes
INT SP_2105-2106		2016331	Indel	G		Yes

Table 4-12: Preliminary whole genome sequence changes in T4JH.

4.9 Discussion

In this study the phenotype and genotype changes in the bioluminescent strain Xen35 used to study *in vivo* disease progression was assessed. This strain showed a decrease in virulence compared to T4NO1, with mice infected with Xen35 showing an increased survival time to those infected with T4NO1. Corresponding to decreased counts in the nasal wash at 24 hours and survival time points and a slower disease progression indicated by a reduced percentage weight loss compared to mice infected with T4NO1. Contrary to this a trend towards an increase in Xen35 adherence capabilities to A549, D562 and HBMEC cells was observed, however this was not significant. This could indicate not an inability of Xen35 to adhere *in vivo* but a decreased ability to disseminate through the host.

One of the key phenotype changes observed in Xen35 was the large decrease in the presence of the pneumococcal pilus on the cell surface. Recent findings have shown that the pneumococcal pilus expression is regulated at the single cell level with a number of cells within a growing population being negative for the pilus (Basset et al., 2011, De Angelis et al., 2011). Xen35 only contains 5.5% of cells in a growing population positive for the pilus on the cell surface compared to that of 30% in TIGR4 stated in the literature, and 20.5% in T4JH and 88% in T4NO1. In *S.pyogenes* the ability to modulate pili at the population level is linked to disease state (Nakata et al., 2009). The fact we see almost an inability for Xen35 to modulate the pilus at the population level may give an indication as to why the strain in less virulent than the wild type. The regulation of this phenomenon is tightly controlled with a number of TCS regulating it suggesting in the pneumococcus it is also an important regulatory mechanism for survival and virulence.

To assess the contributions of any genome changes in Xen35 causing this decrease in virulence/ pilus expression genome sequencing was performed. Whole genome sequencing of Xen35 showed a large number of genome changes. Xen35 was constructed via transformation of Xen7 genomic DNA (bioluminescent version of D39, serotype 2) into TIGR4. In Xen35 along with the *lux* gene insertion (into SP_1914) a large region surrounding these genes in Xen7 was also recombined into TIGR4. This left these genes in Xen35 that of a D39 allele rather

than a TIGR4 allele. Although the majority of these changes were synonymous a number caused amino acid changes in the resulting protein, whether this would affect the functionality of the protein is unknown. Genes in different strains have been shown to act in a strain specific manner so this is definitely possible (Blue & Mitchell., 2003). Also due to this recombination event two genes (SP_1919/ SP_1920) are non-functional in Xen35 due to frameshifts present in the D39 alleles. These genes encode a component of an ABC transporter and transcriptional regulator respectively. Neither gene has been studied for their effect on virulence and therefore their role in Xen35 is unknown. Of the genes recombined in with the *lux* insertion some have been implicated to play a in role in virulence including SP_1909, SP_1913, SP_1923 and SP_1931. SP_1913 although being up regulated in the blood of infected mice contains an authentic frameshift in TIGR4 and therefore is unlikely to play a role in virulence changes in Xen35 (Orihuela, et al., 2004). SP_1923 encodes the pneumococcal toxin pneumolysin (*ply*) which has been shown to be important for lung infection, *ply* contains 1 amino acid change in Xen35, however this change likely doesn't alter *ply* function (Hava & Camilli, 2002). SP_1909 was shown to be up regulated in the CSF of infected rabbits and therefore may play a role in meningitis however this gene was not found to be important in replication during meningitis. This gene also contains only a single amino acid change and again is unlikely to cause the virulence differences in Xen35 (Orihuela et al., 2004, Molzen et al., 2011). SP_1931 has been shown to be important for bacterial replication during meningitis increasing in expression over time, three amino acid changes are observed in this gene and again seems unlikely to cause the huge changes in virulence seen in Xen35 (Molzen et al., 2011).

Xen35 is widely used to assess the virulence of TIGR4 *in vivo* and assess genes in TIGR4 important to virulence (Orihuela et al., 2003, Orihuela et al., 2004). However due to genotype and phenotype changes this perhaps is not the best model. This also shows the importance of proper characterisation of strains genetically manipulated and importance of designing mutants ensuring recombination events only introduce the desired mutations. With the increasing accessibility of genome sequencing and genome sequence data it is becoming easier to assess whole genome changes in mutants and whether SNPs etc may be

introduced due to allele variations between strains, when producing identical mutations in different serotypes.

A number of genome changes in Xen35 were also located outside of the recombination region, although fewer than that seen in this region. Some of these changes could also be mapped to that of what would be seen in D39, indicating perhaps multiple recombination events during a single transformation (Croucher et al., 2012). This could also indicate variation in the original TIGR4 strain used to construct Xen35 (we do not have access to this strain for comparison) to that of the available TIGR4 genome sequence data (Tettelin et al., 2001). Some of these changes are located in genes important for virulence. This included an in frame deletion of the 3rd PASTA domain of the serine/ threonine protein kinase, which was not seen in Xen7 and therefore likely came from the TIGR4 parent of Xen35. There were also SNPs causing introduction of premature stop codons in SpxB and LctO. The *spxB* SNP was also not from Xen7 shown by the fact Xen7 produces similar levels of hydrogen peroxide as T4JH, whereas Xen35 produces none. These findings would also indicate variation not only between different serotypes but that of the same strains. The Xen35 parent TIGR4 seems to contain changes not seen in the TIGR4 strain that was genome sequenced.

This is also validated by some of our data concerning the surface expression of pili on two TIGR4 strains (T4JH and T4NO1). Both show in a growing population variations in the amount of cells positive for the pilus backbone protein on the cell surface. Although not confirmed this is likely due to a SNP present in *rrgC* in T4JH which is not seen in T4NO1. There are further genome changes observed in T4JH which are not present in T4NO1 which further validates the idea that the "same strain" can be genetically different. In this instance the changes lead to differentially regulation of a key virulence factors which may alter virulence. However in this study the virulence of T4JH and T4NO1 were not directly compared.

Of the gene changes observed outwith the *lux* insertion region a number have been shown to play a role in virulence. The genes only containing amino acid changes include SP_0784, SP_0807, SP_0927, SP_1343 and SP_1891 will not be discussed further as their function is likely not perturbed (Hava & Camilli, 2002,

Molzen et al., 2011, Orihuela et al., 2004). A number of genes however do contain changes that make them non-functional and have shown a role in virulence, including SP_0200, SP_0201, SP_0206, SP_0730, SP_1715, SP_1732 and SP_2076. There is a deletion in SP_200/ SP_201 in Xen35, the same deletion lies in the region where both genes overlap causing removal of the stop codon in SP_0200 and introduction of a premature stop codon in SP_0201. This change is seen in the D39 genome sequence data however shows one larger gene encompassing SP_0200 and half of SP_0201 (SPD_0186). Both of these genes were down regulated in the CSF of infected rabbits in TIGR4, and SP_0200 was up regulated on contact to D562 cells suggesting a role of SP_0200 in adherence. SP_0206 contains an insertion in Xen35 causing a frameshift, again in D39 a gene is annotated encompassing SP_0206 and part of SP_0207 (SPD_0191). SP_0206 is up regulated in a mouse model of bacteraemia in TIGR4 so this deletion may contribute to an attenuation of Xen35 invasion into the blood. Although data was not shown, discussed was a statistical decrease in bacterial counts in the blood taken form the tail veins of mice infected with Xen35 24 hours post inoculation. SP_1715 a component of an ABC transporter contains an insertion creating a frameshift, this gene has been noted to play a role in lung infection and meningitis (Hava & Camilli, 2002, Molzen et al., 2011). SP_2076 contains an insertion, this gene is non-functional in TIGR4 however this insertion removes the authentic frameshift restoring its function in Xen35, this gene is important for lung infection in TIGR4 (Hava & Camilli, 2002).

SP_1732 encoding StkP as stated earlier contains an in frame deletion of the third PASTA domain in the N-terminal of the protein. As the protein is still in frame we cannot assess the impact in Xen35 or this change. StkP has been shown to be important for virulence in a bacteraemia and pneumonia models of infection. However Xen35 does not seem attenuated during lung infection and therefore perhaps the deletion in StkP does not perturb its function (Echenique et al. 2004, Hava & Camilli, 2002, Saskova et al. 2007). The role of the deletion in StkP is discussed further in chapter 5 and therefore will not be covered here.

The SNP causing the introduction of a premature stop codon in SpxB may also affect virulence of Xen35. SpxB has been widely studies showing a role in phase variation with cells non-functional in SpxB showing only opaque colonies, a trait seen with Xen35 (data not shown). This gene has been shown to play a role in

colonisation and to a lesser extent a role in replication in the lungs and translocation to the bloodstream (Orihuela et al, 2004, LeMessurier et al, 2006). This gene is also important for competing in the nasopharynx with other coloniser and does this through production of hydrogen peroxide as a by-product of its enzymatic reaction (Pericone et al., 2000). The role of SpxB in Xen35 will be discussed later in this study in chapter 6 and so will not be covered further here.

Microarray analysis was performed on Xen35 comparing it to TIGR4 to assess the expression changes in this strain. A number of the genes shown to be differentially regulated have been shown to play a role in virulence and therefore may contribute to the decrease in virulence of Xen35 *in vivo*. Of these genes SP_0095, SP_0726, SP_0663, SP_1861 and SP_1898 have been shown to be important during lung infection (Hava & Camilli, 2002). SP_0107, SP_0202 and SP_1587 show increased expression in the blood of infected mice and SP_1572 decreased expression. SP_1468 and SP_1587 show increased expression in the CSF of infected rabbits and SP_1572 and SP_1863 showed a decreased expression (Orihuela, et al., 2004). The two genes surrounding the *lux* insertion SP_1914 and SP_1915 were not shown to be important for virulence from the large scale virulence screens or *in vivo* expression experiments. These genes have however been shown to be variable between strains not being present in some, this may indicate a non vital role of these genes in the pneumococcus, and we assume from this are not causing the decrease in virulence *in vivo* of Xen35 (Silva et al., 2006). Interestingly two important virulence factors in the pneumococcus were differentially regulated in Xen35. Up regulation of *psrP* (pneumococcal serine rich repeat protein) and down regulation of the pneumococcal pilus was observed in Xen35.

psrP although only present in roughly 50% of clinical isolates is an important virulence factor in the pneumococcus (Mun et al., 2010). This gene is up regulated 4-6 fold in Xen35 along with its surrounding glycosyltransferases. These genes are found on a large 37kb islet whose presence has been associated with pneumonia causing clinical isolates (Sanchez et al., 2011, Obert et al., 2006) . PsrP has been shown to be important for biofilm formation with biofilms showing a hyper adhesive phenotype to A549 and D562 cell lines (Sanchez et al., 2011). With PsrP able to bind directly to keratin 10 on lung epithelia cells (Rose

et al., 2008, Shivshankar et al., 2009). Although only confirmed at the expression level we assume this increase in expression of *psrP* in Xen35 correlates with that of surface expressed protein, as Xen35 shows an increased biofilm formation (data not shown) and shows a trend towards an increased adherence to A549 cells. A *psrP* homologue in Group B streptococci has been shown to adhere to and aid penetration of the blood brain barrier (Barrier et al., 2009). Although no current data shows association of *psrP* with meningitis in the pneumococcus this could be why a trend towards an increased adherence to HBMEC cells is observed in Xen35.

The pneumococcal pilus another major virulence factor is down regulated 15-25 fold in Xen35. The pneumococcal pilus is encoded on a 14kb islet containing 7 genes, a positive regulator of the operon (*rlrA*), three pilins *rrgA*, *rrgB* and *rrgC* and three sortases *srtB*, *srtC* and *srtD* (Hava & Camilli, 2002). The Hava & Camilli study showed *rlrA* and *srtD* were important during lung infection. Later studies also implicated the pilus in playing a role in colonisation and bacteraemia, as a non piliated TIGR4 strain was out competed in a pneumonia, colonisation and bacteraemia model of infection (Barocchi et al., 2006). The pilus knockout strain also showed an increased survival time compared to its parent (Barocchi et al., 2006). The pilus has also been shown to aid in adherence to A549 cells. In Xen35 a trend towards an increased adherence to all cell lines is observed, which would not be expected due to the decrease in pilus expression, however this is likely the cause of *psrP* which may be able to compensate for the decrease in pilus. *In vivo* analysis did however show a statistical decrease in nasal wash counts of Xen35 infected mice compared to T4NO1 at 24 hours and survival time points and may be due to this decrease in pilus. The decrease in pilus was confirmed as a decrease in the number of cells expressing the pilus on the cell surface and therefore *in vivo* you could envisage fewer Xen35 bacterial cells being able to adhere in the nasopharynx upon inoculation, which causes a lag in disease progression. Xen35 infected mice showed an increased survival time to that of T4NO1 infected mice mimicking the increased survival data of the non piliated TIGR4 strain published.

Due to the large number of genome and expression level changes in Xen35 it is hard to pinpoint the exact culprit of this decreased virulence or if this is multifaceted. To start to narrow down on the factors contributing to the altered

phenotype of Xen35 we further studied the single contribution of a number of genes to the most prominent phenotypic change in Xen35 (the decrease in pilus expression). This includes evaluating the role of StkP, SpxB and LctO in pilus regulation, which are discussed in later chapters. Another potential contributor to this altered pilus expression is the high expression of the *lux* genes and the potential metabolic burden this places on the bacterial cell. The effect this has on pilus expression is also evaluated in chapter 7.

5 Studying the role of StkP in virulence gene regulation.

Aim of this chapter

Eukaryotic like serine/threonine protein kinases have been discovered in a number of bacteria, which function as an alternative signalling system than the highly abundant TCS, described in chapter 1.7.2. The aim of the work described in this chapter was to examine the potential role of the pneumococcal serine/ threonine protein kinase (StkP) in virulence gene regulation, specifically focusing on regulation of the pneumococcal pilus. Serine/threonine protein kinases are known to regulate pili in other species (Panichkin et al. 2006). The genome sequence data of strain Xen35 showed an in frame deletion within the *stkP* gene. Further Xen35 has been shown to have a decrease in pilus expression at the transcript and protein level. In this chapter the potential contribution of the deletion in StkP to the decrease in pilus expression seen in Xen35 is evaluated.

Initial experiments performed were on a knockout of *stkP* constructed in T4NO1. To look at genome wide changes in gene expression microarray analysis was performed followed by RT-PCR to confirm microarray findings on a number of genes of interest. Specifically looking for changes in pilus expression, western blots were performed on T4Δ*stkP* to assess any changes in RrgB levels, followed by FACS analysis to assess the expression of the pilus in the population as a whole.

As the deletion in *stkP* in Xen35 is an in frame deletion the *stkP* knockout is not necessarily representative of this change in Xen35. To elucidate the functionality of the in frame deletion in Xen35 *stkP*, both alleles from Xen35 and T4NO1 were cloned into T4Δ*stkP* at an alternative region in the genome under the control of a strong promoter (P2). Western blotting and FACS was then performed on these strains (T4Δ*stkP*∇ST/ T4Δ*stkP*∇XST) investigating pilus expression, to see how the different alleles are able to complement T4Δ*stkP*.

The role of StkP in adherence in T4NO1 was also assessed and the contribution of the pneumococcal pilus to this validated. Potential variation in adherence to different cell lines was also assessed (HBMEC/ A549/ D562).

5.1 Background

In the pneumococcus there is a single serine/threonine protein kinase (StkP) making it an easy model to study their function (Yeats et al. 2002, Echenique et al. 2004). StkP is highly conserved between pneumococcal strains and although not indispensible clearly plays an important role in survival and virulence (Giefing et al. 2008). The pneumococcal StkP is a global regulator of gene expression (Saskova et al. 2007). Null mutants show a decreased ability to survive under stressful conditions (heat/ osmotic/ oxidative and acid stress) (strain dependant)(Saskova et al. 2007), a reduced transformation efficiency and a decrease in virulence *in vivo* in a bacteraemia and pneumonia model of infection (Echenique et al. 2004, Saskova et al. 2007).

StkP directly regulates genes via phosphorylation of target proteins on a serine or threonine residue (Novakova et al. 2005). StkP is known to phosphorylate a number of pneumococcal protein including two pneumococcal response regulators (RR06, RitR) (Novakova et al. 2010, Novakova et al. 2005, Agarwal et al. 2012, Ulijasz et al. 2009).

Structurally StkP contains a N-terminal kinase domain joined by a hydrophobic linker to a C-terminal extracellular domain (Yeats et al. 2002, Pallova et al. 2007). The extracellular domain consists of four PASTA domains (penicillin binding protein and serine threonine kinase associated domain), which function as a extracellular sensor domain (Maestro et al. 2010). PASTA domains in PBPs function to recognise the amount of unlinked peptidoglycan and regulate via their transpeptidase domain the amount of cross linking (Gordon et al. 2000, Maurer et al. 2012). This is also thought to be the case in StkP with the PASTA domains shown to be able to bind to synthetic peptidoglycan and β-lactam antibiotics (Maestro et al. 2010, Beilharz et al. 2012). The PASTA domains have also been shown to be important for localisation of StkP to the cell septum (Beilharz et al. 2012, Giefing et al. 2010).

StkP functions as a dimer *in vivo* and is found in the genome adjacent to its cognate protein phosphatase which dephosphorylates proteins phosphorylated by the kinase, forming a signalling couple (Pallova et al. 2007, Ulijasz et al. 2009). In the pneumococcus PhpP also affects gene regulation indirectly through

altering the phosphorylation state of key regulatory proteins, which has been shown for RR06 and RitR (Agarwal et al. 2012, Ulijasz et al. 2009). PhpP also regulates a number of important pneumococcal surface proteins and genes involved in cell wall biosynthesis (*cbpA*, *pspA*, *lytA*, *ftsZ*, enolase etc) (Agarwal et al. 2012).

5.2 Gene expression changes seen in T4Δ*stkP*

*5.2.1 Microarray analysis of T4Δ*stkP*

Microarray analysis was performed comparing expression changes in T4Δ*stkP* to its parent strain (T4NO1). StkP is already known to be a global regulator of gene expression as a StkP knockout made in strain CP1015 showed a large number of genes to be differentially regulated (Saskova et al. 2007). However other global gene regulators (TCS) are known to function in a strain specific manner (Hendriksen et al. 2007). Therefore we thought it important to perform microarray analysis on a *stkP* knockout in TIGR4, as this is the background strain of Xen35. This will also give an indication if StkPs regulation is strain specific like PhpPs.

When performing microarray analysis on T4Δ*stkP* a total of 32 genes were differentially regulated, shown in Table 3-2. Microarray analysis indicates StkP likely regulates the biosynthesis of certain cells wall components in TIGR4. In T4Δ*stkP* genes involved in lipoteichoic acid biosynthesis and incorporation of choline into the cell wall components were up regulated. Further SecY the major component of the SecYEG translocase was down regulated in T4Δ*stkP,* implying a role of StkP in regulation of protein translocation onto the cell surface. This translocase is required for transport of the pilins to the cells surface therefore StkP may modulate cell surface pili levels through modulating protein translocation. The serine protease *htrA* was also up regulated in T4Δ*stkP* functioning to maintain protein quality under conditions of cellular stress and is known to play a role in virulence. A number of sugar transporters were also differentially regulated and a number of ribosomal proteins were down regulated. Although a smaller number of genes were statistically differentially regulated than in CP1015 when increasing the P value cut off to 0.1 (10% genes false positive) a total of 188 genes were differentially regulated. Some of these genes are likely true differences. Included in this list was some of the genes that encode the pneumococcal pilus (data not show).

Gene	Description	Abbrev	Fold change
SP_0209	50S ribosomal protein L3. Binds 3' end 23S rRNA, nucleates assembly of 50S subunit.	*rplC*	1.5
SP_0213	30S ribosomal protein S19. complexes with S13 that binds strongly to 16S rRNA.	*rpsS*	1.6
SP_0218	30S ribosomal protein S17, involved in translation fidelity.	*rpsQ*	1.4
SP_0230	Forms heterotrimeric complex in the membrane.	*secY*	1.5
SP_0282	PTS system mannose specific, IID component.	*manN*	1.5
SP_0283	PTS system mannose specific, IIC component.	*manM*	1.5
SP_0284	PTS system mannose specific, IIAB component.	*manL*	1.6
SP_0645	PTS system IIA component, putative.		1.8
SP_0742	Hypothetical protein.		1.3
SP_0801	Hypothetical protein.		1.3
SP_1000	Thioredoxin family protein		1.2
SP_1164	Acetoin dehydrogenase E1 component, alpha subunit, putative.	*acoA*	1.3
SP_1260	Copper homeostasis protein	*cutC*	1.4
SP_1274	Phosphorylcholine metabolism.	*licD2*	1.5
SP_1356	Chlorohydrolase	*trzA*	1.8
SP_1462	Hypothetical protein.		1.9
SP_1599	Mediates pseudouridylation at the tRNA anticodon region.	*truA*	1.6
SP_1646	Metallo-beta-lactamase superfamily protein.		1.31
SP_1734	rRNA methyltransferase.	*rsmB*	6.2
SP_1735	Modifies the free amino group of the aminoacyl moiety of methionyl-tRNA.	*fmt*	4.9
SP_1736	Primosome assembly protein.	*priA*	3.9
SP_1803	Hypothetical protein.		1.5
SP_1884	Trehalose PTS system, IIABC component.		5.2
SP_1972	Hypothetical protein.		1.4
SP_1994	Aminotransferase.	*alaT*	1.4
SP_2086	Phosphate ABC transporter, permease protein.	*pstA*	1.4
SP_2096	M20/ M25/ M40 family peptidase.	*hipO*	1.3
SP_2106	Glycogen phosphorylase family protein.	*malP*	1.6
SP_2107	4-alpha-glucanotransferase.	*malM*	1.6
SP_2175	Incorporate D-alanine into Lipoteichoic acid.	*dltB*	1.2
SP_2239	Serine protease.	*htrA*	2.2

Table 5-1 : Table of genes differentially regulated in T4Δ*stkP*

Table shows genes differentially regulated in T4Δ*stkP* compared to its parent T4NO1 (P<0.05). Fold change represents that seen in T4Δ*stkP* compared to T4NO1, red showing genes up regulated in T4Δ*stkP* and blue are those that are down regulated.

When comparing the T4Δ*stkP* gene expression changes to that of Xen35, only 1 gene was found to be common to both (SP_2106) (Figure 5-1), However this gene was up regulated in Δ*stkP* (+1.6) and down regulated in Xen35 (-1.7). This means that the gene expression changes seen in Xen35 are likely not due to inactivation of StkP.

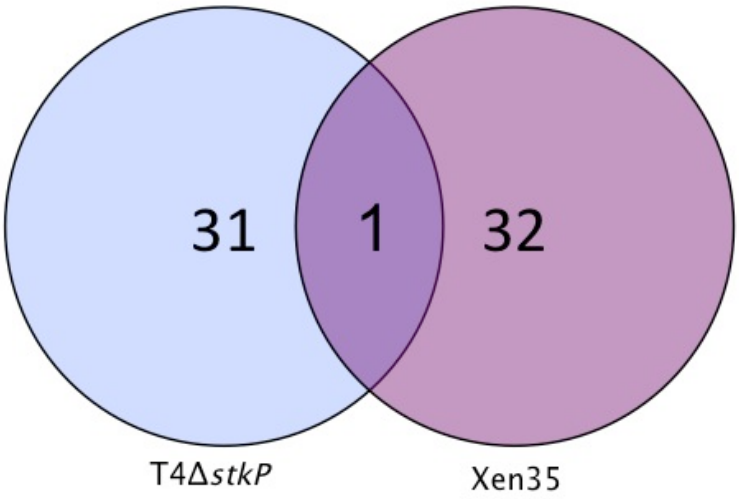


Figure 5-1: Venn diagram of gene expression changes in T4Δ*stkP* and Xen35.

Venn diagram showing genes commonly regulated in T4Δ*stkP* compared to Xen35. Diagram shows only 1 gene common to both evaluated from strains individual gene expression changes compared to their parent strains.

5.2.2 StkP regulates genes in a strain specific manner

Microarray analysis was performed in strain CP1015Δ*stkP* (Saskova et al. 2007). StkPs cognate phosphatase has been shown to regulate genes in a strain specific manner. Therefore this may also be the case for StkP. Using already published data we compared our microarray data to that of the published data using a venn diagram (Figure 5-2) to see if many genes were commonly differentially regulated between the two strains. Although different methodologies were used between the two studies it could give an indication if genes regulated were similar. When comparing the data sets only 3 genes were common between the two strains (SP_0742/ SP_1462/ SP_1803). To ensure there were not a large number of genes similar that just missed our statistical test we increased the P value cut off to 0.1 and compared this data set to that of CP1015Δ*stkP* (Saskova et al. 2007). 188 genes came out in our P<0.1 list (data not shown) (10% are potential false positives). When comparing this gene list to that of the published data only 11 genes were found to be common between the two (appendix VI). Although a number of genes were commonly regulated between the two strains, comparatively to the total number of genes differentially regulated the number is small. This gives a good indication that StkPs regulation is also strain specific.

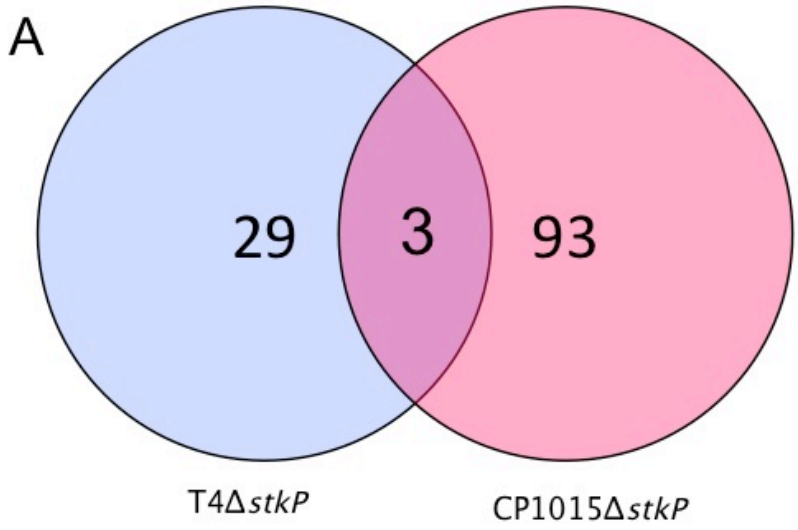


Figure 5-2: Venn diagram of gene commonly regulated by StkP in T4NO1 and CP1015.

Venn diagrams were compiled from our gene expression data in T4Δ*stkP* and that of already published data in CP1015. (A) Compares the gene list In Table 3-2 (P<0.05) to that of published CP1015 gene lists, showing only 3 genes commonly regulated by StkP.

5.2.3 Real-time PCR validation of microarray analysis

RT-PCR (Real-time PCR) was performed on a number of genes to confirm findings from the microarray. Although not in the list above a number of the genes encoding the pneumococcal pilus (SP_0461-63/ SP_0466) came out in the gene list with an increased P value (P<0.1). We therefore decided to perform RT-PCR on the whole pilus islet to see if this was a true difference.

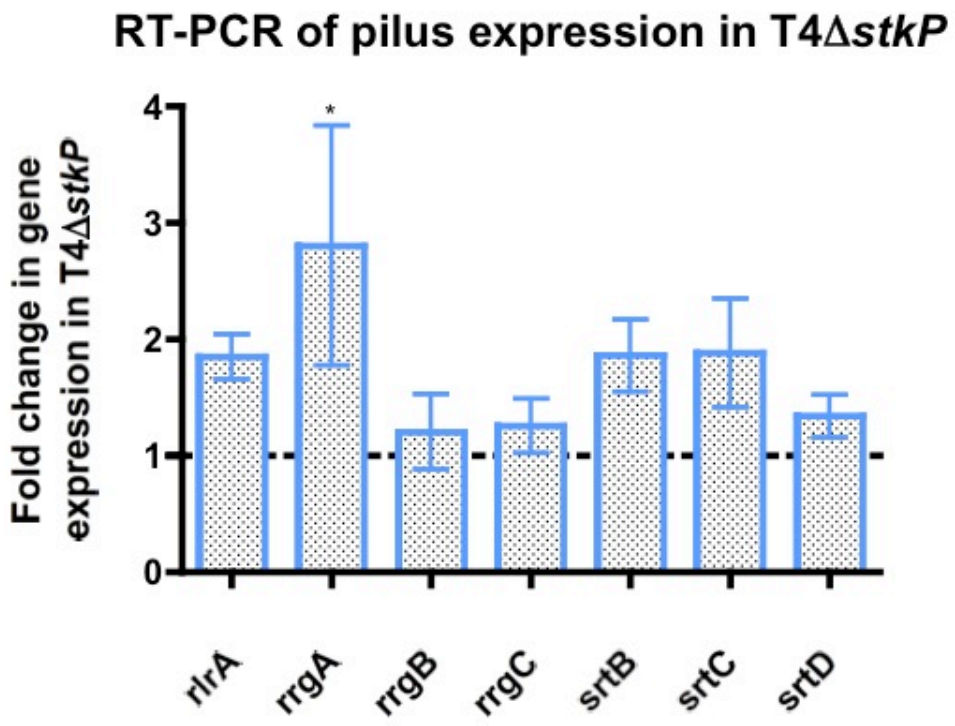


Figure 5-3: RT-PCR graph of pilus expression in T4Δ*stkP*

Graph shows RT-PCR of the whole pilus islet (*rlrA, rrgA, B, C, srtB, C, D*) in T4Δ*stkP*. Fold change represents that of T4Δ*stkP* compared to T4NO1. Each bar represents the average of three replicas (three biological replicas) and errors bars the standard deviation. Statistical analysis was performed by a 1-way ANOVA with a Tukeys testing correction comparing the dCt values of the control strain (TIGR4) to the test strain (T4Δ*stkP*), * P<0.01.

Gene	Microarray	RT-PCR
SP_0461 (*rlrA*)	2.0	1.8
SP_0462 (*rrgA*)	1.7	2.7
SP_0463 (*rrgB*)	1.4	1.2
SP_0464 (*rrgC*)	1.4	1.2
SP_0466 (*srtB*)	1.7	1.8
SP_0467 (*srtC*)	1.3	1.8
SP_0468 (*srtD*)	1.2	1.3

Table 5-2: Comparison of expression changes of the pilus islet in T4Δ*stkP* from microarray and RT-PCR.

Table shows fold changes in gene expression of the whole pilus islet in T4Δ*stkP* compared to T4NO1. Fold changes show that from microarray data and those from RT-PCR.

The RT-PCR of the pilus islet genes in T4Δ*stkP* is shown in Figure 5-3. *rrgA* expression in T4Δ*stkP* was the only gene showing a statistically significant increased expression. *rlrA*, *srtB* and *srtC* showed a trend towards an increase in expression in the StkP deletion mutant, however this was not significant. *rrgB*,

rrgC and *srtD* although potentially showing a very small increase in expression, this again was not significant. Expression values from microarray analysis and RT-PCR were compared in Table 5-2, showing good correlation between both techniques.

StkP has been shown to directly interact with a number of TCS altering their phosphorylation states and therefore their conformation and ability to bind to target genes. Again although not in the list above some of the TCS were shown to be differentially regulated in the P<0.1 gene list (*rr05, rr06, rr10, rr13*). RT-PCR analysis was performed on all the response regulator of the TCS to see if deleting StkP affected their expression.

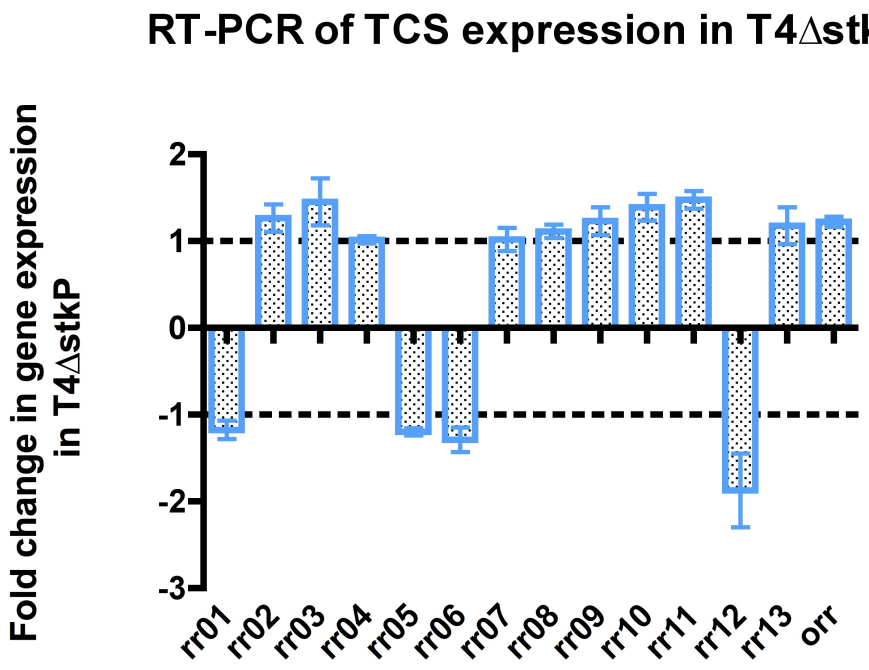


Figure 5-4: RT-PCR graph of TCS expression in T4Δ*stkP*

Graph shows RT-PCR of all the known TCS in S.pneumoniae in T4Δ*stkP*. Fold change represents that of T4Δ*stkP* compared to T4NO1. Each bar represents the average of three replicas (three biological replicas) and errors bars the standard deviation. No statistical difference was observed.

Gene	Microarray	RT-PCR
SP_1633 (*rr01*)	1.2	1.2
SP_1227 (*rr02*)	1.2	1.3
SP_0387 (*rr03*)	1.0	1.4
SP_2082 (*rr04*)	1.0	1.0
SP_0798 (*rr05*)	1.6	1.2
SP_2193 (*rr06*)	1.3	1.3
SP_0156 (*rr07*)	1.4	1.0

SP_0083 (*rr08*)	1.0	1.1
SP_0661 (*rr09*)	1.4	1.2
SP_0603 (*rr10*)	1.2	1.4
SP_2000 (*rr11*)	1.2	1.5
SP_2235 (*rr12*)	1.0	1.8
SP_0526 (*rr13*)	1.2	1.2
SP_0376 (*orr*)	1.1	1.2

Table 5-3: Comparison of expression changes of TCS in T4Δ*stkP* from microarray and RT-PCR.

Table shows fold changes in gene expression of all the TCS in T4Δ*stkP* compared to T4NO1. Fold changes show that from microarray data and those from RT-PCR. Genes highlighted in red are up regulated in T4Δ*stkP* and those in blue down regulated.

The changes in the expression of the TCS are very subtle with no fold change over 2 (Figure 5-4). Of the four TCS that were shown to be differentially expressed by microarray analysis, three showed similar expression levels by RT-PCR (*rr06*, *rr10*, *rr13)* (Table 5-3), although none of the changes were significant. *rr05* when performing RT-PCR showed a change in the opposite direction to that seen in the RT-PCR and therefore is not a true change. Interestingly *rr12* upon RT-PCR seemed to show a trend towards a decreased expression although again this was not significant, and was not seen as significantly differentially regulated in either P<0.05/ P<0.1 lists during microarray analysis.

5.3 Changes in pilus expression in T4Δ*stkP*

To try and confirm any changes in pilus expression in the T4Δ*stkP* strain western blotting was performed using an antibody against the pilus backbone protein (RrgB) to see if there was a noticeable change at the protein level. FACS was also performed to deduce changes in the population of pilus expression. And finally electron microscopy performed to note any obvious changes in the structure or localisation of the pilus in T4Δ*stkP*.

5.3.1 Western blot analysis of T4∆stkP

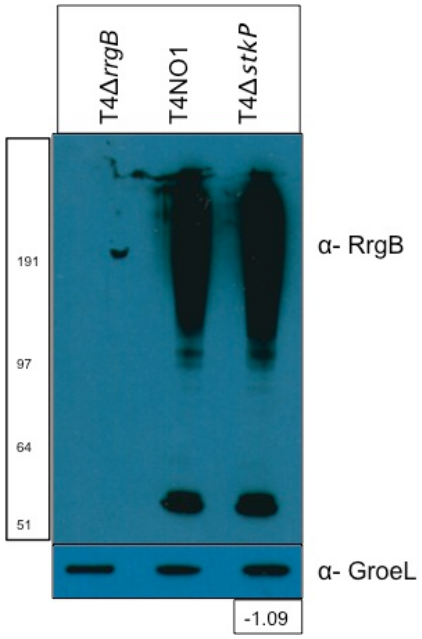


Figure 5-5: Western blot of T4∆*stkP* RrgB expression compared to T4NO1.

Western blotting analysis was performed on T4NO1, T4∆*stkP* and T4∆*rrgB* looking for expression of RrgB in all strains (α-RrgB antibody). Equal protein loading was confirmed by equal expression of GroeL (α-GroeL antibody), normalise against in analysis. Western blotting quantification was performed using ImageJ with fold changes in RrgB expression compared to T4NO1 boxed below the strain.

Western blotting analysis showed no difference between T4NO1 and T4Δ*stkP* in RrgB expression. The high molecular weight ladder of the pilus visually looks more abundant in T4Δ*stkP* compared to T4NO1, however on analysis taking into account the loading control GroeL a slight decrease in RrgB in T4Δ*stkP* was noted.

5.3.2 FACS and fluorescence microscopy analysis of T4∆stkP

FACS (fluorescence activated cell sorting) was performed to further validate changes seen by western blotting. As well as looking at RrgB abundance it will show the number of cells in the population that are RrgB positive. Cells were initially stained using an antibody against the capsule (APC), this population was gated on and from this cells positive for the pilus gated on (FITC). Confirmation of the data seen by FACS analysis was performed via studying the same sample using fluorescence microscopy, visually confirming the FACS findings.

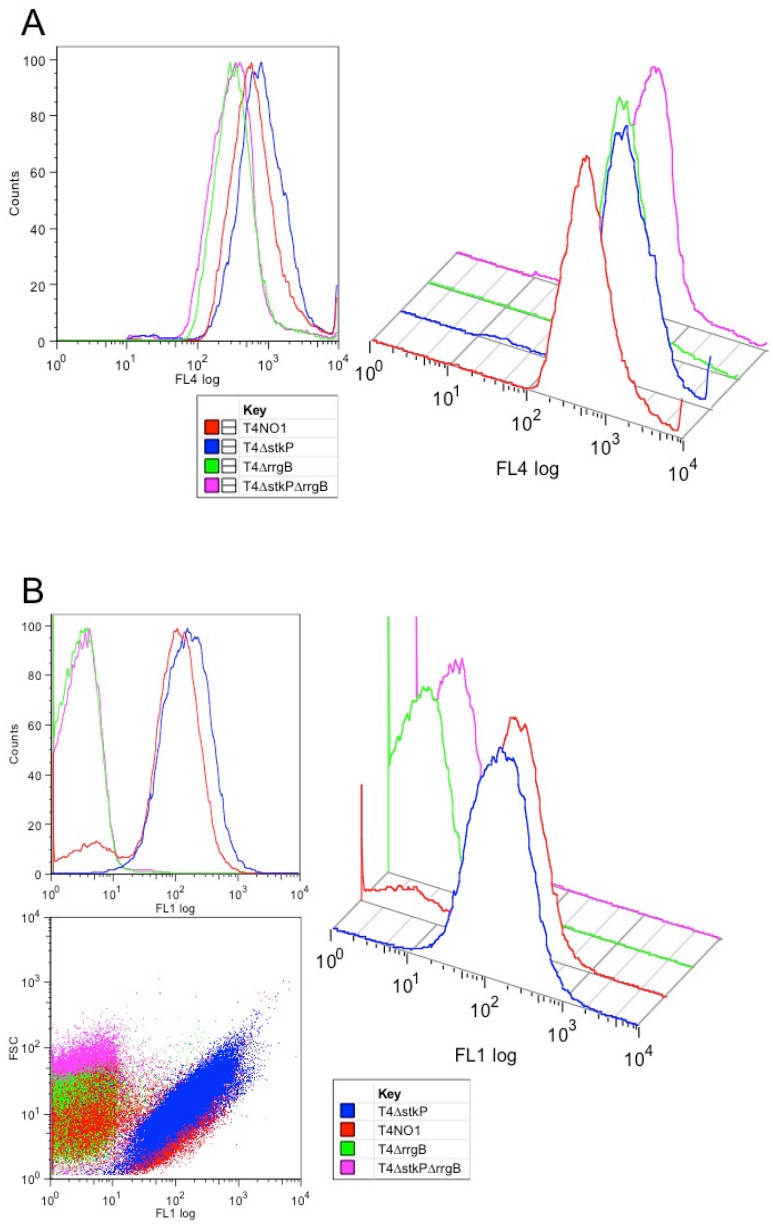


Figure 5-6: FACS analysis of RrgB surface expression in T4Δ*stkP*

FACS was performed on T4Δ*stkP,* T4NO1, T4Δ*stkP*Δ*rrgB* and T4Δ*rrgB*. (A) Shows histograms of the capsule positive populations selected for from samples stained with a capsule antibody, population used for further analysis. (B) Shows the capsule stained populations being gated on for being RrgB positive, histograms/ polychromatic plot show negative (left) and positive (right) RrgB populations in each strain.

Strain	RrgB- (%)	RrgB+ (%)
T4NO1	12.1	87.9
T4Δ*stkP*	0.628	99.4
T4Δ*stkP*Δ*rrgB*	98.3	1.66
T4Δ*rrgB*	98.4	1.6

Table 5-4: Percentage RrgB positive cells in a T4Δ*stkP* population from FACS analysis.

Table shows the percentage RrgB positive and negative cells in a growing bacterial population. FACS analysis was performed in FlowJo.

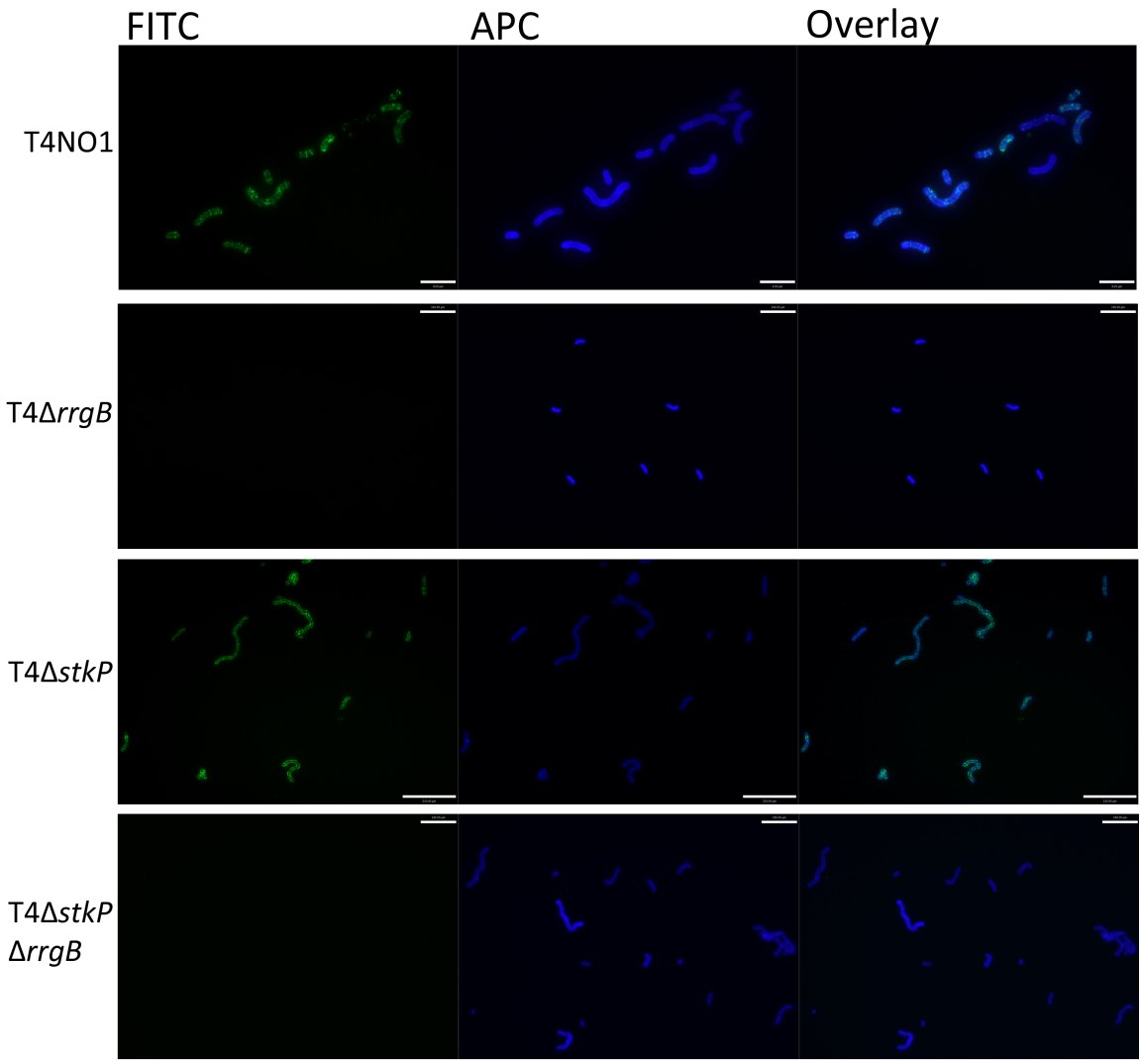


Figure 5-7: Fluorescence microscopy of T4Δ*stkP*.

One representative image of fluorescently labelled T4NO1, T4Δ*rrgB* and T4Δ*stkP* used for FACS analysis. Cells were stained for the presence of RrgB (FITC) and the capsule (APC). Scale bars above represent, T4NO1- 9μm, T4Δ*rrgB* - 140μm, T4Δ*stkP* - 210μm and T4Δ*stkP*Δ*rrgB* - 140μm. The same representative image will be used throughout this study for each strain, more images can be seen in appendix I.

FACS clearly showed a difference in RrgB at the population level in T4Δ*stkP* compared to T4NO1. As already seen in a T4NO1 population when grown in BHI 88% of the cells are positive for the pneumococcal pilus. When removing StkP there is an increase of roughly 10% of cells containing the pilus on the cell surface (Figure 5-6). In StkP 99.4% of a growing population contain the pneumococcal pilus on the cell surface, suggesting a total loss of ability to regulate the amount of the pilus being placed onto the cell surface (Figure 5-6). Visually from the FACS histograms there is a total loss in T4Δ*stkP* of the

population seen on the left hand side representing the pilus negative cells (RrgB negative), which can be seen in T4NO1.

Confirmation of RrgB positive cells is performed via fluorescence microscopy on the same samples used during FACS analysis. The intensity of the RrgB positive population in T4Δ*stkP* and T4NO1 were similar and therefore the amount of pili on a single cell in T4Δ*stkP* is similar to that of T4NO1 (Figure 5-7). Fluorescence microscopy of T4Δ*stkP* showed no cells were pili negative (FITC-) and therefore we assume 100% of T4Δ*stkP* cells contain pili on the cell surface. Both the RrgB knockouts show roughly a 1.6% positive RrgB population however this was not confirmed via fluorescence microscopy (Figure 5-7) and Is likely an artefact of a small amount of non specific binding of the α-RrgB or α- Mouse FITC antibody.

5.3.3 Electron microscopy analysis of T4∆stkP

Electron microscopy was performed on T4Δ*stkP* to evaluate mainly any potential structural changes in the pilus but also any changes in cell morphology already observed in strains when deleting StkP. Microscopy was performed using a α-RrgB primary antibody with a 10nm gold particle conjugate secondary antibody, with pili shown by gold particles labelled along the length of the shaft. Microscopy was also performed on T4Δ*stkP*Δ*rrgB* to further confirm absence of the pilus and no cross reactivity to any other surface proteins. Images were taken between 4000x and 10000x magnification (Figure 5-8).

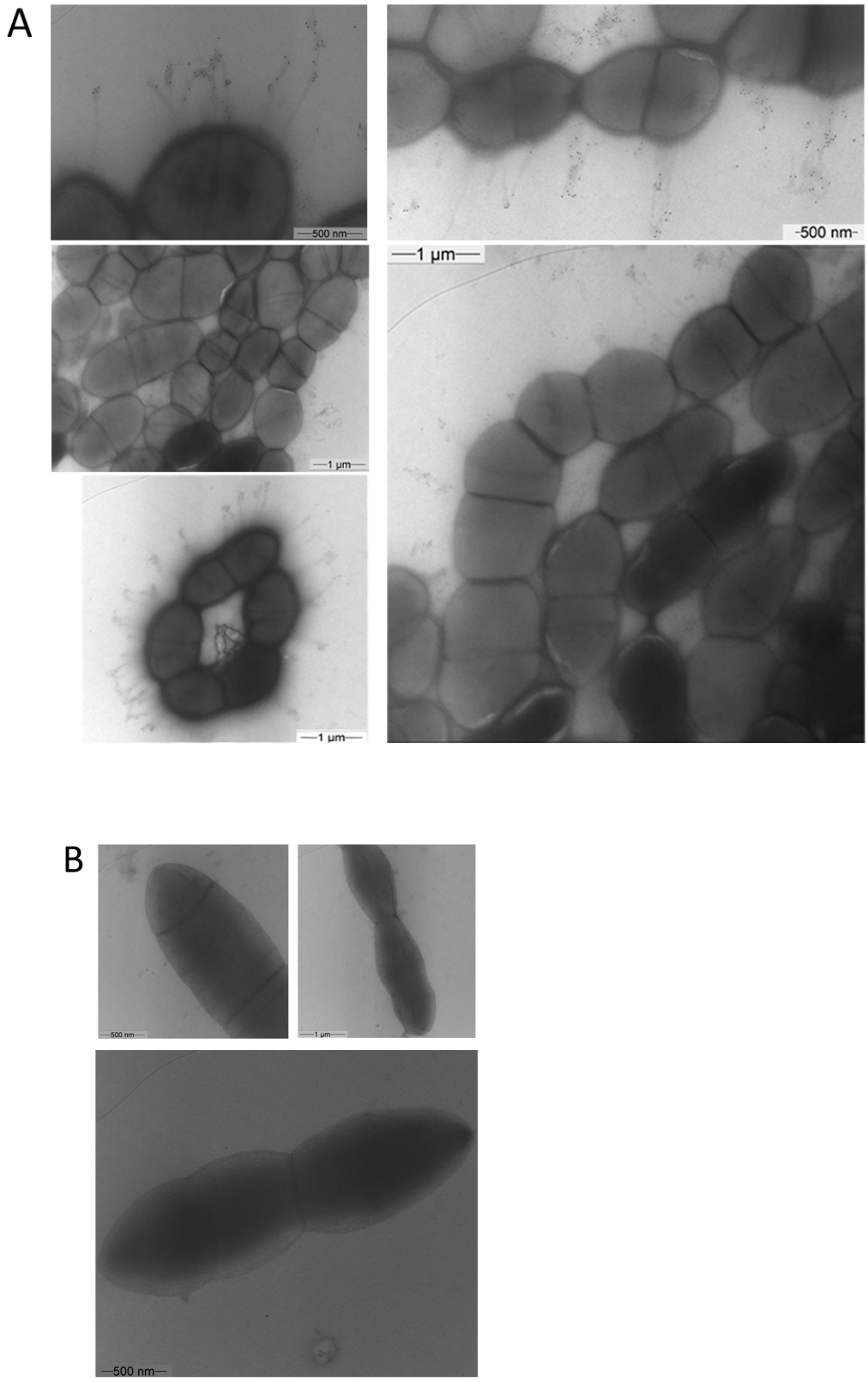


Figure 5-8: Electron microscopy of T4Δ*stkP* and T4Δ*stkP*Δ*rrgB*.

Electron microscopy images of T4Δ*stkP* (A) and T4Δ*stkP*Δ*rrgB* (B). Cells were stained using a α-RrgB antibody and a secondary 10nm gold conjugate antibody. Cells were visualised at 4000-10000x magnifications.

With regards to pili on T4Δ*stkP* cell surface the images clearly show pili on all cells. The number varies from cell to cell with some containing one and others numerous. The projections coming from the cells are not all labelled along the whole pilus shaft compared to T4NO1, which could indicate a change in structure at these regions, which hinders the binding of the RrgB antibody. This could occur through increased RrgA pilin numbers along the RrgB shaft however this has not been validated. These projections are confirmed to be pili as microscopy of T4Δ*stkP*Δ*rrgB* do not show any of these structures (Figure 5-8). There is no discernable difference in the localisation of the pili between the WT train and T4Δ*stkP*.

T4Δ*stkP* was visualised via EM in large clumps (Figure 5-8), which was noticeably different to T4NO1 and T4Δ*stkP*Δ*rrgB,* which formed small clumps/ chains or no clumps respectively. This implicates a role for the pilus in the clumping of T4Δ*stkP*. The inability of T4Δ*stkP* to divide properly probably also plays a role in clumping. Elongated cells with numerous septa and cells with a bulging appearance can be seen in T4Δ*stkP* due to the cells inability to divide properly, a phenotype already noted in the literature. However this phenotype varies with some cells appearing to divide normally and show a regular shape like T4NO1.

5.4 Role of StkP in adherence

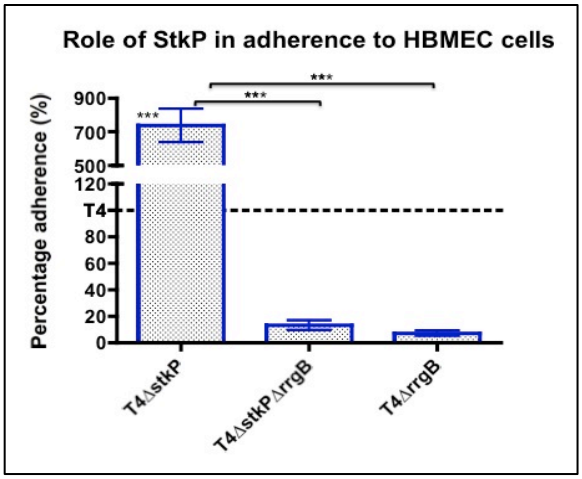
Adherence assays were performed to study the role of StkP in adherence. PhpP has already been shown to play a strain specific role in adherence and therefore as its function is coupled to that of StkP it would be expected that StkP will also alter adherence. Adherence assays were performed on three cell lines to assess if genes regulated by StkP are important at a specific niche. As FACS has indicated an increase in the pilus in T4Δ*stkP* at the population level, the role of pilus in adherence of T4Δ*stkP* was also elucidated. For this adherence assays were also performed on strain T4Δ*stkP*Δ*rrgB* and T4Δ*rrgB*.

5.4.1 Adherence assay analysis of T4△stkP

Adherence assays showed StkP affects adherence, with a statistical increase seen to all cell lines in T4Δ*stkP* (Figure 5-9). However deletion of the pilus (RrgB) in T4Δ*stkP* causes the increase in adherence to be lost, dropping the adherence

capabilities to that similar to T4Δ*rrgB* and T4NO1. The biggest increase in adherence of T4Δ*stkP* is observed to A549 cells (1500%). Upon deletion of the pilus (RrgB) there is a large drop in adherence to levels similar to the WT strain.

A



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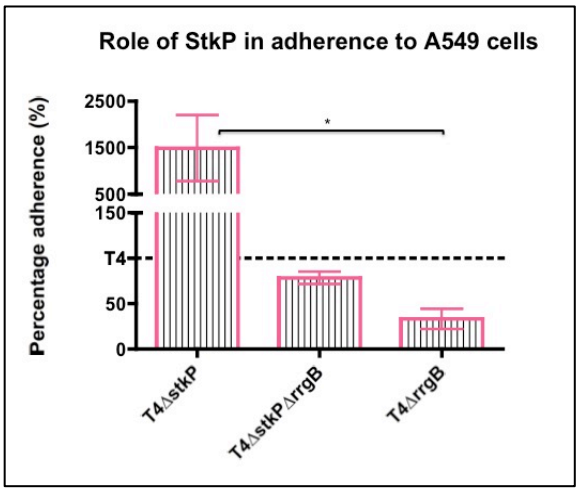

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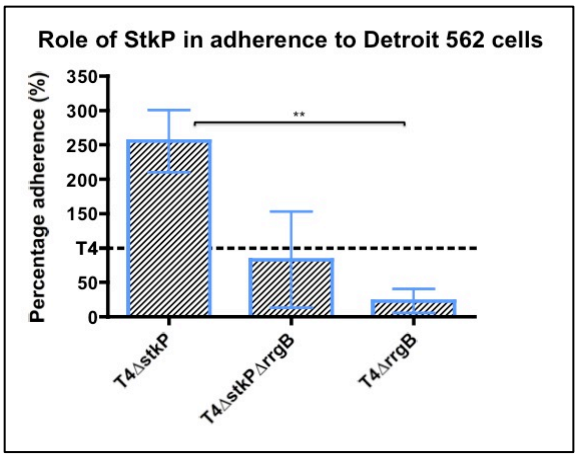


Figure 5-9: Adherence assay of T4Δ*stkP* to different cell lines.

Adherence of strains T4NO1, T4Δ*stkP,* T4Δ*stkP*Δ*rrgB* and *T4*Δ*rrgB* was assessed to HBMEC (A), A549 (B) Detroit 562 (C) cell lines. Data is represented as percentage adherence relative to that of T4NO1 (100%, dashed line), each bar is an average of three replicas and the error bars represent the standard error of the mean. Statistical analysis was performed using a 1-way ANOVA with a Tukeys testing correction, * P<0.05/ ** P<0.001/ *** P<0.0001. * above the bar represent statistical significance compared to T4NO1 (not represented as a bar on the graphs).

5.5 Studying the functionality of PASTA domains in pilus regulation.

As described previously the C-terminal sensor domain of StkP consists of four PASTA domains. In Xen35 the third PASTA domain has been deleted. This still leaves StkP in frame but the functionality of the resulting StkP protein is not known. To study the functionality of Xen35 StkP this *stkP* allele and T4NO1 *stkP* allele were cloned into plasmid pC2LSD P2, placing *stkP* under the control of the strong P2 promoter (pCP2 ST/ pCP2 XST). Once transformed into T4Δ*stkP* this left the *stkP* alleles in the genome between genes SP_1885/ SP_1887 along with the P2 promoter (T4Δ*stkP*∇ST/ T4Δ*stkP*∇XST). Both strains were tested for their ability to complement T4Δ*stkP* in regards to its altered pilus expression. For this western blotting for RrgB was performed as well as FACS to see if there were changes in the population as a whole expressing the pilus. Adherence assays were also performed to see if complementation of T4Δ*stkP* alters the strains ability to adhere to HBMEC and A549 cells.

5.5.1 Western blot analysis of T4∆stkP complements

Western blotting again showed no difference between T4NO1 and T4Δ*stkP* likely due to detection limits of western blotting. However differences in RrgB expression could clearly be seen in T4Δ*stkP*∇ST and analysis showed a 2 fold decrease in RrgB expression compared to T4NO1 (Figure 5-10). T4Δ*stkP*∇XST also showed a decrease in RrgB expression of 1.2 fold compared to T4NO1 (Figure 5-10). This gives an indication that StkP from T4NO1 has increased activity than StkP from Xen35, and therefore the third PASTA domain is important in StkP signalling.

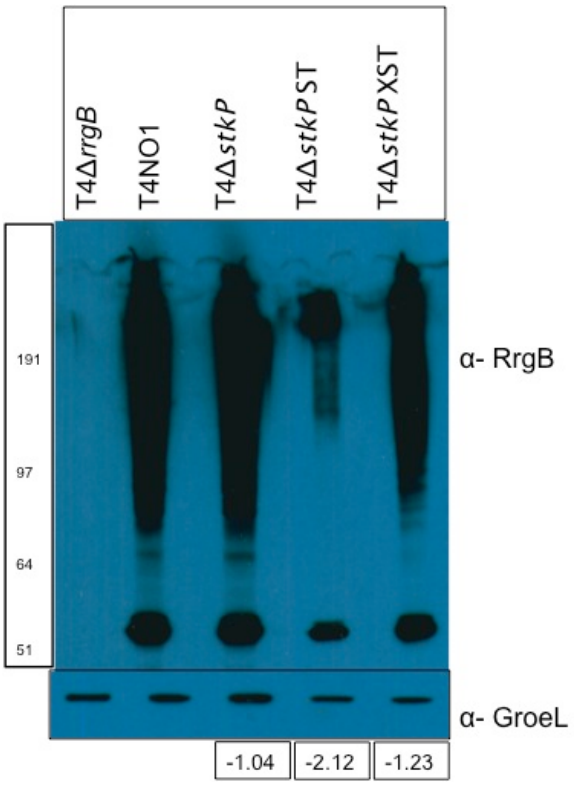


Figure 5-10: Western blot of T4Δ*stkP* complements RrgB expression compared to T4Δ*stkP* and T4NO1.

Western blotting analysis was performed on T4NO1, T4Δ*stkP*, T4Δ*stkP*∇ST, T4Δ*stkP*∇XST and T4Δ*rrgB* analysing expression of RrgB in all strains (α-RrgB antibody). Equal protein loading was confirmed by equal expression of GroeL (α-GroeL antibody), normalise against in analysis. Western blotting quantification was performed using ImageJ with fold changes in RrgB expression compared to T4NO1 boxed below the strain.

5.5.2 FACS and fluorescence microscopy analysis of T4ΔstkP complements.

FACS analysis was also performed on the complement strains to see how pili were distributed throughout the population. FACS analysis clearly showed a large reduction in pilus expressing cells in T4Δ*stkP*∇ST with only 26% of cells remaining pilus positive compared to 100% of T4Δ*stkP* and 88% of T4NO1 cells (Figure 5-11/ Table 5-5). The majority of T4Δ*stkP*∇ST RrgB positive cells also seem to show a large reduction in the amount on a single cells, with just small single pilus positive regions visualised in most cases (Figure 5-12). The exact locality is hard to assess and would require a more sensitive technique. This however may indicate StkP is regulating the positioning of the pilus on the cell surface. However there is not shift to the left of the pilus positive population in

T4Δ*stkP*∇ST observed via FAC, which would indicate that the pili positive cells have a lower fluorescent intensity (less pili) than the other strains tested.

In T4Δ*stkP*∇XST there was also a decrease in the population of pilus expressing cells with only 51% of cells being pilus positive. Indicating Xen35 StkP has roughly half the functionality of T4NO1 StkP containing all four PASTA domains. These cells similarly compared to T4NO1 and T4Δ*stkP* show in the majority of cases pili spread all over the cell surface (Figure 5-12)

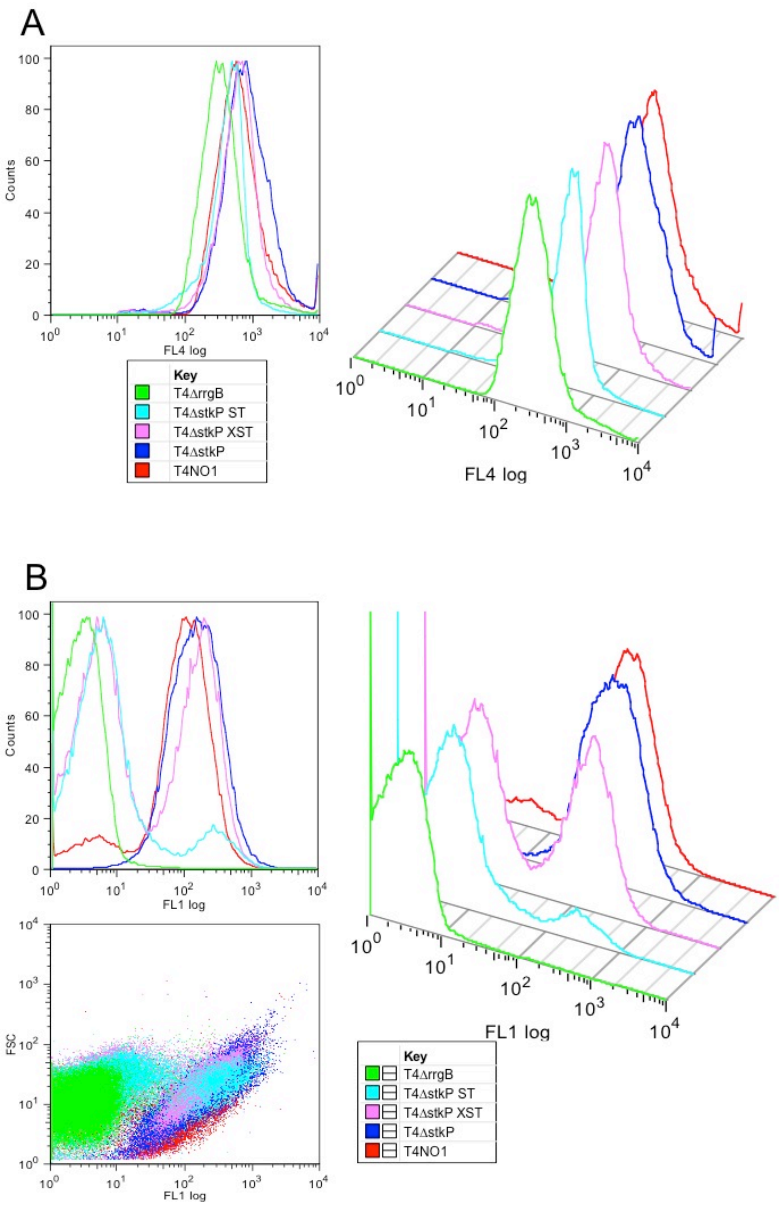


Figure 5-11: FACS analysis of RrgB surface expression in T4Δ*stkP* complements.

FACS was performed on T4Δ*stkP*, T4NO1, T4Δ*stkP*∇ST, T4Δ*stkP*∇XST, T4Δ*rrgB*. (A) Shows histogram of the capsule positive populations selected for from samples stained with a capsule antibody, population used for further analysis. (B) Shows the capsule stained populations being gated on for being RrgB positive, histograms/ polychromatic show negative (left) and positive (right) RrgB populations in each strain.

Strain	RrgB- (%)	RrgB+ (%)
T4NO1	12.1	87.9
T4Δ*stkP*	0.628	99.4
T4Δ*stkP*∇ST	74	26
T4Δ*stkP*∇XST	48.7	51.3
T4Δ*rrgB*	98.4	1.6

Table 5-5: Percentage RrgB positive cells in a T4Δ*stkP* population from FACS analysis.

Table shows the percentage RrgB positive and negative cells in a growing bacterial population. FACS analysis was performed in FlowJo.

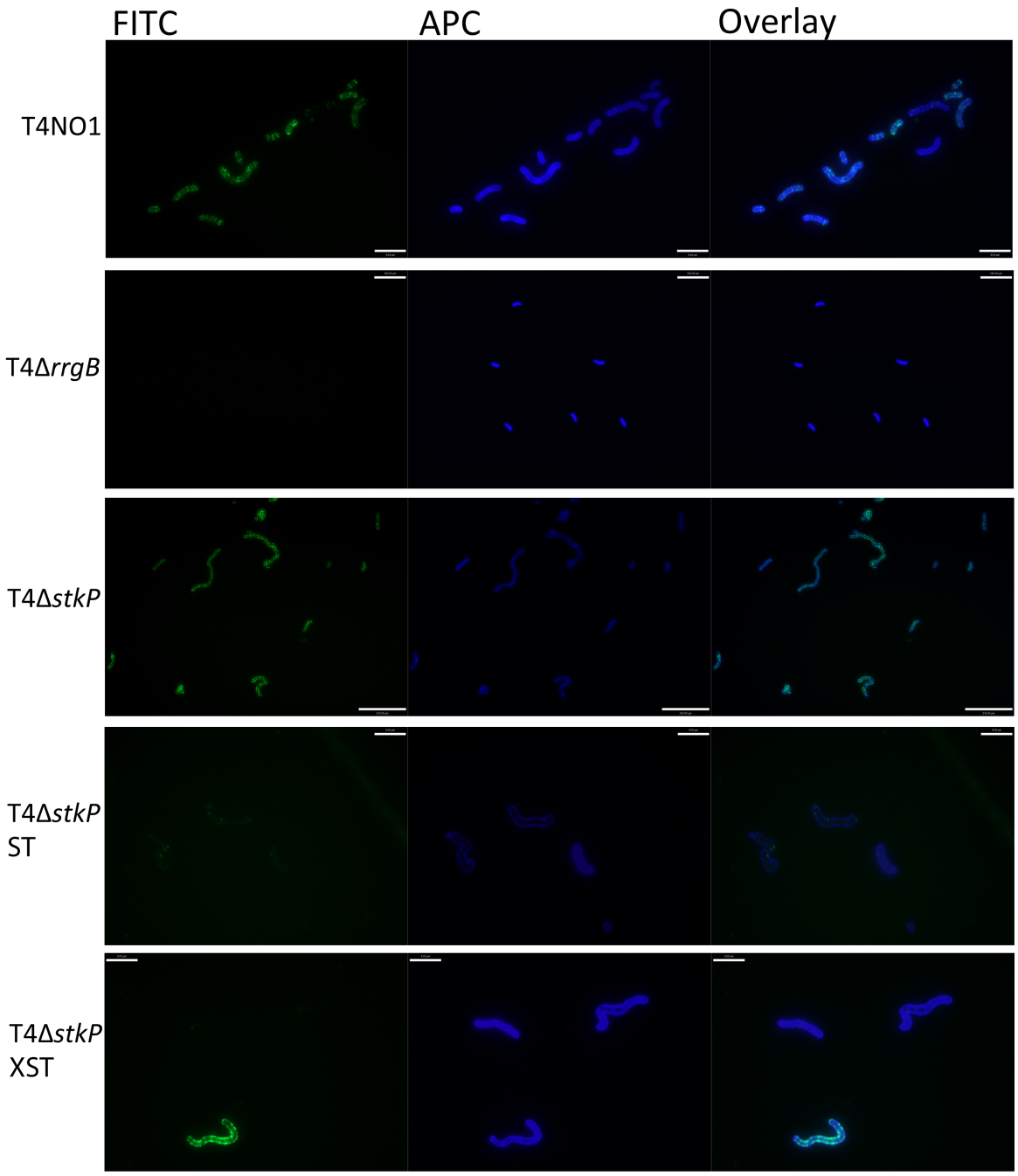


Figure 5-12: Fluorescence microscopy of T4Δ*stkP*∇ST and T4Δ*stkP*∇XST.

One representative image of fluorescently labelled T4NO1, T4Δ*rrgB,* T4Δ*stkP*, T4Δ*stkP*Δ*rrgB,* T4Δ*stkP*∇ST and T4Δ*stkP*∇XST used for FACS analysis. Cells were stained for the presence of RrgB (FITC) and the capsule (APC). Scale bars above represent, T4NO1- 9μm, T4Δ*rrgB* - 140μm, T4Δ*stkP* - 210μm, T4Δ*stkP*∇XST - 9μm and T4Δ*stkP*∇XST - 9μm. The same representative image will be used throughout this study for each strain, more images can be seen in appendix I.

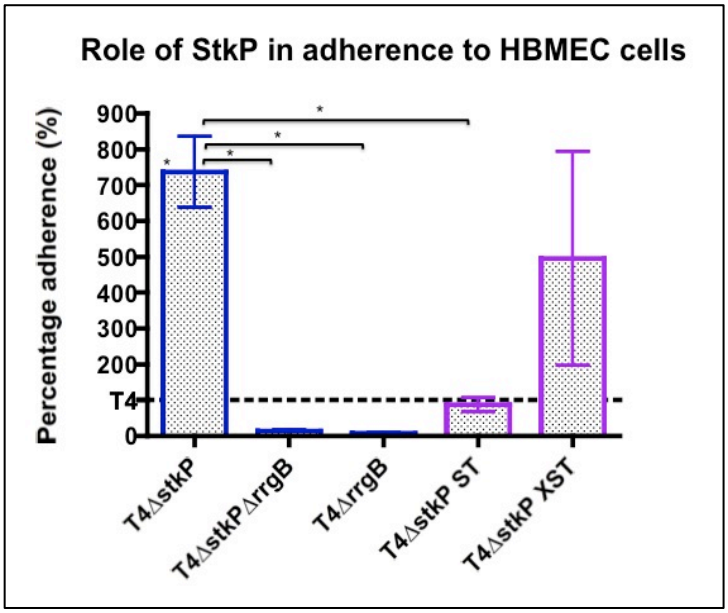
5.5.3 Adherence assay analysis of T4ΔstkP complements

Due to the clear differences seen in pilus expression in T4Δ*stkP*▽ST and T4Δ*stkP*▽XST adherence assays were performed on these strains to HBMEC and A549 cells (Figure 5-13).

Adherence assay data clearly showed a reduced adherence to both cells lines of both strains with complemented StkP. T4Δ*stkP*▽ST showed the biggest statistical decrease in adherence with adherence to both cell lines dropping to nearly that of T4NO1. T4Δ*stkP*▽XST showed a trend towards a drop in adherence compared to T4Δ*stkP*, although this was not statistically significant. This data further validates StkPs role in regulation of the pneumococcal pilus and its importance in adherence to different cell lines. With the pneumococcal pilus in this strain being a large contributing factor to adherence to different cell types.

This data also confirms the importance of the four PASTA domains in StkP as complementation of T4Δ*stkP* with StkP with only three PASTA domains was unable to complement the mutant to the same level as the full length StkP. Why this may be the case is discussed further in section 5.6.

A



B

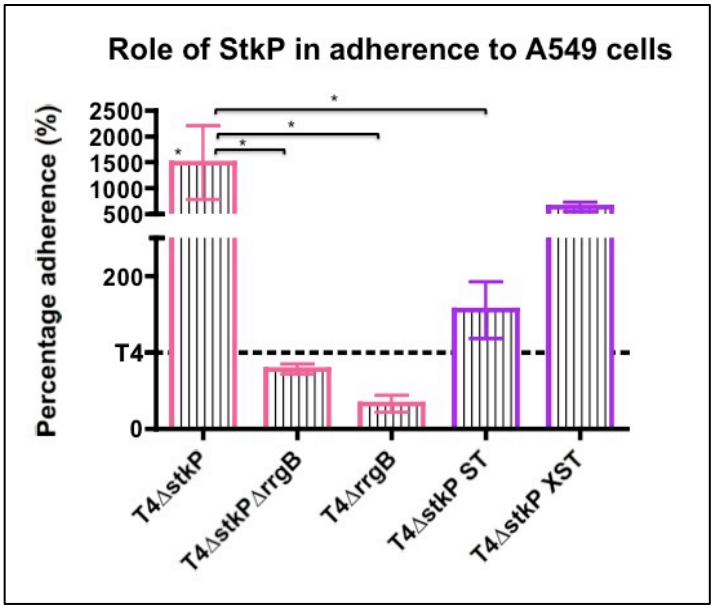


Figure 5-13: Adherence assay of T4Δ*stkP* complements to different cell lines.

Adherence of strains T4NO1, T4Δ*stkP,* T4Δ*stkP*Δ*rrgB,* T4Δ*stkP*∇ST, T4Δ*stkP*∇XST and *T4*Δ*rrgB was assessed* to HBMEC (A), A549 (B) cell lines. Data is represented as percentage adherence relative to that of T4NO1 (100%, dashed line), each bar is an average of three replicas and the error bars represent the standard error of the mean. Statistical analysis was performed using a 1-way ANOVA with a Tukeys testing correction, * P<0.05. * above the bar represent statistical significance compared to T4NO1 (not represented as a bar on the graphs).

5.6 Discussion

StkP has already been shown to be a global regulator of gene expression (Saskova et al. 2007), our data also indicates that this is the case with 32 genes differentially regulated in a *stkP* deletion mutant constructed in T4NO1 (P< 0.05). When decreasing the stringency of analysis a total of 188 genes were differentially regulated (P<0.1). Comparison of these gene lists with genes known to be regulated by StkP in CP1015 showed very few were commonly regulated between the two strains, indicating a strain specific role in regulation of StkP (Saskova et al. 2007). This phenomenon has been shown for alternative signalling systems (TCS) and has recently been shown for StkPs cognate phosphates (PhpP) (Blue et al. 2003, Hendriksen et al. 2007, Agarwal et al. 2012).

Some of the genes shown to be differentially regulated in T4Δ*stkP* were those of genes involved in choline metabolism. Phosphorlycholine is an essential part of the two teichoic acids (TA (Lipoteichoic acid, teichoic acid)) present in the cell wall of the pneumococcus (Tomasz 1967b, Brundish & Baddiley 1968). Removal of choline in growth media results in the hindrance of pneumococcal growth showing altered chain formation, loss of transformability and no autolysis (Tomasz 1967a). Choline metabolism is controlled by two main operons *lic1* and *lic2*. *lic1* operon consists of 5 genes *licABC* involved in choline uptake and *tarI/J* involved in ribitol formation. *lic1* was not shown to be differentially regulated in T4Δ*stkP* (Kharat & Tomasz 2006, Baur et al. 2009). *lic2* consists of 3 genes *licD1/ licD2* involved in attachment of phosphorylcholine to TA and *tacF* involved in transport of TA across the membrane (Damjanovic et al. 2007). *licD2* has been shown to directly affect the amount of choline present in the cell wall with a deletion mutant containing a 50% reduction of the amount of choline in the cell wall (Zhang et al. 1999). *licD2* (P<0.05) and *licD1* (P<0.1) were up regulated on deletion of *stkP*, implicating a role of StkP in regulation of the amount of choline in the cell wall components. *S.pneumoniae* contains a number of important virulence factors, which are dependant on the presence of choline in the cell wall for cell surface attachment (Yother & White 1994, Hakenbeck et al. 2009). These choline binding proteins include a number of adhesins and virulence factors (CbpA, PspA etc) and autolytic enzymes (LytA-D, CbpD) important for cell division and autolysis (Gillespie et al. 1997, Hakenbeck et al. 1983, De Las

Rivas et al. 2002). Altering the amount of cell wall choline will likely effect the abundance of these proteins on the cell surface, determining if they attach or are released into the supernatant (Hakenbeck et al. 1983). The alterations in some of these proteins potentially affected by variations in cell surface choline composition corresponds to the phenotypes changes seen when deleting StkP. T4Δ*stkP* shows reduction in competence, a phenomenon noted upon growth in choline limited media (Tomasz 1967a, Regine Hakenbeck et al. 1983). StkP mutants show a deficiency in ability to divide a phenotype associated to that of a LytB mutant, which may correlate in T4Δ*stkP* with decreased amounts of LytB on the cell surface (Tomasz 1967a, Hakenbeck et al. 1983). Along with choline incorporation into the cell wall *dltB* was also up regulated in T4Δ*stkP,* which is important for LTA biosynthesis (Poyart et al. 2001).

Although TCS CiaRH was not shown to be regulated at the transcript level by StkP other data indicates a potential interaction of StkP and RR05 potentially through phosphorylation as seen with StkP for RR06 and RitR (Agarwal et al. 2012, Ulijasz et al. 2009). A study in 2007 evaluated the direct interaction of CiaR with the promoters of its target genes, which showed a number of similarities to the genes seen to be differentially regulated in T4Δ*stkP* (Halfmann et al. 2007). Of these common genes included were *manM*/ *manM* and *manL* which function as a mannose phosphotransferase system (Cochu et al. 2003), *malM* and *malP* encoding enzymes involved in maltosaccharide metabolism (Nieto et al. 1997). Interestingly the lic1 operon was regulated by CiaR whereas we saw differences in the *lic2* operon, and finally the serine protease *htrA* and *parB* ($P<0.1$) involved in chromosomal segregation (Halfmann et al. 2007, Minnen et al. 2011). From this the hypothesis would be that StkP may also phosphorylate CiaR affecting its binding capacity to these promoter regions causing the changes in gene expression seen in T4Δ*stkP*. For the genes that are know to be regulated by CiaR and were not shown in our Δ*stkP* gene list, it could be that these were not regulated by CiaR in a TIGR4 background.

Along with potentially regulating the attachment of the cell surface proteins to the cell wall our data indicates a potential role of StkP in translocation of proteins onto the cell surface. In T4Δ*stkP secY* was also differentially regulated, which function as a transmembrane pore vital for protein translocation across

the cell membrane. Other members of the translocase *secE* and *secG* were not differentially regulated and normally function on a 1:1:1 ratio with *secY*. *secA* which functions as a protein chaperone was also not differentially regulated (Dalal & Duong 2011). However if this is the case it indicates another level at which StkP Is able to regulate the content of the cell surface. Interestingly recent data has shown that during exponential growth SecY localises to the cell septum at the point where active peptidoglycan synthesis is occurring (Tsui et al. 2011). StkP has already been shown to localise to the midcell and partakes in sensing the cell wall peptidoglycan (Giefing et al. 2010, Maestro et al. 2010). Therefore a role could be envisaged for StkP to act in conjunction with SecY regulating at the expression level the amount of SecY available for protein translocation and therefore the amount of proteins translocated.

Another component shown to interact with the Sec translocase is HtrA a serine protease that works in conjunction with SecA (chaperone for proteins being directed to the SecYEG translocase) (Tsui et al. 2011). HtrA is thought to act here as quality control for the proteins targeted to the translocase and cell surface. HtrA also localises to the cell septum along with SecA and SecY (Tsui et al. 2011). *htrA* is up regulated in T4Δ*stkP* indicating a requirement for protein degradation.

High temperature requirement A (HtrA) protein has been extensively studied in the pneumococcus, functioning as a temperature induced serine protease (Ibrahim et al. 2004a), and in other streptococci has been shown to alter the expression of some cell surface proteins (S. Biswas & I. Biswas 2005). HtrA activity is more evident upon increased temperature and has been shown to help the cell survive environmental stresses (Ibrahim et al. 2004a). HtrA has been shown to be an important virulence factor showing increased survival in a mouse pneumonia and bacteraemia model (Ibrahim et al. 2004b, Ibrahim et al. 2004a). A phenotype of decreased competence associated with HtrA is one that is also seen in T4Δ*stkP* (Sebert et al. 2005,). In T4Δ*stkP* an increase in HtrA expression is observed, which may correlate to a decrease in competence (Sebert et al. 2005). This could potentially be the cause of the decrease in competence in T4Δ*stkP*.

htrA is found adjacent to *parB* in the genome and they are likely expressed as a single operon. ParB has recently been shown to be involved in chromosomal segregation upon replication recruiting condensing to the pneumococcal *oriC* (Minnen et al. 2011). This gene is also up regulated in T4Δ*stkP* (P<0.1), as StkP has been shown to be involved in cell division its not unlikely that it may also regulate proteins involved in chromosome segregation as cell division and DNA segregation would likely be required to work in unison.

We did not study further the affect of the StkP deletion on the presence of choline and choline binding proteins on the cell surface. As the pneumococcal pilus was differentially regulated in the increased P<0.1 list we studied further changes in this virulence factor in T4Δ*stkP*. RT-PCR was performed and confirmed an expression change in *rrgA*, however expression of the other genes on the pilus islet were not shown to be statistically significant. Western blotting for the pilus backbone protein (RrgB) showed no discernable change in T4Δ*stkP* RrgB levels. However FACS analysis looking for the presence of RrgB on the cell surface showed roughly a 10% increase in the number of cells containing the pilus in T4Δ*stkP*. If there was a true decrease in expression of *secY* you would perhaps expect a decrease in proteins on the cell surface, including RrgB. However perhaps such a small fold change is too small to see a large change in phenotype. This increase in pilus shown by FACS was confirmed by fluorescence microscopy and corresponded to 100% of T4Δ*stkP* cells having pili on their cell surface. Therefore StkP seems to regulate whether individual cells produce pili at the population level, which could also be the case for a number of other surface proteins regulated by StkP. StkP cognate phosphatase has shown to regulate a number of cell surface components, as they work as a pair, StkP therefore likely affects oppositely the expression of these surface proteins. Implicating a global role for StkP in regulation of cell surface components, however none of these surface proteins showed expression change from our microarray analysis.

A recent finding has shown that the pneumococcal pilus is regulated at the population level, with within a population two cell types existing, high pilus expressing cells and low pilus expressing cells. As of the moment no genes lieing outwith the pilus islet have been characterised as altering at the population

level the amount of pili that are seen on the cell surface in a growing population (Basset et al. 2011, De Angelis et al. 2011). Between the two populations no expression differences were observed indicating changes in the cell that are expression independent (De Angelis et al. 2011). Further studies showed that this phenotype is modulated via RrgA through its binding to RlrA (Basset et al., 2011, Basset et al., 2012). However there is a large increase in *rrgA* expression in T4Δ*stkP* relative to the expression of *rlrA* therefore it is unlikely StkP modulates its regulation at the population level through altering levels of these two proteins. StkP has been shown to phosphorylate RR06. RR06 has also been shown to repress the expression of the pneumococcal pilus as in a deletion mutant of HK06 an increase in expression of the pilus islet is observed (Rosch et al. 2008). RR06 could potentially be the factor modulating the expression of the pilus at the population level in T4Δ*stkP*.

StkP cognate partner PhpP has been shown to regulate genes that affect adherence to D562 cells in a strain specific manner. Here we show that StkP in T4NO1 also regulates adherence to different cell types. The contribution of the pneumococcal pilus to this increased adherence phenotype in T4Δ*stkP* was also evaluated. The role of StkP in adherence varied between all three cell lines, with the biggest increase in adherence seen to A549 cells with an increase of 1500% compared to T4NO1, HBMEC cells showed an increase of 700% and D562 cells 250%. In HBMEC cells it is clear this increased adherence phenotype is due to the increase in the pneumococcal pilus in T4Δ*stkP,* as upon deletion of the pilus (RrgB) the adherence phenotype is reverted to that of T4Δ*rrgB*. This phenotype is seen for all cell lines, as deletion mutants of RrgB in T4Δ*stkP* are no longer able to adhere to the same level and reverts to the level of adherence of T4Δ*rrgB*. However in A549 cells there is potentially another unknown factor contributing to the increased adherence to this cell line as upon deletion of the pilus (RrgB) the strain does not fully revert to that of T4Δ*rrgB* and has a similar adherence to the WT strain. D562 cells showed the smallest increase in adherence in T4Δ*stkP* perhaps indicating a less important role of the pilus in colonisation to that of pneumonia and meningitis.

Considering there is only a 10% increase in the number of cells containing the pilus in T4Δ*stkP* the huge increase in adherence seems disproportionate relative

to T4NO1. However due to the most recent structure of the pneumococcal pilus it has been shown that RrgA is also able to multimerise and form branches of adhesins (RrgA) coming from varying positions along the RrgB backbone shaft (El Mortaji et al., 2012). As there was a clear increase in *rrgA* expression and likely corresponding protein expression we could hypothesis that as well as the increased amounts of RrgB present in the population on the cell surfaces, these cells may contain increased amounts of branched RrgA molecules, further increasing the adhesive capabilities of the cells. RrgA has also been associated with increased biofilm formation and may promote bacterial cell to cell attachment in T4Δ*stkP* (Muñoz-Elías et al. 2008). Therefore increasing the number of cells adherent during the adherence assays, not only through those that are directly associated with the tissue culture cells but also through bacteria- bacteria interactions. Upon deletion of RrgB this removes the backbone at which RrgA anchors itself to and likely depletes RrgA adhesive capabilities by removing the length at which RrgA can reach beyond the cell wall. Indications that this may be the case was shown by immunogold labelling of pili in T4Δ*stkP* visualised by electron microscopy. With pili from T4Δ*stkP* not always being labelled along the whole length of the RrgB shaft compared to that seen in T4NO1, this could indicate an inability for the antibody to efficiently reach the RrgB backbone due to other proteins blocking them (RrgA). However this could also be a staining problem and would need to be validated further.

To evaluate the role of the deletion of the 3rd PASTA domain in the Xen35 *stkP* allele we looked at its potential role in regulation of the pneumococcal pilus. StkP regulates the pneumococcal pilus however this is opposite to what we would expect to see if this deletion was causing the huge decrease in pilus expression seen in Xen35. However the exact effect the deletion of the 3rd PASTA domain has on StkP in Xen35 is not know. To study this T4Δ*stkP* was complemented with *stkP* amplified from T4NO1 (Four PASTA domains) and Xen35 (three PASTA domains), and both alleles were put under the control of a strong promoter (P2) in T4Δ*stkP*. This resulted in the over expression of these complemented StkP alleles in T4Δ*stkP*. It is assumed these alleles were expressed to the same level in the two strains as they were constructed using the same plasmid, this is currently being validated via western blot for StkP, to

confirm similar expression levels. The role of these different StkP alleles functionality was then assessed for their ability to effect pilus expression.

These experiments further confirmed that StkP regulates the pneumococcal pilus as western blotting showed a clear decrease in RrgB when T4Δ*stkP* was complemented with both alleles. However there was a marked difference in the amount of RrgB between both T4Δ*stkP*∇ST and T4Δ*stkP*∇XST. T4Δ*stkP* complemented with T4 StkP showed a higher reduction in RrgB than that complemented with Xen35 StkP, implying that Xen35 StkP has a reduced functionality and that the 3rd PASTA domain is important for downstream signalling.

FACS performed on these complemented strains also showed that these changes in protein expression are at the population level. With T4Δ*stkP*∇ST now only showing 26% of the population are positive for the pneumococcal pilus compared to 51% of T4Δ*stkP*∇XST to 100% of T4Δ*stkP*. Further studies would need to be performed on these strains to elucidate the downstream gene causing these changes seen in the pilus. Fluorescence microscopy of T4Δ*stkP*∇ST cells also seemed to show a decrease in the amount of pili on each cell compared to that of T4Δ*stkP*∇XST, T4Δ*stkP* and T4NO1. To see if StkP also affects the localisation of the pilus electron microscopy would have to be performed on T4Δ*stkP*∇ST. Yet from this we can say StkP clearly also regulates the amount of pili on a single cell as well as that in the population. Maintaining heterogeneity within a bacterial population is important to allow bacterial survival, the fact StkP modulates this function fits in with the idea that these systems sense the external environment and modulate responses to the outside accordingly. With StkP in each bacterial cell acting according to the environment within which it is present in this instance modulating expression changes depending on the levels of unlinked peptidoglycan on the cell surface. StkP has also been shown to interact with a TCS, which may be the downstream system which modulated the change in pilus expression directly.

Adherence assay were performed on both complement strains using A549 and HBMEC cells. The adherence seen to both cell lines correlated directly to the amount of pili positive cells, with T4Δ*stkP*∇ST showing a large reduction in

adherence to both cell lines. However this was still higher than that of T4Δ*rrgB* and T4Δ*stkP*Δ*rrgB* due to 26% of cells still containing the pilus. T4Δ*stkP*▽XST also showed a trend towards a decrease in adherence to both cell lines compared to T4Δ*stkP* (no significant difference*).* Yet this was still higher than T4Δ*stkP*▽ST due to 51% of cells containing the pilus.

The importance of PASTA domains with regards to the functionality of StkP have been elucidated in Beilharz et al 2012. Mutants constructed removing the PASTA domains were no longer able to localise to the cell septum (Where WT StkP localises too), with mutants containing only the kinase domain or transmembrane and kinase domain showing a cytoplasmic and membrane localisation pattern respectively (Beilharz et al. 2012). When replacing the four PASTA domains of StkP with the Three PASTA domain of PrkC (*B.subtilis*) StkP no longer localised to the cells septum indicating that the PASTA domains are species specific with regards to their substrate binding (Beilharz et al. 2012). Which in this instance is to unlinked peptidoglycan, as blocking these moietys with vancomycin lead to delocalisation of StkP from the midcell (Beilharz et al. 2012). The consequence of the removal of the PASTA domains not only affects StkP localisation but also that of its cognate phosphatase, which localised to the midcell along with StkP in a WT cell, however upon StkP deletion PhpP is only seen in the cytoplasm and therefore requires StkP to function properly (Beilharz et al. 2012). The outcome of this leads to alterations in the protein phosphorylation levels of the targets of StkP and PhpP, which has been shown to be modulated through StkP activity. Beilharz et al 2012 showed blocking the activity of StkP via incubating cells with vancomycin (binds to the substrate of StkP) lead to reduced phosphorylation of protein targets, however incubating cells with ampicillin which binds to the PASTA domains (mimics peptidoglycan substrate) hyperphosphorylation of StkP protein targets is observed. Some of StkPs protein targets are that of cell division proteins (FtsA and DiviVA) which is why when StkP is deleted an altered cell morphology is observed, as deletion of StkP alters the localisation of these cell division proteins which also colocalise at the cell septum with StkP (Beilharz et al. 2012).

As we observed some cells that had an elongated cell morphology for Xen35 we assume this is due to the deletion of the third PASTA domain within StkP, which

is likely a consequence of alteration in the localisation of StkP and cell division protein to their correct cellular positions. Interestingly Beilharz et al 2012 also showed that this elongated morphology is selected against and multiple passages show fewer cells with this morphology, which may be why not all Xen35 cells showed this altered morphology (Beilharz et al. 2012). Further as we know the deletion of PASTA domains alters StkP localisation and interaction with its protein targets, its likely this is also the cause of the altered pilus expression observed in the mutants. RR06 has been shown to regulate the pilus and is a protein target of StkP, its therefore likely that deletion of the third PASTA domain affects the level of phosphorylation of this protein target altering its activity (Agarwal et al. 2012).

StkP clearly plays a multifaceted role in gene regulation and is already know to affect the expression of a large number of genes, and phosphorylate a number of other proteins. In this study we also show a potential role of StkP in regulation of protein translocation through the cell membrane and incorporation of some of these proteins onto the cell wall via regulation of choline abundance in the cells wall TA. Both of these phenomenon fit in with StkPs role in regulation and sensing of cell wall components, however require further study for validation. A role of StkP regulating genes at the population level has also been envisaged, a mode of regulation only recently touched upon in the pneumococcal field. Our studies have also shown that StkP regulates the pneumococcal pilus which has not been shown before and we further validated the importance of the PASTA domains in downstream gene regulation. More studies are required for further confirmation of exactly how the deletion of the third PASTA domain affects StkP functionality.

6 Studying the role of oxidative stress on pilus expression

6.1 Aim of this chapter

The aim of the work described in this chapter is to investigate the potential role of the pneumococcal pyruvate oxidase (SpxB) and lactate oxidase (LctO) in pilus gene regulation. The genome sequence data of Xen35 a bioluminescent strain used to track *in vivo* disease progression contained SNPs in both of these genes, causing the introduction of a premature stop codon, resulting in truncated proteins. Xen35 showed a clear reduction in the expression of the pneumococcal pilus and in this chapter the contribution of these mutations to this phenotype is assessed.

Initial experiments were performed to assess the pilus expression in a SpxB and LctO mutant constructed in T4NO1. For this RT-PCR, western blot analysis and FACS analysis was performed to look at the expression of RrgB in both strains. This analysis was also performed using a double mutant. The expression of the whole pilus islet was assessed in all strains. Adherence assays were also performed to evaluate the role of these genes in adherence and if this is niche specific as SpxB is thought to play an important role during colonisation.

Both genes are involved in growth under aerobic conditions so we sought to assess pilus expression in a wild type strain when grown aerobically and anaerobically. The enzymatic reactions catalysed by both SpxB and LctO lead to production of hydrogen peroxide as a by-product. Therefore the effect of hydrogen peroxide alone on pilus expression was evaluated. For all western blotting and FACS analysis was performed.

6.2 Background

Both SpxB and LctO play a role in energy production in a bacterium that contains no tricarboxylic acid cycle or electron transport chain (Hoskins et al., 2001, Tettelin et al., 2001). *spxB* encodes a pyruvate oxidase which functions to convert pyruvate from glycolysis to acetyl phosphate, which is eventually converted by acetate kinase to acetate to produce ATP (Pericone et al., 2003). The reaction catalysed by SpxB occurs during aerobic growth producing 85% of the cells acetyl phosphate with hydrogen peroxide produced as a by-product (Pericone et al, 2003). *spxB* deletion mutants show reduced levels of ATP production (Pericone et al., 2003). *lctO* encoding a lactate oxidase has not been as extensively studied as SpxB. LctO converts the lactate normally seen as a dead end product of fermentation back to pyruvate which can then be processed by the pyruvate oxidase to acetyl phosphate and eventually ATP (Taniai et al., 2008, Udaka et al., 1959).

Both genes work in a concerted manner under aerobic growth conditions with $1/4^{th}$ of pyruvate converted by SpxB to acetyl phosphate resulting in ATP production (Taniai et al., 2008, Liu et al., 2012). The remaining pyruvate is converted to lactate via lactate dehydrogenase, which acts as a reservoir until the lactate oxidase converts the lactate back to pyruvate resulting in an end product of ATP production (Taniai et al., 2008). This has been hypothesised to maximise ATP production under aerobic growth. Both enzymes are flavoproteins and produce hydrogen peroxide as a by-product of their enzymatic reaction, with SpxB being the main contributor to the high hydrogen peroxide levels produced by the pneumococcus (0.5-2mM) (Liu et al, 2012, Taniai et al., 2008, Pericone et al, 2003). Hydrogen peroxide is an important virulence factor in the pneumococcus, as already discussed in section 1.6.10.

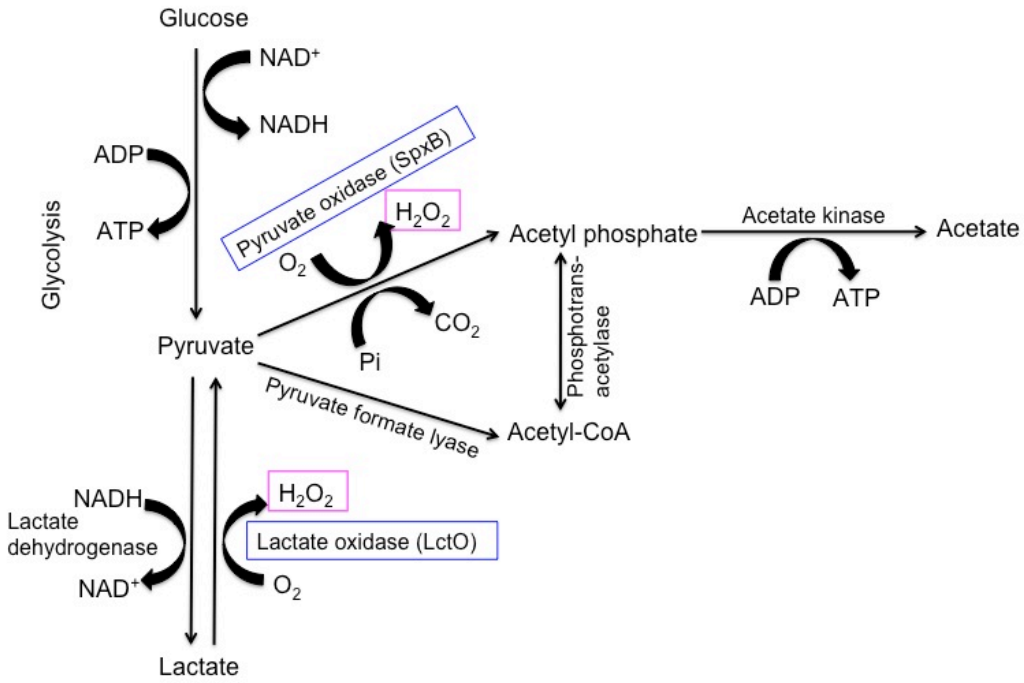


Figure 6-1: Pathway of glucose metabolism in *S.pneumoniae*

(Modified from Taniai et al., 2008) Boxed in blue is the two enzymes discusses in this section. Boxed in pink is the hydrogen peroxide produced as a by-product of said enzymatic reactions

Changes in a number of different genes have been linked to phase variation, including mutations in SpxB (Weiser et al., 1994, Pericone et al., 2000, Ramos-Montañez et al., 2008). For more information see section 1.5.4.

6.3 Role of SpxB and LctO in pilus regulation

To assess the role that SpxB and LctO play in pilus regulation two knockout mutants were constructed. A *spxB* mutant was constructed using a transposon insertion based method and a *lctO* mutant constructed using a splice overlap PCR method to replace the whole gene. Both mutants were constructed in T4NO1. The *spxB* mutant was constructed using a spectinomycin resistance cassette and the *lctO* mutant using a kanamycin resistance cassette. Use of different selection markers allowed construction of a double knockout creating T4Δ*spxB*Δ*lctO*.

*6.3.1 Microarray analysis of T4Δ*spxB*

Initial experiments assessed the expression changes in T4Δ*spxB*, in an attempt to attribute the gene expression changes seen in Xen35 to this deletion. For this microarray analysis was performed on T4Δ*spxB* compared to T4NO1.

Gene	Description	Abbrev	Fold change
SP_0413	Aspartate kinase		1.3
SP_0461	Transcription regulator, putative	*rlrA*	2.1

Table 6-1 : Table of genes differentially regulated in T4Δ*spxB*

Table shows genes differentially regulated in T4Δ*spxB* compared to its parent T4NO1 (P<0.05). Fold change represents that seen in T4Δ*spxB* compared to T4NO1, red showing genes up regulated in T4Δ*spxB* and blue are those that are down regulated.

Microarray analysis showed only two genes differentially regulated between T4NO1 and T4Δ*spxB* (Table 3-2). Neither of these genes were differentially regulated in Xen35 as measured by microarray analysis (P<0.05). However when increasing the P value to 0.1 two genes were commonly down regulated in Xen35 and T4Δ*spxB (*SP_0461 and SP_0462), of which both encode components of the pneumococcal pilus. This gives an indication the non-functional SpxB in Xen35 may contribute to the decreased pilus expression. However it is likely not the only cause as only a 2 fold decrease in *rlrA* expression is observed in T4Δ*spxB* compared to a decrease of 6 fold in Xen35 shown by microarray analysis. Microarray analysis performed on a *spxB* mutant in a R6 strain showed 21 genes differentially regulated compared to its parent (Ramos-Montañez et al., 2008).

6.3.2 Real-time PCR analysis of pilus islet and TCS expression in T4ΔspxB, T4ΔlctO and T4ΔspxBΔlctO

RT-PCR analysis was performed to confirm the change in *rlrA* expression observed in T4Δ*spxB* by microarray analysis, as well as any expression changes seen in the rest of the pilus islet. RT-PCR analysis was also performed on T4Δ*lctO* and T4Δ*spxB*Δ*lctO* knockout strains to assess if LctO also affects pilus expression and if removal of both lead to a similar pilus expression level as observed in Xen35 (Figure 5-3).

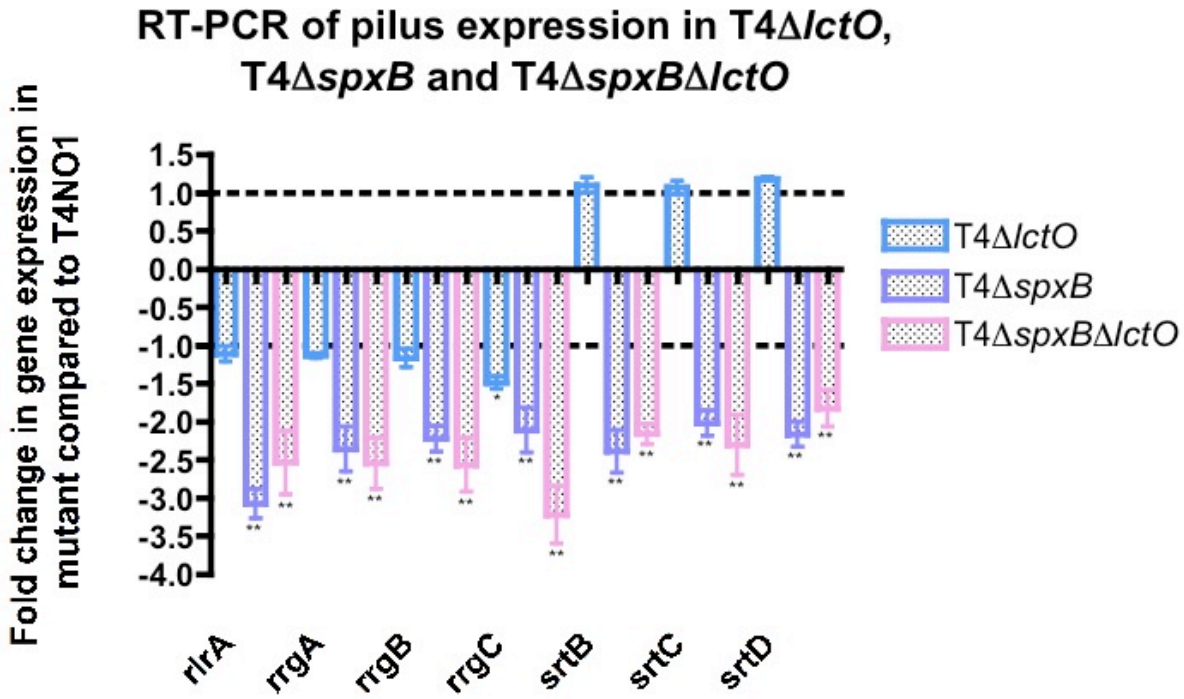


Figure 6-2: RT-PCR graph of pilus expression in T4Δ*spxB,* T4Δ*lctO* and T4Δ*spxB*Δ*lctO.*

Graph shows RT-PCR of the whole pilus islet (*rlrA, rrgA, B, C, srtB, C, D*) in T4Δ*spxB,* T4Δ*lctO* and T4Δ*spxB*Δ*lctO.* Fold change represents that of stated mutant compared to T4NO1. Each bar represents the average of three replicas (three biological replicas) and errors bars the standard deviation. Statistical analysis was performed by a 1-way ANOVA with a Tukeys testing correction comparing the dCt values of the control strain (TIGR4) to the test strain (T4Δ*spxB,* T4Δ*lctO* and T4Δ*spxB*Δ*lctO*), * P<0.05, **P<0.001.

RT-PCR analysis confirmed the difference in pilus expression seen in T4Δ*spxB,* showing roughly a 2-3 fold statistical decrease in expression of all the genes encoded on the pilus islet shown in Figure 5-3. RT-PCR showed perhaps a small drop in expression of some of the pilus islet genes in T4Δ*lctO* compared to T4NO1 however this was not the case for the sortase enzymes and only the

reduced expression of *rrgC* was shown to be statistically significant (Figure 5-3). T4Δ*spxB*Δ*lctO* showed a similar statistical decrease in pilus expression compared to that of T4Δ*spxB*. RT-PCR was also performed on some of the RR of the TCS pairs that have been shown to alter pilus regulation in the pneumococcus, to try and attribute pilus expression changes to that of varied expression of the RR (Figure 5-4).

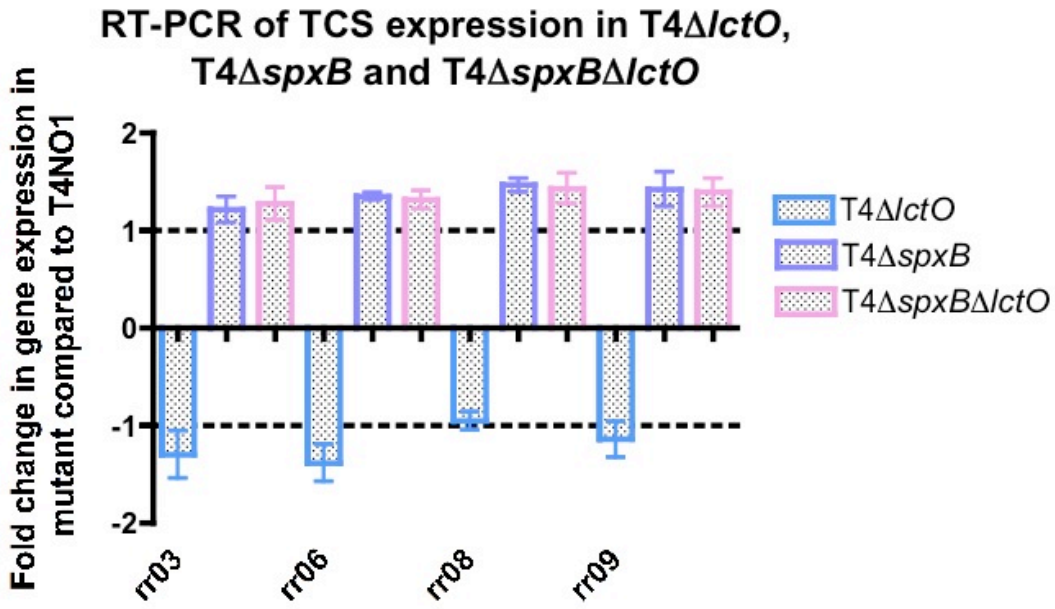


Figure 6-3: RT-PCR graph of TCS expression in T4Δ*spxB,* T4Δ*lctO* and T4Δ*spxB*Δ*lctO*

Graph shows RT-PCR of *rr03*, *rro6*, *rro8* and *rr09* TCS in T4Δ*spxB,* T4Δ*lctO* and T4Δ*spxB*Δ*lctO.* Fold change represents that of the stated mutant compared to T4NO1. Each bar represents the average of three replicas (three biological replicas) and errors bars the standard deviation. No statistical difference were observed.

RT-PCR of some of the TCS shown to alter pilus regulation were all slightly up regulated in T4Δ*spxB* and T4Δ*spxB*Δ*lctO,* however none were statistically significant. T4Δ*lctO* also showed no statistical difference in expression of any of the TCS RR. Due to none of the changes being significant it is unlikely any of these TCS contribute to the altered pilus expression in these strains.

To further confirm changes in pilus expression western blot analysis was performed on all strains comparing the total amount of RrgB (Figure 5-10). FACS analysis was also performed to assess the distribution of the pilus on the cell surface of the bacterial population (Figure 5-6).

6.3.3 Western blot analysis of T4ΔspxB, T4ΔlctO and T4ΔspxBΔlctO

Western blot analysis was performed on T4Δ*spxB*, T4Δ*lctO* and T4Δ*spxB*Δ*lctO* to assess the total amount of RrgB. Western blotting showed differences in the expression of RrgB in T4Δ*spxB*, T4Δ*lctO* and T4Δ*spxB*Δ*lctO* (Figure 5-10). T4Δ*spxB*Δ*lctO* showed a 1.9 fold decrease in RrgB levels compared to T4NO1 and T4Δ*spxB* showed a 1.4 fold decrease. T4Δ*lctO* showed a small decrease of 1.1 fold. These finding correlate well with the RT-PCR values.

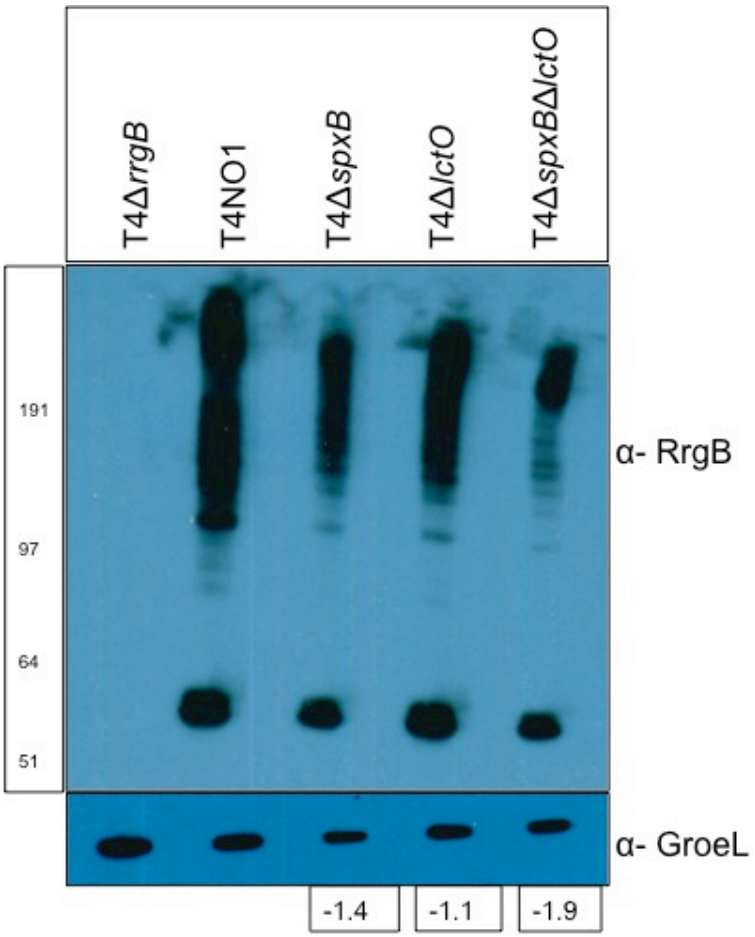


Figure 6-4: Western blot of T4Δ*spxB*, T4Δ*lctO* and T4Δ*spxB*Δ*lctO* RrgB expression compared to T4NO1.

Western blotting analysis was performed on T4NO1, T4Δ*spxB*, T4Δ*lctO,* T4Δ*spxB*Δ*lctO* and T4Δ*rrgB* looking for expression of RrgB in all strains (α-RrgB antibody). Equal protein loading was confirmed by equal expression of GroeL (α-GroeL antibody), normalise against in analysis. Western blotting quantification was performed using ImageJ with fold changes in RrgB expression compared to T4NO1 boxed below the strain. Left hand side numbers represent the size in kDa of proteins run to the same point.

6.3.4 FACS and fluorescence microscopy analysis of T4ΔspxB, T4ΔlctO and T4ΔspxBΔlctO

To further analyse the difference in pilus expression in these strains FACS analysis was performed to assess within a growing population the number of cells positive for RrgB on the cell surface and therefore pilus positive.

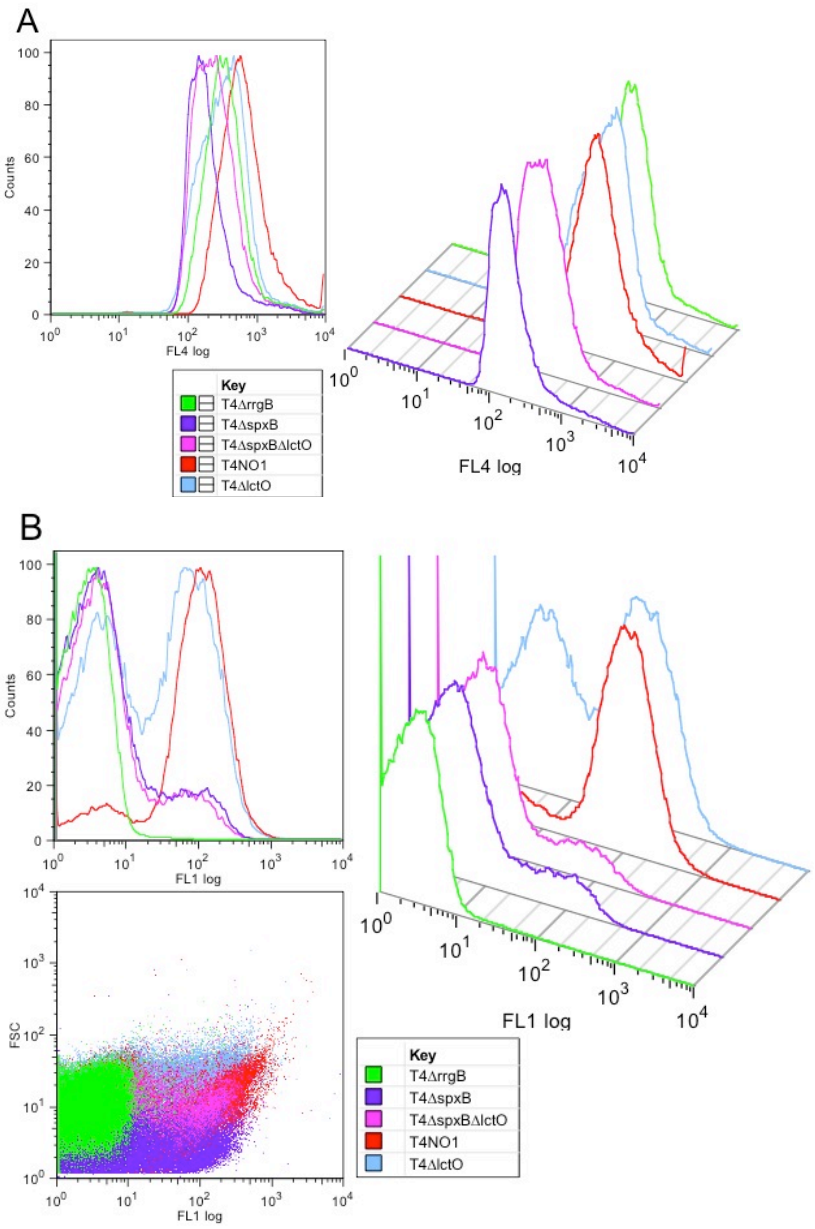


Figure 6-5: FACS analysis of RrgB surface expression in T4Δ*spxB,* T4Δ*lctO* and T4Δ*spxB*Δ*lctO*

FACS was performed on T4Δ*spxB,* T4Δ*lctO,* T4Δ*spxB*Δ*lctO,* T4NO1 and T4Δ*rrgB*. (A) Shows histograms of the capsule positive populations selected for from samples stained with a capsule antibody, population used for further analysis. (B) Shows the capsule stained populations being gated on for being RrgB positive, histograms/ polychromatic plot show negative (left) and positive (right) RrgB populations in each strain.

Strain	RrgB- (%)	RrgB+ (%)
T4NO1	12.1	87.9
T4Δ*spxB*	80.1	19.9
T4Δ*lctO*	43.1	56.9
T4Δ*spxB*Δ*lctO*	81	19
T4Δ*rrgB*	98.4	1.6

Table 6-2: Percentage RrgB positive cells in T4Δ*spxB,* T4Δ*lctO* and T4Δ*spxB*Δ*lctO*

populations from FACS analysis.

Table shows the percentage RrgB positive and negative cells in a growing bacterial population. FACS analysis was performed in FlowJo.

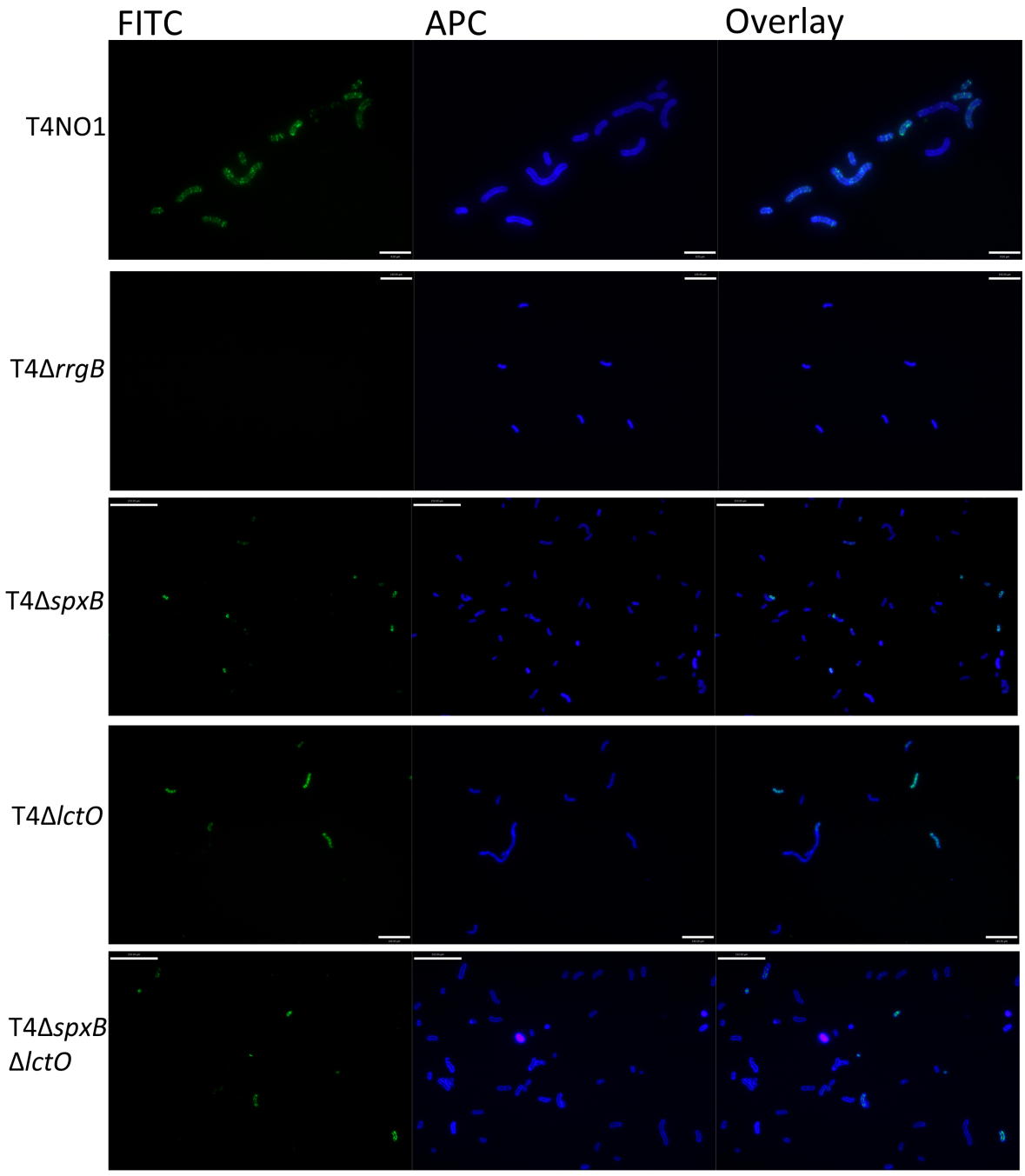


Figure 6-6: Fluorescence microscopy of T4Δ*spxB,* T4Δ*lctO* and T4Δ*spxB*Δ*lctO.*

One representative image of fluorescently labelled T4Δ*spxB,* T4Δ*lctO* and T4Δ*spxB*Δ*lctO* used for FACS analysis. Cells were stained for the presence of RrgB (FITC) and the capsule (APC). Scale bars above represent, T4NO1- 9μm, T4Δ*rrgB* - 140μm, T4Δ*spxB* - 210μm, T4Δ*lctO* - 140μm and T4Δ*spxB*Δ*lctO* - 210μm. The same representative image will be used throughout this study for each strain, more images can be seen in appendix I.

FACS analysis on T4Δ*spxB*, T4Δ*lctO* and T4Δ*spxB*Δ*lctO* showed clear differences in the cell surface exposure of RrgB at the population level compared to T4NO1 (Figure 5-6). There was a large decrease in the number of RrgB positive cells in a growing population of T4Δ*spxB* and T4Δ*spxB*Δ*lctO* both showing only 19-20% RrgB

positive cells compared to that of 88% in a T4NO1 population (Table 6-2). Although there was a difference in RrgB expression seen via western blot there seems to be roughly the same number of cells positive for RrgB in T4Δ*spxB* and T4Δ*spxB*Δ*lctO*. Interestingly there was also a large drop in the number of cells positive for RrgB in T4Δ*lctO* showing 57% of a growing population positive compared to that of 88% of T4NO1 (Table 6-2). As there is only a small drop in RrgB levels in T4Δ*lctO* this would suggest that the reduced pili expression at the population level is not due to this and may be due to another factor, such as reduced protein export to the cell surface. The fact that almost the same percentage of RrgB positive cells is observed in T4Δ*spxB*Δ*lctO* and T4Δ*spxB* would suggest that knocking out *lctO* has no effect on T4Δ*spxB*. And therefore the decrease in the RrgB positive cells in T4Δ*lctO* is likely due to a knock on effects of this deletion to the SpxB catalysed reaction.

Variations in pilus expression via FACS analysis was visualised using fluorescence microscopy of T4Δ*spxB*, T4Δ*lctO* and T4Δ*spxB*Δ*lctO* shown in Figure 6-6.

Due to clear change in the pilus expression at the population level in T4Δ*spxB*, T4Δ*lctO* and T4Δ*spxB*Δ*lctO* we wanted to assess the adherence capabilities to different cell lines which may show potential roles of SpxB and LctO in tissue tropisms during infection.

6.4 Role of SpxB and LctO in adherence

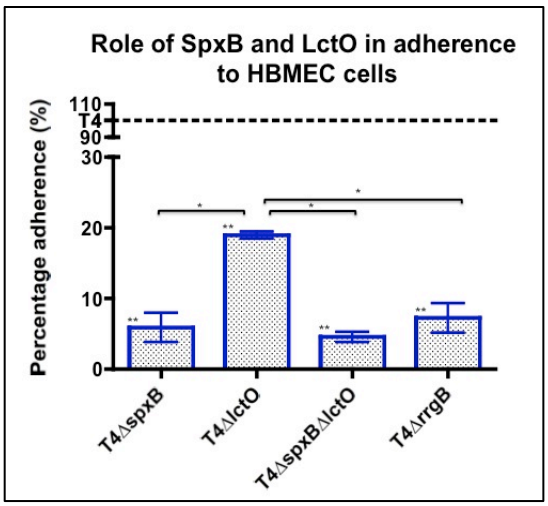
*6.4.1 Adherence assay analysis of T4Δ*spxB, *T4Δ*lctO *and T4Δ*spxBΔlctO

Adherence assays were performed on three different cell lines HBMEC, A549 and Detroit 562 cells to mimic the blood brain barrier, lung and nasopharynx respectively. This assay was performed to assess the adherence capabilities of T4Δ*spxB*, T4Δ*lctO* and T4Δ*spxB*Δ*lctO*.

The adherence assay data shows that T4Δ*spxB* and T4Δ*spxB*Δ*lctO* have reduced adherence capabilities to all cell lines compared to T4NO1. With the largest decrease being to HBMEC cells, showing approximately a 90-95% reduction in adherence compared to that of T4NO1. This level of adherence is similar to a

non pilated strain (Figure 5-9). Interestingly T4Δ*lctO* also showed a reduced ability to adhere to all cell lines compared to T4NO1 (Figure 5-9). The decrease in adherence was similar to that of T4Δ*spxB* and T4Δ*spxB*Δ*lctO* to A549 and D562 cells, with no statistical difference in the adherence capabilities of the three strains. Adherence of T4Δ*lctO* to HBMEC cells however was statistically different to that of T4Δ*spxB* and T4Δ*spxB*Δ*lctO* suggesting SpxB plays a more important role in meningitis, corroborating the ability of transparent variants to cross the blood brain barrier more efficiently (Ring et al., 1998). This difference in the adherence to HBMEC cells correlates well with the number of pilus positive cells within the bacterial populations, with T4Δ*lctO* showing a higher adherence than that of T4Δ*spxB* and T4Δ*spxB*Δ*lctO* as it contains 30-40% more pili positive cells.

A



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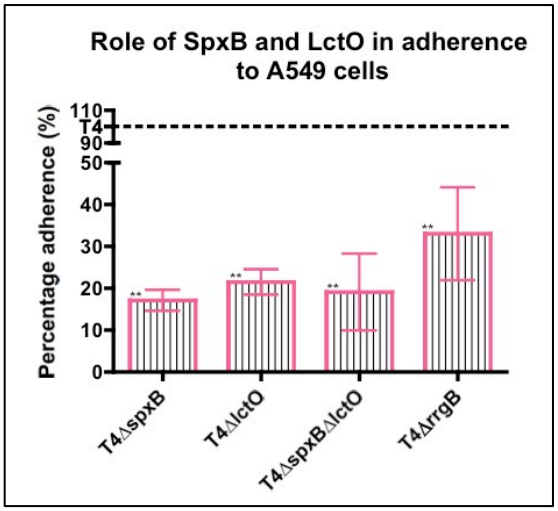

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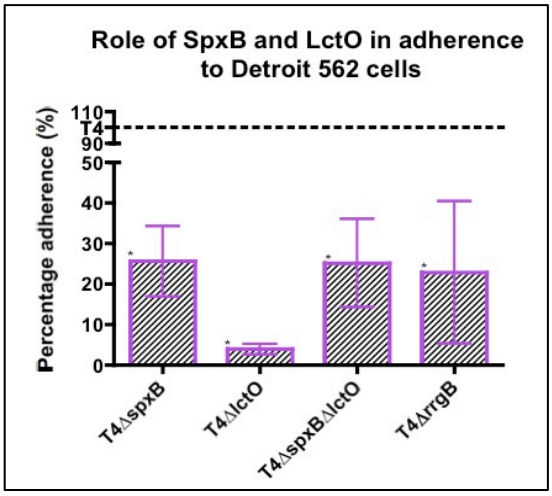


Figure 6-7: Adherence assay of T4Δ*spxB,* T4Δ*lctO* and T4Δ*spxB*Δ*lctO* to different cell lines.

Adherence of strains T4NO1, T4Δ*spxB,* T4Δ*lctO* and T4Δ*spxB*Δ*lctO*, *T4*Δ*rrgB* was assessed to HBMEC (A), A549 (B) Detroit 562 (C) cell lines. Data is represented as percentage adherence relative to that of T4NO1 (100%, dashed line), each bar is an average of three replicas except that of T4Δ*spxB,* T4Δ*lctO* and T4Δ*spxB*Δ*lctO* HBMEC data and T4Δ*lctO* D562 data which is an average of two repeats, the error bars represent the standard error of the mean. Statistical analysis was performed using a 1-way ANOVA with a Tukeys testing correction, * P<0.05/ ** P<0.001. * above the bar represent statistical significance compared to T4NO1 (not represented as a bar on the graphs).

6.5 Variation in pilus expression under different growth conditions

Due to the fact that there is a differences in the expression of the pilus in T4Δ*spxB,* T4Δ*lctO* and T4Δ*spxB*Δ*lctO* which contain knockouts in genes important during aerobic growth, pilus expression under both aerobic and anaerobic growth conditions were evaluated. T4NO1 was either grown statically under aerobic conditions at 37°C or statically anaerobically at 37°C in an anaerobic cabinet. Samples were processed for western blotting or FACS analysis.

6.5.1 Western blot analysis of T4NO1 grown anaerobically

Initial western blot analysis comparing cellular levels of RrgB under the two conditions showed a clear decrease (2.6 fold) in the amount of RrgB when T4NO1 was grown anaerobically compared to aerobically (Figure 6-8).

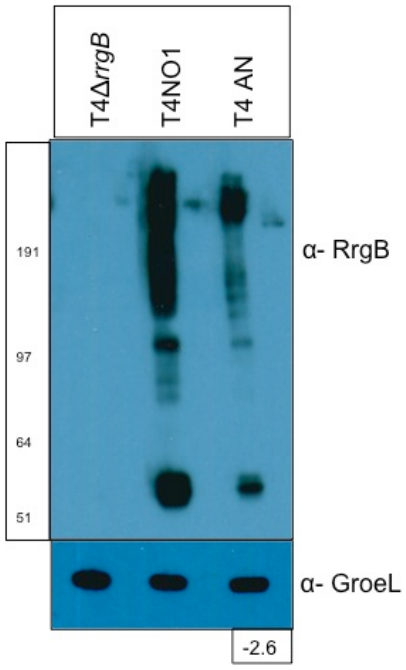


Figure 6-8: Western blot of T4NO1 RrgB expression when grown aerobically or anaerobically.

Western blotting analysis was performed on T4NO1 when grown under aerobic (T4NO1) or anaerobic (T4 AN) conditions looking for expression of RrgB (α-RrgB antibody). Equal protein loading was confirmed by equal expression of GroeL (α-GroeL antibody), normalised against in analysis. Western blotting quantification was performed using ImageJ with fold changes in RrgB expression compared to T4NO1 grown aerobically boxed below the strain. Left hand side numbers represent the size in kDa of proteins run to the same point.

6.5.2 FACS and fluorescence microscopy analysis of T4NO1 grown anaerobically

FACS analysis was performed to assess if the decrease in pilus under anaerobic conditions is due to that of a reduction in the amount of pili on a single cell or if there is a reduction in the number of cells expressing the pilus.

Analysis showed that unlike the reduction at the population level of pili positive cells in T4Δ*spxB,* T4Δ*lctO* and T4Δ*spxB*Δ*lctO,* T4NO1 grown under anaerobic conditions shows around a 1% increase in pilus positive cells and therefore this cannot account for the decrease in expression seen via western blotting (Figure 6-9,Table 6-3). FACS data does however show a clear shift to the left in the FITC pilus positive population grown under anaerobic conditions, suggesting a decrease in the amount of pili on a single cell. No change in the pilus at the population level was confirmed by fluorescence microscopy as seen in Figure 6-10.

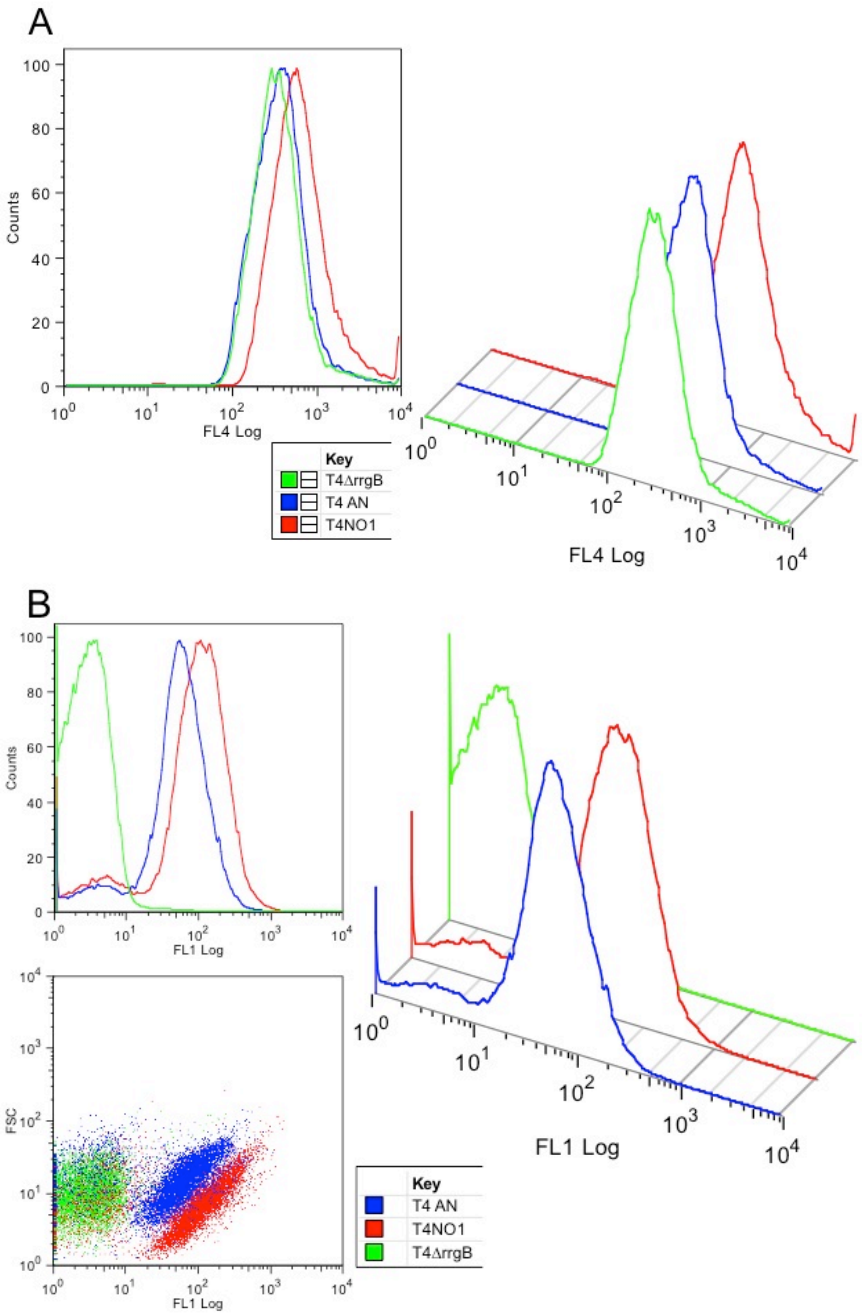


Figure 6-9: FACS analysis of RrgB surface expression in T4NO1 grown anaerobically.

FACS was performed on T4 AN, T4NO1 and T4Δ*rrgB*. (A) Shows histograms of the capsule positive populations selected for from samples stained with a capsule antibody, population used for further analysis. (B) Shows the capsule stained populations being gated on for being RrgB positive, histograms/ polychromatic plot show negative (left) and positive (right) RrgB populations in each strain.

Strain	RrgB- (%)	RrgB+ (%)
T4NO1	12.1	87.9
T4 AN	11.3	88.7
T4Δ*rrgB*	98.4	1.6

Table 6-3: Percentage RrgB positive cells in a T4 AN population from FACS analysis.

Table shows the percentage RrgB positive and negative cells in a growing bacterial population. FACS analysis was performed in FlowJo.

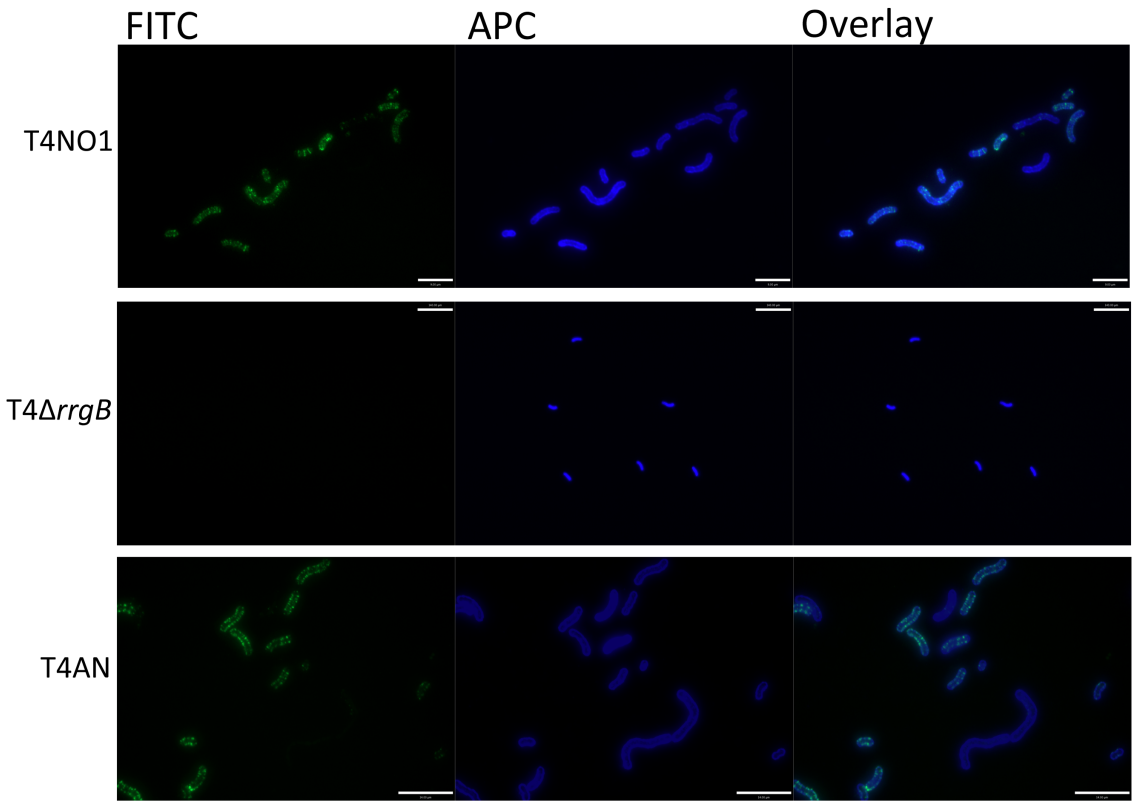


Figure 6-10: Fluorescence microscopy of T4NO1 grown anaerobically.

One representative image of fluorescently labelled T4NO1, T4Δ*rrgB* and T4NO1 grown under anaerobic conditions used for FACS analysis. Cells were stained for the presence of RrgB (FITC) and the capsule (APC). T4NO1- 9μm, T4Δ*rrgB* - 140μm and T4AN - 14μm. The same representative image will be used throughout this study for each strain, more images can be seen in appendix I.

6.6 Role of H_2O_2 in pilus expression

One of the common features of the enzymatic reactions catalysed by SpxB and LctO is that both produce hydrogen peroxide as a by-product, although to varying levels. To confirm this we performed a hydrogen peroxide assay on T4Δ*spxB,* T4Δ*lctO* and T4Δ*spxB*Δ*lctO* using T4NO1 and Xen35 as positive and negative controls respectively (Figure 6-11).

*6.6.1 Hydrogen peroxide production of T4Δ*spxB, *T4Δ*lctO *and T4Δ*spxBΔlctO

Hydrogen peroxide levels were significantly reduced in T4Δ*spxB* compared to T4NO1 with no visible hydrogen peroxide production after 30 minutes. However after 24 hours we can see that T4Δ*spxB* can produce hydrogen peroxide but this is at a much slower rate. SpxB clearly produces the majority of the cellular

hydrogen peroxide however some is still produced by LctO as T4Δ*lctO* also shows a reduction in hydrogen peroxide levels compared to T4NO1 after 30 minutes. Xen35 shows no hydrogen peroxide production after 24 hours due to it containing a non-functional SpxB and LctO. This is also the case for T4Δ*spxB*Δ*lctO* showing no hydrogen peroxide production after incubation for 24 hours.

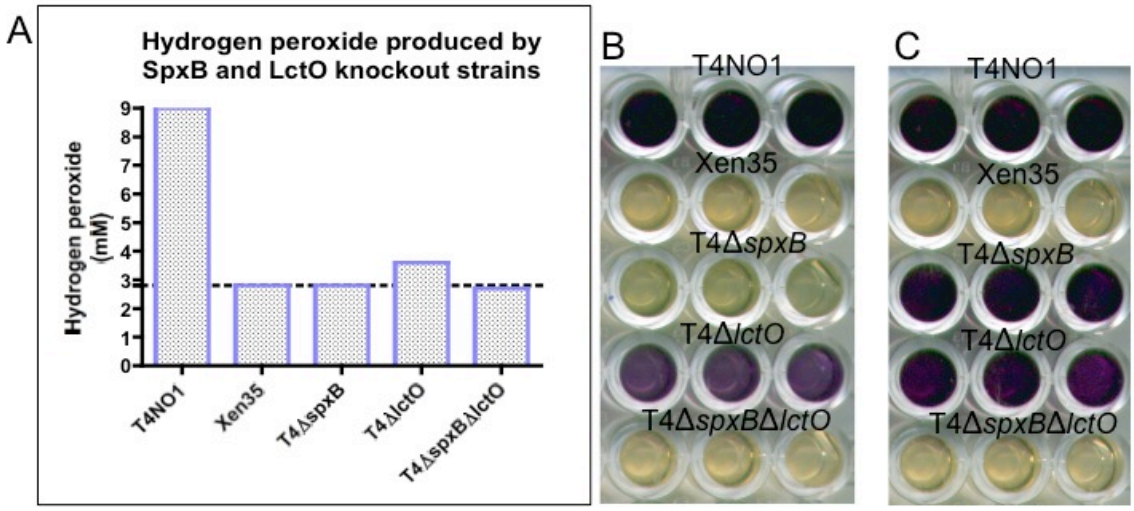


Figure 6-11: Hydrogen peroxide assay of T4Δ*spxB,* T4Δ*lctO* and T4Δ*spxB*Δ*lctO* strains

Each strain was represented in triplicate in the Hydrogen peroxide assay. (A) The graph gives the hydrogen peroxide production in mM of each strain calculated using a standard curve of known hydrogen peroxide concentrations (See Figure 2-14), the dotted line represent the limit of detection. Hydrogen peroxide production above 9mM could not be accurately measured therefore a maximum value of 9mM was assigned. (B) Shows visually the assay performed in a 96 well plate with each strain represented in triplicate after 30 minutes incubation at 37°C. (C) Shows visually the assay performed in a 96 well plate with each strain represented in triplicate after 24 hour incubation at room temperature.

Due to hydrogen peroxide production being a common factor altered in both of these mutants the role of hydrogen peroxide alone on the expression of the pneumococcal pilus was assessed. A culture of T4Δ*spxB,* which has a severely reduced rate of hydrogen peroxide production, was grown and hydrogen peroxide added back in at varying concentrations.

6.6.2 Western blot analysis of T4ΔspxB grown with exogenously added H_2O_2

To assess any variation in pilus expression upon addition of exogenous hydrogen peroxide western blot analysis (Figure 6-12) and FACS analysis (Figure 6-13) was performed on these samples. The H_2O_2 concentrations added to the growing bacterial cultures are noted in the figures as the dilutions used which were made

from a 30% H_2O_2 solution. The final concentrations for each dilution was 9mM (1/10 dilution), 3.6mM (1/25 dilution) and 1.8mM (1/50 dilution). Higher concentrations were tested however this killed the bacterial cells. The lowest concentration is likely similar to the normal levels of exogenous H_2O_2 produced by the cell (0.5-2mM) (Pericone et al., 2003).

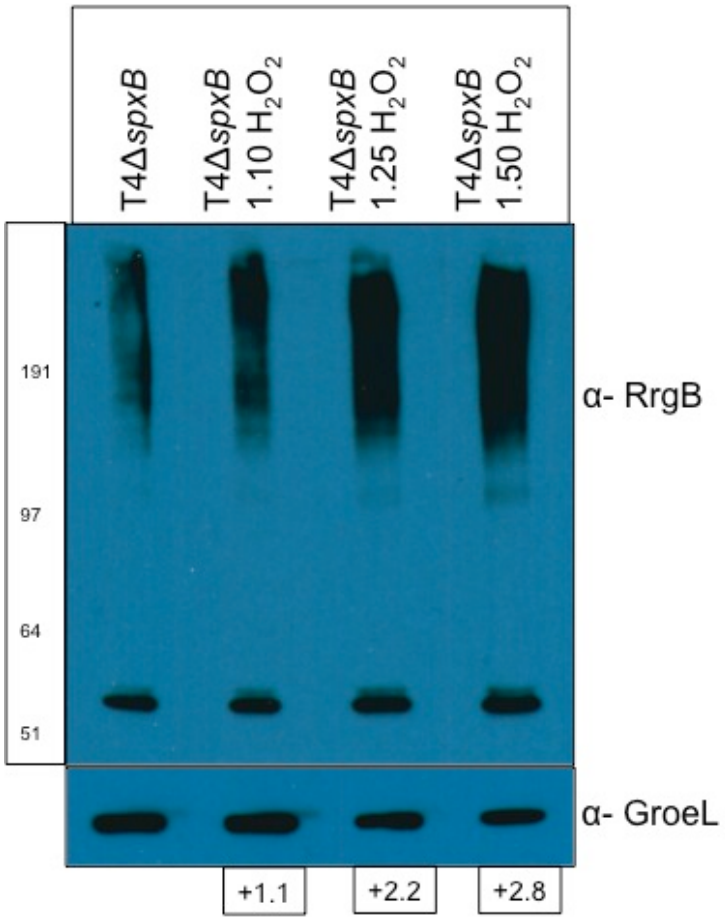


Figure 6-12: Western blot of T4Δ*spxB* RrgB expression when grown in the presence of varying concentrations of H_2O_2.

Western blotting analysis was performed on T4Δ*spxB* when grown in the presence of varying concentrations of H_2O_2 looking for expression of RrgB (α-RrgB antibody). Equal protein loading was confirmed by equal expression of GroeL (α-GroeL antibody), normalised against in analysis. Western blotting quantification was performed using ImageJ. Left hand side numbers represent the size in kDa of proteins run to the same point.

Western blot analysis clearly showed an increase in pilus expression when adding exogenous hydrogen peroxide to a growing culture of T4Δ*spxB,* shown in Figure 6-12. However this was not dose dependant as the highest concentration showed only a 1.1 fold increase In pilus expression, this may be due to toxic effects by H_2O_2 on the cells limiting growth. This was certainly the case at higher concentrations with cells dying (data not shown). However this could also be due

to variation in the western blotting technique. Upon further analysis of lower concentrations we may begin to see a dose dependent increase of pili with increasing amounts of hydrogen peroxide, however this was not assessed.

6.6.3 FACS and fluorescence microscopy analysis of T4ΔspxB grown with exogenously added H_2O_2

FACS analysis confirmed findings seen by western blot showing an increase in pilus positive cells (Figure 6-13). However there is only an increase by roughly 10% of pilus positive cells compared to T4Δ*spxB* and is therefore still much lower than the parent T4NO1 (Figure 6-13). It may be the case that other factors in T4Δ*spxB* are altering pilus expression. Or addition of exogenous hydrogen peroxide does not compensate for the loss of endogenous hydrogen peroxide, which would likely be higher in T4NO1. We also see an increase via FACS in the percentage of RrgB positive cells in the 1.10 H_2O_2 concentration sample, which showed no increase in RrgB levels by western blot analysis. This may suggest that the lower dilutions (1/25 and 1/50) contain higher levels of cellular RrgB but are unable to transport it to the cell surface.

Key points

- Addition of H_2O_2 to a growing T4Δ*spxB* culture increases the cellular amount of RrgB. This is not dose dependant at the concentrations used here, likely due to the toxic effects on the cell at the higher concentrations.

- Although cellular RrgB levels increase, addition of H_2O_2 is unable to fully modulate the RrgB levels present on the cell surface. Perhaps via protein export which may be limited in T4Δ*spxB* due to reduced ATP levels.

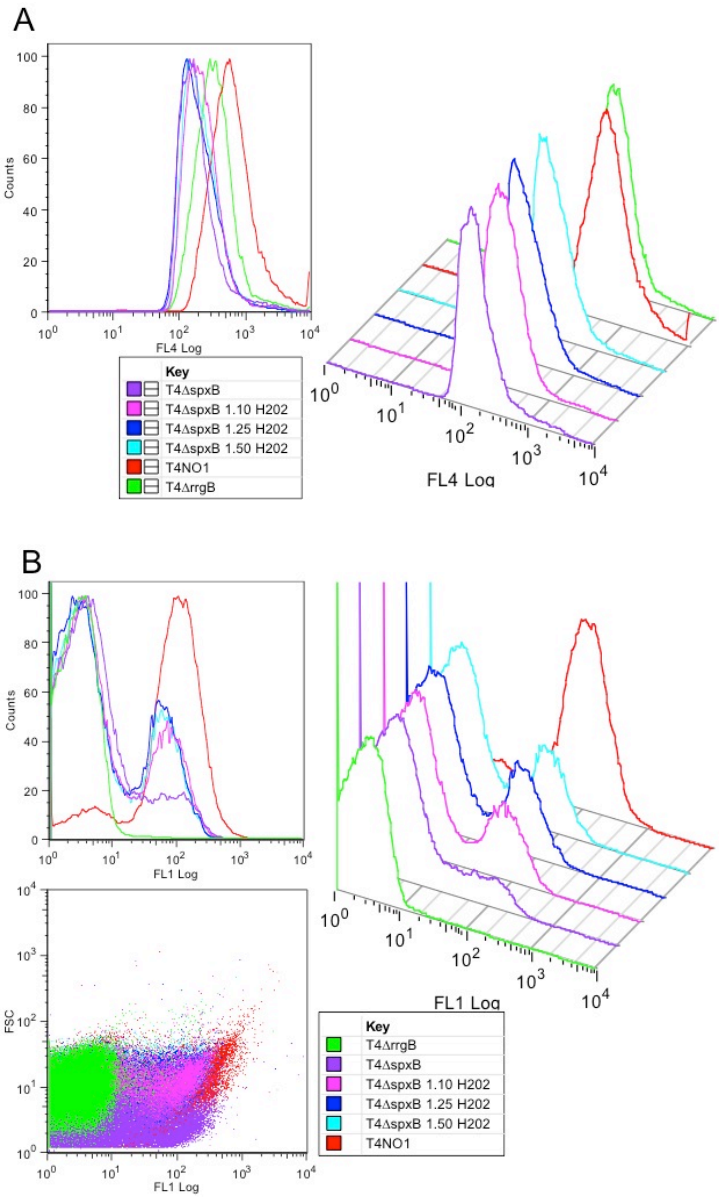


Figure 6-13: FACS analysis of RrgB surface expression in T4Δ*spxB* plus H_2O_2

FACS was performed on T4NO1, T4Δ*spxB*, T4Δ*rrgB* and T4Δ*spxB* grown in the presence of varying concentrations of H_2O_2. (A) Shows histograms of the capsule positive populations selected for from samples stained with a capsule antibody, population used for further analysis. (B) Shows the capsule stained populations being gated on for being RrgB positive, histograms/ polychromatic plot show negative (left) and positive (right) RrgB populations in each strain.

Strain	RrgB- (%)	RrgB+ (%)
T4NO1	12.1	87.9
T4Δ*spxB*	80.1	19.9
T4Δ*spxB* 1.10 H_2O_2	70.8	29.2
T4Δ*spxB* 1.25 H_2O_2	70.3	29.7
T4Δ*spxB* 1.50 H_2O_2	70.9	29.1
T4Δ*rrgB*	98.4	1.6

Table 6-4: Percentage RrgB positive cells in a T4Δ*spxB* population grown in the presence of H_2O_2 from FACS analysis.

Table shows the percentage RrgB positive and negative cells in a growing bacterial population. FACS analysis was performed in FlowJo.

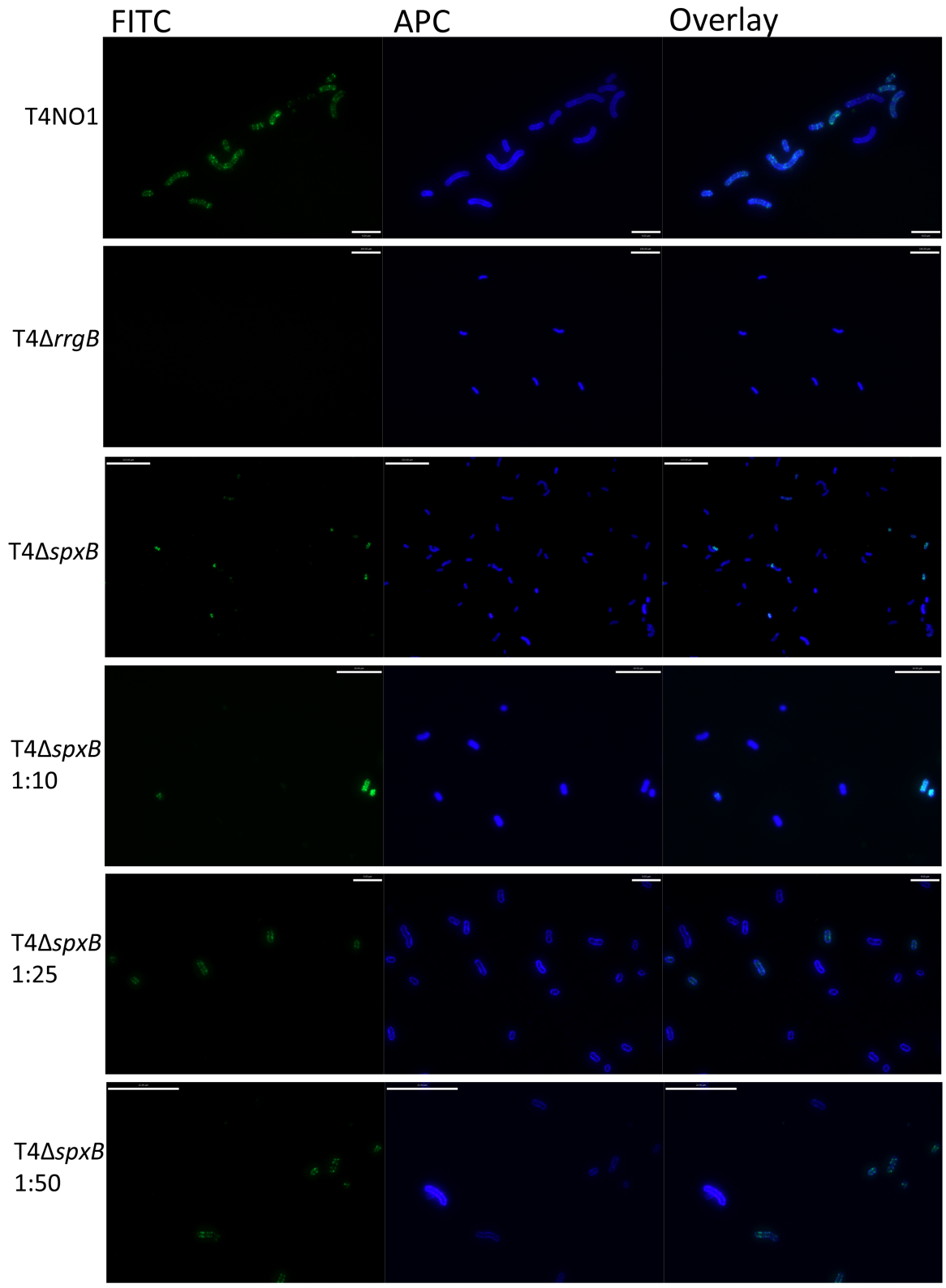


Figure 6-14: Fluorescence microscopy of T4Δ*spxB* grown in the presence of H_2O_2.

One representative image of fluorescently labelled T4NO1, T4Δ*rrgB,* T4Δ*spxB* and T4Δ*spxB* grown in the presence of varying concentrations of H_2O_2, samples used for FACS analysis. Cells were stained for the presence of RrgB (FITC) and the capsule (APC). T4NO1- 9μm, T4Δ*rrgB* - 140μm, T4Δ*spxB* - 210μm, T4Δ*spxB* 1:10 - 14μm, T4Δ*spxB* 1:10 - 9μm and T4Δ*spxB* 1:50- 22μm. The same representative image will be used throughout this study for each strain, more images can be seen in appendix I.

6.7 Discussion

It must initially be noted that the mutants analysed within this chapter were not complemented which is required to ensure the phenotype observed in the mutants strains is due to the deletion of the gene removed and not due to accumulation of other genetic changes. However we can assume the alterations to the levels of hydrogen peroxide is a true consequence of the gene deletions as their known function in the literature is enzymes that produce hydrogen peroxide as a by-product of the reaction. As we have been able to link the levels of hydrogen peroxide to pilus levels it's likely the gene deletions cause the altered pilus expression, but to fully confirm this complementation is required.

SpxB and LctO both play a role in energy production under aerobic conditions (Tettelin et al., 2001). Both are non-functional in the strain Xen35 which shows a reduced virulence and reduced expression of the pilus. SpxB alone has been shown to be important in the ability of the pneumococcus to colonise, replicate in the lungs and translocate to the blood (Orihuela et al., 2004, Spellerberg et al., 1996, Ramos-Montañez et al., 2008, Regev-Yochay et al., 2007). This lack of SpxB is therefore likely to be one of the reasons for the reduced virulence in Xen35. However, other virulence factors are also altered in this strain and the reasons for this reduced virulence is likely multifaceted.

As pilus expression in Xen35 was reduced the contribution of both LctO and SpxB to this decrease was assessed. Western blot analysis and RT-PCR showed a decrease in pilus expression in the *spxB* knockout. This was not the case for the *lctO* knockout. FACS analysis of the knockout strains T4Δ*spxB* showed only 20% of cells were now RrgB positive compared to 88% of the parent strain. Interestingly T4Δ*lctO* also showed a reduction in the percentage of RrgB positive cells with now only 57% positive for RrgB compared to its parent, however no difference in the total cellular amount of RrgB was observed.

The double knockout T4Δ*spxB*Δ*lctO* showed a similar number of RrgB positive cells to that of the single *spxB* knockout and therefore it was concluded that the effects seen on the pilus expression in the *lctO* knockout are likely via having a knock on effect on SpxB functionality. This could occur as SpxB and LctO work in a concerted manner in the pneumococcus, similar to that for *S.mutans* (Liu et

al., 2012, Taniai et al., 2008). Under aerobic conditions only 1/4th of pyruvate is converted to acetyl phosphate and the rest converted to lactate. If lactate oxidase is non-functional then the lactate cannot be converted back to pyruvate, which may be followed by a reduced activity of SpxB as there is less substrate available for its enzymatic reaction (Taniai et al., 2008). In contrast in a SpxB knockout there is no pyruvate converted to acetyl phosphate, which presumably explains the more severe phenotype in the SpxB knockout.

Due to the dependence of pilus expression on SpxB it is predicted that a product of the SpxB reaction may cause the changes in pilus expression. One of these products is acetyl phosphate which is produced from pyruvate via SpxB. This reaction produces 85% of the cells acetyl phosphate (Pericone et al., 2003). Acetyl phosphate has been shown to act as a phosphate donor for phosphorylation of response regulators of TCS pairs. As a number of TCS regulate the pilus in the pneumococcus, this could be responsible for the altered pilus expression (McCleary & Stock, 1994). However if this was the case there should be global expression changes upon deletion of SpxB (as noted in Ramos-Montañez et al., 2008). However this is not the case in our data or in published data and therefore is likely not the cause. Phosphorylation of RR by small phosphate containing molecules has also been shown to be limited to prevent *in vivo* cross talk between the systems (Boll & Hendrixson, 2011).

The end product of the reaction catalysed by SpxB followed by acetate kinase is ATP, vital for cell survival (Ramos-Montañez et al., 2010). The functionality of LctO during aerobic metabolism is thought to maximise ATP production (Taniai et al., 2008). Upon deletion of SpxB there is a severe reduction in ATP production. However some is still produced via conversion of pyruvate to acetyl Co-A via pyruvate formate lyase followed by conversion to acetyl phosphate via phosphotransacetylase. This production of ATP is at a much slower rate than that produced in a cell with a functional SpxB (Pericone et al., 2003, Ramos-Montañez et al., 2008). ATP is vital for a large number of cellular processes, so reduced amount may reduce the overall fitness of the growing cells. Although the assembly of the pneumococcal pilus by sortase enzymes is not energy taxing on the cell, the secretion of proteins to the cell surface is ATP dependent and therefore protein translocation may be reduced in a cell with reduced ATP levels (Lill et al., 1989, Economou & Wickner, 1994). This may account for why we see

no expression change in pilus expression in T4Δ*lctO* yet a clear reduction in the number of cells expressing the pilus on the cell surface compared to the parent.

Another common feature of both of these enzymes is the fact they both produce hydrogen peroxide as a by-product of their reactions. H_2O_2 is an important molecule produced by the pneumococcus as it is able to kill off other colonising bacterial inhabitants of the upper respiratory tract (Pericone et al., 2000, Mcleod & Gordon, 1922). H_2O_2 production however is a double edged sword eventually producing deleterious effects on the pneumococcus which does not produce a catalase enzyme (Tettelin et al., 2001). As both SpxB and LctO function under aerobic conditions we wondered if H_2O_2 production under these conditions acts as a signal to being in the nasopharynx where the bacterium would require up regulation of the cell surface adhesins to aid colonisation.

To assess the effect of hydrogen peroxide alone on the reduced pilus phenotype western blot and FACS analysis was performed assessing whether addition of exogenously added H_2O_2 restores pilus expression in T4Δ*spxB*. Addition of hydrogen peroxide clearly caused an increase in pilus expression with this corresponding to an increase in the number of cells positive for the pilus by roughly 10%. However this was still much lower than the T4NO1 level of 88% positive cells. As previously noted the ability to modulate pilus expression at the population level has been linked in *S.pyogenes* to changes in temperature which are thought to signal to the bacterium where it is present within the host. It may be that in the pneumococcus hydrogen peroxide is the environmental cue which signals to the bacterium that it is present in the nasopharynx where increased adhesive capabilities are required, which is modulated by the pilus. As TCS have been shown in this study to modulate the expression of the pilus at the population level we can hypothesise that this is likely the environmental signal which activates one of the TCS known to modulate pilus expression.

The lack of complete recovery of pilus expression to the WT level could be due to other factors also playing a role. Alternatively it could be that hydrogen peroxide given exogenously is unable to restore the level of endogenous H_2O_2 normally produced within the cell.

The changes seen upon addition of hydrogen peroxide to a growing culture suggests this alone is able to alter cellular functions. We did not assess the mRNA expression level of the pilus after addition of H_2O_2 but increased amounts of protein were seen via western blot. With regards to H_2O_2 or another ROS (reactive oxygen species) acting as a signalling molecule this could be the case as this has been observed in plants, animals and bacteria (see reviews Stone & Yang, 2006 and Lushchak, 2011). Some proteins are able to sense changes in oxidative stress (ROS levels) and alter gene expression by either being oxidised directly by ROS or indirectly affected through reaction with another protein that has an altered confirmation due to interaction with ROS. The levels of intracellular ROS can be modulated by the large number of enzymes used to break down these molecules such as catalases and peroxidises etc (Mishra & Imlay, 2012).

There are a number of bacterial proteins directly modified by ROS including the well studied OxyR transcriptional regulator of *E.coli* which is oxidised by H_2O_2 directly on a cystein residue altering its confirmation and therefore its binding efficiency for target DNA molecules (Toledano et al., 1994, Storz et al., 1990). In *B.subtilis* no homolog for OxyR exists but there are two other peroxide sensing proteins OhrR and PerR. OhrR homologues can also be seen in a number of other bacteria including *S.aureus*, *E.faecalis*, *P.aeruiginosa* and *Agrobacterium tumefaciens* (Chen et al., 2006, Giard et al., 2001, Ochsner et al., 2001, Chuchue et al., 2006). OhrR a member of the MarR family of transcription regulators doesn't recognise H_2O_2 but organic hydroperoxides (ROOH), and regulates the expression of *ohr* a thiol peroxide which catalyses their conversion to a less harmful alcohol (Fuangthong & Helmann, 2002, Panmanee et al., 2002).

PerR homologues have also been seen in a number of bacteria including *S.aureus*, *S.pyogenes*, *Campylobacter jejuni*, *Streptomyces reticuli*, *Streptomyces coelicolor* and *Borrelia burgdorferi* (Horsburgh et al., 2001, King et al., 2000, Van Vliet et al., 1999, Ortiz de Orué Lucana & Schrempf, 2000, Hahn et al., 2000, Boylan et al., 2003). PerR a metallo protein is a member of the ferric uptake regulator family, which contains two metal binding sites. One that binds zinc (structurally required) and another which binds either iron or magnesium which regulate its function. Fe^{2+} or Mn^{2+} when bound act to retain binding of PerR to its target DNA (Herbig & Helmann, 2001). PerR like OxyR

responds to H_2O_2 levels resulting in the metal dependant catalysed oxidation of a histidine residue in PerR resulting in the release of the metal ion and release of PerR from DNA (J.-won Lee & Helmann, 2006). This oxidation is dependent on production of a hydroxyl radical via the Fenton reaction.

Interestingly PerR in *S.pyogenes* has been associated with alterations in pilus expression (Grifantini et al., 2011). Addition of hydrogen peroxide to a WT strain of *S.pyogenes* causes an increase in the expression of the genes required for pilus expression and assembly, of roughly 3-7 fold. This increase in pilus expression is also observed in one of the sortase genes after deletion of *perR*. This would give an indication that recognition of hydrogen peroxide levels by bacteria can lead to alterations in cell surface components. *S.pyogenes* is found in the pharynx causing minor throat infections but can also cause serious soft tissue infections. In the pharynx *S.pyogenes* has been shown to adhere to human tonsil epithelium and it was hypothesised here would require increased levels of pili which would be augmented by sensing by PerR of increased levels of ROS (Abbot et al., 2007). Interestingly as discussed in the Rosch study (2008) the promoter lying upstream of *rlrA* shows high homology to PerR binding sites in other Streptococci, with only a single mismatch between the two (Brenot et al., 2005, Rosch et al., 2008). Currently no homologues of PerR have been found in *S.pneumoniae* and perhaps this pathogen has evolved another means to respond to ROS. With ROS acting as a key signal to being present in the nasopharynx where regulation of cell surface adhesins is required.

Although not associated with PerR increasing levels of hydrogen peroxide have been associated with increase pili expression in the pneumococcus (Rosch et al., 2010). This was observed in a mutant defective in a manganese efflux pump MntE (SP_1552), causing increases in intracellular manganese. Manganese has been shown to affect oxidative stress response, and perhaps in this strain alterations in this causes accumulation of H_2O_2 which in turn alters expression of the pilus (Mcallister et al., 2004, Tseng et al., 2002). SodA (Superoxide dismutase) in the pneumococcus converts superoxide radicals to H_2O_2, this enzyme is manganese dependant and therefore increased levels of intracellular manganese may lead to increase activity of SodA and resulting H_2O_2 production (Yesilkaya et al., 2000).

The role of manganese in pilus regulation was also further confirmed in two studies where deletions of *psaR* resulted in increased expression of the pilus islet genes (Johnston et al., 2006, Hendriksen et al., 2009). PsaR is a transcription regulator which directly regulates a number of genes on the *psa* operon, including those coding for a manganese ABC transporter present (PsaA, PsaB and PsaC). Deletion of PsaR causes an increase in pilus expression, with low Mn^{2+} concentrations associated with high pilus expression and high Mn^{2+} associated with low pilus expression. This is contrary to that seen with high intracellular Mn^{2+} associated with high pilus expression however it is clear manganese directly or indirectly affects pilus expression. PsaD also present on the *psa* operon is thought to function in the breakdown of H_2O_2 which is why deletion mutants show a reduced resistance to oxidative stress (Cha & Kim, 1996, Novak et al., 1998, Johnston et al., 2004, Tseng et al., 2002). However this gene has its own promoter and is not regulated by PsaR and therefore is likely not modulating H_2O_2 levels which is causing the altered pilus expression in the PsaR mutant (Johnston et al., 2006, McAllister et al., 2004, Hendriksen et al., 2009).

merR which has previously been shown to modulate pilus expression was shown to be up regulated in the Hendriksen study (2009) (in a PsaR deletion mutant). However MerR was shown to act as a repressor of the islet and the pilus genes were up regulated in this study, so is likely not the reason for this change (Rosch et al., 2008, Hendriksen et al., 2009). *psaD* is also present on the *psa* operon which encodes a putative thiol peroxide (Novak et al., 1998). Although the Hendriksen (2009) study did not observe any expression changes in *spxB* or *lctO* in the PsaR mutant strain. Deletion mutants in *psaA* have shown to effect the expression of both *spxB* and *lctO* (Ogunniyi et al., 2010). This study however was performed in a non piliated strain (D39) and therefore its effect on pilus expression is unknown. If this was also the case in TIGR4 there could be subtle change in SpxB and LctO levels in the PsaR mutants in the Hendriksen (2009) and Johnston (2006) studies which account for the changes in pilus expression (Johnston et al., 2006, Hendriksen et al., 2009). Both of these studies did not assess the H_2O_2 levels produced in the PsaR mutants.

However in this study we have not assessed whether it is H_2O_2 directly altering pilus expression or if it may be due to a breakdown product of H_2O_2. H_2O_2 itself has a short half life and is rapidly degraded by the Fenton reaction into hydroxyl

radicals and a hydroxide ion (Imlay et al., 1988). The effect of break down products of H_2O_2 on pilus regulation could be assessed further by growing T4Δ*spxB* not only with H_2O_2 but also with iron chelators to block the Fenton reaction or hydroxyl radical scavenger. We would assume that this may be the case as roughly 30 minutes passed after addition of H_2O_2 to the growth media before samples were collected.

The deletion of SpxB has been linked to the ability of the pneumococcus to phase vary, with a single bacterial population consisting of two colony types that of transparent and opaque (Weiser et al., 1994). Transparent colony types have been associated with having a 2.1-3.8 increase in amounts of cell surface teichoic acid compared to opaque types, showing increase ability to colonises the nasopharynx (Kim & Weiser, 1998, Weiser et al., 1994). Whereas opaque phenotype is associated with a 1.2-5.6 fold increase in the amount of capsule on the cell, having an increased virulence during invasive disease and decreased ability to be phagocytosed due to the increased levels of the capsule (Kim & Weiser, 1998, Kim et al., 1999). Along with this a number of variations in the levels of surface proteins has been noticed between the two phenotypes with increased levels of PspA seen in opaque variant and decreased levels of LytA, PpmA and CbpA (Kim & Weiser, 1998, Weiser et al., 1996, Overweg et al., 2000). Increased levels of LytA and CbpA, both choline binding proteins may be due to increased levels of teichoic acid in transparent variants and therefore phosphorylcholine levels creating more anchoring points for these proteins. PpmA (proteinase maturation protein A) is also thought to aid protein maturation on the cell surface and therefore may regulate the abundance of proteins on the cell surface. PpmA has also been shown to play a role in colonisation during infection (Cron et al., 2009).

Our microarray data measuring expression differences between T4NO1 and T4Δ*spxB* did not show any of these genes to be differentially regulated but whether there were differences in protein levels was not assessed. The fact we see difference in a number of surface proteins in the two phenotypes and the fact the transparent phenotype is better equipped during colonisation fits with our data, as the T4Δ*spxB* mutant shows only opaque variants (data not shown), and has reduced levels of the pilus on the cell surface (Ramos-Montañez et al.,

2008). Currently no literature has made a link between phase variation and pilus expression which may be in part due to the fact the majority of studies assessing cell surface constituents in phase variants is performed in non piliated strains. Clearly if there are changes in the capsule levels in T4Δ*spxB* etc this may effect the surface exposure of levels of the pilus and skew the FACS data (Sanchez et al., 2011). However microarray analysis did not indicate differential expression of any of the capsule genes and FACS analysis did not indicate there was an increase in the fluorescence intensity of the capsule stained population, which would be expected if capsule levels were increased in the strains studied.

Another variation that may be affecting the cell surface constituents is that caused by differences in the cell membrane fluidity. This has also been shown to vary between opaque and transparent phenotypes with a lower degree of unsaturated fatty acids seen in opaque variants compared to transparent (Aricha et al., 2004). Variations in the fatty acid saturation of the cell membrane alters membrane fluidity which is important for bacterial adaptation under varying environmental conditions. The ability to regulate the membrane fluidity is called homeoviscous adaptation (Sinensky, 1974). Membrane fluidity can affect the functionality of proteins within the cell membrane including those involved in cell signalling and transport (Lee, 2004). In the pneumococcus variations in the cell membrane fatty acid saturation and H_2O_2 have been directly linked (Pesakhov et al., 2007). FabF an enzyme involved in the fatty acid synthesis is oxidised directly by hydrogen peroxide on a cystein residue which inhibits its activity (Benisty et al., 2010).

This may link in to why we see upon addition of H_2O_2 to a growing culture of T4Δ*spxB* increased levels of pilus on the cell surface. Hydrogen peroxide addition may alter the membrane permeability and directly effect the translocation of proteins through the membrane or alter functionality of proteins retained in the cell membrane that have signalling capabilities. Six pneumococcal TCS have been shown to regulate the pneumococcal pilus, perhaps their signalling functionality is altered upon variation in the membrane fluidity. In the pneumococcus a link between membrane fluidity and variation in surface exposed protein has not been directly assessed and would require further study. Interestingly addition of exogenous H_2O_2 in a SpxB mutant strain was unable to

revert the membrane fatty acid composition to that of the WT strain, suggesting this does not complement to the full extent the levels of endogenous hydrogen peroxide levels (Pesakhov et al., 2007). This as discussed earlier, may be why we only see a slight increase in pilus expression in T4Δ*spxB* upon addition of exogenous hydrogen peroxide.

Contradictory to our hypothesis when grown under anaerobic conditions we did not see a similar effect on pilus expression to that of the T4Δ*spxB* mutant. Pilus expression was reduced suggesting an important role in colonisation, however upon FACS analysis T4NO1 grown anaerobically showed a decrease in the amount of pili on the surface of a single cell. Whereas in T4Δ*spxB* and T4Δ*lctO* we observed a reduction at the population level in the total number of cells positive for pili on the cell surface. H_2O_2 production has been shown to be reduced to almost nothing in pneumococci grown under anaerobic conditions, likely due to reduced activity of SpxB and LctO (Pesakhov et al., 2007). Growth under anaerobic conditions has also been shown to have altered membrane fatty acid saturation due to varying levels of H_2O_2 production (Pesakhov et al., 2007). However the change in fatty acid composition of the membrane during growth anaerobically was more subtle than the huge shift seen upon deletion of SpxB and therefore these differences may account for the differences observed in our data (Pesakhov et al., 2007).

The fact that we see variations in adherence upon deletion of SpxB is not surprising with its role in colonisation is clearly noted in the literature (Spellerberg et al., 1996, Weiser et al., 1996). T4Δ*spxB* showed a statistical decrease in adherence to all cell lines compared to T4NO1, which would suggest a multifaceted role in virulence, which is also clear from the literature. Being important for ATP production within the bacterial cell it would be expected to be the case. T4Δ*spxB*Δ*lctO* also showed a statistical decrease in adherence to all cell lines compared to T4NO1, this was to a similar level as that of T4Δ*spxB*.

Interestingly T4Δ*lctO* also showed a statistically reduced adherence to all cell lines compared to T4NO1. No *in vivo* data is available in the literature assessing the bacterial fitness when this gene is deleted, however due to similar adherence capabilities to T4Δ*spxB* we would expect it to play some role in

virulence. However its role in meningitis is perhaps less than that of T4Δ*spxB* as it showed a statistical increase in adherence to HBMEC cells compared to T4Δ*spxB* and T4Δ*spxB*Δ*lctO*. This difference correlated well with the differing amount of surface exposed RrgB/ pili, again suggesting an important role of the pilus in meningitis. This was also observed when looking at StkP variants expressing different surface levels of RrgB/pili (Chapter 5).

In conclusion we have shown that both SpxB and LctO alter the expression of the pneumococcal pilus at the population level. Microarray data looking at expression differences in T4Δ*spxB* did not give an indication of any other genes that may be affected by this deletion and cause the decrease in expression of the pneumococcal pilus. With regards to our initial question of what contribution these deletions make in Xen35 to the reduced pilus expression, we can conclude from this data they likely cause some of the decreased pilus expression. However the pilus expression seen in Xen35 is still lower than that of T4Δ*spxB* showing 5.5% and 20% cells positive for RrgB on the surface within a population, respectively. This may suggest there are other factors contributing to the reduced pilus expression in Xen35. However it is hard to compare between the two strains as we do not know the level of expression of the pilus in the parent TIGR4 strain Xen35 was constructed in. This could be similar to T4JH which showed only 20.5% of cells positive for RrgB on the cell surface and in that case would equate to around 75% reduction in pilus expressing cells in Xen35, a value that would be similar to that when comparing between T4NO1 and T4Δ*spxB*.

No genome or expression changes were observed in Xen35 in either *mntE*, *psaA*, *psaB*, *psaC* and *psaR*. However microarray data of a MntE mutant grown in the presence of high levels of manganese showed some similar expression changes to those seen in Xen35, but oppositely expressed (Rosch et al., 2010). This included up regulation of SP_515, SP_516, SP_1757, SP_1764-67, SP_1769-71 in Xen35 which were all down regulated in T4Δ*mntE*. SP_0461-64 and SP_0875-77 were up regulated in Xen35 and down regulated in T4Δ*mntE*. Finally SP_1895-98 and SP_1676 were down regulated in both strains. The fact we see no variations in genes which function to modulate intracellular manganese levels in Xen35 would suggest perhaps another common factor between the two strains is causing the common expression changes, which could be due to variations in H_2O_2

production, with none produced in Xen35 and higher levels than the parent TIGR4 strain produced in T4Δ*mntE*. Further studies would be needed to assess whether any genome or expression changes in Xen35 could be altering the ability to regulate manganese uptake/ efflux.

7 Evaluating the potential burden of *lux* gene expression in the pneumococcus

7.1 Aim of this chapter

As seen previously Xen35 shows a reduced pilus expression compared to TIGR4. The work described in this chapter evaluates the role of the *lux* genes insertion and expression with regards to potentially contributing to the reduced pilus expression in Xen35, and causing a metabolic burden on the bacterial cell.

To assess this a new collection of bioluminescent *S.pneumoniae* strains were constructed, placing the *lux* genes at an alternative region in the genome and under the control of different strength promoters, including that of the promoter driving the *lux* gene expression in Xen35. Placing the genes at an alternative, clearly defined location in the chromosome eliminates the risk of effects due to insertion into functional genes e.g. SP_1914 in Xen35. These bioluminescent strains were assessed via western blotting to determine the effect expression of the *lux* genes has on pilus expression (RrgB levels).

One of the constructed strains T4P2 was further characterised by determining the effects the *lux* genes have on whole genome expression changes. For this microarray analysis was performed on T4P2 compared to its parent T4NO1. This data was compared to the genome expression changes observed in Xen35 to see whether common genes are differentially regulated between T4P2 and Xen35. And are therefore likely due to the *lux* genes over expression in both strains. Further the expression of the *lux* genes and pilus genes expression were validated in T4P2 relative to Xen35. T4P2 virulence was also evaluated in a mouse model of infection and compared to the data obtained for T4NO1 and Xen35 virulence. Finally to determine whether expression of the *lux* genes resulted in accumulation of specific genome changes in Xen35, whole genome sequence analysis was performed in T4P2.

7.2 Background

As described previously Xen35 is a bioluminescent serotype 4 *S.pneumoniae* strain, used to visualise *in vivo* disease progression (see section 4.2). Chapter 4 elucidated the genome changes that were present in this strain however the high expression of the *lux* genes alone remains to be evaluated.

The bioluminescence reaction can be seen in Figure 4-1. As well as the requirement for oxygen the rate limiting step for this reaction is production of the long chain aldehyde substrate (RCHO) not normally synthesised within the bacterial cell which is catalysed by the *luxC-E* encoded fatty acid reductase complex, which channelling fatty acids from the fatty acid biosynthesis pathway to produce the substrate for the luciferase (LuxA/ LuxB) (Boylan et al., 1985, Boylan et al., 1989). Initial steps in the synthesis of the aldehyde are catalysed by the transferase (*luxD*), which catalyses the transfer of fatty acyl groups from acyl-ACP or acyl-CoA onto water, resulting in a fatty acid (Byers & Meighen, 1985). The next step involves the synthetase (*luxE),* which only functions when bound to the reductase. The enzyme activates the fatty acid forming a fatty acyl-AMP intermediate bound to the enzyme (Boylan et al., 1985, Boylan et al., 1989). The acyl group is then transferred to the synthetase followed by the reductase, which in turn is reduced to give the aldehyde. During the reactions catalysed by the synthetase and reductase ATP and NADPH are utilised. The reaction equation is shown in Equation 7-1.

$$RCOOH + ATP + NADPH \rightarrow NADP + AMP + PPi + RCHO$$

Equation 7-1: Reaction catalysed by l*uxE* and *luxC*

Reaction catalysed by the synthetase and reductase component of the fatty acid reductase complex, producing the aldehyde substrate for the luciferase (LuxA/ LuxB). RCOOH (fatty acids), ATP (adenosine triphosphate), NADPH (reduced nicotinamide adenine dinucleotide phosphate), NADP (nicotinamide adenine dinucleotide phosphate), AMP (adenosine monophosphate), PPi (pyrophosphate), RCHO (long chain aldehyde)

The reaction also required the constant recycling of FMN to its reduced form $FMNH_2$, which is performed in luminescent bacteria by flavin reductase, which is not encoded on the *lux* operon (reaction is NAD(P)H dependant) (Jablonski & DeLuca, 1977, Michaliszyn et al., 1977). The fact in normally non bioluminescent

bacteria we observe bioluminescence suggests this enzyme is also present, likely due to the requirement for $FMNH_2$ in a number of other biological processes. SP_1627 in *S.pneumoniae* is homologous to a putative NADH flavin reductase. In this instance for $FMNH_2$ production NADH is oxidised reducing FMN giving $FMNH_2$ and NAD^+. The cloning of the *lux* genes into a bacterium that does not normally bioluminesce may pose a metabolic burden to the cell via using ATP for aldehyde production and altering the availability of molecules with reducing power, required for energy production and redox balance.

7.3 Promoter disruption

Initial experiments were performed to confirm the promoter driving the expression of the *lux* genes in Xen35 it that lying downstream of SP_1915. A mutant was constructed in T4NO1 inserting a spectinomycin resistance cassette 20 nucleotides downstream of the start of SP_1915, using a transposon based mutagenesis method. This separated the predicted -10 and -35 sigma70 binding recognition sites from that of the start of the SP_1915 gene (Figure 7-1). This was initially constructed in a TIGR4 background and then PCR amplified from this strain and transformed into Xen35 producing Xen35Δ19. This insertion enabled evaluation of whether this is the promoter driving the expression of the *lux* genes in Xen35.

To assess the ability of Xen35Δ19 to bioluminescence bacterial strains were inoculated at $1x10^6$ cfu/20μl into 180μl of BHI in a black 96 well plate in triplicate, with Xen35 and TIGR4 used as controls. Bioluminescence was measured over time in a plate reader taking measurements every 30 minutes over a period of approximately 10 hours. Average of the three readings was calculated and plotted as shown in Figure 7-2. Insertion of the resistance cassette down stream of SP_1915 removed the ability of the strain to bioluminesce (Figure 7-2), confirming that this promoter drives the high expression of the *lux* genes in Xen35.

Figure 7-1: Schematic diagram of transposon insertion in Xen35Δ19

Diagram of downstream region of gene XEN35_1915 where its predicted promoter lies (BPROM), red box encircles the predicted -10 box and the blue box the -35 box. Blue cross represents the insertion site of the transposon insertion of roughly 1.1kb in size.

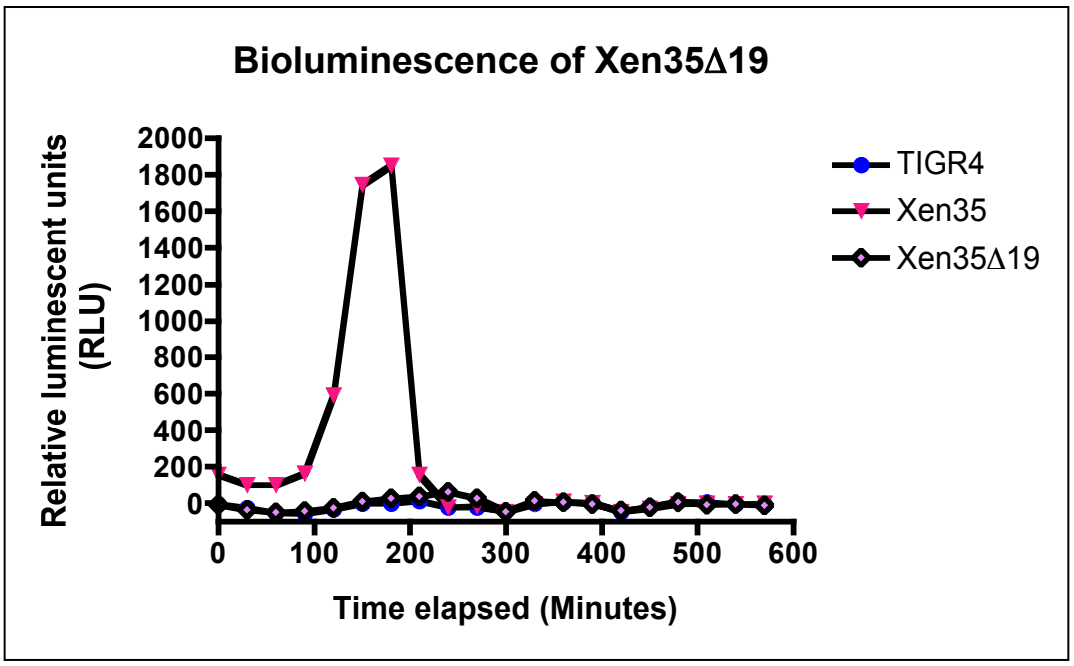


Figure 7-2: Bioluminescence of Xen35Δ19

Graph of bioluminescence of strains Xen35, TIGR4 and Xen35Δ19 over time. Each point on the graph represents the average of a triplicate reading, Readings were taken every 30 minutes.

7.4 Construction of a new bioluminescent strain

To assess the metabolic burden that the high expression of the *lux* genes may have on the bacterial cell a collection of bioluminescent strains were constructed. Initial experiments performed were to clone the *lux* genes (*luxA-E*) into plasmid pCEP2 (Guiral et al., 2006), which placed the genes under the control of a maltose inducible promoter (pCEP2lux). This plasmid was then transformed into T4NO1 and these genes recombined into SP_1886 (IS1167 element, frameshift). This plasmid was designed to allow chromosomal expression of genes and no deleterious effects from affecting adjacent genes, as this insertion is into a non-functional element (Figure 7-3).

Once transformed into T4NO1 the *lux* gene expression was induced using maltose. However no bioluminescence was observed (data not shown). This was attributed to the requirement for a strong promoter and the maltose inducible promoter was not strong enough to drive expression of all five *lux* genes. Therefore bioluminescence produced by this strain was below the detection limit of the plate reader used for analysis.

The maltose inducible promoter was therefore swapped for that of alternative pneumococcal promoters chosen from high expression of the downstream gene shown by RNA-seq analysis, see Table 7-1. Promoter presence was predicted in the upstream regions of the highly expressed genes by using the BPROM internet tool to detect potential sigma70 binding sites. The promoter present upstream of SP_1915 was also chosen as this is the promoter driving the expression of the *lux* genes in Xen35. In some instances the highly expressed gene contained no promoter directly upstream and therefore the expression of the surrounding genes was gauged to assess whether the gene is contained within an operon. In this instance the nearest upstream promoter was chosen as for SP_0236. Site directed mutagenesis was performed on pCEP2lux to create a restriction site upstream of the maltose inducible promoter to allow excision and replacement with the new promoters, which was performed for all chosen promoters. Plasmids constructed were then transformed into T4NO1 creating strains T4P1, T4P2, T4P3, T4P4 and T4P19. These strains were initially assessed for their ability to bioluminesce.

Promoter	**Gene number**	**Gene function**	**RPKM expression value**	**Promoter 5' of gene**
P1	SP_1489	Elongation factor Tu	8,057.38	SP_1489
P2	SP_2012	GAPDH	5,126.58	SP_2012
P3	SP_1128	Enolase	3,202.48	SP_1128
P4	SP_0236	DNA directed RNA polymerase alpha	3,178.91	SP_0232
P19	SP_1915	Hypothetical protein	34.84	SP_1915

Table 7-1: Table of promoters used to drive expression of the *lux* genes

Table of promoters chosen for cloning into pC2LSD upstream of the *lux* genes, and information regarding their expression in TIGR4.

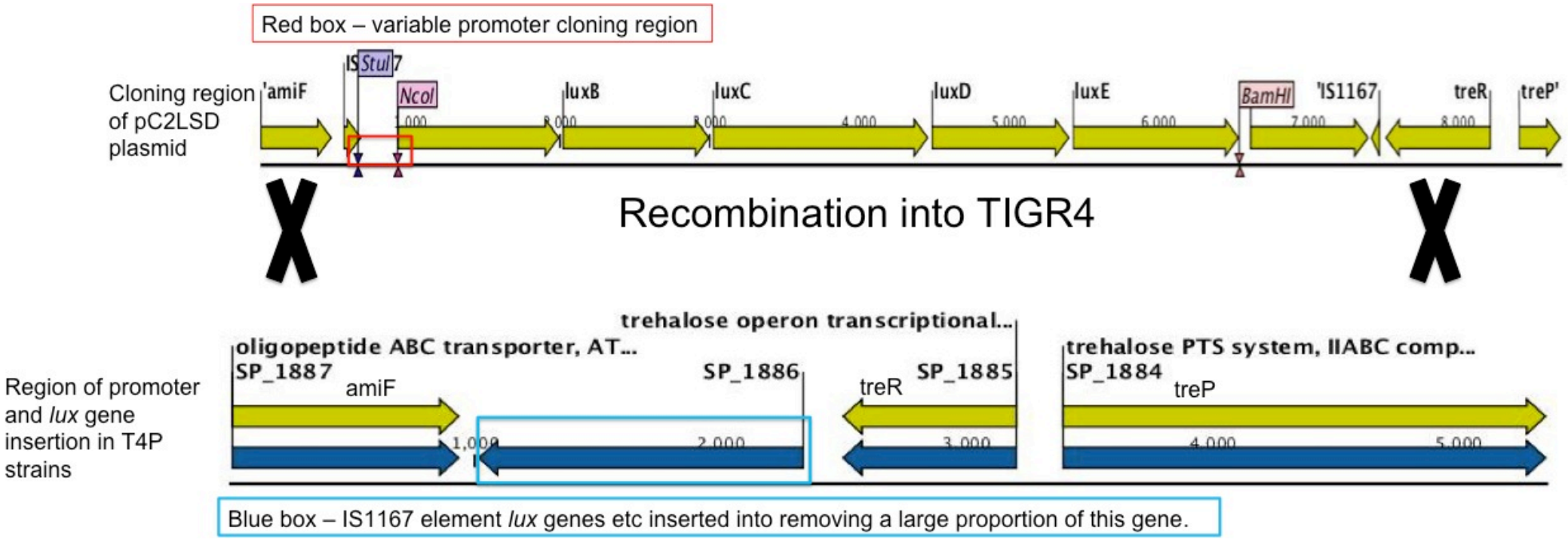


Figure 7-3: Schematic diagram of cloning region in pC2LSD plasmid and recombination site into TIGR4.

7.4.1 Bioluminescence of T4P strains

The different T4P strains were assessed for their ability to bioluminesce, which gives an indication of the strength of the promoter placed upstream of the *lux* genes. Bacterial strains were inoculated at $1x10^6$ cfu/20μl into 180μl of BHI in a black 96 well plate in triplicate. Bioluminescence was measured over time in a plate reader taking measurements every 20 minutes over a period of 10 hours. Average of the three readings was calculated and plotted as shown in Figure 7-4.

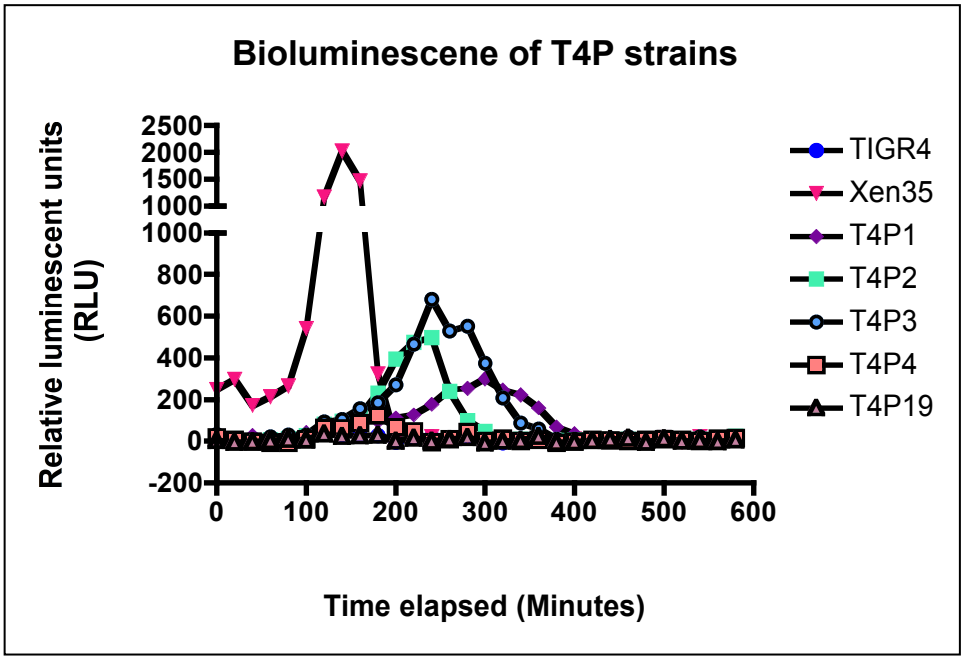


Figure 7-4: Bioluminescence of T4P strains

Graph of bioluminescence of Xen35, TIGR4 and the T4P strains over time. Each point on the graph represents the average of a triplicate reading, Readings were taken every 20 minutes.

T4NO1 was used as a negative control for bioluminescence measurement and Xen35 as a positive control. All T4P1-4 strains showed bioluminescence using the plate reader, however to varying extents. T4P2 and T4P3 showed the highest bioluminescence, T4P1 showed slightly lower levels and T4P4 bioluminescence levels were almost undetectable. When comparing these strains to Xen35 none of them were as bioluminescent with T4P3 and T4P2 showing roughly 3-4 times less bioluminescence respectively. T4P19 although containing the promoter that drives the *lux* gene expression in Xen35 showed no bioluminescence. This would indicate that some of the other genome changes seen in Xen35 effect the

activity of this promoter, which is why the bioluminescence is so bright in Xen35 and other Xen strains.

7.4.2 Western blot analysis of T4P strains

Western blot analysis was performed on strains T4P1-4 to assess the effect of *lux* gene expression on RrgB expression, as RrgB levels were severely reduced in Xen35.

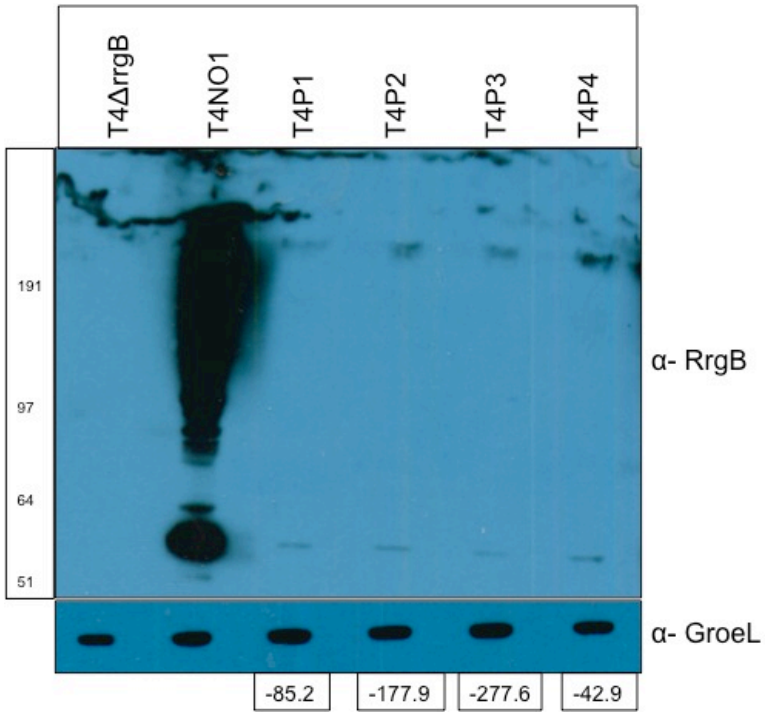


Figure 7-5: Western blot of T4P strains RrgB expression compared to T4NO1.

Western blotting analysis was performed on T4NO1, T4P1, P2, P3, P4 and T4Δ*rrgB* looking for expression of RrgB in all strains (α-RrgB antibody). Equal protein loading was confirmed by equal expression of GroeL (α-GroeL antibody), normalise against in analysis. Western blotting quantification was performed using ImageJ with fold changes in RrgB expression compared to T4NO1 boxed below the strain. Left hand side numbers represent the size in kDa of proteins run to the same point.

Expression of the pilus backbone protein (RrgB) showed a large decrease in the RrgB levels in all T4P strains, shown in Figure 7-5. All strains showed a decrease of 40-280 fold in RrgB pilus protein levels compared to T4NO1. Xen35 was not included in the analysis so the relative RrgB levels in these strains and Xen35 may vary. The fold change observed in these strains is much higher than that observed in Xen35. However Xen35 was compared against T4JH and the T4P strains were constructed in T4NO1. Pilus expression varies between the two

TIGR4 strains with roughly a four fold increase in T4NO1 pilus positive cells compared to T4JH, which may account for why a bigger drop in RrgB expression is observed in the T4P strains.

7.4.3 Hydrogen peroxide production of T4P strains

Levels of hydrogen peroxide were measured to see whether expression of the *lux* genes effects hydrogen peroxide production. In Xen35 there is no hydrogen peroxide production due to the SNPs located in *spxB* and *lctO* resulting in truncated proteins. Both of these protein are important during aerobic growth. As both are non-functional in Xen35 this may indicate they were non-functional/ had a reduced functionality anyway and therefore mutations were able to accumulated in these genes.

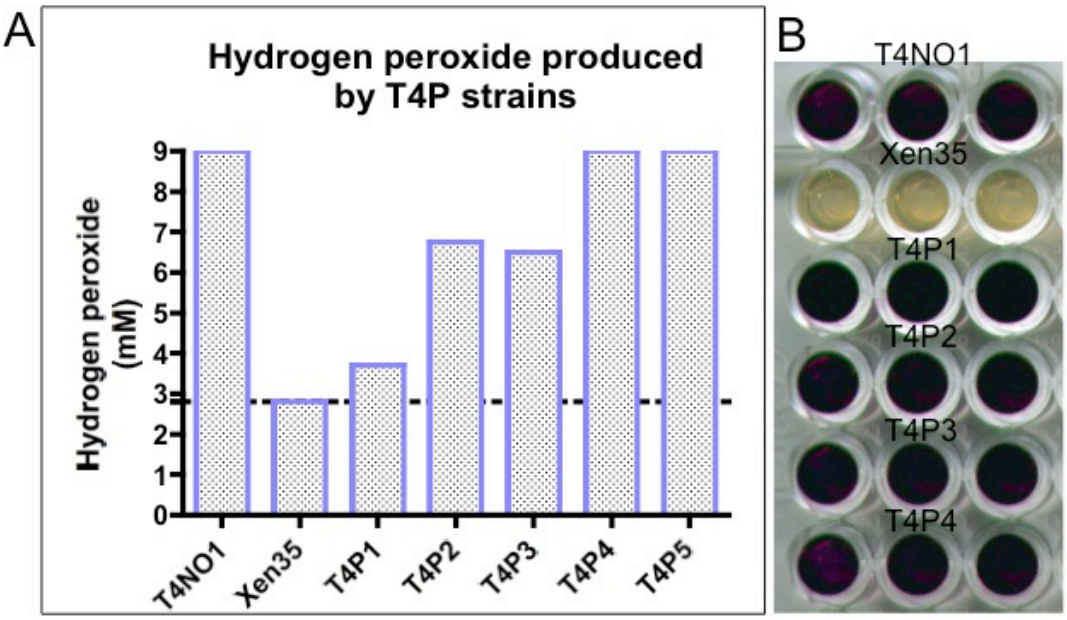


Figure 7-6: Hydrogen peroxide production of T4P strains

Each strain was represented in triplicate in the Hydrogen peroxide assay. (A) The graph gives the hydrogen peroxide production in mM of each strain calculated using a standard curve of known hydrogen peroxide concentrations (See Figure 2-14), the dotted line represent the limit of detection. Hydrogen peroxide production above 9mM could not be accurately measured therefore a maximum value of 9mM was assigned. (B) Shows visually the assay performed in a 96 well plate with each strain represented in triplicate.

SpxB is functional in the T4P strains shown from Figure 7-6, however all show reduced levels of hydrogen peroxide production compared to T4NO1. This may suggest SpxB has a reduced function in these strains. However even after

deletion of SpxB we did not observe the same level of reduced pilus expression that we see in Xen35 and the T4P strains. Therefore there must be another factor causing this reduction in pilus expression in the T4P strains and Xen35, which is in some way linked to high expression of the *lux* genes.

Only one of the T4P strains was chosen for further analysis, so further experiments were performed on T4P2.

7.5 Characterisation of T4P2

7.5.1 FACS and fluorescence microscopy analysis of T4P2

Differences seen in pilus expression in T4P2 were further confirmed by FACS analysis and fluorescence microscopy. This assessed if the difference in RrgB expression is that of a reduction in the RrgB levels on a single cells or that of a reduction in the number of pili positive cells within the population as a whole.

FACS analysis confirmed the reduction in RrgB and like Xen35 this reduction was at the population level (Figure 7-7, Figure 7-8), with now only 7.6% of T4P2 cells expressing RrgB on their cell surface compared to that of its parent T4NO1 (88% RrgB+) (Table 7-2). A population of Xen35 however still contained fewer RrgB+ cells (5.4%). As observed for some Xen35 pili positive cells, some T4P2 cells contained pili just at set location on the cell and a small number also showed pili over the whole cell surface (Figure 7-8). The level of expression of the *lux* genes and pilus islet genes in T4P2 were analysed comparing their expression levels to Xen35.

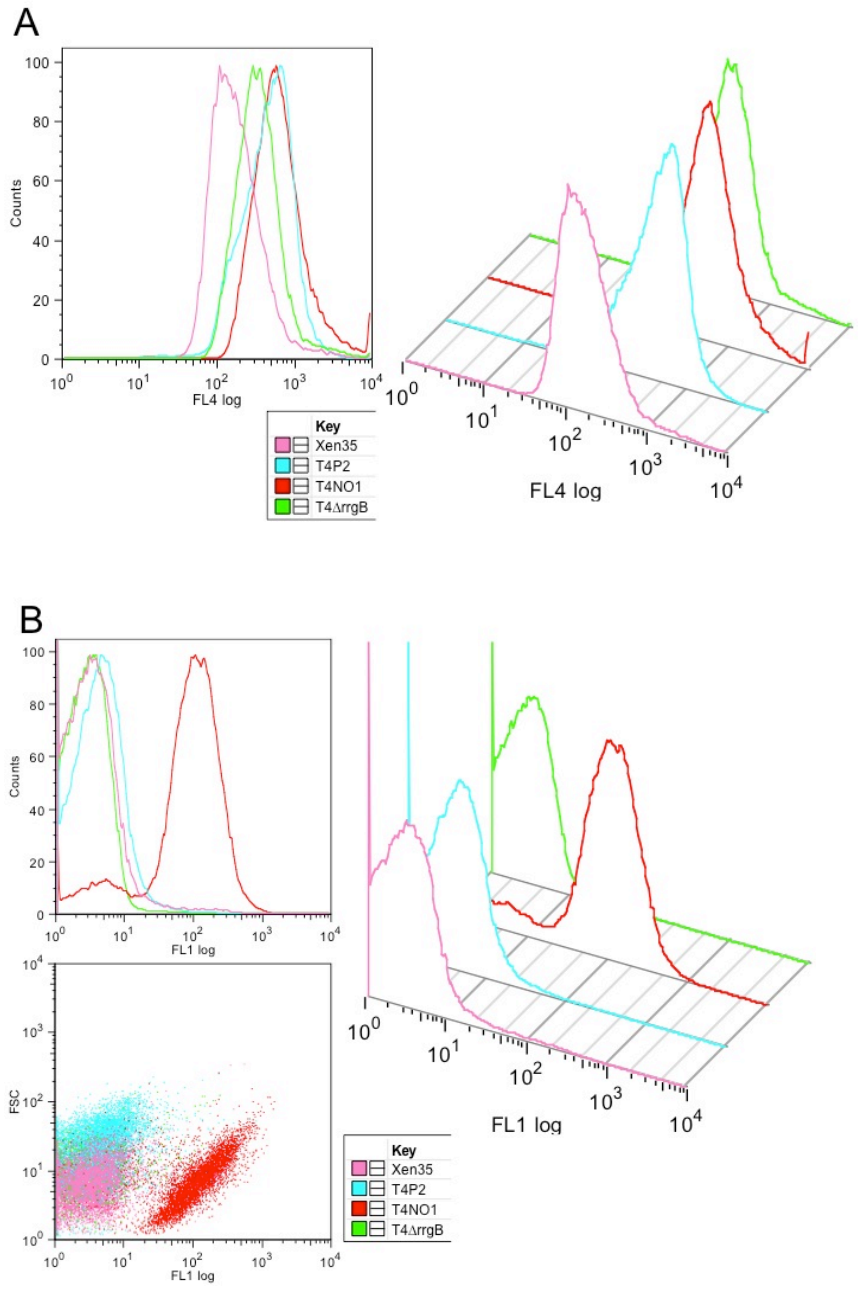


Figure 7-7: FACS analysis of RrgB surface expression in T4P2

FACS was performed on T4P2, T4NO1, Xen35 and T4Δ*rrgB*. (A) Shows histograms of the capsule positive populations selected for from samples stained with a capsule antibody, population used for further analysis. (B) Shows the capsule stained populations being gated on for being RrgB positive, histograms/ polychromatic plot show negative (left) and positive (right) RrgB populations in each strain.

Strain	RrgB- (%)	RrgB+ (%)
T4NO1	12.1	87.9
T4P2	92.4	7.6
Xen35	94.7	5.4
T4Δ*rrgB*	98.4	1.6

Table 7-2: Percentage RrgB positive cells in a T4P2 population from FACS analysis.

Table shows the percentage RrgB positive and negative cells in a growing bacterial population. FACS analysis was performed in FlowJo.

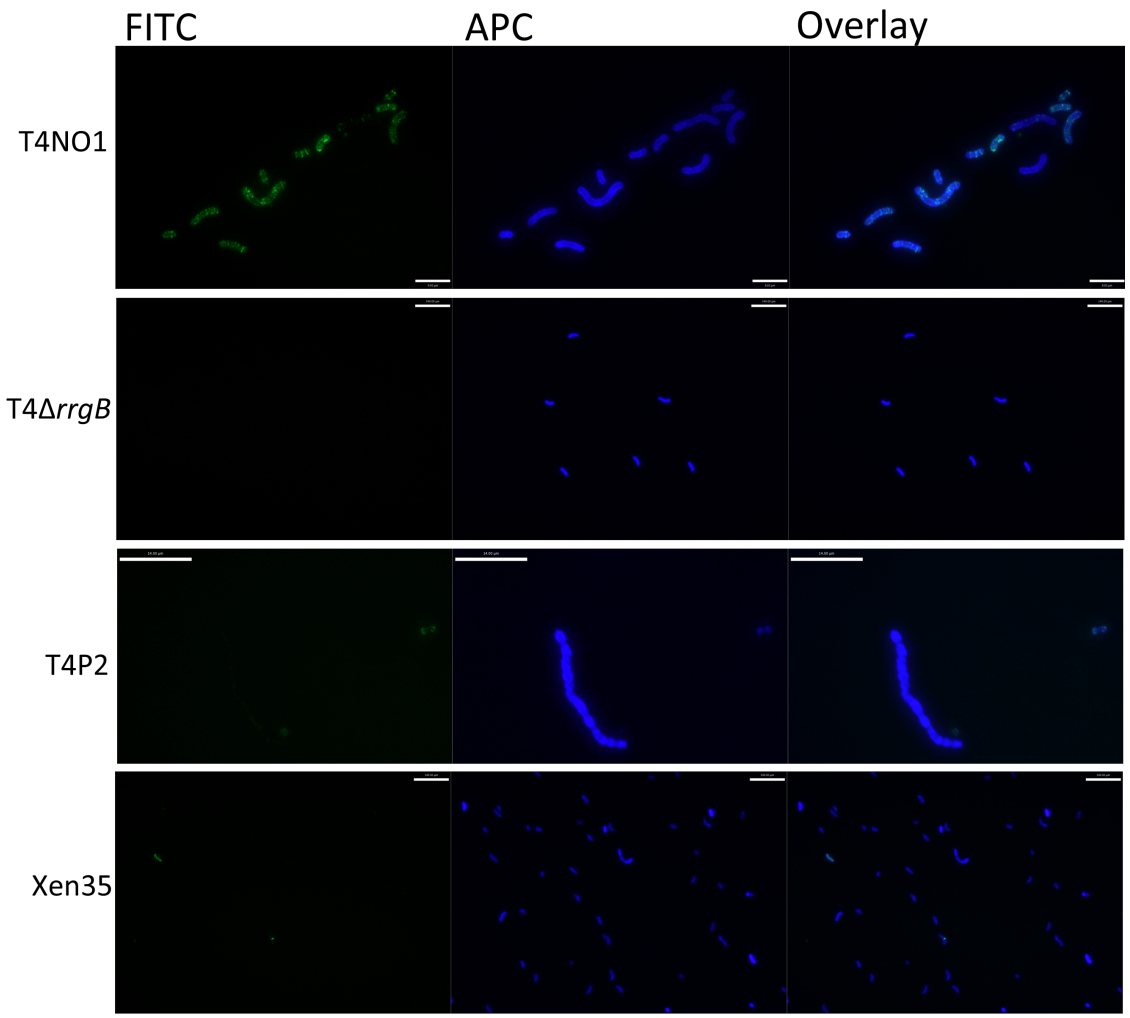


Figure 7-8: Fluorescence microscopy of T4P2.

One representative image of fluorescently labelled T4NO1, T4Δ*rrgB*, Xen35 and T4P2 used for FACS analysis. Cells were stained for the presence of RrgB (FITC) and the capsule (APC). Scale bars above represent, T4NO1-9μm, T4Δ*rrgB* - 140μm, T4P2 - 14μm and Xen35 - 140μm. The same representative image will be used throughout this study for each strain, more images can be seen in appendix I.

7.5.2 Lux gene and pilus expression in T4P2

RT-PCR analysis was performed to measure the expression levels of the *lux* genes (*luxA-E*) and the pilus islet in T4P2 relative to that of Xen35.

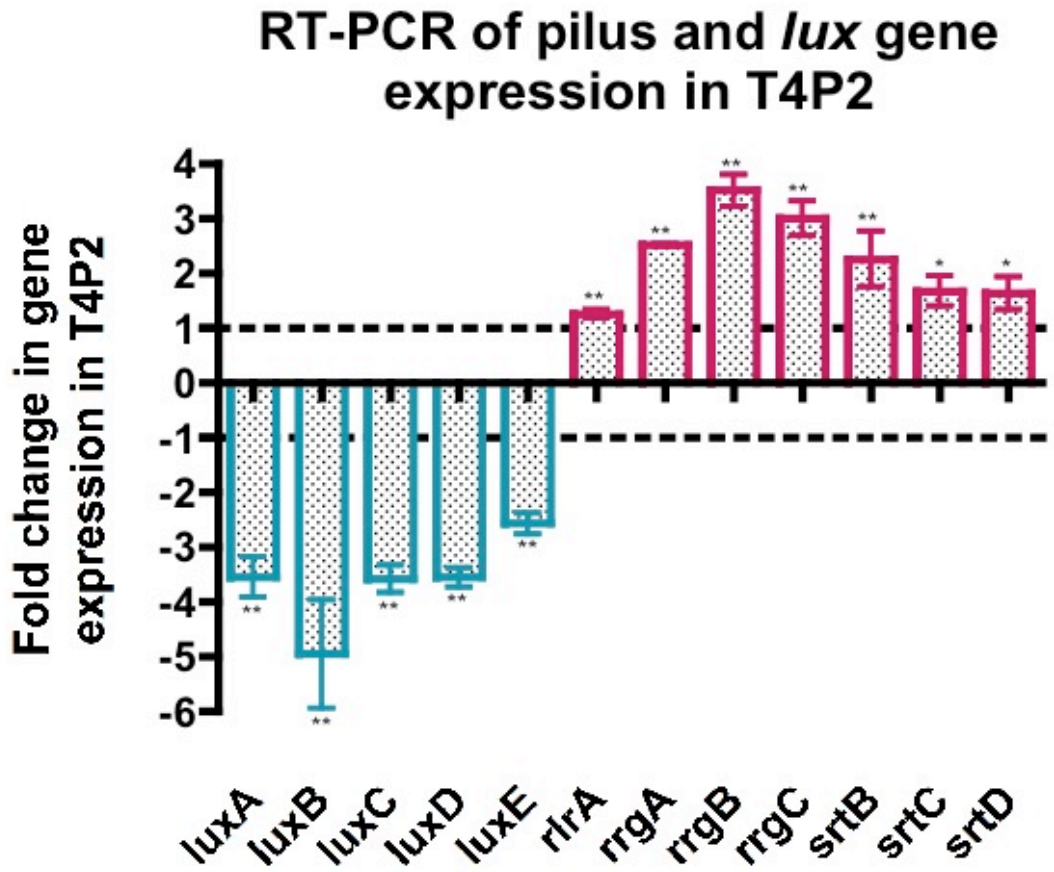


Figure 7-9: RT-PCR graph of *lux* and pilus genes expression in T4P2

Graph shows RT-PCR of *luxA-E* and the whole pilus islet (*rlrA, rrgA, rrgB, rrgC, srtB, srtC, srtD*) in T4P2. Fold change represents that of T4P2 compared to Xen35. Each bar represents the average of three replicas (three biological replicas) and error bars the standard deviation. Statistical analysis was performed by a 1-way ANOVA with a Tukeys testing correction comparing the dCt values of the control strain (Xen35) to the test strain (T4P2), * P<0.05, **P<0.001.

RT-PCR showed roughly a 3-5 fold decrease in the expression of the *lux* genes in T4P2 compared to Xen35, this correlates well with the difference in bioluminescence levels produced between the two strains, shown in Figure 7-4. Interestingly this also correlates with an increase in pilus expression of 2-4 fold seen in T4P2 compared to Xen35. This would indicate a potential metabolic burden on the cell of expressing the *lux* genes, with higher expression of the *lux* genes resulting in decreased pilus expression.

7.5.3 Microarray analysis of T4P2

Microarray analysis was performed on T4P2 to assess whole genome expression changes compared to T4NO1, this will also enable evaluation of the contribution

of the expression of the *lux* genes to variations in gene expression in T4P2 and Xen35.

Gene	Description	Abbrev	Fold change
SP_0453	Amino acid ABC transporter, permease protein.		1.2
SP_0461	Transcriptional regulator, pilus islet	*rlrA*	36.3
SP_0494	CTP synthase, catalyses amination of UTP to CTP	*pyrG*	1.4
SP_1071	ABC transporter ATP binding protein.		1.8
SP_1296	Hypothetical protein		1.5
SP_1356	Chlorohydrolase, Atz/Trz family protein		2.1
SP_1500	Amino acid ABC transporter, amino acid binding protein.		1.7
SP_1501	Amino acid ABC transporter, ATP binding protein.		1.8
SP_1576	Homoserine-o succinyltransferase, methionine biosynthesis.		1.1
SP_1578	Hypothetical protein		1.3
SP_1675	ROK family protein		3.0
SP_1676	N-acetlyneuraminate lyase		2.8
SP_1682	Sugar ABC transporter, permease protein		2.8
SP_1684	PTS system, IIBC component		2.4
SP_1688	ABC transporter permease protein		4.3
SP_1816	Anthranilite synthase component II	*trpG*	3.2
SP_1852	Galactose-1-phosphate uridylyltransferase, galactose metabolism.		3.7
SP_1874	Ribosomal large subunit pseudouridine synthase		2.2
SP_1875	Functions during chromosome segregation.	*scpB*	2.0
SP_1878	CBS domain containing protein		1.9
SP_1879	Hypothetical protein		2.0
SP_1880	Putative deoxyribonucleotide triphosphate pyrophosphatase		2.1
SP_1882	Hypothetical protein		4.1
SP_1894	Sucrose phosphorylase	*gtfA*	3.0
SP_1895	Sugar ABC transporter, permease protein	*rafE*	3.0
SP_1897	Sugar ABC transporter, sugar binding protein	*rafG*	4.4
SP_2000	DNA binding response regulator	*rr11*	1.8
SP_2196	ABC transporter, ATP binding protein.		1.6

Table 7-3: Genes differentially regulated in T4P2

Table shows genes differentially regulated in T4P2 compared to its parent T4NO1 (P<0.05). Fold change represents that seen in T4P2 compared to T4NO1, red showing genes up regulated in T4P2 and blue are those that are down regulated.

A total of 28 genes were shown to be differentially regulated between T4P2 and T4NO1 shown in Table 7-3. Included in this are a number that have been shown to play a role in virulence. SP_0494 and SP_1356 have been shown to play a role in meningitis (Molzen et al., 2011). SP_0461, SP_0494, SP_1816 and SP_1879 are important for lung infection (Hava & Camilli, 2002). SP_1356 is up regulated upon contact with D562 cells, SP_1578, 1852, 1875 and 1878 are up regulated in the blood of infected mice and SP_1675 and SP_2196 are up regulated in the CSF of infected rabbits (Orihuela et al., 2004).

The genes differentially regulated in the T4P2 were compared to those that were differentially regulated in Xen35, with common genes likely due to expression of the *lux* genes. A comparison was made between the P<0.05 (Figure 7-10) and P<0.1 (appendix VII) gene lists from microarray analysis of both strains compared to TIGR4. Comparison was performed using a venn diagram.

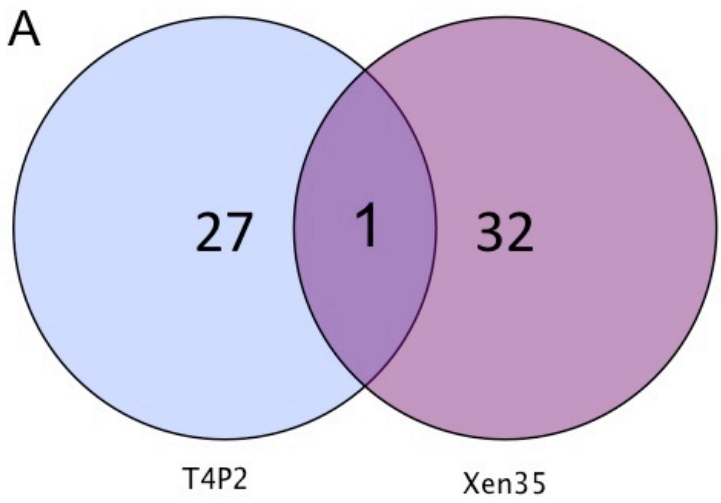


Figure 7-10: Venn diagram of gene expression changes in T4P2 and Xen35.

Venn diagram showing genes commonly regulated in T4P2 compared to Xen35. Diagram shows only 1 gene (P<0.05) (A) and 16 genes (P<0.1) (B) common to both evaluated from strains individual gene expression changes compared to TIGR4.

When comparing gene expression changes between T4P2 and Xen35 only 1 gene was commonly regulated in both (P<0.05) SP_1895, a component of a sugar ABC transporter. SP_1895 was down regulated in Xen35 and T4P2 5 and 3 fold respectively. When comparing the gene lists with an increased P value (P<0.1) there were a further 15 genes commonly differentially regulated in the two strains shown in appendix VII. Of the genes a number are regulated oppositely in the two strains and therefore are likely not genes regulated commonly by the *lux* genes. The genes that were common to both and aren't oppositely regulated include SP_0461-64 (the pilus pilins and transcription regulator), SP_1895-98 (sugar ABC transporter genes and a alpha galactosidase) and SP_2193 (*rr06,* response regulator).

7.5.4 Real-time PCR validation of microarray analysis in T4P2

Microarray analysis was confirmed by RT-PCR analysis of the genes encoded on the pilus islet. Microarray analysis showed a large decrease in pilus expression in T4P2 compared to T4NO1. This decrease is larger than that of Xen35, but this is due to the fact Xen35 pilus expression was compared to that of T4JH, which shows roughly a 4 fold decrease in pilus expression compared to T4NO1. As T4P2 was constructed in T4NO1 this is the appropriate comparison.

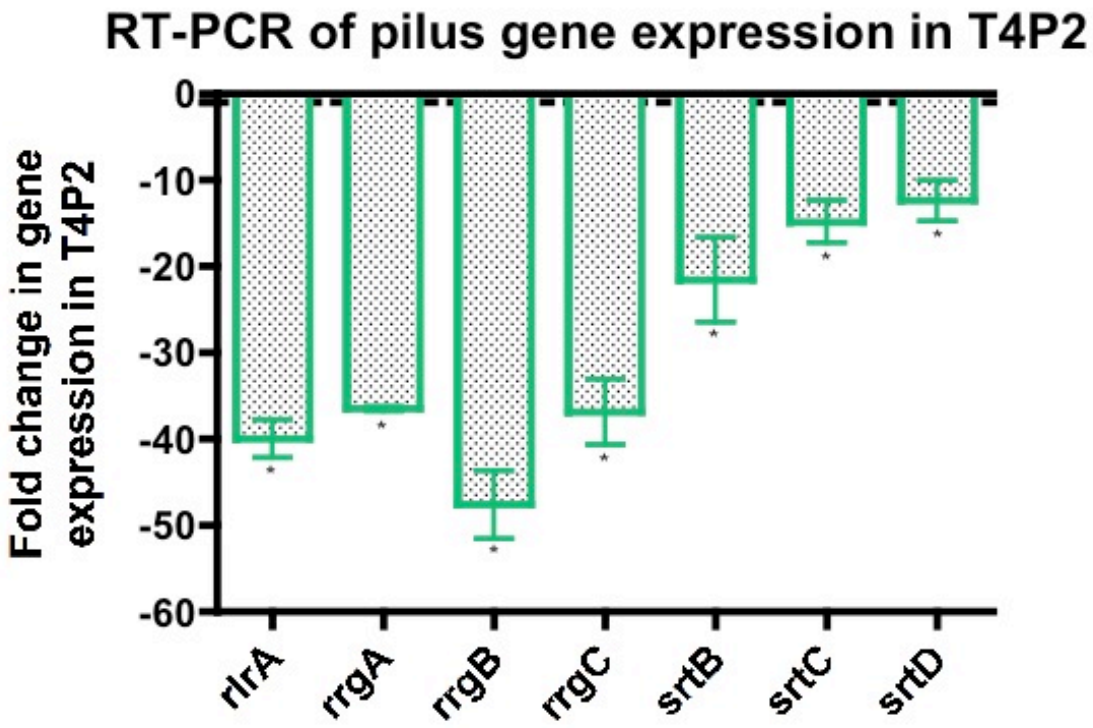


Figure 7-11: RT-PCR graph of pilus expression in T4P2

Graph shows RT-PCR of the whole pilus islet (*rlrA, rrgA, B, C, srtB, C, D*) in T4P2. Fold change represents that of T4P2 compared to T4NO1. Each bar represents the average of three replicas (three biological replicas) and error bars the standard deviation. Statistical analysis was performed by a 1-way ANOVA with a Tukeys testing correction comparing the dCt values of the control strain (TIGR4) to the test strain (T4P2), * P<0.001.

Gene	Microarray	RT-PCR
SP_0461 (*rlrA*)	36.3	39.9
SP_0462 (*rrgA*)	24.9	36.5
SP_0463 (*rrgB*)	25.1	47.5
SP_0464 (*rrgC*)	23.2	36.8
SP_0466 (*srtB*)	5.0	21.3
SP_0467 (*srtC*)	9.3	14.7
SP_0468 (*srtD*)	13.5	12.3

Table 7-4: Comparison of expression changes of the pilus islet in T4P2 from microarray and RT-PCR analysis.

Table shows fold changes in gene expression of the whole pilus islet in T4P2 compared to T4NO1. Fold changes show that from microarray data and those from RT-PCR.

RT-PCR further confirmed the down regulation of the pneumococcal pilus in T4P2 showing a 35-50 fold down regulation of the genes that encode the pilins and the transcriptional regulator and a 10-20 fold down regulation of the sortase enzymes (Figure 7-11). Comparison of the pilus islet expression by microarray and RT-PCR analysis showed very similar expression levels, confirming this change (Table 7-4).

7.5.5 In vivo analysis of T4P2

To assess the virulence of T4P2 *in vivo* a mouse model of infection was performed as described previously for T4NO1 and Xen35 (experimental design identical to that of Xen35 vs. T4NO1). The data below is that of the T4NO1 and Xen35 data already presented above and comparisons made between these data sets and T4P2. In this data set statistical analysis was performed comparing T4NO1 and T4P2/ Xen35 and T4P2 only as the T4NO1 and Xen35 comparison have been assessed earlier in this study.

Initial analysis was performed looking at the survival of MF1 mice infected with T4P2 and the percentage weight loss of these mice over time (Figure 7-12). Mice infected with T4P2 had a statistically significantly longer survival time than mice infected with T4NO1. However their survival time was similar to that of mice infected with Xen35, showing no statistical difference between the two (Figure 7-12). Percentage weight loss of mice infected with T4P2 was statistically lower than that of mice infected with T4NO1 suggesting a slower disease progression in this strain compared to T4NO1 (Figure 7-12). Mice infected with Xen35 showed a similar percentage weight loss to that of T4P2 infected mice indicating a similar disease progression.

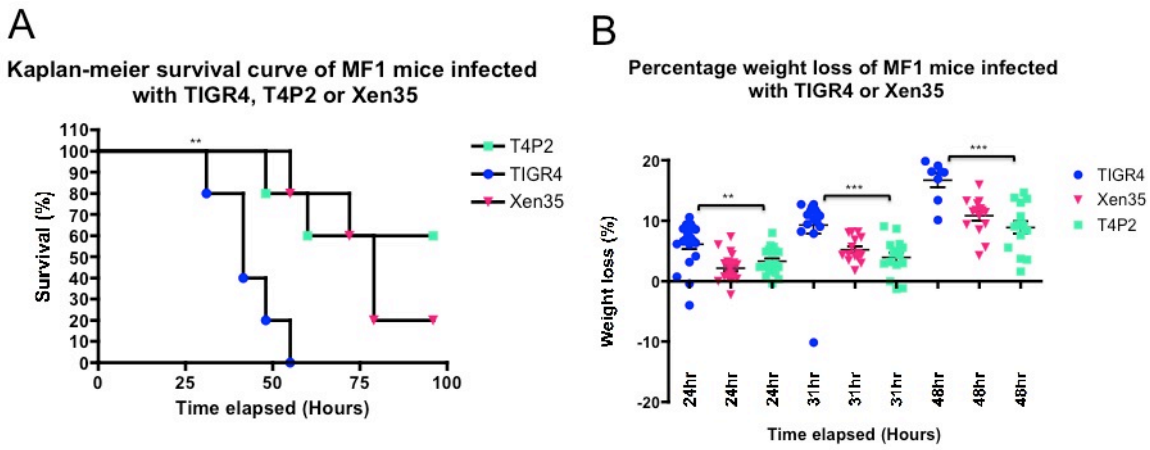
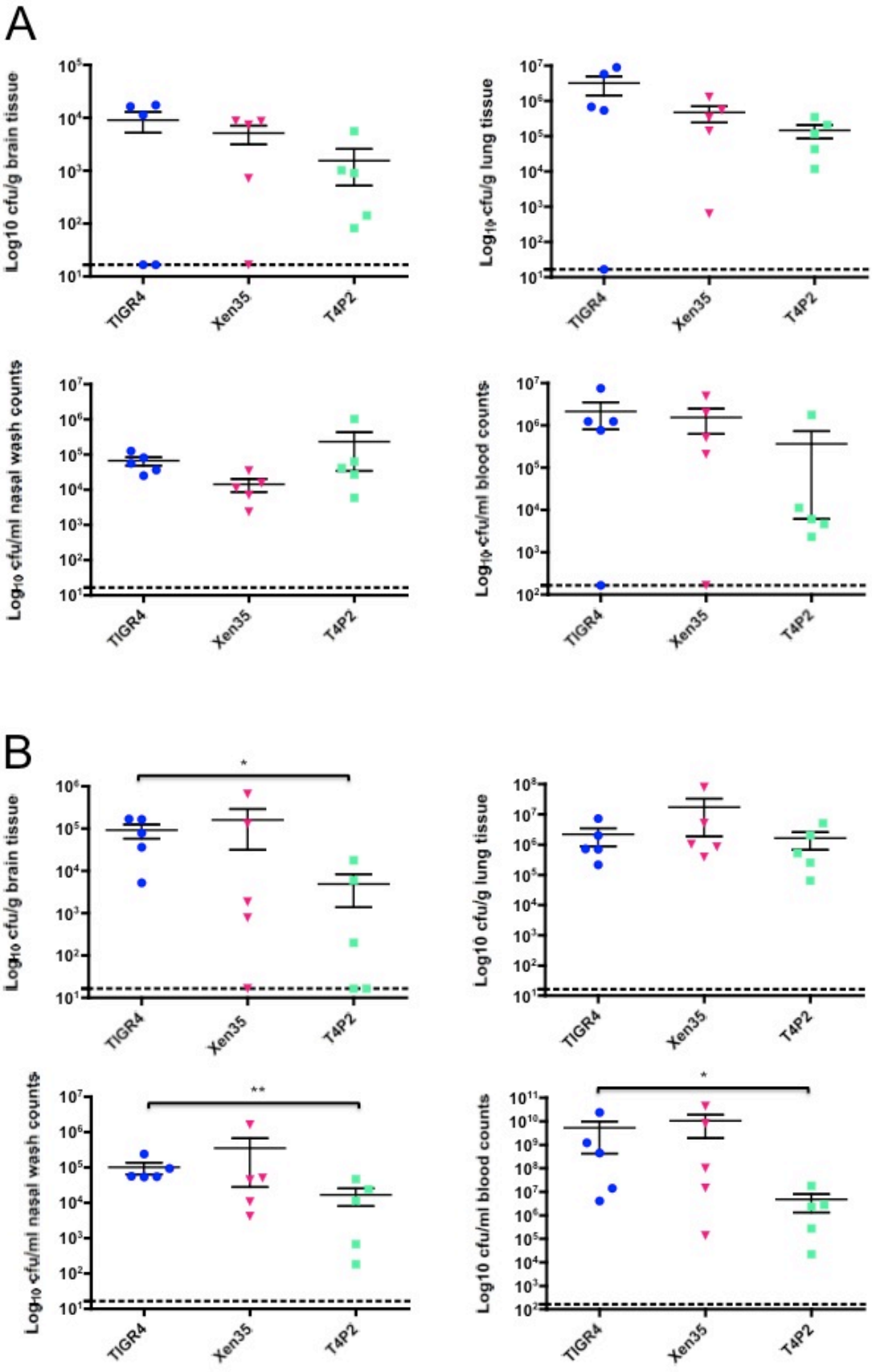


Figure 7-12: Survival and weight loss of mice infected with T4P2

(A) Shows percentage survival of mice infected with T4NO1, Xen35 or T4P2 over time, statistical analysis was performed comparing T4P2 to T4NO1 or T4P2 to Xen35 using a logrank Test, ** P<0.01. ** above the strain indicated a statistical difference compared to T4P2. (B) Shows percentage weight loss of mice infected with Xen35, T4NO1 or T4P2. Statistical analysis was performed using a non-parametric Mann-Whitney two sample rank test, **P< 0.01 and ***P<0.001.

To assess at what point during infection this attenuation of T4P2 is occurring enumerated of the bacterial load in the mouse lungs, brain, blood and nasal wash over time was performed.

At 24 hours post infection there seemed to be no statistical difference in counts from any organs/ bodily fluids when comparing T4P2 to T4NO1 and Xen35. However there is a trend towards lower counts in all compared to T4NO1 except the nasal wash (Figure 7-13). At later time points there are some clear differences in virulence between T4P2 and T4NO1/Xen35. At 48 hours and survival time points there is a statistical difference in bacterial counts in the nasal wash, blood and brain of T4P2 infected mice compared to T4NO1, at 72 hours post infection bacterial counts in the blood and brain were statistically lower compared to T4NO1. This may suggest an inability of T4P2 to spread into the blood and to the brain. The decrease in colonisation is likely due to the decrease in pilus as T4P2 has a functional SpxB confirmed by hydrogen peroxide production (Figure 7-6). Interestingly at this time point there was also a statistical decrease in bacterial counts in the brain, blood and lungs of T4P2 infected mice compared to Xen35, suggesting a decrease in virulence of T4P2 compared to Xen35 (Figure 7-13). T4P2 infected mice were imaged at each time point to visually assess disease progression, shown in Figure 7-14.


A
Log10 cfu/g brain tissue
Log10 cfu/g lung tissue
Log10 cfu/ml nasal wash counts
Log10 cfu/ml blood counts
TIGR4
Xen35
T4P2
B
Log10 cfu/g brain tissue
Log10 cfu/g lung tissue
Log10 cfu/ml nasal wash counts
Log10 cfu/ml blood counts
*
**
*
TIGR4
Xen35
T4P2

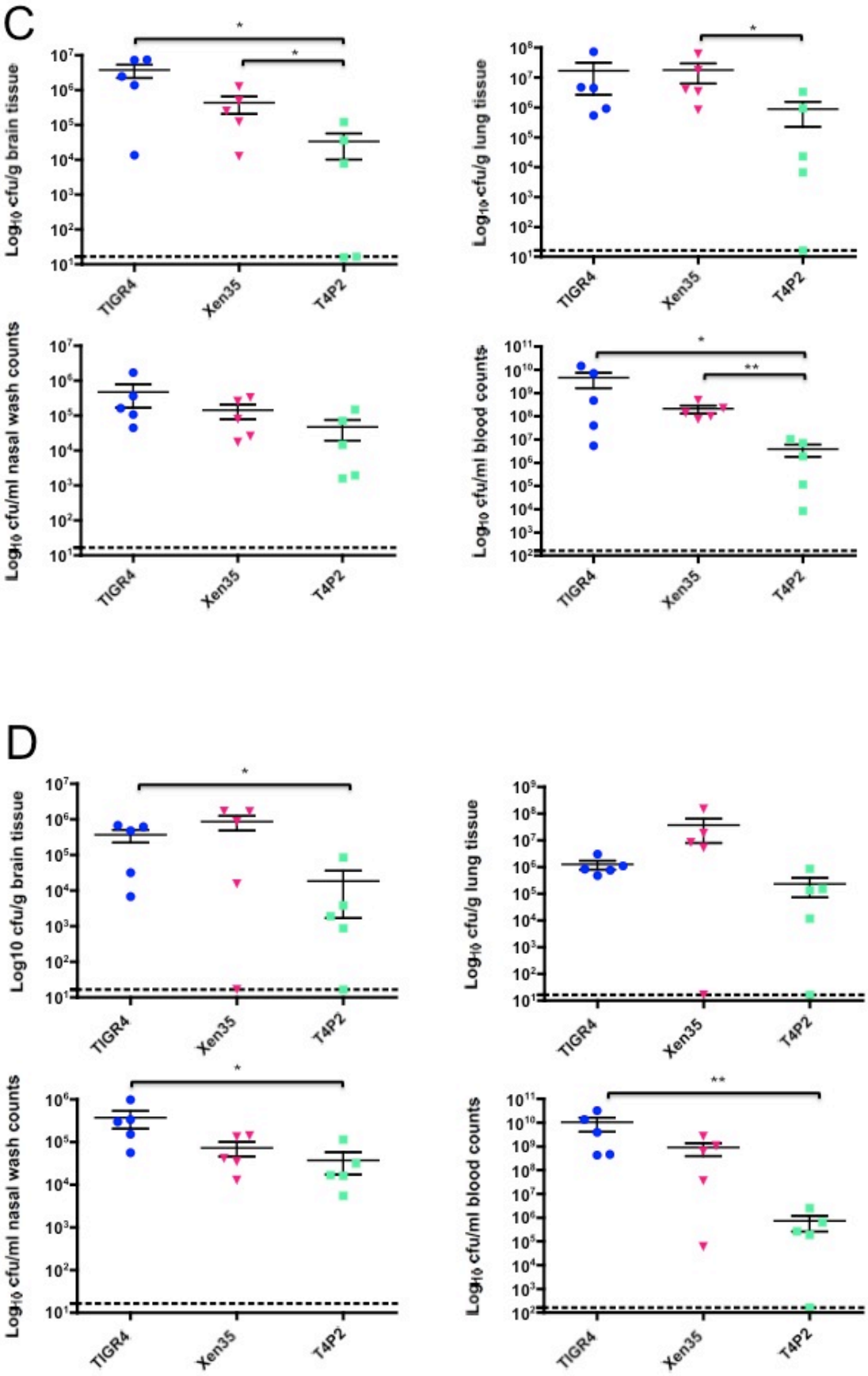


Figure 7-13: Bacterial counts in organs and bodily fluids of MF1 mice infected with Xen35, T4NO1 or T4P2.

Bacterial counts were enumerated from brain, lungs, nasal wash and blood of mice infected with either Xen35, T4NO1 or T4P2. Counts were enumerated from 5 mice for each strain over varying time points, graph (A) represent the 24 hour post infection time point, (B) 48 hours, (C) 72 hours and (D) the survival time point. Statistical analysis was performed comparing T4NO1 to T4P2 or Xen35 to T4P2 using a non-parametric Mann-Whitney two sample rank test, *P< 0.05, ** P<0.01.

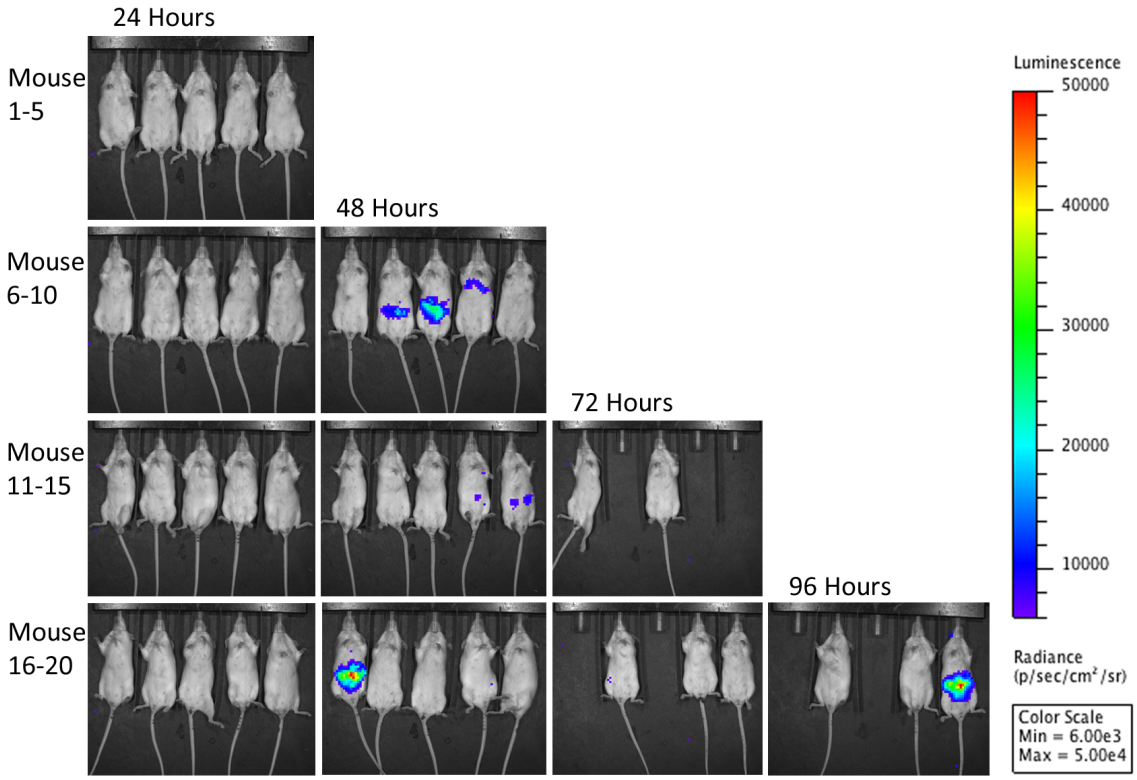


Figure 7-14: Images of mice infected with T4P2

Images of mice infected with T4P2 over time showing *in vivo* infection in the lungs and abdomen of some mice. Images were acquired using the IVIS spectrum *in vivo* imaging system, imaging for 5 minutes for each. Hours above the images indicate that of the time elapsed since inoculation. Numbers to the side indicate the mice numbers in each group. If mice are not present in the image they have been culled.

7.6 T4P2 whole genome sequence

Whole genome sequence analysis was performed on T4P2 to assess whether expression of the *lux* genes leads to accumulation of genetic changes. Preliminary whole genome sequence changes of T4P2 compared to the genome sequence data available at NCBI (NC_003028) can be seen in Table 7-5. Preliminary analysis included reference assembling the sequence reads of T4P2 to the available genome sequence (NC_003028), followed by SNP and indel testing and assessing if there are any low coverage regions. This analysis was

performed by Dr Andrea Mitchell. Only three genome changes were observed in T4P2 compared to the genome sequenced strain. One of these changes included a low coverage region in gene SP_1886, which is where the *lux* genes have been inserted, as these would not align to the TIGR4 genome sequence. A second low coverage regions was observed in the intergenic region between genes SP_1199-SP_1200, this was also observed in the parent strain of T4P2 (T4NO1) so this change likely came from this. Finally the indel in the intergenic region between SP_1777 and SP_1778 was not observed in T4NO1. Interestingly this change was observed in T4JH and Xen35. Whether this change is linked to any biological function would require further study. Interestingly there were two genome changes in T4NO1 that were not observed in T4P2.

Gene	Information	Position TIGR4	Variant		In T4NO1?
INT SP_1199-1200			Low coverage		Yes
INT SP_1777-1778		1696086	Indel	T	No
SP_1886	New lux gene insertion site		Low coverage		No

Table 7-5: Preliminary whole genome sequence changes in T4P2

The fact only a small number of genome changes were observed in T4P2 would suggest the reason for the reduced pilus expression in this strain is solely due to the metabolic burden expression of the *lux* genes places on the cell. In this experiment T4P2 had only been passaged once whereas it could be that some of the genome sequence changes in Xen35 were able to accumulate over time if passaged multiple times.

7.7 Discussion

To assess the metabolic burden of bioluminescence on the bacterial cells the *lux* genes were placed at a different genomic region (into SP_1886) to that in Xen35 and expressed at a high level using different pneumococcal promoters. Inserting the genes into a different genomic region removes any potential effects to pilus regulation the *lux* insertion site has in Xen35, such as SP_1914 becoming non-functional and the high expression of SP_1915. The plasmids constructed only contained roughly 2kb of pneumococcal DNA required for recombination either side of the cloning site, which removed the potential contributions of introducing other changes via recombining in large amounts of surrounding DNA, which occurred in Xen35 (Guiral et al., 2006).

Xen35Δ19 containing a transposon insertion downstream of XEN35_1915 (SP_1915) confirmed the promoter downstream of SP_1915 was that driving the *lux* gene expression, as no bioluminescence was observed in this strain. The new collection of bioluminescent strains produced include T4P19 which contained the SP_1915 promoter in front of the *lux* genes. However this strain showed no bioluminescence suggesting this promoter alone is unable to drive the high expression of the *lux* genes. This may indicate disruption in Xen35 of some regulatory network surrounding the *lux* insertion site, which results in the high expression of SP_1915. Perhaps though perturbing the function of a repressor of this gene via the SNPs/ indels recombined in from Xen7 surrounding the *lux* insertion site. Both SP_1919 and SP_1920 are non-functional in Xen35 due to changes recombined in from Xen7, however further study would need to be done to assess a potential role of these genes in regulation of SP_1915.

Bioluminescence produced by all T4P strains was observed however to varying levels, with T4P2 and T4P3 showing the highest levels. Yet this was still lower than that observed in Xen35. Western blot analysis for expression of the pilus backbone protein RrgB showed a large decrease in RrgB levels in all T4P strains compared to T4NO1, showing a similar reduction in RrgB levels to that seen in Xen35. However the RrgB levels were not directly compared by western blot. The fold change observed in RrgB levels compared to T4NO1 correlated well with the level of bioluminescence with T4P3 showing the largest decrease in RrgB levels and the highest bioluminescence, followed by T4P2, T4P1 and T4P4.

T4P19 showed no difference in RrgB levels compared to T4NO1 (data not shown), and no bioluminescence. In T4P1-4 hydrogen peroxide levels were reduced by around 25%-50% compared to T4NO1, which may suggest a decreased functionality of SpxB.

T4P2 was taken forward for further analysis. RT-PCR comparing the pilus islet genes and *lux* genes expression in T4P2 relative to Xen35 further validated the likelihood that the *lux* gene expression puts a metabolic burden on the bacterial cell. Observed was a 3-5 fold reduction of the *lux* gene expression in T4P2 compared to Xen35, corroborated by a lower bioluminescence in T4P2 seen on the plate reader. Simultaneously T4P2 has a 2-4 fold increase in expression of the pilus islet genes compared to Xen35. This would indicate that the expression of the *lux* genes places a metabolic burden on the cell as the lower levels of *lux* gene expression in T4P2 correlates to an increased expression of the pilus islet genes.

Although T4P2 was not complemented to ensure the change in pilus expression is due to *lux* expression we assume that the expression of the *lux* genes is the cause of the phenotypic change and not due to any other genetic changes as all mutant strains showed the same phenotype. Further whole genome sequencing was performed on T4P2 to show one a very small number of genetic changes had occurred in the strain which did not seem to be linked to pilus expression.

Further analysis by FACS showed as in Xen35, T4P2 had a large decrease in the number of cells positive for RrgB on the cell surface, with only 7.6% of cells RrgB positive compared to 88% in T4NO1. In T4P2 only 2% more cells were RrgB positive than Xen35. For both in the majority of instances pili were only observed at set locations on the bacterial cell rather than spread over the whole cell as seen in T4NO1.

To assess genes commonly effected by the *lux* gene expression comparison of the genes differentially regulated in T4P2 and Xen35 were compared. Using both the $P<0.05$ and $P<0.1$ genes lists a total of 8 genes were shown to be commonly regulated including the pilus islet genes *rlrA*, *rrgA*, *rrgB*, *rrgC* and SP_1895, SP_1896, SP_1897, SP_1898 and SP_2193. SP_1895-97 encode an ABC transporter enabling growth on raffinose, stachyose and melibiose (Bidossi et al., 2012).

SP_1896 and SP_1898 were also shown to be important for lung infection (Hava & Camilli, 2002). The response regulator SP_2193 was also commonly regulated in both T4P2 and Xen35 and has been shown to regulate the pneumococcal pilus and is therefore likely the cause of the decrease in pilus in both strains (Rosch et al., 2008). However this difference was not shown to be statistically significant by RT-PCR in Xen35 and has not been validated by this method in T4P2. Further analysis of the expression of *rr06* in the other T4P strains may give a clearer indication if this is a common feature of all strains. If this is the case RR06 in this instance acts as a repressor of the pilus islet genes, as shown in the literature (Rosch et al., 2008).

How the expression of the *lux* genes may have such a pronounced effect on the bacterial cell is likely due to two factors: through utilisation of ATP for aldehyde production and a requirement for large amounts of reducing power for the recycling of FMN to $FMNH_2$ (Jablonski & DeLuca, 1977, Boylan et al., 1985). Natural bioluminescent bacteria are equipped for this however in normally non bioluminescent bacteria expression of the *lux* genes may lead to depletion of ATP levels and altered redox balance, leading to alterations in normal cell functioning and virulence.

The production of energy in the pneumococcus is strictly fermentative via glycolysis followed by pyruvate metabolism to lactate, acetate, formate or ethanol, with no genes found encoding the required enzymes for the TCA cycle of electron transport chain (Hoskins et al., 2001, Tettelin et al., 2001), see Figure 7-15. Glycolysis yields two ATP molecules, two NADH molecules and two molecules of pyruvate. Pyruvate fermentation follows glycolysis, under both anaerobic and aerobic conditions. During fermentation pyruvate is converted to lactate via lactate dehydrogenase (NADH dependant), simultaneously NADH is converted to NAD^+ required for glycolysis. Under aerobic conditions there is a shift to mixed acid fermentation resulting in acetate, formate and ethanol production rather than lactate (Yesilkaya et al., 2009). During this pyruvate is either processed via SpxB (pyruvate oxidase) under aerobic conditions to acetyl phosphate followed by conversion to acetate via AckA (acetate kinase). The latter reaction producing ATP as a by-product, which contributes to the majority of the cells ATP production, ATP levels severely drop upon deletion of SpxB or AckA (Ramos-Montañez et al., 2010, Pericone et al, 2003). Alternatively

pyruvate is converted to Acetyl CoA and formate via pyruvate formate lyase, acetyl CoA can then either be converted to acetyl phosphate by Pta (phosphotransacetylase) and processed by AckA, converted to ethanol via acetaldehyde CoA/ alcohol dehydrogenase or used as a precursor for fatty acid biosynthesis (Yesilkaya et al., 2009). Lactate is not a dead end metabolic product in the pneumococcus and under aerobic conditions can be converted back to pyruvate via LctO (lactate oxidase), which is thought to enable further processing by SpxB and AckA for increased ATP production (Taniai et al., 2008).

The ability to maintain the redox balance within the cell is vital for proper cellular growth and functioning. NADH and NAD^+ are required during carbon catabolism and the constant recycling between the two factors must be maintained. During glycolysis NAD^+ is oxidised producing its reduced equivalent NADH, which is recycled back to NAD^+ during fermentation of pyruvate to lactate by lactate dehydrogenase. As described earlier one of the requirements of the bioluminescence reaction is the availability of $FMNH_2$ (reduced riboflavin), which is oxidised during the reaction giving FMN, recycling between the two via flavin reductase is NAD(P)H dependent (Jablonski & DeLuca, 1977, Michaliszyn et al., 1977). In the pneumococcus the NADH dependant flavin reductase upon conversion of FMN to $FMNH_2$ results in NADH oxidation giving NAD^+, potentially altering the levels of NAD^+ relative to NADH.

Variations in redox balance may affect the functionality of NADH dependant enzymes such as NADH oxidase in the pneumococcus. NADH oxidase converts oxygen to water helping prevent the toxic effects oxygen may have on the cell (Auzat et al., 1999). If this enzyme is unable to function oxygen may be broken down into harmful reactive oxygen species such as superoxide anions/hydroxyl radicals, causing harm to the bacterial cell.

Changes in redox balance have been shown to cause variation in gram positive and gram negative bacterial metabolism (Bennett & San, 2005, Berrıos-Rivera et al., 2002, Ramos et al., 2004, Neves et al., 2002, Vemuri et al., 2006). Over expression of a NAD^+ dependant formate dehydrogenase from *Candida boidinii* in *E.coli,* causes an increase in NADH levels, which results in a shift in the final carbon catabolite concentrations both under aerobic and anaerobic growth conditions (Berrıos-Rivera et al., 2002). In this instance increased levels of NADH

caused a shift away from fermentation (lactate production) and resulted in increased levels of ethanol and acetate production. Further studies expressing NADH oxidase from *S.pneumoniae* in *E.coli* resulted in the oxidisation of NADH into NAD^+ (Vemuri et al., 2006). NADH levels are thought to act as a sensor which when NADH levels are high metabolism is directed into acetate production to prevent further accumulation of NADH produced from the TCA cycle. Over expression of NADH oxidase, results in reduced NADH levels causing a shift to glucose metabolism via the TCA cycle. Under these conditions a 70% increase in glucose uptake was observed, confirming a clear link between NADH levels and glucose metabolism.

Disruption in catabolism has been shown in the pneumococcus to alter regulation of large groups of genes, altering virulence and regulation of virulence factors. Deletion of the global regulator of catabolite metabolism CcpA in D39 shows altered expression levels of up to 19% of genes in the genome (Carvalho et al., 2011). Along with differential regulation of genes involved in metabolism, there was differential regulation of a number of TCS, transcription regulators (*codY)*, *psaR* the capsule and surface proteins *pcpA, nanA* and *nanB* and the *lic1* operon regulating choline metabolism. The D39 genome does not encode the pneumococcal pilus and therefore would not be seen in this study (Lanie et al., 2007). Virulence studies showed a reduced virulence of *ccpA* mutants compared to their parent strain (TIGR4), with *ccpA* mutants being out competed in a pneumonia and colonisation model of infection (Iyer et al., 2005).

From this a hypothesis has been drawn where upon expression of the *lux* genes in *S.pneumoniae* there is a change in the redox balance within the cell, due to the requirement for the oxidation of NADH occurring during the production of $FMNH_2$. How this may affect the NAD^+/NADH balance is unknown. This change could lead to an increased shift towards lactic acid fermentation if NAD^+ levels were increased, as in *E.coli* upon decreased NAD^+ levels there was a shift away from fermentation (lactate production). This may be why there is reduced hydrogen peroxide production in T4P2 as SpxB has a reduced activity.

Fermentation resulting in mainly lactate formation occurs to a greater extent under anaerobic condition which in the pneumococcus would likely signal being present in low oxygen environments, for instance in the blood. Under these

conditions you would perhaps need reduced levels of adhesins such as the pilus, and variations in redox balance under these conditions may be the trigger for its down regulation. The fact we observe up regulation of *rr06* in Xen35 and T4P2 which is known to regulate the pilus would suggest this factor may be causing the pilus down regulation in both strains. However currently it is not known whether RR06 modulates pilus expression in its phosphorylated or non phosphorylated form and therefore the change in expression levels may not directly correlate to its ability to regulate the pilus if it is not present in the correct form. The external stimulus required for the activation of *rr06* is unknown and may be via sensing intracellular redox potential. There is some evidence to back up this theory as deletion of RR06 in D39 causes a 6 fold increase in the expression of a NADPH dependant flavin mononucleotide reductase, confirming a link between RR06 expression and redox potential (Standish et al., 2007). Redox potential varies depending on the usage of different sugars during growth and it could be envisaged during *in vivo* growth different carbon sources are available, with free sugars in short supply, but glycoproteins readily available (Philips et al., 2003, Voynow & Rubin, 2009). Availability of different sugars could indicate the niche in which the bacterium is present via the redox potential of the cell, which in turn leads to regulation of virulence factors required for survival within that niche.

Shifts in metabolism will also affect the ATP pool present within the cell, which is required for a large number of cellular processes and cell survival. The majority of the ATP produced within the pneumococcus is synthesised under aerobic conditions via SpxB and AckA. If metabolism is shifted towards lactate fermentation lower ATP amounts will be produced. Coupled to this is the fact aldehyde production for the bioluminescence reaction requires ATP, further depleting the intracellular ATP stores. Mutations in AckA (acetate kinase) have been shown to be unstable and result in spontaneous mutation in SpxB or SpxR (SpxB regulator) (Ramos-Montañez et al., 2010). This may be due to the requirement for ATP to repair the cellular damage created via H_2O_2 production, as cellular ATP levels are severely reduced in AckA mutants (Ramos-Montañez et al., 2010). This may be why we see SNPs in both SpxB and LctO in Xen35 as both produce hydrogen peroxide as a by-product of their reactions, and likely reduced ATP levels. There is no SNP in *spxB* or *lctO* in T4P2, therefore expression of the

lux genes which may lead to reduced ATP levels may not be the cause of the SNP in *spxB* and *lctO* in Xen35.

One of the TCS known to regulate the pneumococcal pilus (HK03) was shown to be down regulated upon deletion of *spxB*, *ackA* and *pta*, however differential regulation of this TCS was not observed in Xen35 and T4P2 so is likely not the factor causing the pilus repression in these strains (Rosch et al., 2008, Yesilkaya et al., 2009).

The idea that reduced ATP levels are causing this reduced pilus expression in the both bioluminescent strain is also supported by data in previous chapters. A SpxB mutant showed only 20% pili positive cells compared to 88% in T4NO1 and SpxB mutants have been shown to have reduced ATP levels (Pericone et al., 2003). The LctO mutant also has reduced pili expression with only 57% of cells pili positive, upon deletion of SpxB in this strain the same number of pili positive cells as a single SpxB mutant is observed, suggesting the reduced pili phenotype in the LctO mutant is acting via knock on effects to SpxB. If LctO functions to convert lactate back to pyruvate to be processed via SpxB, in a knockout this would not occur and perhaps lead to reduced ATP levels within the cell. If this is the case in the bioluminescent strains there must be less ATP available than in the SpxB and LctO mutants as both strains have lower number of pili positive cells (Xen35 5.4% and T4P2 7.6%). Differential expression of any other surface proteins is not observed in both bioluminescent strains, which would suggest the pilus alone is dependant on the reduced ATP levels, or resulting from a knock on effect this has to the cell. In *S.aureus* deletion of PknB a serine/threonine protein kinase showed a significant alteration in the cellular levels of NADH and FMN as well as intermediates required during glycolysis, cell wall biosynthesis and fatty acid biosynthesis (Liebeke et al., 2010). As hypothesised for SpxB and LctO the mutation seen in StkP in Xen35 may have occurred in this strain due to metabolic stresses on the cell, which required a reduction in StkP functionality.

To validate what is happening in T4P2 and Xen35 with regards to having a potentially altered metabolism, metabolomic analysis could be performed, which was not done due to time limitations. This would enable validation of what the effect the *lux* genes expression has on levels of different metabolites within the cell. This could also be performed on strain T4Δ*SpxB* and T4Δ*lctO*

which would allow us to understand if variations in pilus expression in these strains are due to the same effect on metabolism changes or due to variations in hydrogen peroxide levels, ATP levels, or both.

From this it is not surprising a reduced virulence is observed in T4P2 as seen in Xen35. T4P2 infected mice showed a statistical increase in survival time compared to T4NO1, and a slower disease progression deduced from a statistically reduced percentage weight loss compared to T4NO1 at all time points. No difference in organ, nasal wash and blood bacterial counts was observed at 24 hours post infection when compared to Xen35 and T4NO1, yet at 48 hours post inoculation and survival time points nasal wash bacterial counts were lower compared to T4NO1 and blood and brain counts at 48, 72 and survival time points. Interestingly there was also a significant decrease in bacterial counts in the brain, lungs and blood at 72 hours post inoculation in T4P2 infected mice compared to Xen35. This would indicate Xen35 is more virulent than T4P2.

Interestingly unlike Xen35, T4P2 only contains a small number of genome changes compared to its parent T4NO1. One is a SNP in an intergenic region of which its function is unknown. This SNP is also found in Xen35 so could be linked to changes in pilus expression, however it is also found in T4JH. The other change is a region of low coverage, which was expected as this is the insertion site of the *lux* genes (SP_1886) in T4P2. This data further validates that the reduced expression of the pilus is due to a metabolic burden placed on the cell. It was also hypothesised that the metabolic stress on the cell may promote the accumulation of specific SNPs and indels, due to the fact such a large number of genome changes were observed in Xen35. This seems not to be the case for T4P2 however it may be that over time SNPs would accumulate in this strain in genes such as SpxB. As the genome sequence of T4P2 was performed on the strain taken only a single passage from the original constructed strain, it may be that this has not had time to occur.

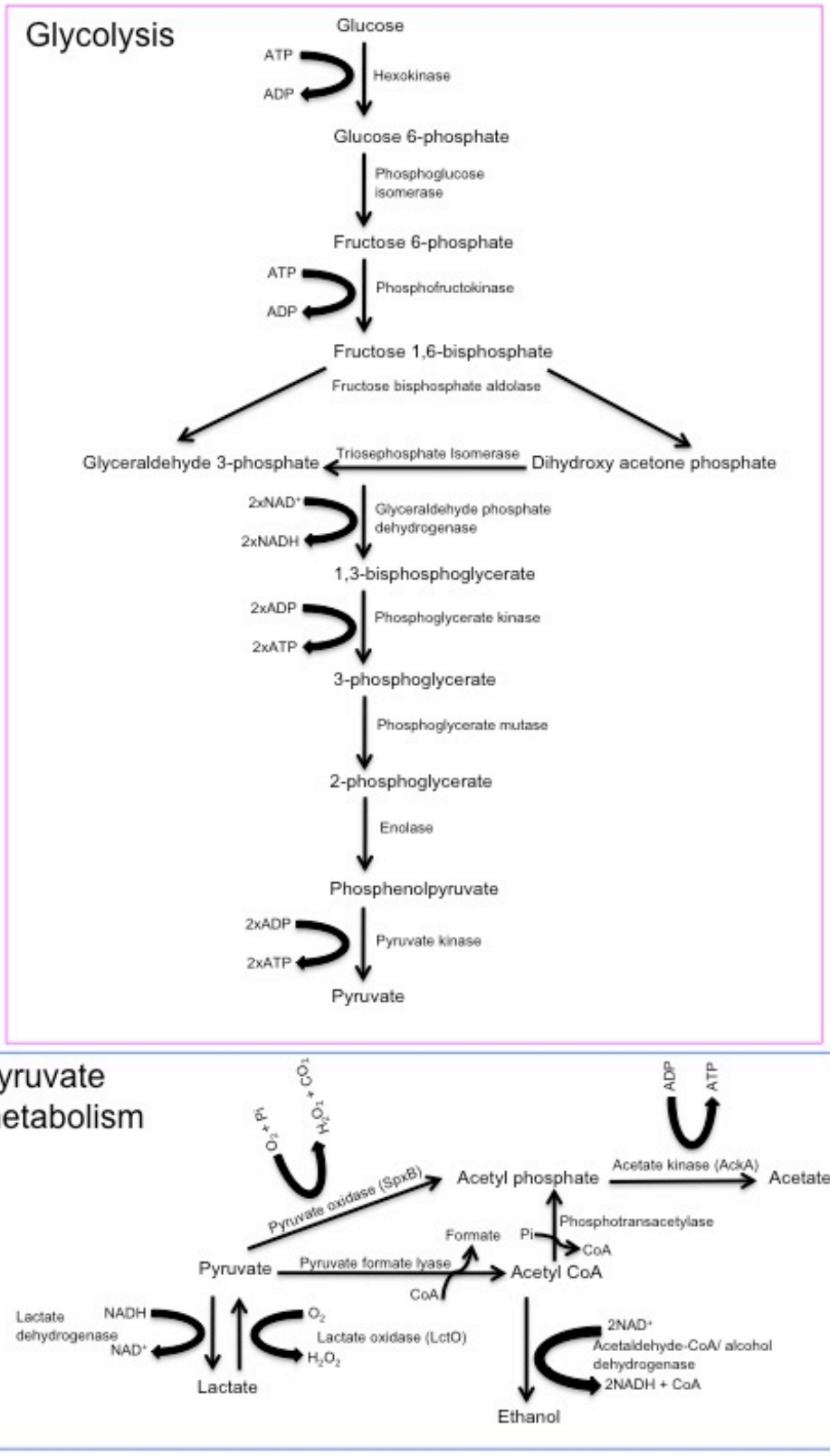


Figure 7-15: Glycolysis and Pyruvate metabolism in *S.pneumoniae*

Diagram of glycolysis and pyruvate metabolism in *S.pneumoniae*, adapted from (Carvalho et al., 2011).

8 Final discussion

8.1 Final Discussion

The key aims of this study were to evaluate genes regulated by some of the key signalling systems used by the pneumococcus to adapt to different niches in response to external stimuli. This study has hopefully made a contribution to the current understanding of how these systems work, what genes they regulate and gives examples of external stimuli these systems may respond to.

One key element to take from this study is the substantiation it provides to show the complexity of the signal transduction pathways in the pneumococcus, especially with regards to regulation by TCS. In the literature it has already been shown that TCS12 and TCS13 are able to converge on the same promoter of an ABC transporter and a number of the other systems have been implicated to regulate other systems, shown by microarray analysis (Knutsen et al., 2004, Mccluskey et al., 2004, Peterson et al., 2004). In this study a similar phenomenon was observed between TCS08 and TCS09 where some genes were commonly regulated by both but in most instances they regulated their own collection of genes. In a double knockout a large number of genes were shown to be regulated which were not observed in the single knockouts alone. This could indicate the systems are able to compensate for the deletion of the other. However, another reason for this may be that deletion of both systems puts a stress on the cell which leads to differential expression of these genes. Our current data is unable to distinguish between these two phenomenon and further study is required to evaluate this.

Although no clear conclusions can be made about the potential cross regulation of these systems and whether a hierarchy exists it is clear these systems are connected in some way, whether that be responding to a similar stimulus or regulating each other etc. In the literature expression analysis on TCS mutants are often performed under different conditions and in different background strains. This is the first study to assess the whole genome expression changes in more than once mutant and a double mutant. This study only assessed the expression changes upon deletion of these TCS and further studies into the genes directly regulated by each system, whether these genes are regulated by the RR in the phosphorylated or non phosphorylated form, protein-protein interactions

of these systems and the external stimulus activating the system need to be performed.

An alternative signalling system was also assessed in this study looking at the genes it regulates. The serine/ threonine protein kinase although the only signalling system of its type in the pneumococcus is able to interact with the TCS (Agarwal et al., 2012, Ulijasz et al., 2009). No differential regulation of the pneumococcal RR was observed in a StkP deletion mutant however this system has been shown to phosphorylate two of these RR (Agarwal et al., 2012, Ulijasz et al., 2009). This further adds another layer of complexity to the regulation of genes by both of these systems. The fact deletion of the HK of TCS pairs results in a less severe phenotype of a number of systems would suggest these are not as vital (Throup et al., 2000). This would also suggest that the RR can function without the requirement of phosphorylation by its cognate kinase and is perhaps phosphorylated by another means. Although currently only evaluated for two RR it may be that all the pneumococcal RR are also phosphorylated by StkP, which is able to modulate the binding affinity to certain promoter targets. This could allow different groups of genes to be regulated by each system in its different forms which would include the non phosphorylated form, the phosphorylation on an aspartate residue (typically by the HK), phosphorylation on a threonine (or serine) residue (by StkP) and potentially phosphorylation of both at once.

Evidence this may be the case for other RR has come from this study. Although RR05 was not differentially regulated in the StkP knockout, a number of genes were shown to be directly regulated by RR05, through the binding of RR05 to the upstream promoter regions, which were also differentially regulated in T4Δ*stkP*. This may also be the case for RR02 as homologues of this RR in *S.pyogenes* are phosphorylated by its serine/ threonine protein kinase (ST-STK) (Agarwal et al., 2011).

A further role attributed to StkP in this study is to modulate protein translocation to the cell wall. SecY of the SecYEG translocase was down regulated in the StkP deletion mutant. Interestingly this resulted in an increase in pili on the cell surface so it could be that deletion of StkP results in a dysregulation of cell wall constituents. StkP has been shown to localise to the cell septum and is important for cell division (Giefing et al. 2010, Maestro et al.

2010). Through the use of its extracellular PASTA domains which can bind to peptidoglycan it can perhaps sense the presence of cell wall surface components and modulate from this the level of protein translocation to the cell surface (Beilharz et al. 2012). Interestingly also differentially regulate in the StkP deletion mutant was HtrA a serine protease and ParB a chromosomal division protein (Ibrahim et al. 2004a, Minnen et al. 2011). HtrA has recently been shown to colocalise with SecA (a protein chaperone) to the midcell/ septum during cell division. SecA functions as a chaperone for proteins being directed to the SecYEG translocase (Tsui et al. 2011). From this data it could be inferred that these above protein all function together localising to the midcell/ septum during cell division. This protein complex contains StkP which functions to sense the changes in the cell wall. Which relays information to the SecA, SecYEG and HtrA protein complex, modulating its activity. This complex would be present at the midcell/ septum which is the point at which new protein are placed onto the cell wall, which is also the site at which new peptidoglycan biosynthesis occurs (Tsui et al. 2011). ParB which is also regulated by StkP could also functions alongside this complex, as you would expect chromosome segregation to occur alongside cell division (Minnen et al. 2011).

Another key feature elucidated in this study is the potential impact that genome changes may have on the bacterial cell with regards to gene regulation and regulation of key virulence factors. The new era of affordable genome sequencing enables better validation of genetically manipulated strains with regards to assessing that they only contain the desired mutation. The genome sequence of Xen35 has clearly shown that the genetic manipulation of strains can lead to major genome changes depending on how the strain has been constructed. This leads to a big issue about strains used in studies that have been genetically manipulated and whether along with the desired mutation other genome change have also occurred. Perhaps through multiple recombination events if transformed with large PCR products or gDNA. In the literature there is also evidence that deletion of some genes results in accumulation of genomic changes in other genes, which are required to counteract the deleterious effect on the cell of the required deletion. This has been observed in deletion mutants of AckA (Acetate kinase) which when deleted acquires mutations in SpxB or SpxR (Ramos-Montañez et al., 2010). This

phenomenon has also been shown to occur in deletion mutants of CodY (Caymaris et al., 2010).

One example of how genetic manipulation of a strain can severely affect its phenotype includes Xen35 (the bioluminescent version of TIGR4), which had acquired a large number of genetic changes, some in part due to the construction of the strain, which highlights the need to ensure when genetically manipulating strains that only the desired genetic mutations are introduced. Other genetic changes in this strain were likely due to variations in the genome of the parent strain Xen35 was constructed in (TIGR4) which can vary as noted below. Changes at the genome level in this strain likely contributed to the reduced virulence of the strain and the changes in gene expression observed in this strain compared to TIGR4.

However, in this instance the drop in pilus expression observed in Xen35 was not due to the genome changes observed and was shown to be due to the over expression of the *lux* genes (genes required for bioluminescence). This also gives an insight into the potential effects of expression of foreign genes in a non native species, and whether these constructed strains used for *in vivo* disease progression etc can really give a true picture of disease caused by these strains. In this instance the expression of these genes resulted in a reduction in the expression and level of a key virulence factor the pilus (adhesin) on the cell surface in Xen35. Perhaps in the pneumococcus an alternative reporter gene should be used for this type of study as expression of and/ or the enzymatic reactions catalysed by the *lux* genes/ LuxA-E respectively alter other biological functions within the cell. As no other cell surface virulence factors were shown to be commonly differentially regulated in Xen35 and T4P2 it is assumed the over expression of the *lux* genes specifically effects a biological function that is linked to how the pilus is regulated, which our current studies did not identify.

This study also sheds light on the genetic diversity between the supposed same strain. Globally TIGR4 a serotype 4 *S.pneumoniae* strain is used as a laboratory standard to enable comparisons between work performed in different laboratories. In this study two un-manipulated TIGR4 strains were found to vary at the genome level and variations between the two altered expression of a key cell surface adhesin. Therefore studies performed in different background

strains (TIGR4 etc in different laboratories) may result in different findings if genome changes are present that affect the expression of the genes being assessed. In this instance it is not known whether the regulation of the pilus islet by TCS in T4JH would be effected by the SNP present in RrgC.

It is assumed that this SNP which causes an amino acid change in RrgC in T4JH is the reason for the variations in the level of pilus expression at the population level. With T4NO1 showing 88% of cells to be pili positive whereas in T4JH only 20.5% are pili positive. Why this single amino acid change would cause reduced expression of the pilus suggests RrgC also plays a regulatory role in pilus expression. It is not known to have any DNA binding functions and therefore may modulate expression changes through some sort of protein-protein interaction. This type of regulation has been seen for RrgA which binds to RlrA (Basset et al., 2011, Basset et al., 2012). It may be that RrgC also binds to RlrA or perhaps to RrgA modulating its regulatory function. The pneumococcal orphan response regulator RitR has been shown to form a protein-protein interaction with PhpP (Ulijasz et al., 2009). Therefore perhaps other response regulators are also regulated in this manner. The three TCS evaluated in this study have all been shown to regulate pilus expression at the population level so perhaps RrgC modulates the function of one of these proteins through a protein-protein interaction. It may be that the pilus islet itself is temporally regulated as it would be unnecessary for the bacteria to produce RrgA and RrgB if there is no way to anchor the proteins to the cell wall. Perhaps the SNP in RrgC in T4JH renders the protein unable to anchor properly to the cell wall of hinders transport to the cell surface at which point there is a checkpoint which initiates synthesis of the other pilins. This may be hindered in T4JH which is why there is fewer cells at the population level that are pili positive.

This study has clearly shown that expression of the pilus likely poses a metabolic burden on the cell. T4NO1 was originally isolated from a case of IPD, isolated from the blood of a 30 year old male from Norway and was found to be highly invasive in a mouse model of infection (Aaberge et al., 1995, Tettelin et al., 2001). This strain was used in these studies only one passage from the original strain and therefore likely retained the phenotypic traits of the strain taken from the infected male. Whereas T4JH is a lab strain and may perhaps have acquired mutations in the pilus islet due to the metabolic burden it places on

the cell, which render its unable to express high levels of pili as this trait is not required in lab strains.

As already mentioned these two strains differ in the amount of pili expressed at the population level, with T4JH having 20.5% of cells pili positive whereas T4NO1 has 88% of cells pili positive. This ability to regulate the pilus at the population level may be key to why there has been no clear association of strains that carry pili with increased incidence of IPD, yet it has been shown that it must convey some advantage otherwise strains wouldn't have it (Moschioni et al., 2008, Regev-Yochay et al., 2010). Although strains may have the pilus islet all studies assessing its association with strains able to cause IPD do not assess the level at which each strain expresses the pilus (Moschioni et al., 2008, Regev-Yochay et al., 2010). Which has been shown in this study to vary even between two closely related TIGR4 strains. Therefore strains that are able to express the pilus on the surface of a larger proportion of cells may be more invasive and are associated with higher levels of IPD. No animal data is currently available comparing the virulence of T4NO1 and T4JH. Data to support this idea is seen when RR09 is deleted in T4JH which results in 91% of cells become pili positive. Adherence assay data of this strain shows a large increase in adherence to all cell lines tested. Therefore it would be assumed T4NO1 is able to adhere better than T4JH as it contains roughly four times more pili on the cell surface and in the host perhaps causes a more severe disease. However, more pili does not necessarily lead to increased adherence as observed for T4Δ*rr086* which had a similar number of pili on the cell surface as T4Δ*rr098* yet showed no increase in adherence compared to the parent. This strain however also showed when stained with FITC for the pilus a lower fluorescence intensity than that of pili positive cells in the WT strain suggesting in this strain there are fewer pili per cell or the pili are shorter.

From this study more factors shown to alter pilus expression have been identified including the serine/ threonine protein kinase, pyruvate oxidase and lactate oxidase. These add to the six TCS that have been shown to alter pilus expression, three transcription regulators and the differential expression observed in response to vancomycin stress (Haas et al., 2004, Haas et al., 2005, Hendriksen et al., 2007, Hendriksen et al., 2009, Hemsley et al., 2003, Johnston

et al., 2006, Rosch et al., 2008, Sebert et al., 2002, Song et al., 2009). Due to the large number of genes that regulate this islet it has been concluded that pilus expression is probably closely linked to the overall fitness of the cells as a number of the genes shown to regulate the pilus are involved in modulating and responding to changes in cell wall integrity and energy production. This includes StkP, which regulates genes important for cell wall integrity, RR03, (binds directly to the *rlrA* promoter) responds to cell envelope stress, and post treatment with vancomycin (induce cell wall stress) the pilus is down regulated (Giefing et al., 2010, Haas et al., 2005, Rosch et al., 2008, Suntharalingam et al., 2009). The role of LctO and SpxB in pilus regulation supports this idea further with regards to pilus regulation being linked to the fitness of the cell. As both show reduced pilus expression when they are deleted and both are important for energy production during aerobic metabolism, with knockouts of SpxB shown to have reduced cellular levels of ATP (Pericone et al., 2003, Taniai et al., 2008). This is further validated by the fact the over expression of the *lux* genes also causes reduction in pilus expression which is assumed to lead to reduced ATP levels in the cell, however this would need further confirmation. If this is the case it may explain why only 30% of strains contain the PI-1 encoded islet as some strains may not be able to generate enough energy to maintain all the required cellular processes as well as maintain large cell surface structures. This may also be the case for other large cell surface proteins such as PsrP, which is only present in roughly 50% of strains (Munoz-Almagro et al., 2010).

The exact function of the pilus in the pneumococcus still remains elusive, however it likely plays an important function. In other bacteria functions have been assigned to pili which may play a similar role in the pneumococcus. For instance pili in *S.pyogenes* have been shown to aid in resistance to phagocytosis and killing by neutrophils (Maisey et al., 2009). During invasive disease neutrophils and macrophages have been shown to be key factors in clearance of the pneumococcus from the lung (Kadioglu & Andrew, 2004). Neutrophils function in one way through release of reactive oxygen species including hydrogen peroxide, which have bactericidal effects on bacterial cells (Kadioglu & Andrew, 2004). It could therefore be envisaged that the release of hydrogen peroxide by neutrophils could induce pilus expression in the pneumococcus, which helps protect the bacteria from killing.

To conclude, this study has offered a further insight into the complexity of gene regulation by signal transduction systems in the pneumococcus. It has offered insights into not only the regulatory mechanisms used to modulate gene expression but also the potential to regulate protein export. Another key feature touched upon is the requirement for a metabolically fit cell and its importance in maintaining normal cellular functioning. One major finding to be taken from this study is the potential impact of a small number of genetic changes between two very closely related strains and how this may have a big impact on the phenotype of the strain. This study has hopefully contributed to a better understanding of gene regulation in the pneumococcus.

Appendices

Appendices can be found on a CD at the back of this thesis. The contents included in this are noted below.

Appendix I

Fluorescent microscopy images of capsule and pili stained bacteria, including 3-4 representative fluorescent images and DIC images of T4NO1, T4JH, Xen35, T4P2, T4Δ*stkP*, T4Δ*stkP*∇ST, T4Δ*stkP*∇XST, T4Δ*spxB*, T4Δ*lctO*, T4Δ*spxB*Δ*lctO*, T4Δ*rr06*, T4Δ*rr08*, T4Δ*rr09*, T4Δ*rr086*, T4Δ*rr096*, T4Δ*rr098*, T4Δ*rr0986*, T4AN and T4Δ*spxB* grown in the presence of varying hydrogen peroxide concentrations.

Appendix II

Graph directly comparing RR expression in T4Δ*rr08,* T4Δ*rr09* and T4Δ*rr098,* deduced from RT-PCR analysis. Expression values are located below in a table showing the average fold change in RR expression in the TCS mutants stated above.

Appendix III

Table summarising all the genome changes in Xen35 compared to the TIGR4 genome sequence (NC_003028).

Appendix IIII

Table of genes differentially regulated in Xen35 compared to T4JH (P<0.1).

Appendix V

Venn diagram comparing the genes differentially regulated in T4Δ*rr08*, T4Δ*rr09* and T4Δ*rr098* via microarray analysis when compared to T4JH, comparison was performed using the P<0.1 genes lists. A table of the genes found to be commonly differentially regulated in all three strains was compiled comparing the expression differences.

Appendix VI

Venn diagram comparing the genes commonly differentially regulated in StkP mutant strains in a T4NO1 background (this study) and CP1015 (published data) via microarray analysis, the data from this study shows a comparison using the P<0.1 genes lists. The commonly regulated genes are noted below the diagram.

Appendix VII

Venn diagram comparing the genes differentially regulated in Xen35 and T4P2 via microarray analysis when compared to T4JH and T4NO1 respectively, comparison was performed using the P<0.1 genes lists. A table of the genes found to be commonly differentially regulated between the two strains was compiled comparing the expression differences below.

Future work

With regards to further assessing gene regulation by TCS future work includes assessing the genes regulated by each TCS through direct binding of the RR to the upstream promoter regions. And assessing whether these RR form any protein-protein interactions with each other or other key proteins. Little is known about the external stimulus which activates the majority of these systems and this knowledge is vital for a better understand of where genes regulated by these systems are important *in vivo,* studies into this are also required. With regards to their regulation of the pilus more information is required as to whether RR08 and RR09 bind directly on the pilus islet promoters, and whether the RR that modulate this activity compete for binding, or bind to different promoter sites within the islet.

To further evaluate the metabolic burden placed on the cell by expression of the *lux* genes metabolomic analysis could be performed. This will evaluate if expression of these genes alters levels of a certain metabolites such as ATP, which may accounts for the pilus expression changes. This analysis could also be performed on Xen35, T4Δ*spxB*, T4Δ*lctO* and T4Δ*spxB*Δ*lctO* which may give an indication if the reason for the reduced pili expression in all strains is linked to alterations of the same factor.

Other key studies include assessing further the function of the pilus in the pneumococcus, whether like in *S.pyogenes* it is able to protect against death by neutrophils. Important studies could also be performed into elucidating whether clinical isolates vary in their pili expression at the population level and if this correlates with invasive disease potential.

Further studies need to also be performed to assess whether StkP is also able to modulate other cell surface components and whether this is performed via regulating protein translocation. If this is the case, whether StkP modulated this activity through direct interactions with the SecYEG translocase at the cell septum during cell division needs to be evaluated. And further whether this potential protein complex also contains HtrA, SecA and ParB.

Conference contributions

Presenting author underlined

Jenny Herbert, Andrea Mitchell, Jiangtao Ma, Tim Mitchell. The pneumococcal pilus: another layer of complexity. (Poster 2011) Europneumo, Amsterdam, Holland.

Jenny Herbert, Andrea Mitchell, Jiangtao Ma, Kirsty Ross-Hutchinson, Tim Mitchell. Genetic regulation of pilus production in *Streptococcus pneumoniae*. (Poster 2010) Society of General Microbiology, Nottingham, England

Jenny Herbert, Andrea Mitchell, Jiangtao Ma, Tim Mitchell. Genetic regulation of pilus production in *Streptococcus pneumoniae*. (Poster presentation 2010) 7th International Symposium on Pneumococci and Pneumococcal Disease, Tel-Aviv, Israel.

Jenny Herbert, Tim Mitchell. Role of Two-component system nine in repression of the pilus in *Streptococcus pneumoniae*. (Oral presentation 2009) Europneumo, Bern, Switzerland.

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