Genetic Variation and Virulence of

Streptococcus pneumoniae

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Author's declaration

This thesis is the original work of the author unless otherwise stated.

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Abstract

Streptococcus pneumoniae or pneumococcus is included among major human pathogens and is responsible for a number of diseases including life-threatening conditions such as pneumonia, meningitis and sepsis. Though pneumococcal vaccines are available, they provide limited coverage against infections as pneumococcus shows extensive variation, which also allows escape from vaccines and antibiotic resistance. It is armed with several virulence factors including capsule, surface proteins, enzymes and toxins, which are variably expressed and altogether determine pneumococcal virulence.

The aim of this project was to study pneumococcal genetic variation and its effect on virulence, with a focus on pneumococcal capsule, which is considered the major determinant of virulence and is involved in interaction with host immune system. It is the target for current vaccines and at least 93 pneumococcal serotypes are known, which differ in pathogenicity.

To study the effect of capsule on the pneumococcal virulence, capsule-switch mutants were constructed in three genetic backgrounds; TIGR4 (serotype 4, virulent), 403 (serotype 4, avirulent) and D39 (serotype 2, virulent) and were studied for variation in their *in vivo* and *in vitro* characteristics. These mutants were compared with their parent strains and other mutants for effects of capsule switching on their growth, formation of capsular polysaccharide, capsular thickness, chain formation and virulence in murine models of infection using MF1 mice. Significant differences were observed in behaviour of parent and mutant strains.

To develop a broader insight into pneumococcal virulence, avirulent derivative of strain TIGR4, 403 was genome sequenced and compared with TIGR4 for genetic mutations. To study differences in gene expression both the strains were also compared using microarrays. Genome analysis revealed only few mutations in strain 403 but microarray experiments showed 288 genes to be expressed differently in strain 403.

Strain 403 was also tested as live attenuated vaccine to see if it could provide protection against the same and different serotypes, as it can be used as a vehicle for delivery of different polysaccharides to the host body along with the whole set of pneumococcal antigenome. Vaccine trials of 403 were not very fruitful as it failed to provide any protection through intranasal route though partial protection was observed in mice vaccinated intraperitoneally with significant differences in levels of bacteraemia, survival, weight and temperature losses on challenging with homologous strain.

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Abbreviations

°C	Degrees Celsius
-/-	Deficient
μl	Micro litre
μm	Micro meter
μM	Micro Molar
Ab	Antibody
AOM	Acute Otitis Media
BAB	Blood Agar Base
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
cfu	Colony forming unit
CPS	Capsular Polysaccharide
CSF	Cerebrospinal Fluid
Csp	Competence stimulating peptide.
DBPS	Dulbecco's Phosphate Buffer Saline
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide Triphosphate

Fig	Figure
g	Gram
g	Centrifugal Force
h	Hour
HIV	Human Immunodeficiency Virus
hpi	Hour post infection/inoculation
HRP	Horse Radish Peroxidase
IFNγ	Interferon gamma
IPD	Invasive Pneumococcal Disease
Ig	Immunoglobulin
IL	Interleukin
IN.	Intranasal
IP	Intraperitoneal
Kb	Kilobase
kDa	Kilodalton
j	Janus strain
L	Litre
M	Molar
mg	Milligram(s)

min	Minute(s)
ml	Millilitre
MLST	Multi-Locus Sequence Typing
mM	Millimolar
MW	Molecular Weight
NETs	Neutrophilic Extracellular Traps
ng	Nanogram
NK	Natural Killer
nm	Nanometre
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PCV	Pneumococcal Conjugate Vaccine
PPV	Pneumococcal Polysaccharide Vaccine
PS	Polysaccharide
rpm	Revolutions per minute
RT	Room Temperature (~20°C)
SEM	Standard Error of the Mean
sec	Second (s)

SSC	Standard Saline Citrate
ST	Sequence Type
TEM	Transmission Electron Microscope
T_H	Helper T cells
T_R	Regulatory T cells
T_C	Cytotoxic T cells
TNF	Tumour Necrosis Factor
U	Units
V	Volts
WT	Wild type
WBC	White blood cells

1 Introduction:

Streptococcus pneumoniae, commonly known as pneumococcus, is a facultative anaerobe that is gram-positive and grows in pairs and short chains. It is considered among major human pathogens and is responsible for several diseases including life-threatening conditions such as meningitis, pneumonia and sepsis (O'Brien *et al.*, 2009). Pneumococcal diseases cause considerable financial loss to the society and mainly affect children, the elderly and immunocompromised patients. It is a highly transformable bacterium, which shows extensive variation and plasticity of its genome (Golubchik *et al.*, 2012; Croucher *et al.*, 2011).

Though pneumococcal research has completed more than hundred years and it has played a major role in the development of our insight into nature of genetic material, genetic transformation, infectious diseases and development of antibiotic resistance, pneumococcal diseases still cause significant morbidity and mortality around the world (O'Brien *et al.*, 2009; Black *et al.*, 2003; Austrian, 1981). The high transformability of pneumococcus has resulted in evolution of antibiotic-resistant strains that can mutate and avoid effects of vaccines (Brueggemann *et al.*, 2007; Cartwright, 2002).

1.1 Classification of pneumococcus:

S. pneumoniae is closely related to *Streptococcus mitis* and *Streptococcus oralis* which reside in nasopharyngeal niches, though it can be differentiated from them on the basis of sensitivity to optochin, solubility in bile and specific anti-

pneumococcal antibodies (Kilian *et al.*, 2008; Whatmore *et al.*, 2000). It has been classified into at least 93 serotypes on the basis of capsular structure and Quellung reactions, which can be visualized as capsular swelling and bacterial agglutination when specific antibodies are used against homologous capsular components (Calix & Nahm, 2010; Park *et al.*, 2007; Pai *et al.*, 2006; Henrichsen, 1999). These serotypes vary in their distribution and virulence (Calix & Nahm, 2010; Park *et al.*, 2007). Since serotyping with anti-sera is expensive and cross-reactivity occurs, cheaper and more specific methods based on molecular techniques have been developed to type pneumococci with accuracy (Siira *et al.*, 2012; Pai *et al.*, 2006; Lawrence *et al.*, 2003). Though it has been an extremely useful tool for pneumococcal characterization, it has its limitations and provides only a broader classification scheme, which has encouraged the development of better molecular characterization techniques for the study of pneumococcal molecular epidemiology and ecology. One of them is multi-locus sequence typing (MLST), which was described by Enright and Spratt (Enright & Spratt, 1998). It allows genetic identification of isolates by the matching of internal sequence of seven housekeeping gene loci, which are:

i. Glucose kinase (*gki)*
ii. Shikimate dehydrogenase (*aroE)*
iii. Glucose-6-phosphate dehydrogenase *(gdh),*
iv. Signal peptidase I (*spi)*
v. *T*ransketolase *(recP)*
vi. D-alanine-D-alanine ligase (*ddl)*
vii. Xanthine phosphoribosyltransferase (*xpt)*.

Each allele is assigned a unique number after matching with sequences in online MLST database. This unique number is then compared to those for other isolates in the database and each isolate is assigned a specific sequence type (ST). As a

result isolates with all seven identical alleles of these 7 housekeeping gene loci share the same ST. It is a powerful technique for online molecular characterization of bacterial isolates and provides an electronic portable method of comparison of evolutionary relationship of different species (Enright & Spratt, 1999). It has added a substantial amount of information about pneumococcal taxonomy and population dynamics. Utilizing information obtained from MLST, it was reported that not only a variety of STs are present in each serotype, but also some STs are found in more than one serotype, indicating that pneumococci can switch their capsule type by acquiring capsules of different serotypes from other strains (Pai *et al.*, 2006; Mcgee *et al.*, 2001; Coffey *et al.*, 1998a; Coffey *et al.*, 1996; Coffey *et al.*, 1991; Munoz *et al.*, 1991). It was also reported that certain serotypes and STs were more related to invasive disease as compared to others (Hanage *et al.*, 2005).

This diversity in the pneumococcal genome, shown by molecular characterization has been explained by Distributed Genome Hypothesis (DGH), which proposes that pneumococci share a supragenome that enables them to shuffle their genetic contents in response to environmental stimuli (Hiller *et al.*, 2007). Pneumococcus acquires new genetic material by different methods including horizontal gene transfer, the environment or from bacteriophages (Thomas *et al.*, 2005). This transfer not only takes place within the species but can also involve different bacteria residing in the same niche (Hakenbeck *et al.*, 1999). Besides having a core genome, they possess additional genes, which may be strain specific. These genes code for virulence factors that can be expressed in variable manner according to conditions of host environment (Hiller *et al.*, 2007).

As a result of this extensive variation in pneumococcal genome, despite all classification schemes, exact characterization of *S. pneumoniae* strains is only possible through whole genome sequencing, as strains that share the same serotype and ST not only vary in spectrum of symptoms they produce, but also differ in host selection (Forbes *et al.*, 2008; Sjostrom *et al.*, 2006). As a part of this project we sequenced the complete genome of an avirulent type 4 strain and compared it with previously published TIGR4 genome to study genes involved in pathogenicity of serotype 4 pneumococci.

1.2 Carriage dynamics of pneumococcus:

S. pneumoniae is among predominant colonizers of human nasopharynx. Pneumococcal carriage rates have been reported to be different among various studies and populations (Bogaert *et al.*, 2004) and carriage prevalence is quite high in developing countries as compared to developed ones, which results in higher exposure to different strains (Scott, 2007). Colonization may begin very early in life and up to 95% of healthy individuals can be colonized in the first three years, who can carry up to four different serotypes simultaneously (Obaro & Adegbola, 2002). This nasopharyngeal colonization may begin as soon as the very first day after birth, though duration of carriage may vary and can decline with increasing age (Gray *et al.*, 1979). It may be related to a number of risk factors such as use of antibiotics, smoking, asthma, ethnicity, over-crowding and proximity of adults with children (Bogaert *et al.*, 2004).

S. pneumoniae is accompanied by *Neisseria, Haemophilus, Staphylococcus* and other *Streptococcus* species in the nasopharynx (Bogaert *et al.*, 2004), which creates a highly competitive environment for bacterial growth. Pneumococcus

has developed certain features, which provides a competitive advantage over other colonizers of nasopharynx such as neuraminidase and hydrogen peroxide production, which inhibits their growth (Bogaert *et al.*, 2004; Shakhnovich *et al.*, 2002; Pericone *et al.*, 2002; Pericone *et al.*, 2000). Though pneumococcal colonization is an important event in disease development, it also protects against occupation of nasopharyngeal spaces by other pathogens, reducing their chance of causing invasive diseases (Bogaert *et al.*, 2004).

1.3 Pneumococcal diseases and their burden:

S. pneumoniae causes a number of diseases ranging from life-threatening pneumonia, meningitis and septicemia to painful otitis media and sinusitis (Cartwright, 2002). These diseases not only cause great economic burden on the society but also cause considerable mortality around the world, especially in developing countries (Black *et al.*, 2003). According to World Health Organization (WHO) estimates of 2005, pneumococcal diseases kill 1.6 million humans annually, while the worst affected are children as their yearly mortality count due to pneumococcal diseases is about 0.7–1 million (WHO, 2008) and these diseases have an estimated death toll of approximately 11% (8–12%) of all deaths in non-HIV positive children less than 6 years of age (O'Brien *et al.*, 2009). Another commonly affected group is the elderly, whose susceptibility to contract pneumococcal diseases increases due to decreasing immunity and antibody counts with increasing age (Simell *et al.*, 2008). Chances of getting disease also increase in presence of co-morbidities, such as cardiac or renal disease, especially in hospitalized patients, where there is a very high rate of mortality among patients acquiring pneumococcal infections nosocomially. Influenza has also been shown to increase pneumococcal disease (Bogaert *et al.*,

2004; McCullers & Rehg, 2002) resulting in higher mortality during epidemics and pandemics (Brundage & Shanks, 2008).

1.3.1 Pneumonia and bacteraemia:

S. pneumoniae is the most common cause of infectious pneumonia acquired in the community and is responsible for up to 35% of cases in adults (Moine *et al.*, 1995). It claims approximately 20% of all childhood deaths, chiefly in developing countries (Black *et al.*, 2003). It is characterized by inflammation of lung parenchyma accompanied by consolidation and exudation of alveolar spaces, which obstructs optimum gaseous exchange between lungs and bloodstream (van der Poll & Opal, 2009).

A number of factors predispose an individual to pneumococcal pneumonia such as extremes of age, pulmonary, cardiac, hepatic or neurological disease, smoking, cancer, HIV, diabetes, alcohol abuse, recent hospitalization and previous pneumonia (Cardozo *et al.*, 2008). Pneumonia may lead to bacteraemia resulting in sepsis and death (Laterre *et al.*, 2005), though bacteraemia occurring independently is not uncommon (Myers & Gervaix, 2007; Kaplan *et al.*, 1998).

1.3.2 Meningitis:

Inflammation of protective membranes of brain and spinal cord along with infection of cerebrospinal fluid (CSF) as a result of pneumococcal infection is called pneumococcal meningitis (Mook-Kanamori *et al.*, 2011). It is a serious and life threatening condition, characterized by a range of symptoms including

headaches, stiffening of the neck, seizures and coma, which may lead to death. Mortality ranges from 16-37%, with residual neurological sequel in 32-50% (Kastenbauer & Pfister, 2003; Durand *et al.*, 1993). Meningitis is generally preceded by initial pneumococcal infection elsewhere, in about 30% of cases by acute otitis media and in about 18% by pneumonia (Ostergaard *et al.*, 2005), though pneumococci also have the ability to invade and infect central nervous system directly through olfactory neurons (Van Ginkel *et al.*, 2003).

1.3.3 Acute otitis media (AOM)

Acute otitis media is (AOM) the commonest but relatively benign pneumococcal infection of middle ear cavity (Mahadevan *et al.*, 2012; Monasta *et al.*, 2012; Hausdorff *et al.*, 2002). It is a cause of great economic burden on the society and in the US alone, costs about $5 billion per annum (Bondy *et al.*, 2000).

1.3.4 Other pneumococcal infections:

Pneumococci are also responsible for some other infections which are relatively less common. These infections include a number of relatively benign clinical problems, which are discussed in the following paragraphs.

Infection and inflammation of conjunctiva, the transparent membranous covering of eye, is called conjunctivitis. Conjunctivitis is generally caused by non-typable pneumococci (Williamson *et al.*, 2008; Porat *et al.*, 2006), which mainly occurs as outbreaks (Martin *et al.*, 2003) though sporadic cases have been reported (Porat *et al.*, 2006).

Pneumococci can also cause inflammation of internal layer of heart, called endocarditis, which may involve heart valves and inter-ventricular septum. It may occur between 0.8-3.4% of patients having pneumococcal bacteraemia (Lindberg & Fangel, 1999). Though it is not very common the outcome may be fatal in up to 40-50% cases despite appropriate antibiotic therapy (Bruyn *et al.*, 1990).

Pneumococcal infections can also lead to collection of pus in brain (cerebral abscesses) or lungs (empyema), which are quite uncommon but generally result in high morbidity and mortality (Carpenter *et al.*, 2007; Eastham *et al.*, 2004; Gransden *et al.*, 1985).

The problem of pneumococcal diseases is complicated because of the emergence of antibiotic resistant strains and their intercontinental spread (Jacobs, 2008; Van Bambeke *et al.*, 2007; Jacobs, 2004) which might limit our choice of antibiotics in future.

1.4 Pneumococcal virulence factors:

Streptococcus pneumoniae is equipped with a large armamentarium of virulence factors, which are important for successful host colonization and subsequent invasive disease. These will be discussed in following sections.

1.4.1 Capsule:

The capsule is a polysaccharide covering of the pneumococcal cell and is of prime importance as it is considered a major determinant of virulence. It not

only helps to evade opsonins, inhibits complement activity and protects the pneumococcus from phagocytosis but also helps them avoid mucosal clearance by electrostatic repulsion (Melin *et al.*, 2010; Henriques-Normark & Normark, 2010; Nelson *et al.*, 2007). It is a complex structure and contains multiple sugars, in form of a sugar backbone containing side chains. The backbone depends upon type, number and ring-size of monosaccharides, type and orientation of linkages, which are repeated numerous times to form structure of capsular polysaccharide (CPS). Most commonly occurring monosaccharides include α or ß-D-glucose, D-galactose, L-rhamnose and N-acetyl-α or ß-D-glucosamine (Bentley *et al.*, 2006).

The capsular locus is an extremely variable region and sequencing performed on 90 serotypes by Bentley and co-workers has revealed its genetic diversity and shown that the capsular genes are almost always found between genes *dexB* and *aliA* and vary in size between 10 and 30 kb. Analysis of *cps* loci also shows presence of several different forms of each of important enzyme classes related to capsule synthesis. Annotated proteins were also assembled into homology groups and various groups were observed including polysaccharide polymerases, flippases and a large number of transferases, responsible for variable expression of different components of capsule. A large number of mobile elements was also noted (Bentley *et al.*, 2006). Analysis has also shown that the 5′ end contains some conserved genes (*cpsA, cpsB, cpsC, cpsD* and *cpsE)*, related to processing, regulation and export of constituent sugars of capsular polysaccharide and and these sugars may have a role in attachment of polysaccharide to bacterial cell wall (Eberhardt *et al.*, 2012).

Pneumococcus is capable of switching capsular type by horizontal gene transfer, which also allows it to escape from vaccines that are directed at particular capsular types (Coffey *et al.*, 1998a). This has resulted in evolution of pneumococcal isolates of particular STs with several different serotypes (Coffey *et al.*, 1999; Coffey *et al.*, 1998b; Munoz *et al.*, 1991), though capsule switching can also result in decreased virulence (Kelly *et al.*, 1994). Different serotypes differ in their capacity to cause disease, and although we know that there are at least 93 serotypes, only 20% of these cause the majority of pneumococcal diseases (Hausdorff *et al.*, 2000a; Hausdorff *et al.*, 2000b).

The highly conserved arrangement of *cps* genes amongst all strains helps naturally transformable pneumococcus to switch serotype through recombination (Brueggemann *et al.*, 2007). This genetic process can be imitated and otherwise isogenic capsule switch mutants can be constructed in the laboratory using various techniques (Sung *et al.*, 2001; Kelly *et al.*, 1994). These mutants can be used as an experimental tool to study effect of capsule switching on virulence and other biological properties.

In this project, capsule switch mutants were constructed in serotype 4 and serotype 2 genetic backgrounds. These mutants were isogenic except capsular type and were used for studying variation in pneumococcal biology in relation to polysaccharide capsule.

Pneumococci are also capable of altering their capsule expression in response to the environment, which results in phase variation. Decreased expression results in a transparent phenotype, which is mainly adapted for colonization and is more transformable (Weiser & Kapoor, 1999). Opaque colonies have higher level of

capsular expression and are more suitable for survival in blood and are more resistant to phagocytosis (Bruckner *et al.*, 2004; Obaro & Adegbola, 2002; Weiser *et al.*, 1994). The transition between these phenotypes is considered an important factor that is related to change from carriage to invasive phenotype (Hammerschmidt *et al.*, 2005). It has been reported that pneumococcal strains isolated from blood of infected animals had significantly increased levels of capsular mRNA as compared to those growing *in vitro* (Ogunniyi *et al.*, 2002). It was also observed that metabolically cheaper capsule types tend to be thicker and thus more likely to offer resistance against host immune response, which might lead to persistence in nasopharynx and subsequent infection (Weinberger *et al.*, 2009).

The pneumococcal capsule is very important from an immunological point of view as it enables the body to identify invading organisms and mount an appropriate immune response. It is the main target for present polysaccharide and conjugate polysaccharide vaccines, which have been developed against several serotypes.

We have also constructed capsule switch mutants of avirulent serotype 4 strain 403 to study and compare effects of capsular switching on bacterial growth.

1.4.2 Pneumococcal surface protein C (PspC)

PspC is a highly variable choline binding surface protein, also called CbpA, PbcA, SpsA and Hic (Iannelli *et al.*, 2002). PspC proteins have several functions, which include binding complement factors C3, factor H and secretory portion of IgA (Dave *et al.*, 2001; Janulczyk *et al.*, 2000). It also affects bacterial adherence

to pulmonary epithelium, colonization of nasopharyngeal tissue and invasion (Rosenow *et al.*, 1997).

The contribution of PspC *in* pneumococcal virulence can vary among different strains (Kerr *et al.*, 2006). It was reported that there is some variation in role of PspC among different strains as a *PspC* knock out in type 2, 3,19F and 4 had different effects on virulence in different serotypes (Kerr *et al.*, 2006). It was an interesting finding that no significant difference was observed in the survival rate of mice infected with type 2, 3 and 19F wild type and *PspC*-deficient mutants respectively, while survival time significantly increased in case of type 4 mutants (Kerr *et al.*, 2006). It has been considered as a candidate for inclusion in protein-based vaccines.

1.4.3 Pneumolysin:

Pneumolysin is an important pore-forming toxin. It has a major role in pneumococcal pathogenesis (Mitchell & Andrew, 1997; Andrew *et al.*, 1997) as it causes host cell lysis in many tissues (Hirst *et al.*, 2003; Zysk *et al.*, 2001; Zysk *et al.*, 2000; Mohammed *et al.*, 1999; Feldman *et al.*, 1990). It also targets the immune system in various ways and induces neutrophil necrosis (Zysk *et al.*, 2000), inhibits dendritic cell maturation and induces their apoptosis (Littmann *et al.*, 2009). It inhibits degranulation of monocytes and stimulates release of IL-1ß and TNF-α to prevent pneumococcal clearance (Houldsworth *et al.*, 1994; Nandoskar *et al.*, 1986) and inhibits lymphocyte response to infection. It can also activate complement through classical pathway without involvement of specific antibodies (Mitchell *et al.*, 1991; Paton *et al.*, 1984). It is well

conserved (Kadioglu *et al.*, 2008) and is considered as a vaccine candidate for protein-based vaccines of the next generation.

1.4.4 Pneumococcal surface protein A (PspA)

PspA is another choline binding protein associated with virulence of pneumococcus and another candidate for protein based vaccines (Arulanandam *et al.*, 2001). *PspA* is also highly variable and is coded by mosaic genes, which have undergone extensive recombination (Heeg *et al.*, 2007; Mollerach *et al.*, 2004). It is considered to inhibit uptake of bacteria by phagocytosis (McDaniel *et al.*, 1987) and prevents deposition of complement and its activation (Ren *et al.*, 2003). It also interacts with lactoferrin and protects bacteria from apolactoferrin mediated killing (Shaper *et al.*, 2004; Hammerschmidt *et al.*, 1999).

These virulence factors with other important determinants of virulence are summarized in Table 1.1.

Table 1.1.Summary of pneumococcal virulence factors.

Virulence Factor	Description
Capsular polysaccharide	At least 93 serotypes (Calix & Nahm, 2010; Park *et al.*, 2007) and many non-typable strains (Park *et al.*, 2012). Resists complement, protects pneumococcus from phagocytosis (Melin *et al.*, 2010; Hyams *et al.*, 2010a; Hyams *et al.*, 2010b). Resists killing of phagocytosed bacteria (Peppoloni *et al.*, 2010). Influence growth *in vitro* (Hathaway *et al.*, 2012). Helps to avoid mucosal clearance by electrostatic repulsion and helps in colonization (Nelson *et al.*, 2007). Important for development of immunity (Cohen *et al.*, 2012).
Cell wall and its components	Made up of peptidoglycans and teicholic acids (Bui *et al.*, 2012) though chemical structure may vary among strains (Draing *et al.*, 2006). Provides anchorage to the capsule (Eberhardt *et al.*, 2012) .
PspC (CbpA)	Highly variable (Iannelli *et al.*, 2002). Binds factor H (Dave *et al.*, 2001), which is a regulator of complement and has a role in adherence (Agarwal *et al.*, 2010). Effect varies with strain (Kerr *et al.*, 2006) Down-regulates classical pathway (Dieudonne-Vatran *et al.*, 2009).
Choline binding proteins B,C,D,E,F,G	CbpD, CbpG, CbpE have role in colonization (Gosink *et al.*, 2000). Role in biofilm formation (Moscoso *et al.*, 2006). Role in adhesion (Rosenow *et al.*, 1997). CbpF Inhibits LytC and regulates pneumococcal autolysis (Molina *et al.*, 2009). CbpD is responsible for fratricide (Eldholm *et al.*, 2009).

Pneumolysin	Interacts with complement (Yuste *et al.*, 2005; Mitchell *et al.*, 1991; Paton *et al.*, 1984) . Cause cell damage and destroys ciliary activity (Zysk *et al.*, 2000; Mohammed *et al.*, 1999; Mitchell & Andrew, 1997; Feldman *et al.*, 1990).
Autolysins	Role in biofilm formation (Moscoso *et al.*, 2006). Cell wall degradation and remodeling, chain splitting(Las Rivas *et al.*, 2002). LytA and LytC are responsible for fratricide (Eldholm *et al.*, 2009). LytB and LytC , have role in colonization (Gosink *et al.*, 2000). Lyt A is responsible for autolysis (Weiser *et al.*, 1996).
Hyaluronidase	Increases epithelial damage caused by pneumolysin (Feldman *et al.*, 2007).
Neuraminidases	Exoglycosidase that desialates host protective proteins (Dalia *et al.*, 2010; Burnaugh *et al.*, 2008; King *et al.*, 2006). Presence may vary among the strains (Pettigrew *et al.*, 2006). Also protects against colonization with other bacteria (Shakhnovich *et al.*, 2002). Facilitates biofilm formation (Soong *et al.*, 2006). Assists in invasion of neuroendothelium (Uchiyama *et al.*, 2009).
Hydrogen peroxide	Causes epithelial damage and ciliary slowing (Feldman *et al.*, 2002). Inhibits other nasopharyngeal colonizers (Pericone *et al.*, 2000).
Pneumococcal surface protein A	Highly variable (Heeg *et al.*, 2007; Hollingshead *et al.*, 2000). Inhibition of complement activation (Tu *et al.*, 1999) and deposition (Yuste *et al.*, 2005). Binds human apolactotoferring and lactoferrin and protects against phagocytosis (Shaper *et al.*, 2004; Hakansson *et al.*, 2001).
Pneumococcal surface adhesin A	Highly variable (Berry & Paton, 1996). Protects against oxidative stress (Johnston *et al.*, 2004).

Pneumococcal histidine triad proteins	PhtA, PhtB and PhtD are required for lung infection (Hava & Camilli, 2002). Inhibit complement (Ogunniyi *et al.*, 2009).
Pili	Host cell adhesion (Barocchi *et al.*, 2006).

1.5 Immunity against pneumococcus:

Host defenses against pneumococcus utilize both arms of immune system and cell mediated and humoral mechanisms work in co-ordination for development of immune response against the organism (Paterson & Orihuela, 2010; Malley, 2010). The roles of cell mediated and humoral mechanisms are briefly discussed below:

1.5.1 Cellular Immunity:

Neutrophils are phagocytic cells, which are the first responders to bacterial invasion. As soon as pneumococcus is phagocytosed by neutrophils, lysosomes fuse with phagosome and convert it into phagolysosome, which mediates bacterial killing by oxidative and non-oxidative mechanisms (Standish & Weiser, 2009; Klebanoff, 2005). Neutrophils also kill bacteria extracellularly by releasing DNA, histones, and other proteins forming neutrophilic extracellular traps (NETs), which can bind bacteria and are bactericidal (Mori *et al.*, 2012; Wartha *et al.*, 2007; Urban *et al.*, 2006). They play a significant role in controlling pneumococcal infections as their impaired recruitment have been reported to result in severe pneumococcal pneumonia in mice (Nakasone *et al.*, 2007; Sun *et al.*, 2007)

Another group of phagocytic cells, which provide immunity against pneumococci are alveolar macrophages that engulf opsonized bacteria (Jonsson *et al.*, 1985), release proinflammatory cytokines and express various receptors that play a vital role in development of immunity (Koppe *et al.*, 2012; Paterson & Orihuela, 2010; Koppel *et al.*, 2008). Importance of their role can be understood by the

reports that the incidence of pneumonia was found to be higher in patients with impaired macrophage activity because of smoking cigarettes (Phipps *et al.*, 2010). It was also shown that macrophage dysfunction leads to higher mortality in animal models (Arredouani *et al.*, 2006; Arredouani *et al.*, 2004), while their depletion reduces survival (Traeger *et al.*, 2009).

Adaptive immune response is mediated by T lymphocytes, which interact with dendritic cells and produce cellular immunity against pneumococci (Palucka & Banchereau, 2002). These T lymphocytes are of three main types, helper T cells (T_H), cytotoxic T cells (T_c) and regulatory T (T_R) cells. Helper T cells are divided into three subtypes known as T_H1, T_H2 and T_H17, which develop from naïve T_H cells into one of these subtypes depending upon cytokine environment. T_H1 are produced mainly in response to viruses and intracellular bacteria that stimulate macrophages or natural killer (NK) cells and promote cellular immunity, which has been shown to provide protection in humans. T_H2 mediated response is directed against toxins, allergens and parasites. It acts by induction of cytokines, which promote production of antibodies by B cells and release of inflammatory mediators by mast cells. These cells produce IFNγ, which contributes towards the development of immunity against pneumococci and other bacteria. T_H17 response develops under the influence of a number of extracellular and intracellular bacteria and fungi. It mainly controls the balance between humoral and cell mediated immune responses and forms a bridge between these two by monocyte, macrophage and neutrophil recruitment. They also have a role in production of anti-bacterial peptides by epithelial cells (Malley, 2010; Peck & Mellins, 2010; Zhang *et al.*, 2009).

1.5.2 Humoral immunity and B lymphocytes

B lymphocytes are nonphagocytic cells which are responsible for development of humoral responses against pneumococcal antigens (Moens *et al.*, 2008). These responses are very important for protection against pneumococcal diseases, as has been shown by successful utilization of capsular polysaccharide based vaccines and demonstration of protective antibodies against capsule and other antigens (Lipsitch *et al.*, 2005). Some of these responses are antigen dependent as capsular polysaccharide stimulates differentiated B cells independent of T cells, which results in failure of induction in young children (Casal & Tarrago, 2003). In contrast, immune response against proteins is T cell dependent and stimulated B cells can develop into plasma cells and generate memory cells, resulting in antibody production, which has been reported in case of a wide range of pneumococcal proteins (Giefing *et al.*, 2008; Ogunniyi *et al.*, 2007; Jomaa *et al.*, 2006; Brown *et al.*, 2001; Briles *et al.*, 2000). These antibodies have a significant contribution in development of immune response against pneumococci as they perform opsonization (Plotkin, 2008) and complement activation (Baxendale *et al.*, 2008; Brown *et al.*, 2002). Patients with disorders related to antibody production have been reported to have increased susceptibility to pneumococcal infections (Phipps *et al.*, 2010; Yuste *et al.*, 2008).

1.5.3 Role of complement:

The complement system is an antimicrobial system that consists of numerous serum and membrane proteins, which participate in host defence by acting as opsonins, bactericidal and chemoattractive agents. These proteins remain

present in blood in inactive form and can be activated by a number of factors. There are three pathways that activate complement called classical, alternative and mannose-binding lectin pathways (Walport, 2001a; Walport, 2001b). Several pneumococcal proteins such as PspA, pneumolysin and phtD interact with complement and interfere with its function. Regardless of the activated pathway, the end result of complement activation is deposition of C3 convertase on pathogen surface, leading to breakdown of C3 into C3a and b, which finally leads to cleavage of C5 that draws neutrophils and macrophages (Hawlisch & Kohl, 2006). Further assembly of component proteins and their insertion on the pathogen membrane results in its perforation, resulting in an influx of water and solutes into the cell causing bacterial cell lysis (Walport, 2001a). Phagocytosis of *S. pneumoniae* has also been reported to be complement dependent (Yuste *et al.*, 2008), which is mainly activated by classical pathway in case of pneumococcal infection (Brown *et al.*, 2002). The importance of the complement system and classical pathway has been described in a number of studies and it was observed that patients with dysfunctional complement have an increased tendency to develop pneumococcal disease (Yuste *et al.*, 2008; Brown *et al.*, 2002; Roy *et al.*, 2002)., while the classical pathway was reported to be the most important in murine models of pneumococcal infections (Brown *et al.*, 2002).

1.6 Pneumococcal vaccines:

Vaccines have been used for pneumococcal disease control for a very long time. Current vaccines against pneumococci employ capsular polysaccharide (CPS), which induces serotype-specific antibodies to activate and fix complement and induce opsonisation and phagocytosis by host phagocytes (Plotkin, 2008). There

are two main vaccination strategies against pneumococcal infections, which are the use of purified CPS based pneumococcal capsular polysaccharide (PPV) and pneumococcal conjugate vaccines (PCV) that are prepared by conjugating CPS to immunogenic carrier proteins (Artz *et al.*, 2003).

PPV and PCV have certain drawbacks and certain advantages over each other. PCV, unlike PPV is known to reduce carriage as well as invasive pneumococcal disease (IPD) by included serotypes (O'Brien & Dagan, 2003; Obaro & Adegbola, 2002). The PPV23 vaccine serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F covers for most of (>90%) of IPD in developed world. Multivalent vaccines were found to be providing protection in about 80% of cases against invasive disease by serotypes, which are included in vaccine (Austrian, 1981). A major limitation of PPV23 is that it is only effective in children older than two years of age and adults, as children younger than two are unable to mount an appropriate immune response to a polysaccharide vaccine because of insufficient development of immune mechanisms.

PCV7 is used in the prevention of invasive disease in children younger than two years. It consists of capsular polysaccharide from serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F combined with detoxified diptheria toxin (CRM147). This conjugation results in a T-cell mediated response. Introduction of PCV7 in vaccination program in the US in 2000 resulted in significant reduction in rate of incidence of pneumococcal diseases within a few years (Benninger, 2008; Fletcher & Fritzell, 2007; Mahon *et al.*, 2006), even in HIV positive infants (Klugman *et al.*, 2003) and elderly over 65 due to development of herd immunity (Mcbean *et al.*, 2005).

A major problem with these vaccines is serotype replacement, which allows pneumococci to gradually escape the effects of vaccines by replacing capsular genes included in vaccines with those of uncovered serotypes (Brueggemann *et al.*, 2007). These bacteria can acquire a different type of capsule to escape vaccine-effects and continue to cause disease as has been observed with multidrug resistant clone 23F (Croucher *et al.*, 2009; Coffey *et al.*, 1998b; Munoz *et al.*, 1991) whose variants have been found expressing different capsular types such as 19F, 14, 19A, 9N, 3 and serogroup 6 (Mcgee *et al.*, 2001). Similar capsular switching have been reported for serotype 14 and 19A variants (Coffey *et al.*, 1999; Coffey *et al.*, 1998a; Coffey *et al.*, 1998b). These variants arise from capsular switching among different strains due to recombinational events in capsular biosynthesis *cps* operon (Coffey *et al.*, 1999; Coffey *et al.*, 1998a; Coffey *et al.*, 1998b). These changes in the capsule change the serotypes carried in the nasopharynx, which results in transmission of serotypes not included in the vaccine and rise in pneumococcal infections in the community (Brueggemann *et al.*, 2007; Spratt & Greenwood, 2000).

This phenomenon has been reported from around the globe as in Alaska native children, a 96% decrease in vaccine serotypes was noted from 2004, which was also accompanied by a 141% increase in non-vaccine serotypes, mainly due to infection by 19A (Singleton *et al.*, 2007).

There are serious concerns that limited coverage by these vaccines could result in spread of non-vaccine serotypes, which would render these vaccines ineffective in populations.

A much better strategy would be to introduce vaccines that can provide serotype independent protection. Such vaccines may be protein based, so that they can prevent pneumococcal disease independent of serotype.

The use of protein-antigens, which are conserved across serotypes, could offer serotype-independent protection in all age groups for vaccines of the future. Surface exposed proteins are used to generate protective response and opsonophagocytosis of bacteria to clear them from host tissues though cytoplasmic proteins are more conserved (Ogunniyi *et al.*, 2007; Jomaa *et al.*, 2006; Brown *et al.*, 2001).

Another possibility is of use of live attenuated vaccines. Use of strains containing deletions of major determinants of virulence has been noted to offer significant protection in mice and capsule deleted strains were able to generate serotype independent immunity (Roche *et al.*, 2007).

Whole-cell killed vaccines have also been tested and unencapsulated killed intranasal pneumococcal vaccine using cholera toxin as adjuvant was found to be highly effective in preventing colonization and also conferred protection against infection through other routes (Malley *et al.*, 2004). A similar vaccine using an aluminium derived adjuvant, when given as injection induced both cell mediated and humoral immunity with a 30-fold higher antibody response as compared to previous approach (Malley & Anderson, 2012)

1.7 Aims of the project:

Aims of this project were to investigate:

1. The effect of capsular variation on pneumococcal biology.

   For this purpose otherwise isogenic mutants were constructed in three strains TIGR4, D39 and 403 expressing various serotypes, and were compared for variation in *in vitro* and *in vivo* characteristics.

2. The genomic diversity in closely related strains of *Streptococcus pneumoniae* in order to understand the affects of mutations on phenotype.

   Non virulent type 4 strain 403 was genome sequenced and compared for differences with previously sequenced virulent serotype 4 strain TIGR4. Microarray analysis was also performed to study variation of gene expression among the two strains.

3. The possibility of clinical application of capsule switching phenomenon.

   Avirulent strain 403 was also tested as a live attenuated vaccine without using any adjuvant. It was planned that its capsule switch mutants would also be tested as vaccines as a combination of live attenuated and whole cell killed vaccine. Since 403 could not provide sufficient protection on its own, the capsule switch mutants were not tested as live attenuated vaccine.

2 Materials and methods:

2.1 Bacterial strains:

To study the effect of capsule switching on different genetic backgrounds, different bacterial strains were selected as recipient and donors of capsule genes on the basis of difference of their virulence and capsular polysaccharide structure.

2.1.1 Wild type (WT) and capsule knock out bacterial strains:

Three strains serotype 4 strain TIGR4, serotype 2 strain D39 and serotype 4 strain 403 with different virulence and capsular structure were used in this study as the recepients of capsule genes. Among these, TIGR4 is the most virulent, D39 has intermediate virulence and 403 is an avirulent strain.

Highly virulent serotype 8 strain ATCC6308, virulent serotype 3 strain OXC141 and D39 were used as capsule donors. Bioluminescent virulent serotype 3 strain A66.1 Xen10 was used for challenging mice vaccinated with 403.

These strains and their capsule knock out mutants are summarized in Table 2.1.

Table 2.1 Wild-type and capsule knock out strains used in this study:

Strain	Serotype	Description
TIGR4	Serotype 4	Wild type virulent strain
403	Serotype 4	Avirulent strain derived from TIGR4
D39	Serotype 2	Wild type virulent strain
OXC141	Serotype 3	Wild type virulent strain
ATCC6308	Serotype 8	Wild type highly virulent strain
TIGR4j (P1672)	_	Capsule knock-out TIGR4
403j	_	Capsule knock-out 403
D39j	_	Capsule knock-out D39
A66.1 Xen10	Serotype 3	Virulent, Bioluminescent

2.1.2 Capsule switch strains:

S. pneumoniae strains were modified using Janus technique so that they remain isogenic except for capsule locus (Trzcinski *et al.*, 2003; Sung *et al.*, 2001). This technique has the advantage of double selection as it allows selection for incorporation of the cassette accompanied by the capsule knock out as well as the loss of Janus cassette resulting from capsule replacement (Trzcinski *et al.*, 2003; Sung *et al.*, 2001). It is a bicistronic cassette, which allows selection for its acquisition as well as removal and contains kanamycin resistance gene *aphIII* and streptomycin sensitivity gene *rpsL* as shown in Fig 2.1.

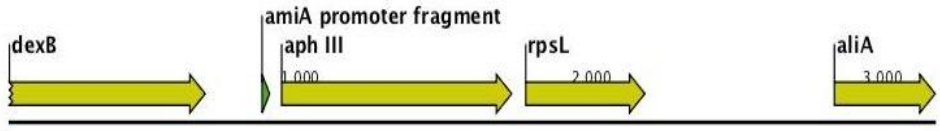


Figure 2.1. Schematic diagram of Janus cassette containing kanamycin resistance gene *aphIII* and gene *rpsL* conferring sensitivity to streptomycin. Cassette is ligated with genes *dexB* and *aliA,* which flank capsular locus in *S. pneumoniae.* Figure constructed using CLC Genomics workbench(CLC Bio, Denmark)

Using the Janus method, bacteria are first transformed with *rpsL* gene, which confers recessive streptomycin resistance in target bacteria as shown in Fig 2.2 A. When these bacteria are transformed with dominant allele *rpsLR*$^{+}$, present in Janus cassette along with Kanamycin resistance cassette, they become sensitive to streptomycin and can be selected for kanamycin resistance. Once this cassette is replaced, transformants can be selected for redevelopment of resistance to streptomycin and loss of kanamycin resistance (Sung *et al.*, 2001) as shown in Fig 2.2 B and C.

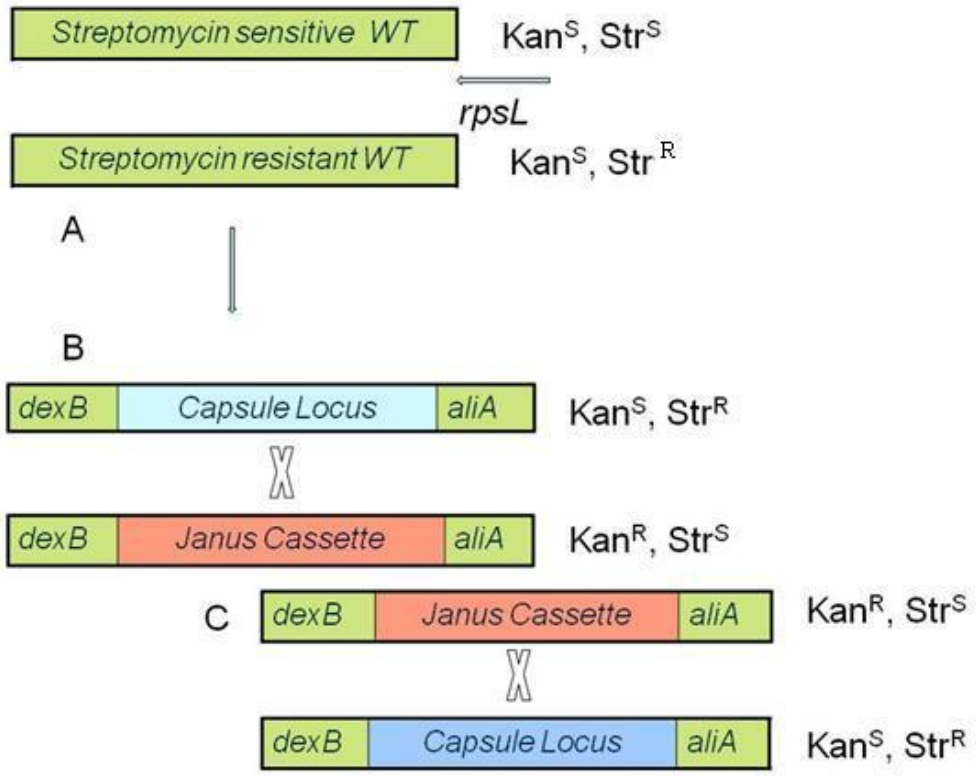


Figure 2.2. Mechanism of Janus transformation. (A) Streptomycin sensitive wild type bacteria can be transformed with *rpsL* cassette conferring streptomycin resistance. (B) Janus cassette flanked with identical genes can be used to replace the target gene, which also confers kanamycin resistance and streptomycin sensitivity. (C) Replacement of Janus cassette by another gene confers streptomycin sensitivity and kanamycin resistance. (Modified from Sung *et al* 2001)

403, TIGR4 and D39 capsule genetic loci were replaced with this cassette, which was then replaced with different type of capsules as explained in Section 3.1.

Switching of capsule genes was confirmed by Quellung reaction to check capsular serotype and correct positioning of capsule locus was confirmed by PCR with forward primers designed against specific genes present in respective serotypes and reverse primer against *aliA*, which is common flanking gene in all serotypes as shown in Fig 2.3.

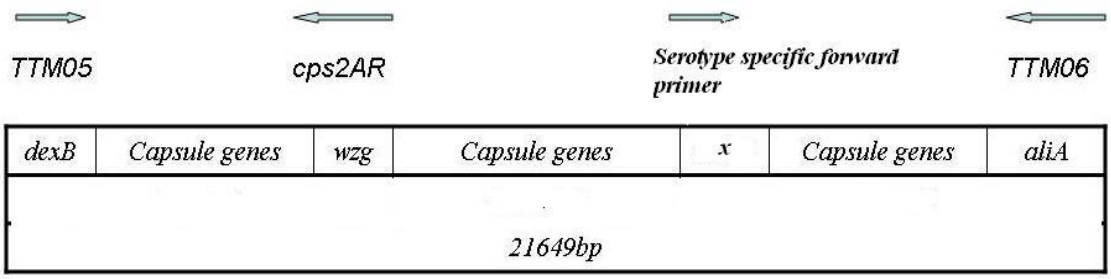


Figure 2.3. Schematic diagram showing method of primer design for confirmation of correct placement of capsule locus. TTM05 is forward primer designed against *dexB while cps2AR* is reverse primer designed inside capsule locus. TTM06 is reverse primer designed against *aliA* gene while the forward primer is serotype specific, used to confirm correct serotype and its proper placement in recipient genome.

Mutant capsule switch strains are summarized in Table 2.2.

Table 2.2. Capsular switch mutants strain.

Strains	Description
TIGR4^{2} (TIGR4^{2}3x)*	Otherwise isogenic TIGR4 expressing serotype 2 capsule
TIGR4^{3} (TIGR4^{3}3x)*	Otherwise isogenic TIGR4 expressing serotype 3 capsule
TIGR4^{8} (TIGR4^{8}3x)*	Otherwise isogenic TIGR4 expressing serotype 8 capsule
TIGR4^{4} (P1702)	Otherwise isogenic TIGR4 retransformed with serotype 4 capsule
403^{2} (403^{2}3x)*	Otherwise isogenic 403 expressing serotype 2 capsule
403^{3}(403^{3}3x)*	Otherwise isogenic 403 expressing serotype 3 capsule
403^{8}(403^{8}3x)*	Otherwise isogenic 403 expressing serotype 8 capsule
D39^{8}(D39^{8}3x)*	D39 expressing serotype 8 capsule

*All 3x strains are three times back crossed strains.

2.2 Growth conditions:

Pneumococcal strains were statically grown from a single colony in Brain Heart Infusion (BHI) broth (Oxoid, UK) at 37°C to mid log phase (OD_{600nm} 0.6) and stored at -80°C in 1ml aliquots in BHI containing 15% glycerol (Sigma-Aldrich, UK) or in form of bead stocks (Pro-Lab Diagnostics, UK). Strain purity was verified by streaking the culture on Blood Agar Base (BAB) (Oxoid, UK) supplemented with 5% horse blood (E&O Laboratories, UK) on the next day and optochin sensitivity was confirmed (Mast diagnostics, UK). Antibiotic concentrations used in selective media were 500 µg/ml kanamycin (Sigma-Aldrich, UK), 300 µg/ml streptomycin (Sigma-Aldrich, UK), 3 µg/ml chloramphenicol (Sigma-Aldrich, UK) and 1 µg/ml erythromycin (Sigma-Aldrich, UK).

2.3 Preparation of pneumococcal genomic DNA:

Strains were grown statically overnight at 37°C in 20ml BHI, and culture was centrifuged at 4000 x g at 4°C for 15 min to pellet the cells. A BAB plate was aseptically streaked with culture to confirm purity and identity using an optochin disc (Mast diagnostics, UK) before centrifugation. The pellet was then resuspended in 1ml lysis buffer (10mM Tris, 100mM EDTA, 0.5% SDS) and incubated for an hour at 37°C. Proteinase K (Invitrogen, UK) was added to attain a concentration of 20µg/ml and was incubated for three hrs at 50°C. RNase A (Invitrogen, UK) was then added to a final concentration of 20µg/ml, and samples were incubated for half hour at 37°C. Each sample was mixed gently with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) (Sigma-Aldrich, UK) and centrifuged at 12,000 x g for 3 min. Upper phase was separated and placed into a fresh tube of 1.5ml. 0.2 volumes of 10M ammonium acetate

(Sigma-Aldrich, UK) and 600µl analytical reagent grade absolute ethanol (Fisher Scientific, UK,) were added to samples and mixed gently. Tubes were centrifuged at 12,000 x g for half an hour to form a DNA pellet. Supernatant was decanted and pellet was dried in air for half an hour to remove any remaining ethanol and later suspended in 300µl PCR water and incubated at 65^{o}C for 10 min, with intermittent mixing. The quality of DNA was assessed by running the DNA on 0.7% agarose gel with SYBR® Safe DNA Gel Stain (Life technologies, UK) and visualization using a Bio-Rad® Gel doc system (Bio-Rad, UK). DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Agilent Technologies, UK)

Samples were then stored at 4^{o}C if to be used shortly or -20 ^{o}C for long term storage.

2.4 Transformation conditions:

Bacteria were statically grown to OD_{600} 0.1 in BHI containing 0.1 mM $CaCl_2$. Competence was induced in TIGR4, 403 and their mutants by using 100ng/ml competence-stimulating peptide 2 (csp-2) while in D39 and its derivatives using 100ng/ml csp-1. Bacteria were incubated 15 min at 37 ^{o}C. One µg/ml DNA was used in transformation steps both for chromosomal DNA and purified PCR products.

2.5 Optochin sensitivity:

All parent and mutant strains were tested for optochin sensitivity to confirm the purity of strains by plating colonies on blood agar and placing 5 µg optochin disc

(Oxoid, UK). Plates were incubated overnight at 37 ^{o}C to confirm a zone of growth inhibition around the disc.

2.6 Serotyping:

Capsular serotype of parents and transformants were determined by the Quellung test with serotype specific typing sera (Statens Serum Institute, Denmark). Equal amount of bacterial suspension was mixed with type specific antiserum (Statens Serum Institute, Denmark), incubated for 10 min and examined under the microscope using an oil immersion lens at 1000X for capsule swelling. Agglutination was also observed macroscopically.

2.7 PCR amplification:

PCR amplification was performed to amplify the Janus cassette and confirmation of correct placement of transformed DNA using 150 ng of genomic DNA in 50 µl mixture comprising of 10 µl buffer, 5 µl 2mM dNTPs and 100nM each primer with 4.5 U of Taq DNA polymerase (Invitrogen, USA) or 5 µl buffer, 5 µl 2mM dNTPs and 100nM each primer with 0.5 U of Pfu DNA polymerase (Stratagene, USA). PCR reaction consisted of initial denaturation of 3 min at 95°C and 30 cycles with every step comprising of 30 sec at 95°C, 25 sec at 5°C less than annealing temperature of primers, and 1 min/1000 base pairs of estimated product length at 72°C and a final incubation at 72°C for 4 min.

Table 2.3. Primers used in this study.

Primer	Sequence	Description	Reference
CPSF	5'-GACCGTCGCTTCCTAGTT-3'	Forward primer *dexB* to amplify Janus cassette	This study
CPSR4	5'-AGCCTTAGCAGTTGTCAAAT-3'	Reverse primer *aliA* to amplify Janus cassette	This study
TTM05	5'-AAGGTGAGGAGATTGGGATGA-3'	Forward primer *dexB* to confirm Janus cassette placement	(Trzcinski *et al.*, 2003)
TTM06	5'-TGTCGCAGCCTTAGCAGTTG-3'	Reverse primer *aliA* to confirm Janus cassette placement	(Trzcinski *et al.*, 2003)
Cps2AR	5'-CTGCCAAGTAAGACGAACTC-3'	Reverse primer *wzg* to confirm capsule placement	This study
CPS1rmlDF1	5'-TCAAGCCAGTAGATTCCAGT-3'	Forward primer rmlD specific for serotype 1 and 2.	This study
Cps3wchEF	5'-TTTCCAGACATAAACCATCCATCCGA-3'	Forward primer wchE specific for serotype 3	This study
Cps8wzy	5'-AGCTTGGTCTATGTATGCG-3'	Forward primer wzyE specific for serotype 8	This study
Cps1A	5'-CGACCGTCGCTTCCTAGTTGTGGCTAAC-3'	Forward primer *dexB* to confirm capsule placement	Bentley *et al.*, 2006
Cps1B	5'-GTCTTGAGCTTTGACTGCCGCGTATTCT-3'	Reverse primer *aliA* to confirm capsule placement	Bentley *et al.*, 2006

2.8 Janus Intermediates:

Capsule knock-out strains were constructed using the Janus technique as discussed in Section 2.1.2 (Sung *et al.*, 2001). These knock-outs could be selected for the acquisition of capsule genes from other strains on blood agar plates supplemented with streptomycin.

2.8.1 Janus Intermediate in 403 and TIGR4:

The Janus intermediate strain of 403 was a kind gift from Professor Marc Lipsitch of Harvard School of Public Health (U.S) and TIGR4 from Dr. Jeremy Brown of University College London (U.K).

2.8.2 Construction of Janus Intermediate in D39:

Unencapsulated D39 was constructed using Janus cassette from TIGR4j. The cassette was amplified using primers CPSF and CPSR4 against *dexB* and *aliA* and was cleaned using PCR purification kit (Qiagen,UK). Strain D39 was statically grown to OD_{600} 0.1 at 37^{o}C in BHI containing 1mM $CaCl_2$, incubated 15 min with 100ng/ml csp-1 and was transformed with a gene conferring streptomycin resistance *rpslR*$^{+}$. Suspension was then incubated for 2 hrs before plating on to BAB plates supplemented with 300 µg/ml streptomycin (Sigma-Aldrich, UK). streptomycin resistant colonies were picked up and were replated on BAB plates supplemented with 300 µg/ml streptomycin (Sigma-Aldrich, UK) with an optochin disc (Mast Diagnostics, UK) to confirm purity and identity. Streptomycin resistant strain D39S was statically grown to OD 0.1 at 37^{o} in BHI containing 1mM $CaCl_2$, incubated 15 min with csp-1 and was transformed with 1µg purified Janus cassette. Suspension was incubated for another 2 hrs before plating on to BAB

agar plates supplemented with 500µg/ml kanamycin (Sigma-Aldrich, UK). Colonies were picked up and were re-confirmed by using two sets of primers, original primers CPSF, CPSR4 used for amplification of cassette and primers cps1A and cps1B in flanking sequence of *cps* locus. Confirmed colonies were saved and purified PCR product from one of them was sent for sequencing to confirm insertion site of Janus cassette.

2.9 Construction of *cps* transformants;

Cps transformants were constructed in strains TIGR4j, 403j and D39j using genomic DNA from the following strains. They are summarized in Table 2.4.

Table 2.4. Capsule donor strains

Strain	Type of capsule
D39	Serotype 2
OXC141	Serotype 3
TIGR4	Serotype 4
ATCC6308	Serotype 8

For each transformation genomic DNA from the donor strain was transformed into the capsule knock-out strains (Section 2.4) and 50 µl of the suspension was plated on to the streptomycin supplemented BAB plates. For each capsular transformation, up to 24 morphologically different colonies were picked from the plates, incubated overnight and streaked on kanamycin and streptomycin plates simultaneously to confirm the loss of kanamycin resistance and redevelopment of streptomycin sensitivity. The process is schematically summarized in Fig 2.4.

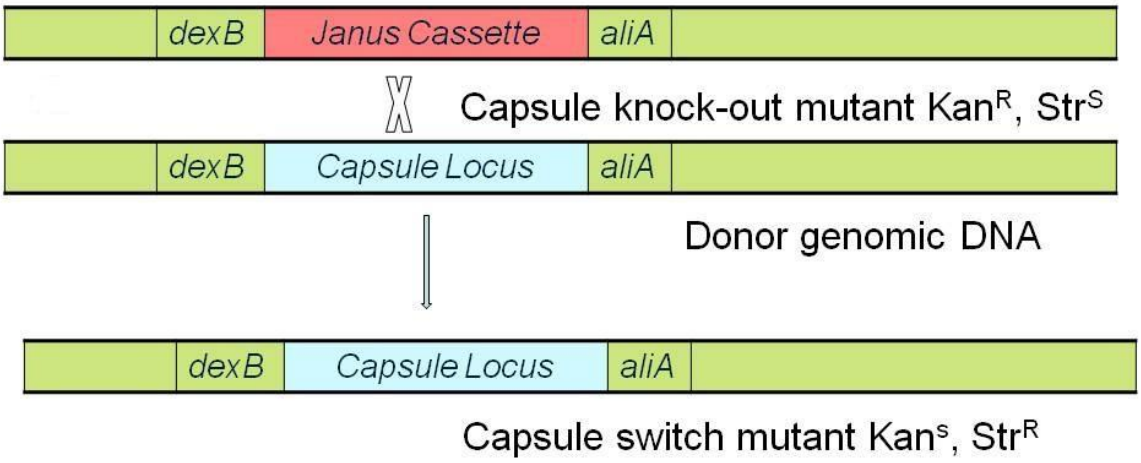


Figure 2.4. Schematic diagram explaining generation of capsule switch mutant. Capsule knock-out mutant (Kan^R, Str^S) is transformed with the genomic DNA from the donor strain that removes Janus cassette resulting in Kan^S, Str^R phenotype that can be selected on streptomycin-supplemented BAB plates.

Quellung reaction was performed to confirm the capsular type. DNA was purified from that mutant and used to retransform the unencapsulated janus strain into an encapsulated strain again. This back crossing was repeated thrice to construct three times backcross (3x back-cross) transformants (Fig 2.5). Mutant strains constructed using the Janus technique are summarized in Table 2.2.

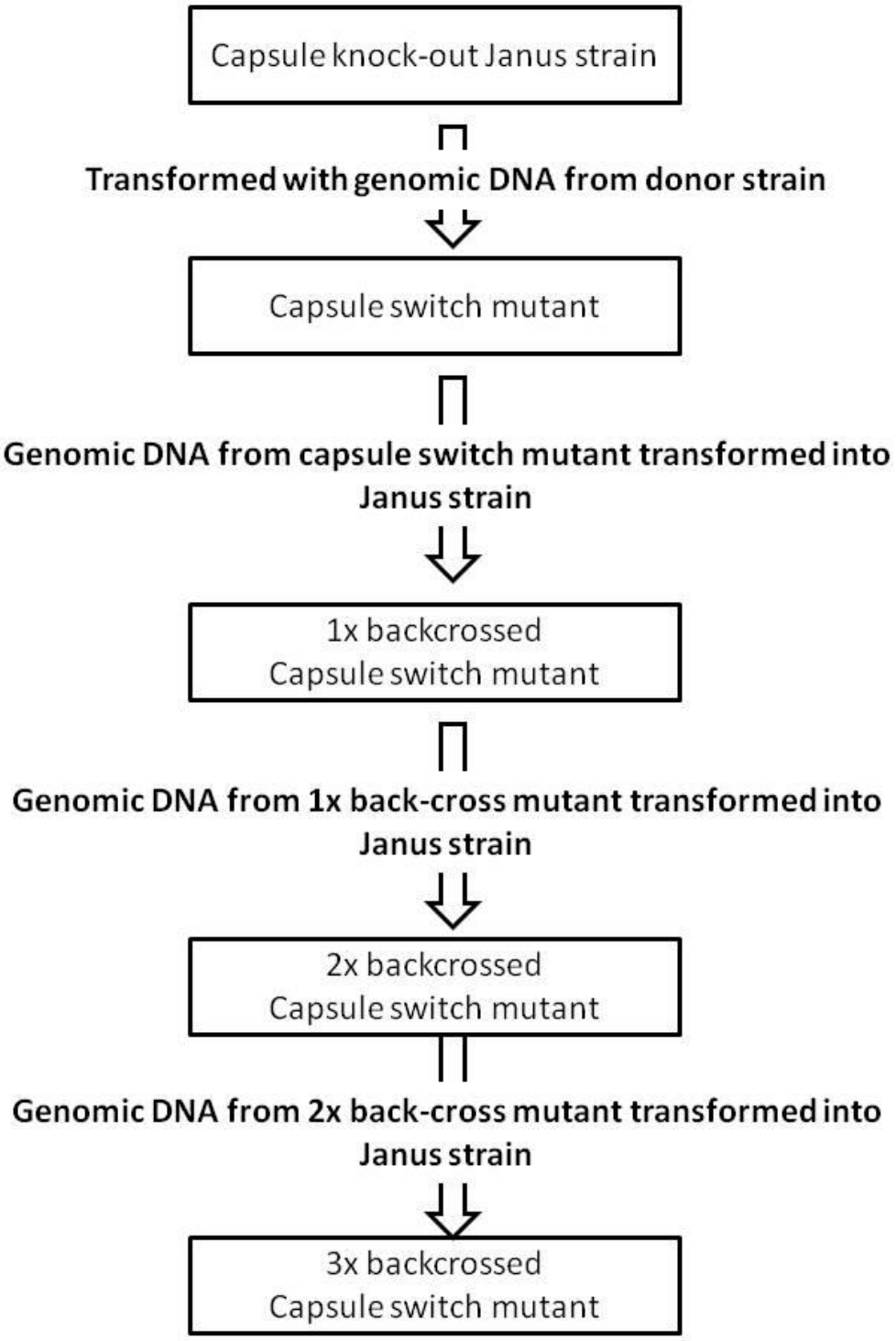


Figure 2.5. Flow-dagram showing process of back-crossing to produce 3x backcrossed mutant strains.

2.10 Characterization of mutant strains:

2.10.1 Growth curves:

To compare the effect of capsule switching on growth of the mutant strains, growth rates of parent, capsule switch and capsule donor strains in BHI (Sigma-Alrdich, UK) were investigated and compared by constructing growth curves using three replicates. Approximately 1 x 10^5 colony forming units/ml (cfu/ml) were taken in 20 ml BHI and growth rates were monitored by reading OD_{600} every two hrs for the first four hrs and every hour then onwards for up to 12 hrs. Blood agar plates were inoculated for calculation of viable counts. Growth curves were plotted using these viable counts in GraphPad Prism 4 (GraphPad Software, USA).

2.10.2 Sample preparation for electron microscopy:

To compare the effect of capsule switching on capsular thickness, electron microscopy was performed at the EM facility at University of Glasgow with the help of Ms. Margaret Mullin and Dr. Lawrence Tetley. The samples were prepared by Ms. Margaret Mullin using the following method.

Bacteria were grown to OD_{600} 0.4 and centrifuged at 2400 x g for 15 min. Pallets were resuspended in 1ml PBS and washed thrice. The samples were transported to the EM facility and were incubated in a solution of 2% paraformaldehyde (Sigma, UK) and 2.0% glutaraldehyde (Sigma, UK) in 0.15M cacodylate buffer containing 0.15% ruthenium red (Agar Sc, UK) and 0.0075% lysine (Sigma, UK) for 30 min on ice. Samples were washed with 0.15M sodium cacodylate and

incubated in 2% paraformaldehyde, 2.0% glutaldehyde containing 0.15% ruthenium red in cacodylate buffer for one and a half hour on ice. Following three washes with 0.15M sodium cacodylate for 5 mins, samples were fixed with 1% osmium tetroxide and 0.15M sodium cacodylate for an hour on ice. Samples were washed thrice with distilled water and were left in the dark in 0.5% uranyl acetate in distilled water for an hour and washed with distilled water twice for a minute. Samples were dehydrated with ethanol according to the following scheme.

0% Ethanol	2x5mins
50% Ethanol	2x5mins
70% Ethanol	2x5mins
90% Ethanol	2x5mins
Absolute Ethanol	4x5mins
Dried Absolute Ethanol	4x5mins

Dehydrated samples were then given four washes of five min each with propylene oxide and left on overnight incubation with propylene oxide/Epon812 resin 1:1mix. Samples were washed with Epon 812 resin several times the next day and were embedded in fresh Epon resin and polymerised at 60ºC for up to 48 hrs. These specimens were ultrathin sectioned (60-70nm thickness) and were contrast stained in 2% methanolic uranyl acetate for 5 min and Reynolds lead citrate for another 5 min. Images were captured by Ms. Margaret Mullin on a LEO912 AB transmission electron microscope (TEM).

2.10.3 Measurement of capsule thickness:

Mean capsular thickness was calculated from measuring the completely extended capsular fibres at 3-7 points/bacterium in five bacteria. Measurements were taken using image processing software iTEM (Olympus Soft Imaging Solutions, Germany) on the areas where cell wall boundaries could be traced easily.

2.10.4 Measurement of amount of capsule:

To compare the effect of capsule switching on the amount of capsular polysaccharide, amount of capsular polysaccharide was calculated with the help of semi-quantitative Stains-All assay using Stains-All stain (Sigma, UK) for detecting acidic polysaccharides (Hathaway *et al.*, 2007; Hammerschmidt *et al.*, 2005). Bacteria were cultured in BHI to OD_{600} 0.3 and 0.6, 20µl was removed and diluted in PBS for plating and quantification. Four ml culture was centrifuged at 5000 x g for 10 min, washed twice with PBS and resuspended in 0.5 ml water.

A solution was freshly prepared with 20 mg 1-ethyl-2 (3-(1-ethylnaphthho-(1,2-d) thiazolin-2-ylidene)-2 methylpropenyl) naphthho-(1,2-d) thiazolium bromide, 60µl glacial acetic acid and 100 ml 50% formamide and 2ml was added to sample and OD_{640} was determined. Two ml Stains-All solution was used as a blank. Values for 10^9 cfu were calculated from obtained readings.

2.10.5 Transformation efficiency:

To compare the effect of capsule switching on the transformability of pneumococci, transformation efficiency was calculated by transforming similar number of bacteria under similar growth conditions with 200ng plasmid

pVA838/ml and selecting for development of erythromycin resistance. Experiment was repeated five times and total colonies were used to calculate number of colonies/ng of plasmid DNA.

2.10.6 Chain formation in selected strains:

To study the effect of different capsules on bacterial chain formation, number of bacteria associating in particular types of chains was calculated. For counting number of bacteria associated to form a particular type of chain, all bacteria in chains in phase were counted in ten fields at early, mid and late log phase. These chains were broadly classified in three groups short (2-4), medium length (5-10) and long chains (11+). Since there was no major difference in three different growth phases except for increase in total bacterial count, only early log phase readings were considered for statistical analysis.

2.11 Mouse infection studies

2.11.1 Mice

Animal experiments were performed in 6-8 weeks old out-bred MF1 mice obtained from Harlan Olac (UK). Experiments were performed according to the UK Animals Act 1986 (Scientific Procedures Act) under Home Office project and personal license, approval was taken from the University of Glasgow Ethics Committee. Mice were acclimatized for one week before the procedure and were housed at 20-22ºC with a 12h light/dark cycle. Mice were challenged at 16-18 weeks of age.

2.11.2 Preparation of standard inocula:

Single colonies of *S. pneumoniae* serotype 4 strain TIGR4, serotype 3 strains Xen 10 A66.1 and OXC141, and serotype 8 strain ATCC6308 were grown up to mid-log phase in BHI and I ml aliquots were stored in 10% glycerol at -80ºC. After 24 hrs, viable counts were performed and $5x10^6$ cfu/200µl were injected intraperitoneally (IP) into an MF1 mouse, which was sacrificed after 6 hrs under general anaesthesia by terminal exsanguination via cardiac puncture. Blood was incubated overnight in 20 ml BHI at 37ºC and plated onto BAB plates to check sterility.

To prepare standard inocula, a 1:50 dilution of overnight culture was grown at 37ºC to an OD_{600nm} 0.6, inoculated in pre-warmed BHI containing 15% fetal calf serum (FCS) and frozen at -80ºC in cryovials. Cultures were checked for purity and cfu/ml in each culture was calculated using following method:

After freezing for 24 hours, three vials for each strain were defrosted rapidly for 2 min in a 37ºC water bath and were centrifuged for 5 min at 13000 x g at room temperature in a bench-top centrifuge. The supernatant was decanted and pellet was resuspended in 1ml PBS. 1:10 dilutions were made in sterile Dulbecco's Phosphate Buffer Saline (DPBS) (Sigma-Aldrich, UK) in a round-bottomed 96 well plate from 10^{-1} to 10^{-8}. Three spots of 20µl from each dilution were spotted onto BAB plates, which were divided into eight sectors and allowed to dry. Plates were incubated overnight in a candle jar at 37ºC and sector where there were 10-70 colonies/20µl was used to calculate cfu/ml in following steps:

- The average is calculated in the first step:

  [(Spot 1) + (Spot 2) + (Spot 3)] /3 = average/60µl

- Average/ml is calculated as:

  [average/60µl] x 50(cfu/ml) x dilution = average/ml

2.11.3 Dose preparation of avirulent 403 strains:

S. *pneumoniae* strain 403 was found to be avirulent in mice so glycerol stocks were used to immunize the mice. Single colony was selected and grown up to mid-log phase in BHI and I ml aliquots were stored in 10% glycerol at -80ºC. After 24 hrs, viable counts were performed and cultures were streaked on BAB plates to check for purity and optochin sensitivity. Colony forming units/ml in each culture was calculated using previously described method.

2.12 Vaccination of mice with 403 strains:

2.12.1 Vaccination of mice with live 403 strains:

Twelve week old MF1 mice were caged into group sizes of 10 and were bled from the lateral tail vein a day before immunization for obtaining baseline blood. Blood was then left overnight to clot at room temperature and was centrifuged the next morning at 13000 x g for 5 min. Serum was then stored in a fresh tube and was frozen at -80ºC.

For IN vaccination, mice were anaesthetised and vaccinated IN with 10µl across both nares either with sterile DPBS or 10^5 cfu of strain 403. Three vaccinations were performed at fortnightly interval.

For IP vaccination, mice were anaesthetized and vaccinated IP with 10µl into the peritoneal cavity either with sterile DPBS or 10^5 live 403 strains. Three vaccinations were performed at fortnightly intervals.

Bleeds were taken and mice were left for a further fortnight before challenge with the virulent strains.

2.12.2 Challenge of mice with virulent pneumococcal strains:

Mice wer challenged with strains TIGR4, ATCC6308 and A66.1 Xen 10 to assess the protection provided by the vaccination. Mice were also challenged with the capsule donor and capsule switch strains OXC141, D39, TIGR4^2, TIGR4^3, TIGR4^8, P1702 and D39^8 to assess the effect of capsule switching on the virulence of these strains. Mice were inoculated and bled by Dr. Kirsty Ross, Dr. Carol McInally and Mr. Ryan Ritchie.

Standard inocula were thawed and a dose was prepared right before challenging mice by diluting in sterile DPBS. Viable counts were assessed just before and after the challenge and compared to ensure that number of viable inoculum counts remained the same during challenge and whether the correct dose was given.

2.12.2.1 Intranasal challenge:

Mice were anaesthetized with 3.5% isofluorane/1.5% oxygen (1.5 litre/min) (Astra-Zeneca, UK) until loss of limb movement reflex. Mice were challenged IN with required dose in 50µl of sterile DPBS and 25µl was administered to each nare. Mice were then relocated to their cages and were left to recover in ventral position within their cage.

2.12.2.2 Intraperitoneal challenge:

IP challenge with the virulent pneumococcal strains was carried out by scuffing the mice at neck and administration of injection with the specific dose in 200µl of sterile DPBS into the peritoneal cavity.

2.12.3 Imaging with bioluminescent strains:

Imaging was performed by Dr. Kirsty Ross and Mr. Ryan Ritchie. Mice challenged with bioluminescent strain Xen 35 A66.1 were anaesthetised and positioned within IVIS Spectrum (IVIS: Caliper Life Sciences, UK) imaging chamber inside masks with a constant flow of anaesthetic. Living Image® 3.1 software (Caliper Life Sciences, UK) was used for image acquisition. Mice were exposed for five min before acquisition of initial bioluminescent images on large binning and field of view, which were kept consistent in all figures. Further images were taken with adjusted settings in case if there were saturated pixels in the regions of interest.

2.12.4 Retrieval of blood for viable counts from infected mice:

After each challenge with the virulent strain, mice were bled from the tail vein at 6 hourly intervals post infection (hpi) to monitor development of bacteræmia and progression of infection. Blood samples were promptly diluted in sterile DPBS to prevent clotting.

2.12.5 Plating of the blood for viable counts:

Blood samples were immediately plated on BAB plates and counted the next day. For the purpose of statistical analysis, samples below detection limits were ascribed value just beneath the limits of detection, which depends upon the number of spots and the dilution factor. As 1 cfu from three spots of dilution 10^{-1} equals 166.6 cfu/ml or $\log_{10}$ 2.22, or 1 cfu from three spots of neat dilution equals 16.6 cfu/ml or $\log_{10}$ 1.22 therefore the cfu/ml of blood is plotted as a log value with a detection limit of 2.2.

2.12.6 Survival assessment and management of clinical symptoms during infection:

For assessment of progression of disease, a clinical scoring system is utilized in our lab instead of taking death as end point. It consists of monitoring for hunching, lethargy and piloerection. On reaching a point where an animal doesn't move when encouraged, it is considered moribund and is humanely culled using a Schedule 1 method. Animals are also culled if they lose 20% of their body weight during the course of the infection. If an animal dies between the two readings, survival time is calculated as intermediate time between the

two time points, when animal was last scored and the time it was found dead to approximate time when animal became moribund.

2.13 Western blots:

TIGR4 whole cell lysate was prepared by growing bacteria to OD_{600} 0.6 in a 30 ml culture and sonicating after 2x centrifugation and washing with PBS. Loading buffer was added to samples, and were incubated at 70ºC for 10 min. Precast gels (Invitrogen, UK) were used to run the samples. Running tank was filled with MES running buffer and 13 µl of sample + 2 µl of marker was added to the gel tank and was then run for 1 hr at 150V. Gels were equilibrated in transfer buffer and run with 100 µl antioxidant at 100V for 1 hour to transfer to Hybond-C nitrocellulose membrane (Amersham Biosciences, UK). They were blocked overnight in 3% skimmed milk in 0.25mM Tris-NaCl (pH 7.4) with shaking overnight and then incubated for 2 hrs with shaking in 3% skimmed milk at 37ºC with 1:100 mouse sera. Membranes were rinsed 3x in 0.25mM Tris-NaCl (pH 7.4), incubated in 3% skimmed milk with 1:1000 HRP-linked secondary anti-mouse IgG (Amersham Biosciences, UK) and washed 4x with 0.25mM Tris-NaCl and developed by placing in 4-Chloro-1- naphthol developing solution. Distilled water was used to stop the reaction.

2.14 Genome sequencing, microarrays and bioinformatic and statistical analysis:

2.14.1 Complete genome sequencing:

Whole genome sequencing of strain 403 was carried at The Sir Henry Wellcome Functional Genomics Facility, University of Glasgow by Dr. Pawel Herzyk and Ms. Julie Galbraith using Illumina® genome analyzer and paired end-sequencing method was used. Using CLC Bio® Genomics Workbench, genomic alignments were performed by Dr. Andrea Mitchell between complete genome sequence of TIGR4 strain and consensus sequence, which was generated for strain 403.

2.14.2 Microarray experiments:

2.14.2.1 RNA extraction:

Bacteria were grown in 15ml BHI at 37°C until an OD_{600} 0.6 was reached. Purity of culture was checked by aseptically streaking 10µl on to 5% blood agar and incubating overnight at 37°C. Ten ml of culture was centrifuged at 5027 x g for 5 min at room temperature in 15 ml centrifuge tubes. The supernatant was discarded and pellet was frozen in liquid nitrogen immediately and stored at -80°C. For extraction, lysozyme TE buffer was prepared freshly using 10µl 1M Tris HCl (pH8.0) (Ambion/Applied Biosystems, UK), 2µl of 0.5M EDTA (pH8.0)(Ambion/Applied Biosystems, United Kingdom) and 15mg of lysozyme (Sigma-Aldrich, UK) in 1ml of nuclease free water (Ambion/Applied Biosystems,UK). Two hundred µl of buffer (15 mg/ml) was added to the pellet and vortexed for 10 sec in a rotamixer (Hook and Tucker Instruments, UK) and

incubated at room temperature. Samples were incubated for 15 min and vortexed for 10 sec every 2 min. Extraction was carried out using a Qiagen RNeasy Mini Kit (Qiagen, UK) according to manufacturer's protocol. Five µl of the extract was separated for quality assessment on Agilent 2100 bioanalyser (Agilent Technologies, UK). An integrity number above 9 was set as an indicator of high enough quality. Quantification was performed using Nanodrop ND-1000 spectrophotometer (Agilent Technologies, UK). Remaining RNA was stored at -80°C

2.14.2.2 Microarray analysis of RNA from Strain 403:

Expression experiments on strain 403 were performed in triplicates by Dr. Jenny Herbert (different broth cultures grown to mid log phase) by hybridizing 403 RNA against RNA from TIGR4 grown to mid-log phase. Reactions were performed as per manufacturer's protocols. One Cy3 and one Cy5 labeled cDNA sample (2-10µg) was prepared for each microarray by incubating at 70°C for 10 min with 1µl random primers (Invitrogen, UK) and made up to 11µl with nuclease free water (Ambion/ Applied Biosciences, UK). Samples were snap cooled on ice and centrifuged. Hundred mM dithiothritol (DTT) 2.5µl, 5xFirst strand buffer (Invitrogen, UK) 5µl, dNTPs (containing 5mM dATP, 5mM dGTP, 5mM dTTP and 2mM dCTP) 2.3µl, Cy3 or Cy5 dCTP (GE Healthcare, UK) 1.7µl and 200U/µl SuperScript II (Invitrogen, UK) 2.5µl were added to each sample from TIGR4 and 403 and solution was then incubated for 10 min in dark at 25°C and then at 42°C for 90 min in a Techegene thermal cycler (Bibby Scientific, UK).

Prehybridization solution (20 x Standard Saline Citrate (SSC) (Ambion/Applied Biosciences, UK) 8.75 ml, 20% SDS (Ambion/Applied Biosciences, UK) 250µl, 100mg/ml bovine serum albumin (Sigma-Aldrich, UK) 5ml and sterile double

distilled water (to make up 50ml) was preheated to 65°C and samples were soaked in a Coplin jar (Fisher Scientific, UK) for 20 min and placed in a Techne Hybridizer HB-1D (Bibby Scientific, UK). Prehybridized arrays were washed for 1 minute with 400ml double distilled water and then with 400ml propan-2-ol (VWR International, USA) for another minute. Each array was centrifuged for 5 min at 1200 x g then stored in a box.

Labelled samples were combined into a Qiagen mini-elute purification column (Qiagen, UK) and eluted according to manufacturer's protocol. 14.9 µl of Cy3/Cy5 labelled sample was combined with 4.6µl of 20xSSC and 3.5µl of 2% SDS and the solution was heated at 95°C for 2 min in a Techegene thermal cycler (Bibby Scientific, Uk). Arrays were covered by lifter slips (Erie Scientific Company, USA), and DNA samples were pipetted under, and array slides were then carefully placed into hybridization cassette, sealed and then incubated in water in dark at 65^{o}C for 20 hrs in a Techne-Hybridiser HB-1D (Techne, USA).

After hybridization was complete, slides were first washed with shaking in a pre-heated (65^{o}C) buffer solution (1xSSC buffer, 0.05% SDS,) for 2 min and then again with a second buffer solution (0.06xSSC buffer) for 4 min followed by centrifugation at 800 x g for 5 min.

2.14.2.3 Normalization for analysis of RNA expression:

The arrays were scanned with ScanArray Express ™ (Packard Biosciences Biochip Technologies, USA) and generated TIFF images were entered into Bluefuse (BlueGnome Ltd , UK) for Microarrays 3.5 © BlueGnome Ltd using Channel 1 for Cy3 labelled image and Channel 2 for Cy5 labelled image. Gridmap files were obtained from Bacterial Microarray Group at St George's (BUG@S). Unreliable

results with a confidence estimate < 0.1 due to poor hybridization were excluded.

Normalization was performed by importing the Output_fused.xls files into Genespring GX 7.3.1 (Agilent Technologies, USA) for triplicate samples. Dye swap procedure was not used. Data generated by Genespring GX 7.3.1(Agilent Technologies, USA) was statistically analyzed using ANOVA. Gene lists were then imported into Microsoft Office Excel, (Microsoft, UK) for comparison.

2.14.3 Bioinformatics and statistical analysis:

All bioinformatics work including genome comparison and primer designs were performed using CLC Bio® Genomics Workbench. Sequence searches were performed using search engines from Kyoto Encyclopedia for genes and genomes (KEGG) (http://www.genome.jp/kegg), National Center for Biotechnology Information (NCBI) (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi) and The comprehensive microbial resource (CMR) (cmr.jcvi.org).

All statistical analysis was performed in Graph Pad prism. (GraphPad Prism 4.0, USA).

3 Construction of capsular switch mutants:

Pneumococcal capsule is an important virulence factor and is necessary for invasion and pathogenesis. It protects pneumococcus from the host immune system by multiple mechanisms including evasion of opsonins, inhibition of complement activity, protection from phagocytosis and provides resistance from killing after phagocytosis (Peppoloni *et al.*, 2010; Hyams *et al.*, 2010a; Melin *et al.*, 2010). It is electrostatically charged and helps them to avoid the mucosal clearance by electrostatic repulsion (Nelson *et al.*, 2007). It is a complex structure and is made up of multiple sugars bonded with each other to form a complete protective layer around the bacterial cell (Bentley *et al.*, 2006).

There are at least 93 known serotypes of pneumococcal capsule and it has been reported that pneumococci can switch between different serotypes under influence of environmental stress or therapeutic interventions (Golubchik *et al.*, 2012; Croucher *et al.*, 2011; Brueggemann *et al.*, 2007; Munoz *et al.*, 1991; Coffey *et al.*, 1991). The above studies report natural capsular switching in a number of strains though enough data is not available to indicate whether capsular switching is a universal phenomenon or if only a few strains can switch capsules. Many studies indicate that natural capsular switching events are not uncommon in the evolutionary history of pneumococcus. A study analyzing 252 isolates from Scotland reported frequent capsular switching by analyzing number of serotypes expressed by particular sequence types. Many sequence types such as ST 311 (Serotypes 23, 23F, 3 and 9V), ST199 (Serotypes 15, 15B and 9A), ST66 (Serotypes 9N, 9V), ST156 (Serotypes 14,9V and non-typable), ST113 (Serotypes 11A, 18C and non-typable) were found to be expressing more than two serotypes, indicating capsular switching among these strains (Jefferies *et al.*,

2004). Another study in Finland, which analyzed 224 isolates from different cases in Finland also reported a number of sequence types such as ST156 (Serotypes 14,9V,19F), ST162 (9V,19F,14), ST199(15,19A,19F,35F) to be expressing more than two capsule types (Hanage *et al.*, 2005). As many as nine different serotypes have been reported to be expressed by the multidrug resistant clone named Spain^{23F}-1 including serotypes 6B, 9V, 14 19A, 19F, and 23F, 3, 9N, and 15B. Another penicillin-resistant clone Spain 9V-3 was reported to be expressing serotypes 14, 9A,11 and 19 (Croucher *et al.*, 2011; Porat *et al.*, 2006; Mcgee *et al.*, 2001). A detailed study comprising of 240 isolates of Spanish 23F clone showed multiple capsule switching events, with one giving rise to whole sub-population of serotype 19A vaccine-escape mutants in the US (Croucher *et al.*, 2011). Similar findings have been reported in many other studies, which indicate that capsule switching is an important evolutionary phenomenon as it provides an important escape route from vaccines and other therapeutic interventions (Brueggemann *et al.*, 2007; Sandgren *et al.*, 2004; Porat *et al.*, 2004; Ramirez & Tomasz, 1999; Nesin *et al.*, 1998) as it can also be accompanied by acquisition of antibiotic resistance elements (Brueggemann *et al.*, 2007)

Analysis of the effects of capsule switching on pneumococcal biology in these naturally occurring mutants can be difficult and misleading due to the possibility of the presence of other unrelated genes as a result of acquisition of multiple genetic fragments. To circumvent this issue, capsule switch mutants have been constructed, which remain isogenic except for the transformed capsule locus. These mutants have been used to study various aspects of pneumococcal biology. Using the capsule switch mutants, it was reported by Battig and co-workers that the pneumococcal capsule affects rate of growth in capsule switch

mutants (Battig *et al.*, 2006; Sjostrom *et al.*, 2006). It has also been shown to affect pneumococcal virulence in a background-dependent manner by Kelly and co-workers (Kelly *et al.*, 1994), while Sjostrom and co-workers showed that the capsular type is also related to colonization or invasion phenotype (Sjostrom *et al.*, 2006).

To study the capsular effects on pneumococcal biology, several studies have utilized capsule-switch mutants and reported various effects of capsule switching on the pneumococcal behaviour (Trzcinski *et al.*, 2003; Kelly *et al.*, 1994), though data on virulence still remain deficient.

To study role of capsule switching in pneumococcal virulence and other aspects of pneumococcal biology, mutants were constructed in three different backgrounds, the serotype 4 virulent strain TIGR4, serotype 2 virulent strain D39 and the avirulent serotype 4 strain 403, expressing different capsule types.

3.1 Janus intermediate strains:

In this study capsule switch mutants were constructed using Janus technique (Sung *et al.*, 2001), which has advantage of double selection over previously used techniques of capsule knock out and capsule replacement (Pearce *et al.*, 2002). It is a bicistronic cassette, which allows selection for its acquisition as well as removal and contains kanamycin resistance gene *aphIII* and streptomycin sensitivity gene *rpsL* as discussed in Section 2.1.2.

The cassette was amplified and flanked with fragments of genes *dexB* and *aliA*, from *S. pneumoniae* strain R6, so that it can be used to knock out capsule locus (Trzcinski *et al.*, 2003). In this study the cassette was obtained from the Janus strain kindly provided by Professor Mark Lipsitch and was used to construct capsule-knock out mutant in serotype 2 strain D39. The transformation removed the type 2 *cps* locus that is 20kb in size and contains 17 genes as shown in Fig 3.1.

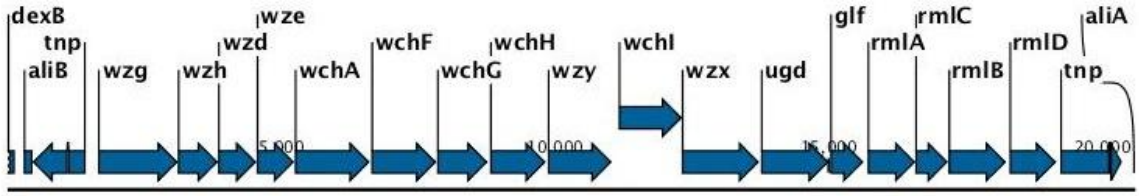


Figure 3.1. Schematic diagram of capsular locus of serotype 2 strain D39, which was replaced by Janus cassette. It is 20kb in length and contains 18 genes. Janus cassette, which was used to knock out capsule locus, was designed with flanking genes *dexB* and *aliA*. . Figure constructed using CLC Genomics workbench (CLC Bio, Denmark)

Streptomycin resistant type 2 strain D39S was constructed from D39 and was transformed with Janus cassette to obtain unencapsulated mutant D39J. The insertion of the cassette knocked out 20kb *cps* locus of D39S as schematically shown in Fig 3.2.

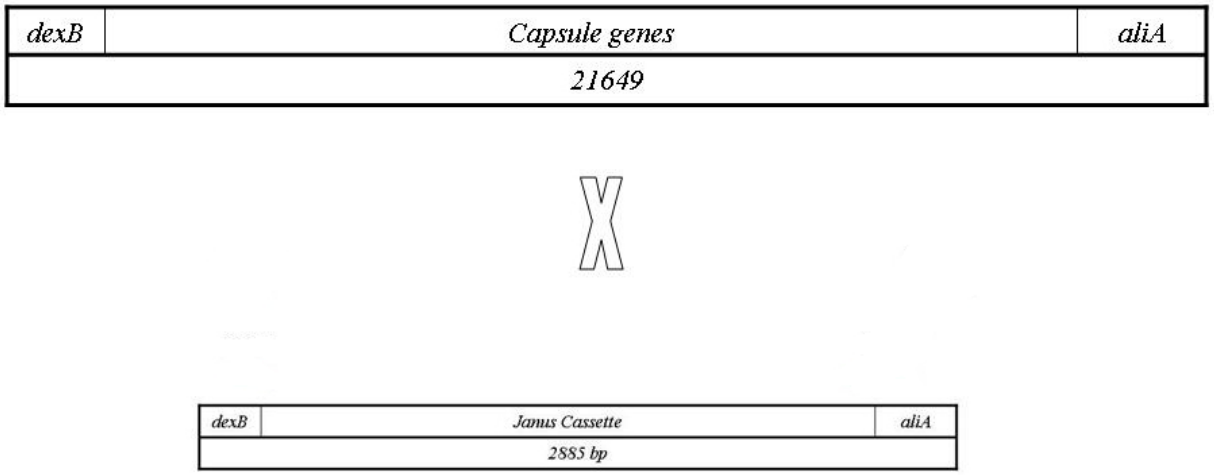


Figure 3.2. Schematic diagram showing replacement of serotype 2 D39 capsule locus by Janus cassette.

Other intermediate strains used for construction of capsule switching 403 Janus intermediate and TIGR4 Janus intermediate were kindly gifted by Professor Mark Lipsitch and Dr. Jeremy S. Brown respectively.

3.2 Construction of capsule switch mutants

Capsule switch mutants expressing serotype 2, 3 and 8 capsules were constructed in three different backgrounds. These mutant strains and their intermediates constructed during the back crossing process are summarized in Tables 3.1, 3.2 and 3.3.

Table 3.1. Capsular switch mutants in TIGR4 background.

Strains	Description
TIGR4^{2}o	TIGR4j derivative of *kan-rpsL*+ ::*cps2* by transformation with D39 chromosomal DNA. Km^{s} Sm^{r}.
TIGR4^{2}1x	TIGR4j derivative of *kan-rpsL*+ ::*cps2* by transformation with TIGR4^{2}o chromosomal DNA. Km^{s} Sm^{r}. 1x backcross transformant.
TIGR4^{2} 2x	TIGR4j derivative of *kan-rpsL*+ ::*cps2* by transformation with TIGR4^{2}1x chromosomal DNA. Km^{s} Sm^{r}. 2x backcross transformant.
TIGR4^{2} 3x	TIGR4j derivative of *kan-rpsL*+ ::*cps2* by transformation with TIGR4^{2}2x chromosomal DNA. Km^{s} Sm^{r}. 3x backcross transformant.
TIGR4^{3}o	TIGR4j derivative of *kan-rpsL*+ ::*cps3* by transformation with OXC141 chromosomal DNA. Km^{s} Sm^{r}.
TIGR4^{3}1x	TIGR4j derivative of *kan-rpsL*+ ::*cps3* by transformation with TIGR4^{3}o chromosomal DNA. Km^{s} Sm^{r}. 1x backcross transformant.
TIGR4^{3} 2x	TIGR4j derivative of *kan-rpsL*+ ::*cps3* by transformation with TIGR4^{3}1x chromosomal DNA. Km^{s} Sm^{r}. 2x backcross transformant.
TIGR4^{3} 3x	TIGR4j derivative of *kan-rpsL*+ ::*cps3* by transformation with TIGR4^{3}2x chromosomal DNA. Km^{s} Sm^{r}. 3x backcross transformant.
TIGR4^{8}o	TIGR4j derivative of *kan-rpsL*+ ::*cps8* by transformation with ATCC6308 chromosomal DNA. Km^{s} Sm^{r}.
TIGR4^{8}1x	TIGR4j derivative of *kan-rpsL*+ ::*cps8* by transformation with TIGR4^{8}o chromosomal DNA. Km^{s} Sm^{r}. 1x backcross transformant.
TIGR4^{8} 2x	TIGR4j derivative of *kan-rpsL*+ ::*cps8* by transformation with TIGR4^{8}1x chromosomal DNA. Km^{s} Sm^{r}. 2x backcross transformant.
TIGR4^{8} 3x	TIGR4j derivative of *kan-rpsL*+ ::*cps8* by transformation with TIGR4^{8}2x chromosomal DNA. Km^{s} Sm^{r}. 3x backcross transformant.

Table 3.2. Capsular switch mutants in D39 background.

Strains	Description
D39S	D39 but Sm^r by transformation with streptomycin resistance cassette. Km^s Sm^r
D39j	D39 but *cps::kan-rpsL*$^+$ Km^r Sm^s
$D39^8$o	D39j derivative of *kan-rpsL+* ::*cps8* by transformation with ATCC6308 chromosomal DNA. Km^s Sm^r.
$D39^8$1x	D39j derivative of *kan-rpsL+* ::*cps8* by transformation with $D39^8$o chromosomal DNA. Km^s Sm^r. 1x backcross transformant.
$D39^8$ 2x	D39j derivative of *kan-rpsL+* ::*cps8* by transformation with $D39^8$1x chromosomal DNA. Km^s Sm^r. 2x backcross transformant.
$D39^8$ 3x	D39j derivative of *kan-rpsL+* ::*cps8* by transformation with $D39^8$2x chromosomal DNA. Km^s Sm^r. 3x backcross transformant.

Table 3.3. Capsular switch mutants in 403 background.

Strains	Description
403^{2}o	403j derivative of *kan-rpsL+* ::*cps2* by transformation with D39 chromosomal DNA. Km^{s} Sm^{r}.
403^{2}1x	403j derivative of *kan-rpsL+* ::*cps2* by transformation with 403^{2}ochromosomal DNA. Km^{s} Sm^{r}. 1x backcross transformant.
403^{2} 2x	403j derivative of *kan-rpsL+* ::*cps2* by transformation with 403^{2}1x chromosomal DNA. Km^{s} Sm^{r}. 2x backcross transformant.
403^{2} 3x	403j derivative of *kan-rpsL+* ::*cps2* by transformation with 403^{2}2x chromosomal DNA. Km^{s} Sm^{r}. 3x backcross transformant.
403^{3}o	403j derivative of *kan-rpsL+* ::*cps3* by transformation with OXC141 chromosomal DNA. Km^{s} Sm^{r}.
403^{3}1x	403j derivative of *kan-rpsL+* ::*cps3* by transformation with 403^{3}o chromosomal DNA. Km^{s} Sm^{r}. 1x backcross transformant.
403^{3} 2x	403j derivative of *kan-rpsL+* ::*cps3* by transformation with 403^{3}1x chromosomal DNA. Km^{s} Sm^{r}. 2x backcross transformant.
403^{3} 3x	403j derivative of *kan-rpsL+* ::*cps3* by transformation with 403^{3}2x chromosomal DNA. Km^{s} Sm^{r}. 3x backcross transformant.
403^{8}o	403j derivative of *kan-rpsL+* ::*cps8* by transformation with ATCC6308 chromosomal DNA. Km^{s} Sm^{r}.
403^{8}1x	403j derivative of *kan-rpsL+* ::*cps8* by transformation with 403^{8}o chromosomal DNA. Km^{s} Sm^{r}. 1x backcross transformant.
403^{8} 2x	403j derivative of *kan-rpsL+* ::*cps8* by transformation with 403^{8}1x chromosomal DNA. Km^{s} Sm^{r}. 2x backcross transformant.
403^{8} 3x	403j derivative of *kan-rpsL+* ::*cps8* by transformation with 403^{8}2x chromosomal DNA. Km^{s} Sm^{r}. 3x backcross transformant.

The placement and the position of *cps* locus in mutants were confirmed in three steps.

1. Redevelopment of kanamycin sensitivity with simultaneous reacquisition of streptomycin resistance.

2. Quellung reaction and agglutination confirming the gain of particular capsule. Agglutination is shown in Fig 3.3

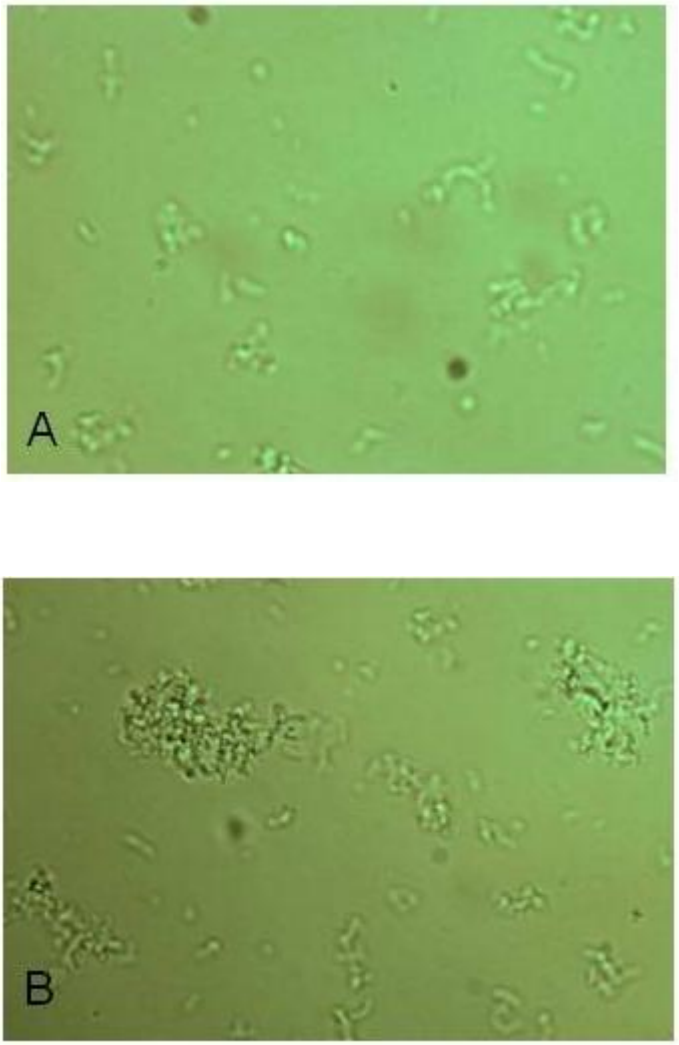


Figure 3.3. Agglutination reaction confirming presence of type 3 capsule in 403[3] resulting from reaction with serotype 3 antiserum (Statens Serum Institute, Denmark). (A) Negative control showing 403[3] in PBS. (B) Positive control showing serotype 3 mutant TIGR4[3] mixed with antiserum.

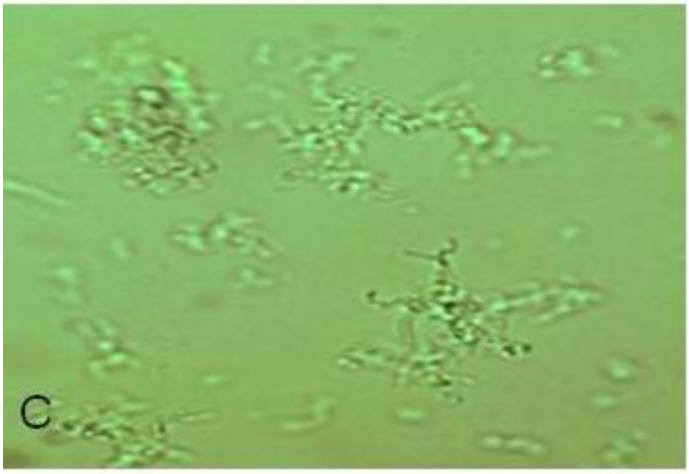
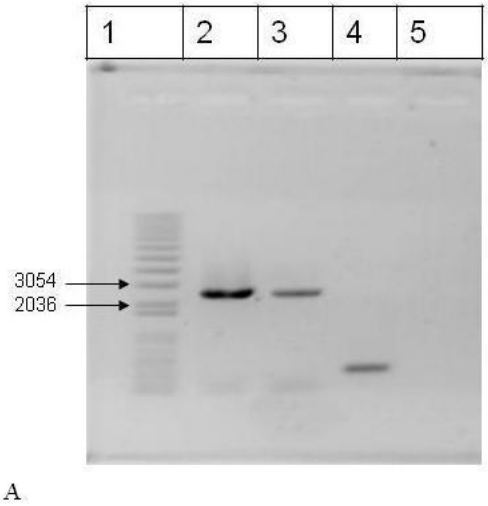


Figure 3.3. Agglutination reaction confirming presence of type 3 capsule in 403[3] resulting from reaction with serotype 3 antiserum (Statens Serum Institute, Denmark). (C) Test strain 403[3] mixed with antiserum. Micrograph taken by Zeiss Acroscope Microscope at 40x.

3. PCR to confirm the presence and position of the cps locus were performed on each side, with one primer in the flanking region and other serotype specific primer within the capsule locus as shown in Figure 3.4 A and B (Schematic diagram in Section 2.1.2).



A

Figure 3.4. (A) Gel photo of PCR amplification for confirmation of transformation of serotype 8 capsule in D39 showing a 2.5 kbp PCR fragment amplified with primers TTM05 and cps2AR confirming the position and placement of the left flank.
Lane 1: 1 kb ladder
Lane 2: PCR fragment from capsule donor strain ATCC6308 genomic DNA amplified with primer pairs TTM05 (*dexB*) and cps2AR (*wzg*).
Lane 3: PCR fragment from recipient strain D39^{8} genomic DNA amplified with primer pairs TTM05 (*dexB*) and cps2AR (*wzg*) confirming identity of left flank in donor and recipient strains.
Lane 4: Positive control pneumolysin.
Lane 5: Negative control with no DNA.

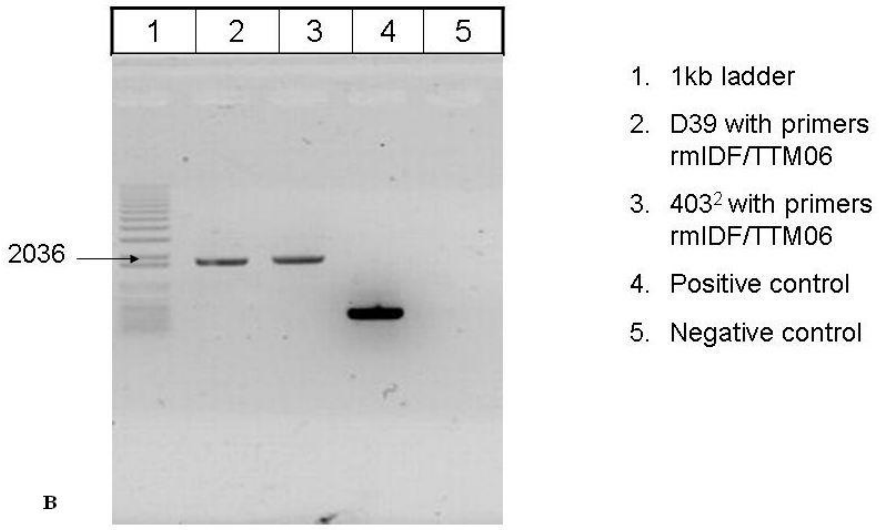


Figure 3.4. (B) Gel photo of PCR amplification for confirmation of the transformation of type 2 capsule in 403 showing approximately 2 kbp PCR fragment amplified with primers TTM06 and rmlDF confirming position and placement of right flank along with confirmation of serotype.
Lane 1: 1 kb ladder
Lane 2: PCR fragment from capsule donor strain D39 genomic DNA amplified with primer pairs rmlDF (*rmlD*) present in serotype 2 capsule locus and TTM06 (*aliA*).
Lane 3: PCR fragment from the recipient strain 403[2] genomic DNA amplified with primer pairs rmlDF (*rmlD*) present in serotype 2 capsule locus and TTM06 (*aliA*).
Lane 4: Positive control pneumolysin
Lane 5: Negative control with no DNA.

3.3 Capsule switch mutants:

Capsular switch mutants were constructed in D39, TIGR4 and 403 to study effects of capsule switching on pneumococcal biology.

3.3.1 Type 2 capsule switch mutants:

Type 2 capsule switch mutants were constructed in 403 and TIGR4 with genomic DNA extracted from D39. The position of capsule was confirmed using primer sets as shown in Fig 3.5.

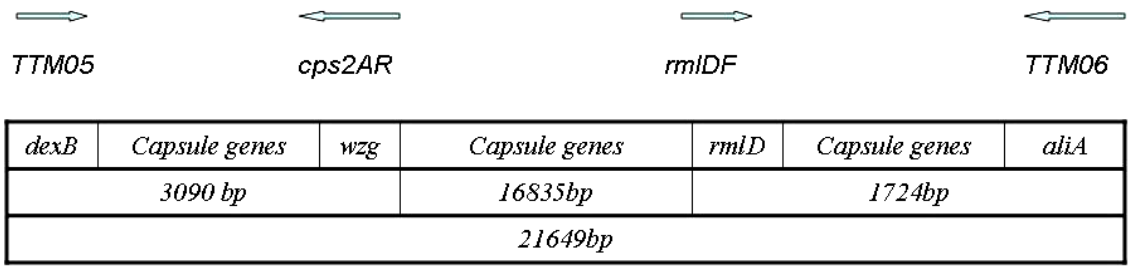


Figure. 3.5. Schematic diagram of serotype 2 capsule locus. The locus is approximately 21kbp and consists of 17 genes. Primers were designed to confirm presence and position of capsule along with the particular serotype.

3.3.2 Type 3 capsule switch mutants:

Type 3 capsule switch mutants were constructed in 403 and TIGR4 with genomic DNA extracted from OXC141. These mutant forms large and mucoid colonies and could be identified with unaided eye (Fig 3.6).

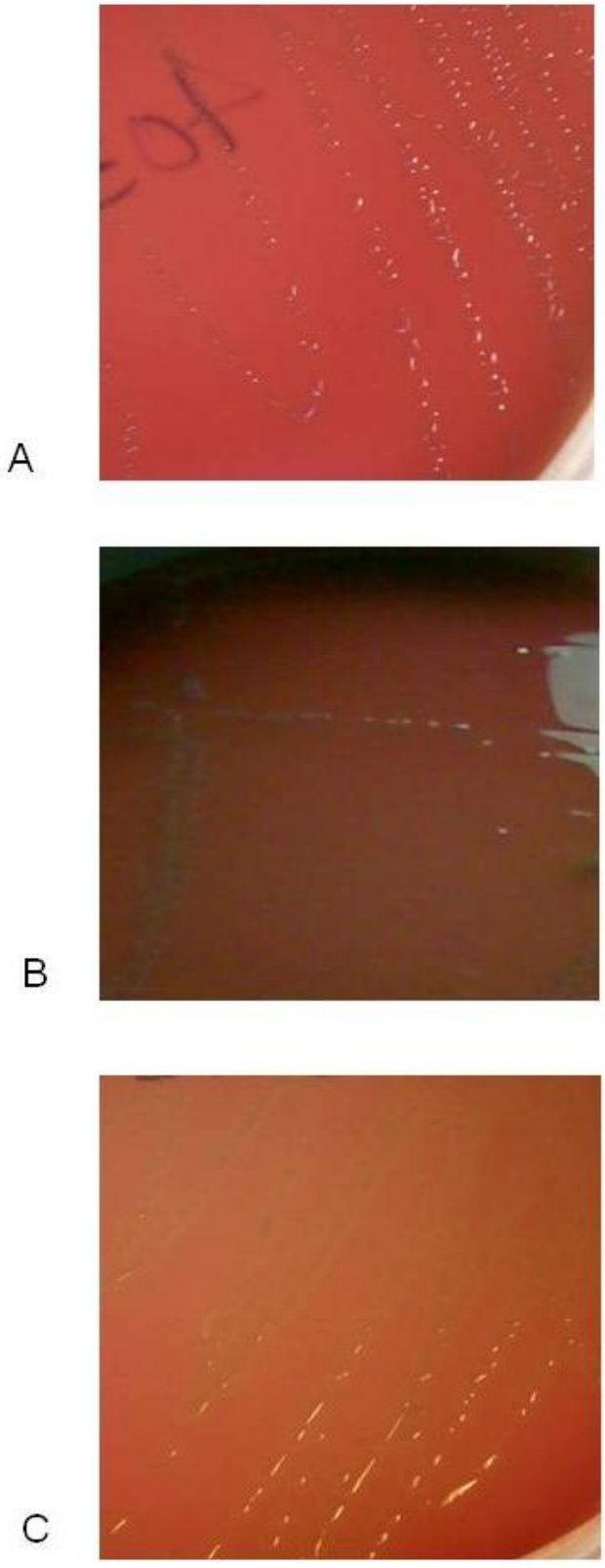


Figure 3.6. Comparison of colony morphology 403,403[3] and OXC141 grown on BAB plates supplemented with 5% Horse blood. (A) 403[3] (B) 403 (C) OXC141

Position of capsule was confirmed using primer sets as shown in Fig 3.7.

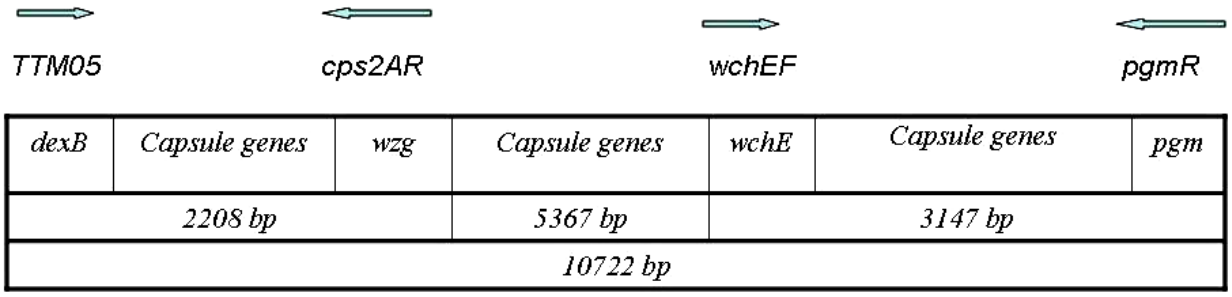


Figure. 3.7. Schematic diagram of serotype 3 capsule locus. The locus is 10 kbp and consists of 9 genes. Primers were designed to confirm presence and position of capsule along with serotype

3.3.3 TIGR4 type 8 capsular switch mutants:

Type 8 capsule switch mutants were constructed in 403, TIGR4 and D39 using genomic DNA from serotype 8 strain ATCC6308. These mutants also formed mucoid colonies and could be identified with unaided eye. The capsule position was confirmed using serotype primer sets as shown in Fig 3.8.

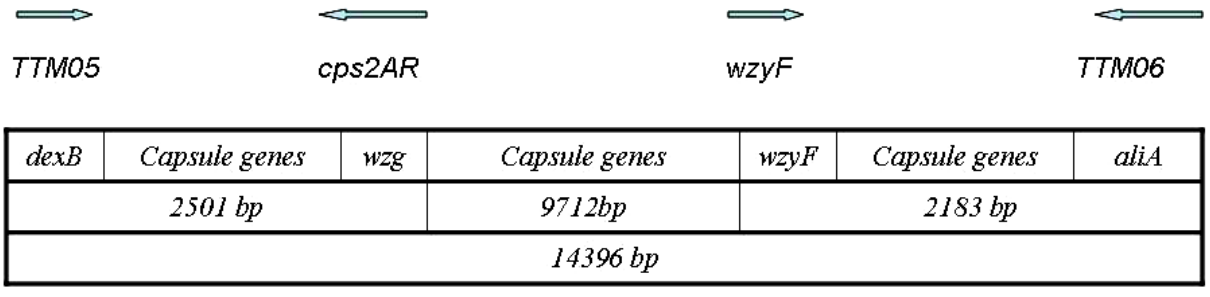


Figure 3.8. Schematic diagram of serotype 8 capsule locus. The locus is 14 kbp and consists of 14 genes. Primers were designed to confirm presence and position of capsule along with serotype.

All type 8 mutants were observed to be forming longer chains as compared to parent strains as shown in Fig 3.9.

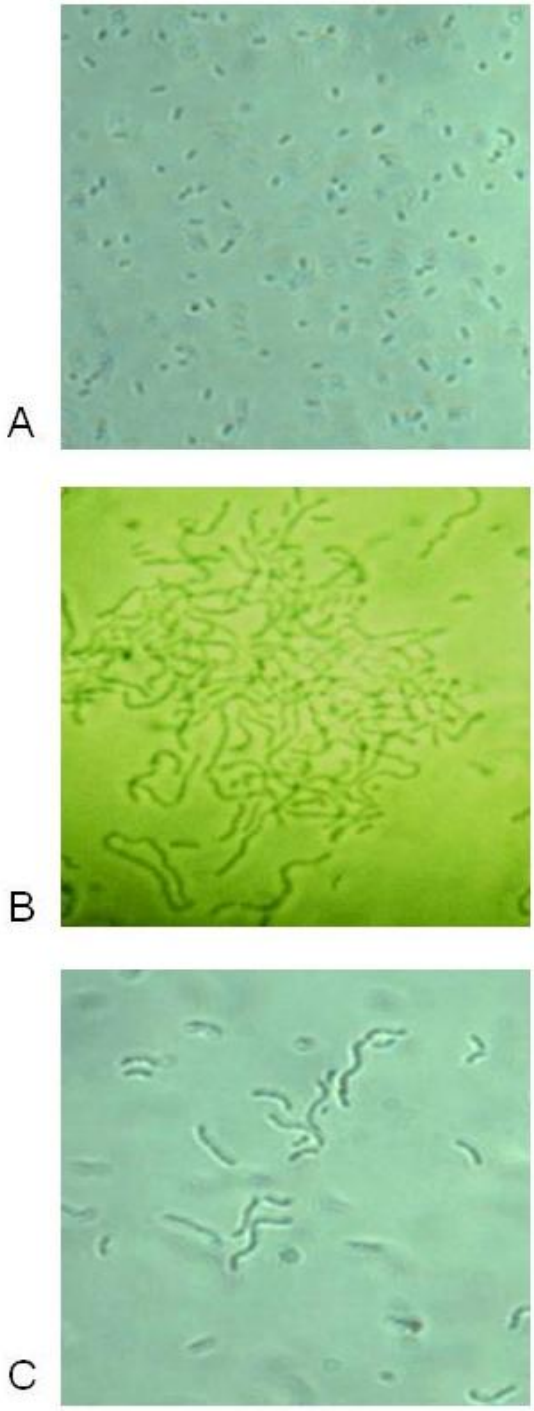


Figure 3.9. Micrographs of strains associating in chains. (A) D39 diplococci (B) D39^{8} long chains (C) ATCC6308 long chains. Micrograph taken with Zeiss Acroscope Microscope at 40x.

These capsule switch mutants were used in a number of downstream experiments to study effect of capsule switching on various *in vitro* and *in vivo* pneumococcal characteristics.

3.4 Discussion:

Capsular switching has an important role in pneumococcal pathogenesis as it provides an opportunity for them to escape from effects of vaccines, which specifically target pneumococcal capsules. Capsular switching events have been reported to occur in several studies as a result of environmental stresses (Croucher *et al.*, 2011; Brueggemann *et al.*, 2007). These events are among countless transformations, which occur in pneumococci and contribute towards their diversity. Pneumococci may acquire other genes along with the capsule genes that may contribute to a particular phenotype (Croucher *et al.*, 2011; Brueggemann *et al.*, 2007). Because of the possibility of the presence of multiple genetic fragments, analysis of capsule switching and its effects on pneumococcal biology in naturally occurring capsule switch mutants may be misleading, as otherwise otherwise isogenic mutants are required to study the effects of capsule switching. These mutants were constructed with or without using Janus technique, within the same and different genetic backgrounds and their variation in growth, colonization and virulence was studied (Trzcinski *et al.*, 2004; Kelly *et al.*, 1994). One of the studies reported that the type of capsule and genetic background both are important for pathogenesis and capsular switching may or not increase pathogenicity of the organism, though

the study was done in different genetic backgrounds (Kelly *et al.*, 1994). Another study showed that type of capsule affects the rate of growth and capsule switch mutants could colonize mouse nasopharynx but did not describe the effects of capsule switching on virulence (Trzcinski *et al.*, 2003).

Available literature also shows that naturally occurring capsule switching is more frequently reported for certain sequence types while a lot of others show more stable populations (Hanage *et al.*, 2005; Jefferies *et al.*, 2004). It is not clear whether capsule switching is only limited to certain clones or if it is a more widespread phenomenon. There is a possibility that switching capsule to certain types could grossly affect phenotype and reduce virulence, which might limit its spread and further propagation in the population as correlation of capsule type with *in vitro* growth rate has been reported, which itself is related to property of the strains being carriage or invasive (Battig *et al.*, 2006)

To study these processes in detail capsular switch mutants were constructed in three different back grounds, which could be compared with one another for a number of *in vivo* and *in vitro* characteristics and possible use as a live attenuated vaccine.

Though characterization of these mutants is discussed in the next chapter in detail some striking differences were observed in the mutants expressing different capsular types. All the serotype 8 mutants, regardless of their genetic background were found to be associating in long chains of about 20 pneumococci. This behaviour was similar to the capsule donor strain ATCC6308, which tends to form long chains and is a highly virulent strain. Long chains have been associated with certain mutations (Barendt *et al.*, 2009) and have been reported to favour colonization (Rodriguez *et al.*, 2012). It has also been

reported that pneumococci and other streptococci form very long chains when they are grown in immune serum (Ekstedt & Stollerman, 1960) and shorter chains are more suitable for invasive phenotype (Dalia *et al.*, 2012). Though the chain length might have an association with the invasiveness of strains but the *in vivo* behaviour has not been reported in the literature.

Another remarkable feature was noted in TIGR4^2, which formed colonies which resembled unencapsulated pneumococci. There is a possibility that due to the difference in the biochemical structure of serotype 2 and serotype 4 capsules TIGR4^2 is unable to sythesize enough capsular polysaccharide.

TIGR4^3 and 403^3 were found to phenotypically similar to their capsule donor strains OXC141, as compare to the parent strains, which might be due to the simple structure of serotype 3 capsule and relatively short *cps* locus of only 10kb. There is a possibility that it can be easily incorporated in other serotypes but it has been reported to affect different serotype backgrounds differently as Kelly and co-workers reported it to be detrimental to the serotype 5 and serotype 6B strains while Nesin and co-workers observed it to be beneficial for serotype 23F as it increased the virulence of the recipient strain.

The effect of capsule switching on pneumococcal biology can be found in the following chapter which also includes a detailed discussion on capsule switch mutants previously used in different experiments.

4 Effect of capsule on pneumococcal biology

The capsule is an important virulence factor for pneumococci. It has at least 93 serotypes, which are structurally different from each other (Calix & Nahm, 2010; Park *et al.*, 2007; Bentley *et al.*, 2006). Pneumococci have the ability to switch their capsule, which is clinically important as it may result in failure of current pneumococcal vaccines that target the polysaccharide capsule. The biological process of capsule switching is not understood very clearly as it is not known if capsule switching is a universal phenomenon or if it can occur only with certain serotypes and certain genetic backgrounds and whether all genetic backgrounds can express all types of capsules. To study effects of capsule switching on pneumococcal biology, experiments were performed to observe effects of different capsules on same and different genetic backgrounds.

4.1 Effect of capsule switching on pneumococcal growth:

Pneumococcal growth *in vitro*, especially length of lag phase, has been observed to be associated not only with capsule serotype but also with the virulence of pneumococcal strains (Battig *et al.*, 2006). Capsule type has been reported to affect pneumococcal growth (Hathaway *et al.*, 2012), while deletion of capsule genes have also been reported to extend duration of lag phase of growth *in vitro* (Battig & Muhlemann, 2007). Capsule switch mutants were studied for effect of different capsules in isogenic backgrounds TIGR4 and 403 by using viable counts as indicator of growth and no differences were found in the duration of lag phase of parent strains and capsule switch mutants (Fig4.1).

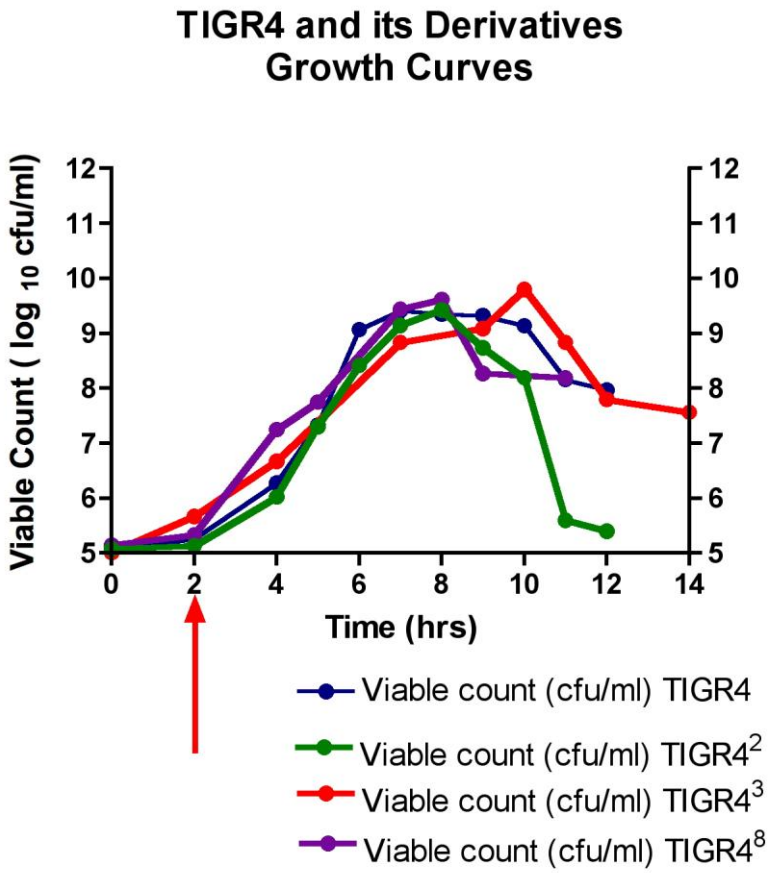


Figure 4.1 Length of lag phase of capsule switch mutants measured by viable counts using three replicate experiments under identical conditions starting with 10^5 cfu. Red arrow marks the end of lag phase in growth curve. Growth curves plotted in Graph pad Prism (GraphPad Prism 4.0, USA).
(A) Similar duration of lag phase in serotype 4 virulent strain TIGR4 and its capsule switch mutants.
$TIGR4^2$: Otherwise isogenic TIGR4 capsule switch mutant expressing serotype 2 capsule
$TIGR4^3$: Otherwise isogenic TIGR4 capsule switch mutant expressing serotype 3 capsule
$TIGR4^8$: Otherwise isogenic TIGR4 capsule switch mutant expressing serotype 8 capsule

The statistical analysis of the slopes of growth curves also did not show any difference in the log phase of growth between capsule switch mutants and TIGR4 as summarized in Table 4.1.

Table 4.1. Linear Regression analysis of the slope of the curve during the log phase of growth in TIGR4 and the capsule switch strains

Strain	Slope 10^8	95% confidence interval 10^8	P values versus TIGR4
TIGR4	6.11	2.03-10.2	
TIGR4^2	6.18	2.66-9.69	0.5216
TIGR4^3	5.94	2.34-9.55	0.8995
TIGR4 ^{8}	7.08	4.93-9.22	0.4459
P1672	5.42	3.14-7.69	0.5932

Analysis performed with GraphPad Prism 4.0, USA)
TIGR4: Virulent type 4 strain
TIGR4^2: Otherwise isogenic TIGR4 capsule switch mutant expressing serotype 2 capsule.
TIGR4^3: Otherwise isogenic TIGR4 capsule switch mutant expressing serotype 3 capsule.
TIGR4^8: Otherwise isogenic TIGR4 capsule switch mutant expressing serotype 8 capsule.
P1672: Unencapsulated TIGR4

The extension of lag phase with deletion of capsule, as has been reported for strain D39 (Battig & Muhlemann, 2007) was also not seen in case of capsule knock out strain of TIGR4 (P1672). Comparison of un-encapsulated TIGR4 retransformed with serotype 4 capsule (P1702) also did not show any difference in growth pattern of the two strains (Fig4.2).

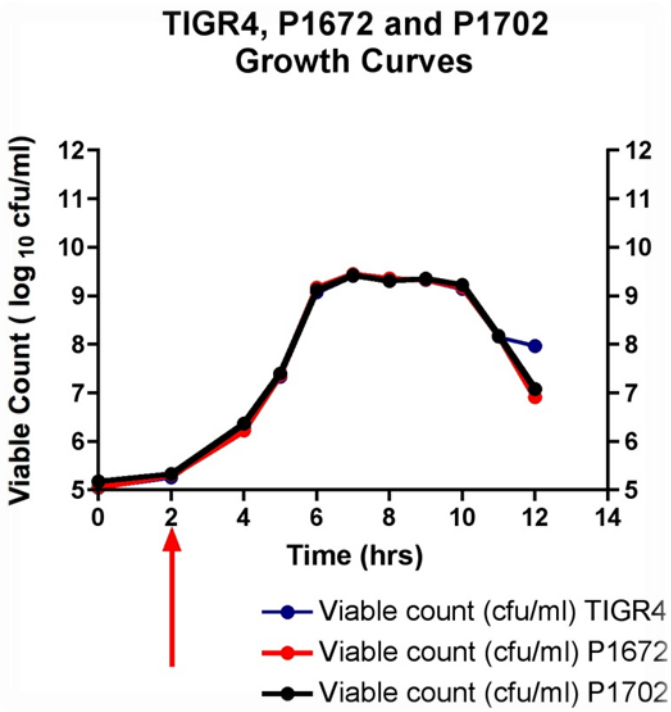
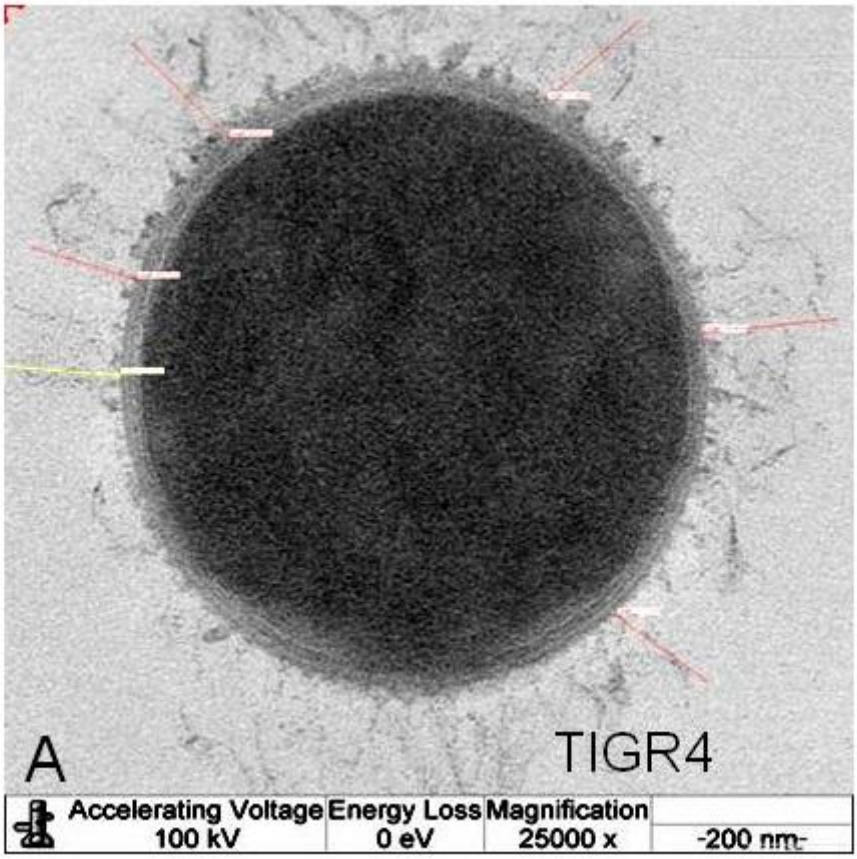
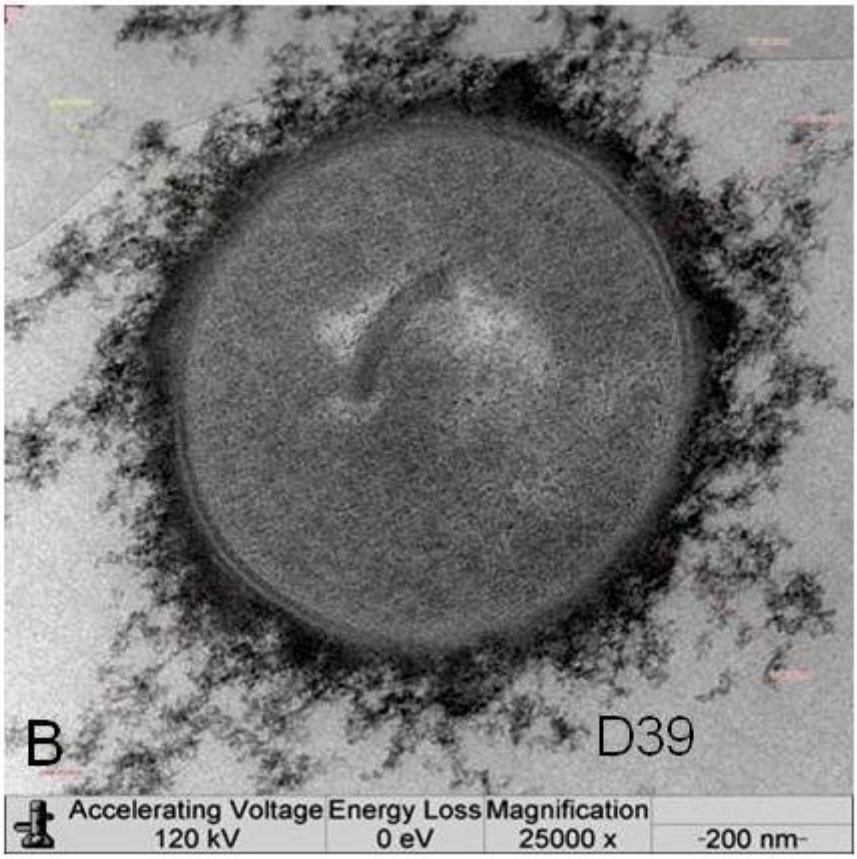


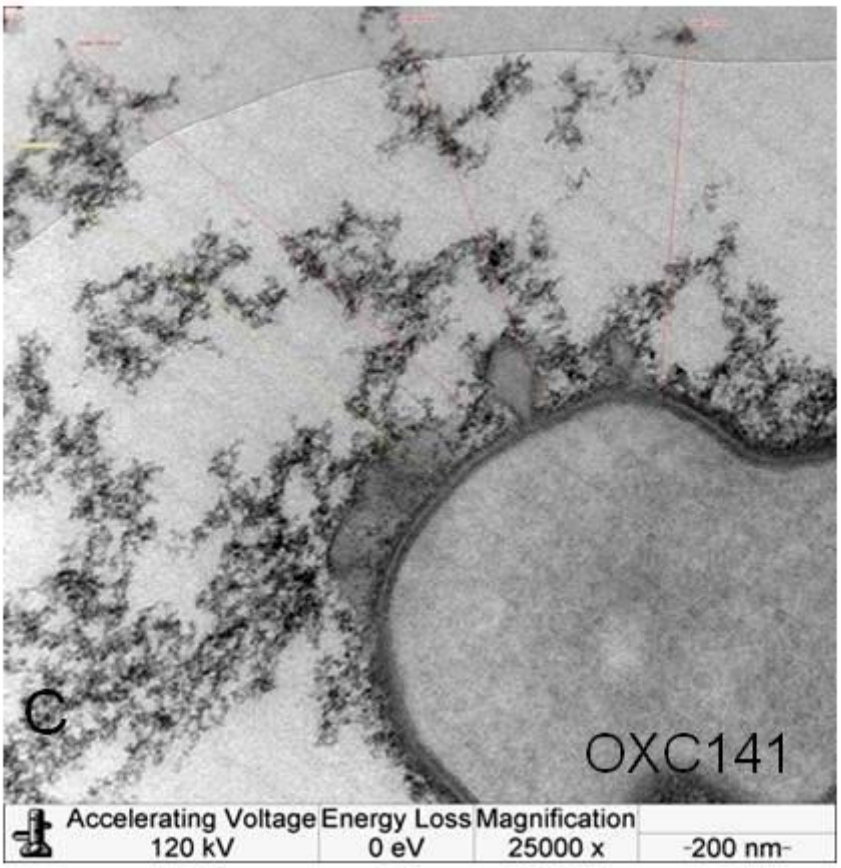
Figure 4.2 Comparison of the length of lag phase of capsule in TIGR4, unencapsulated TIGR4 (P1672) and TIGR4 retransformed with serotype 4 capsule. Curves were plotted using viable counts. Red arrow shows end of lag phase. Data were plotted in Graph Pad Prism. (GraphPad Prism 4.0, USA).

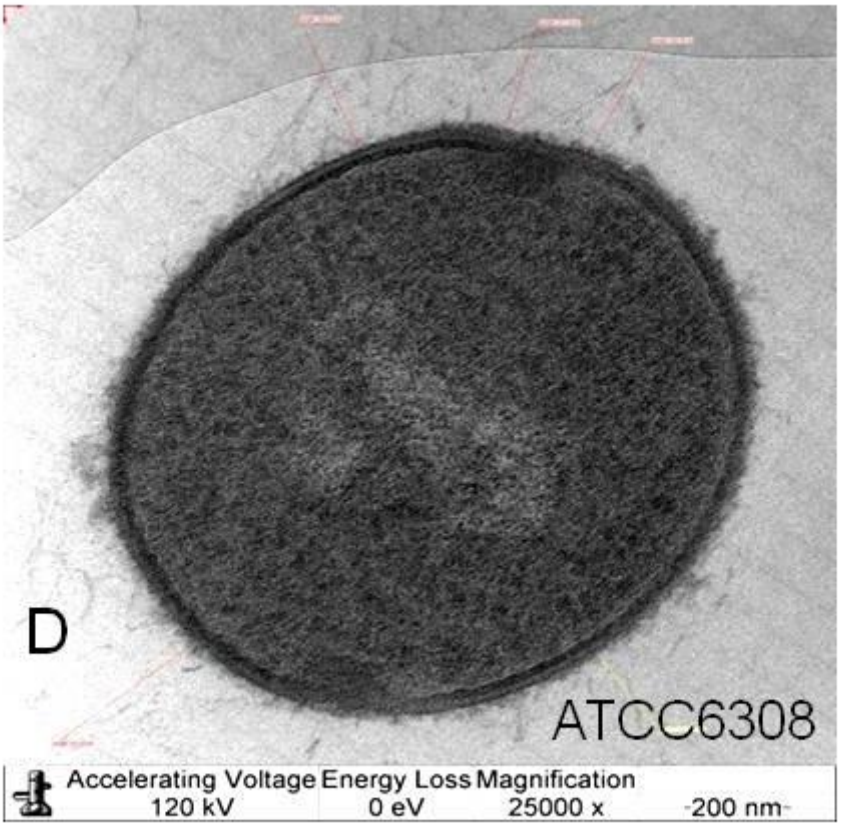
4.2 Effect of capsule switching on capsule-thickness:

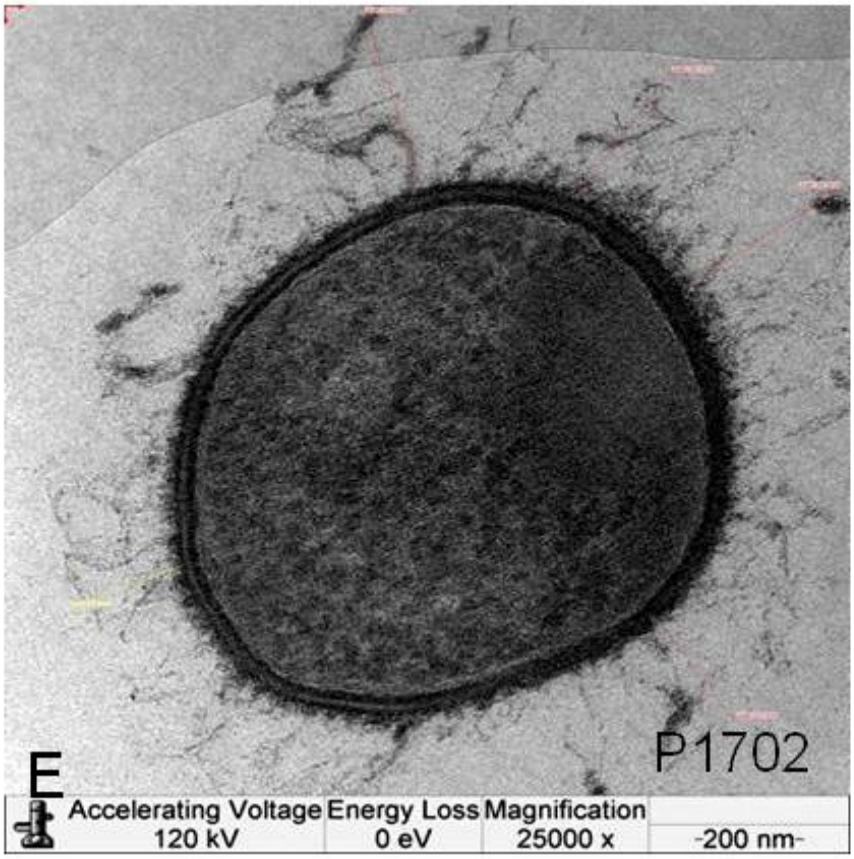
TIGR4, D39 and their capsule switch mutants were studied under electron microscope Leo 912AB (Leo, USA) to observe structural details of capsules of parent strains and their capsule switch mutants. Electron micrographs are shown in Fig 4.3.

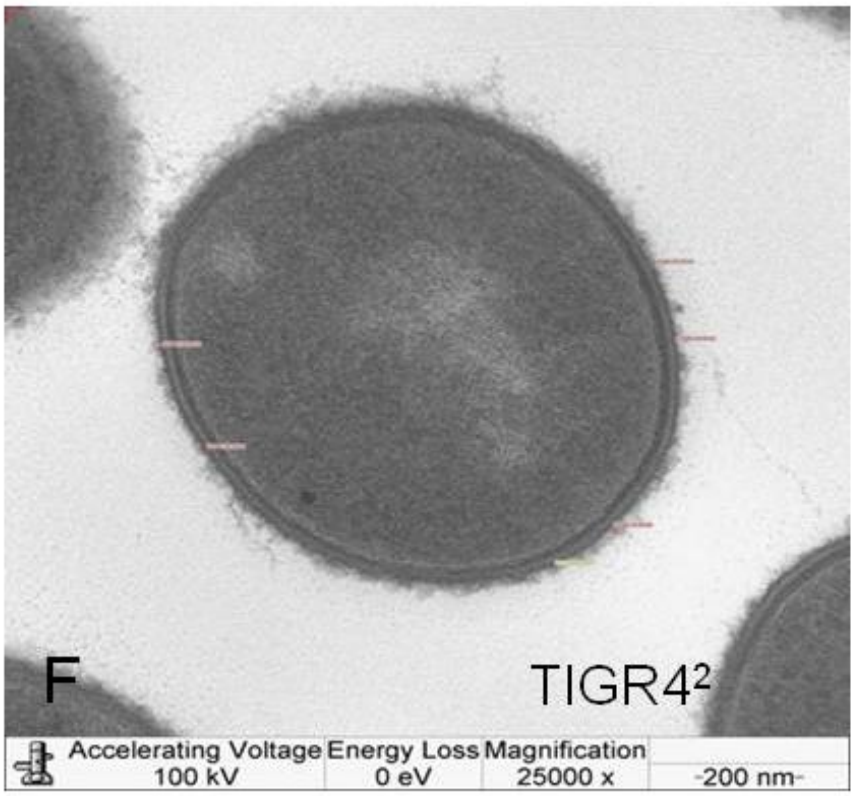

A
TIGR4
Accelerating Voltage
100 kV
Energy Loss
0 eV
Magnification
25000 x
-200 nm-

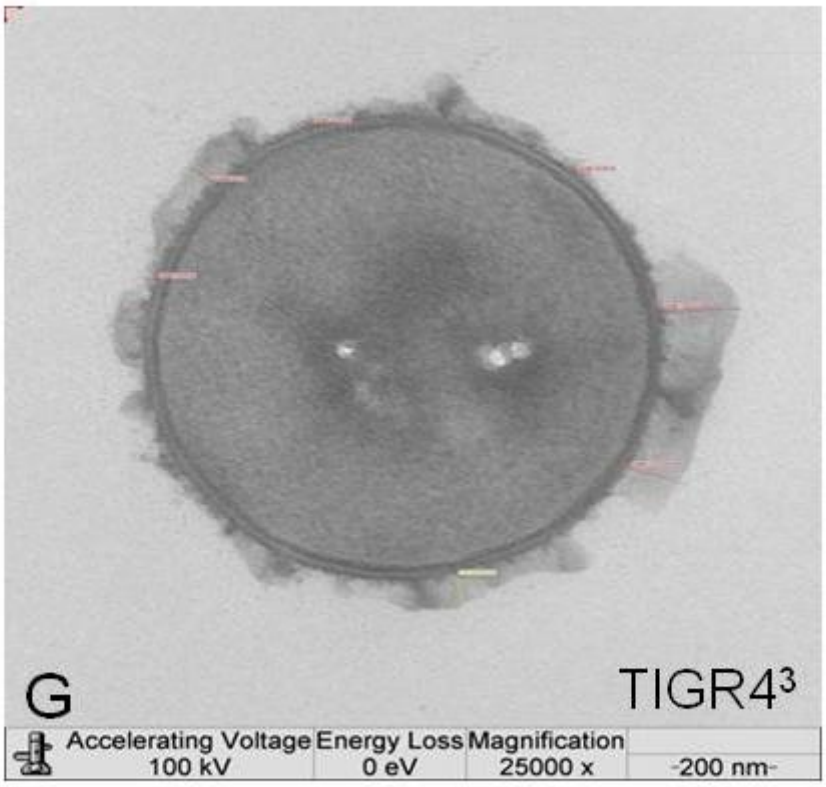

B
D39
Accelerating Voltage
120 kV
Energy Loss
0 eV
Magnification
25000 x
-200 nm-

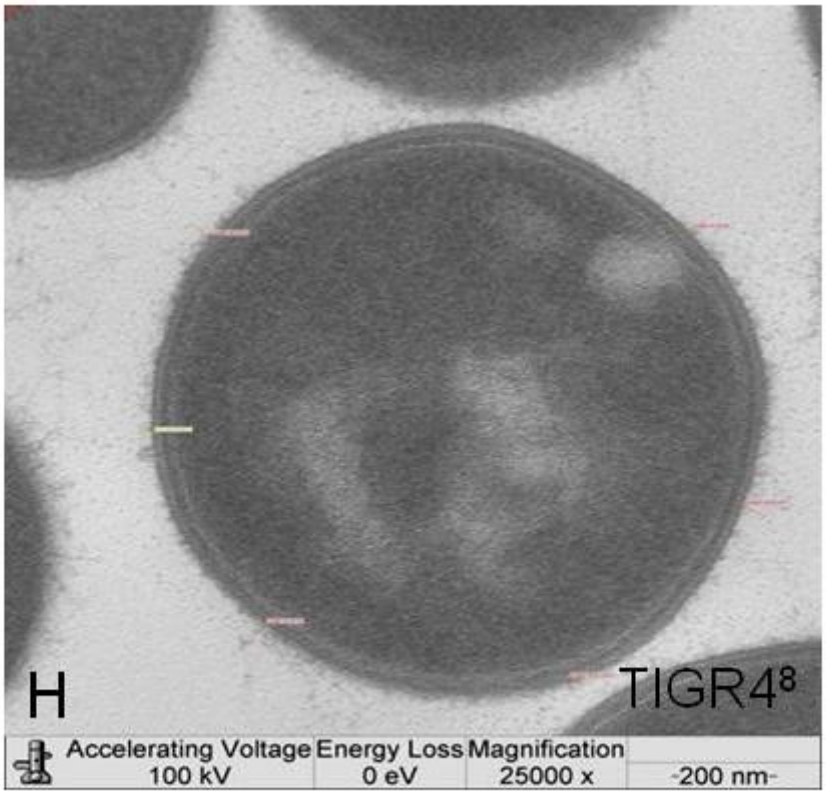

C
OXC141
Accelerating Voltage
120 kV
Energy Loss
0 eV
Magnification
25000 x
-200 nm-

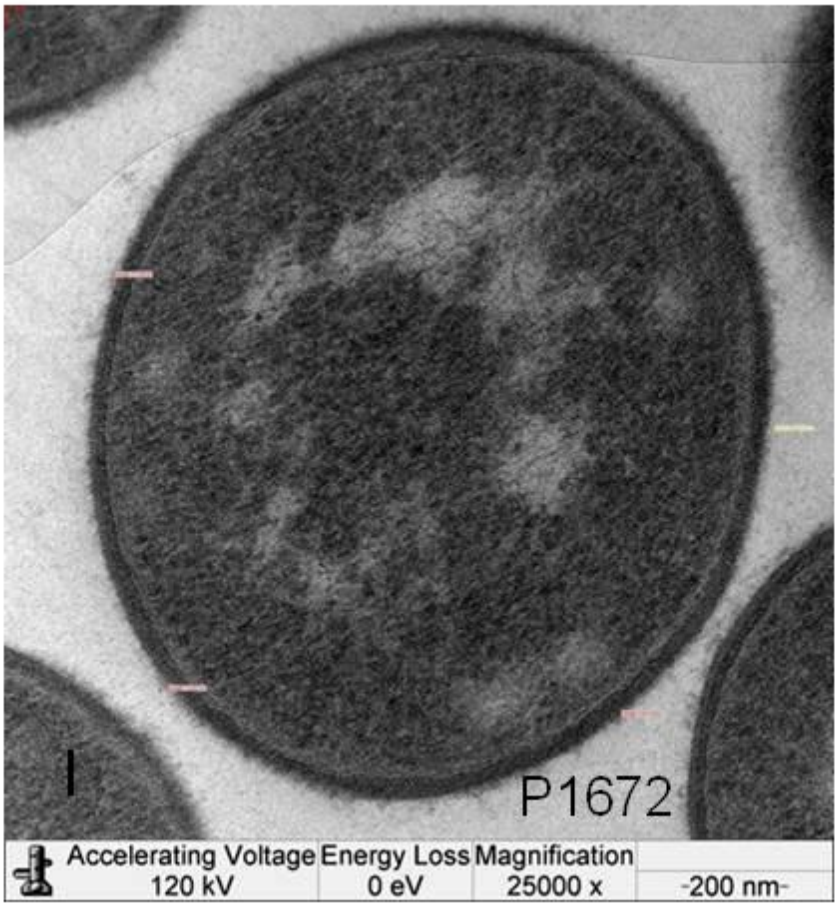

D
ATCC6308
Accelerating Voltage
120 kV
Energy Loss
0 eV
Magnification
25000 x
-200 nm-

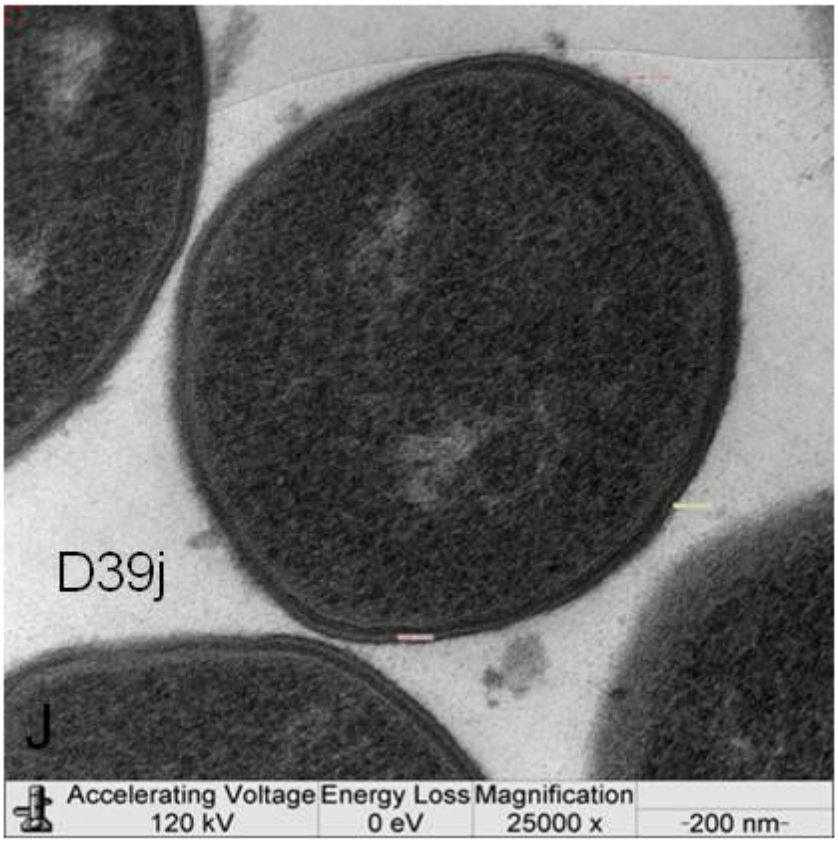

E
P1702
Accelerating Voltage
120 kV
Energy Loss
0 eV
Magnification
25000 x
-200 nm-


F
TIGR4²
Accelerating Voltage
100 kV
Energy Loss
0 eV
Magnification
25000 x
-200 nm-


G
TIGR4³
Accelerating Voltage
100 kV
Energy Loss
0 eV
Magnification
25000 x
-200 nm-


H
TIGR4⁸
Accelerating Voltage
100 kV
Energy Loss
0 eV
Magnification
25000 x
-200 nm-


I
P1672
Accelerating Voltage
120 kV
Energy Loss
0 eV
Magnification
25000 x
-200 nm-


D39j
J
Accelerating Voltage
120 kV
Energy Loss
0 eV
Magnification
25000 x
-200 nm-

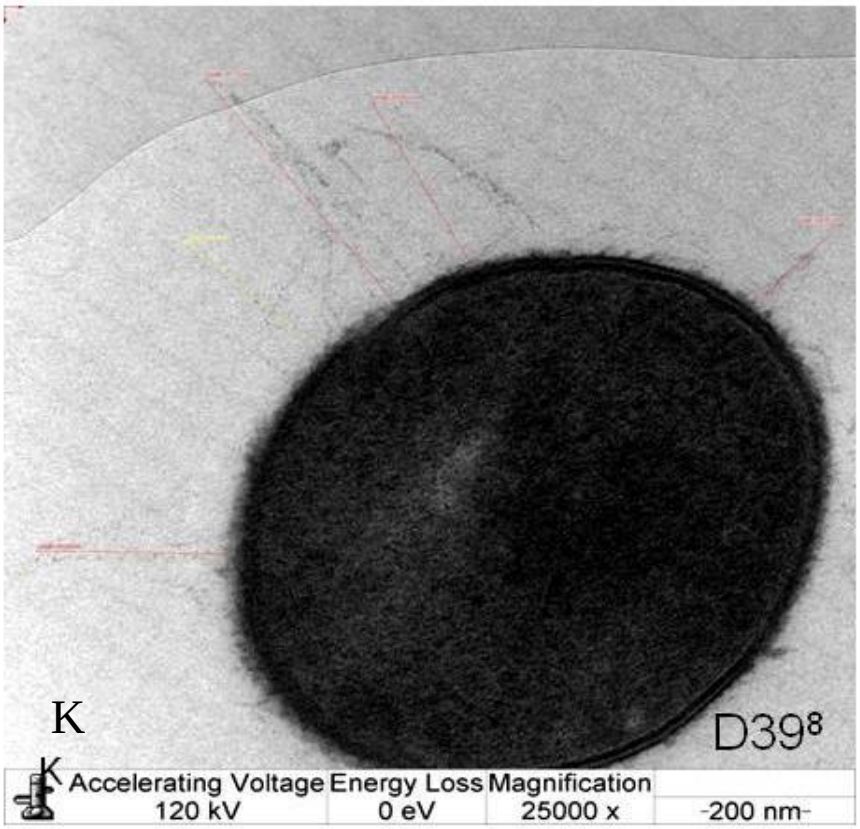


Figure 4.3. Transmission Electron micrographs showing capsule switch, capsule donor and unencapsulated strains obtained with electron microscope Leo 912AB (Leo, USA).
(A) Serotype 4 virulent strain TIGR4.
(B) Serotype 2 virulent strain D39
(C) Serotype 3 virulent strain OXC141
(D) Serotype 8 virulent strain ATCC6308
(E) TIGR4 retransformed with serotype 4 capsule P1702
(F)TIGR4^{2}: Otherwise isogenic TIGR4 capsule switch mutant expressing serotype 2 capsule.
(G) TIGR4^{3}: Otherwise isogenic TIGR4 capsule switch mutant expressing serotype 3 capsule.
(H) TIGR4^{8}: Otherwise isogenic TIGR4 capsule switch mutant expressing serotype 8 capsule.
(I)P1672: Capsule knock out strain TIGR4.
(J) D39j: Capsule knock out strain D39.
(K) D39^{8}: Otherwise isogenic D39 capsule switch mutant expressing serotype 8 capsule.

TIGR4 strain retransformed with serotype 4 capsule (P1702) was used as positive control to see if switching capsule and replacing it back with same type of capsule has any effect on pneumococcal capsule formation, while capsule knock out strains from D39 and TIGR4 were used as negative controls.

Mean capsular thickness was calculated from measuring completely extended capsular fibres at 3-7 points/bacterium in five bacteria. Measurements were taken using image processing software iTEM (Olympus Soft Imaging Solutions, Germany) on areas where cell wall boundaries could be traced easily.

Measurements taken on TIGR4 and P1702 showed that thickness of type 4 capsule was the same as P1702 and there was no significant difference between them (Fig 4.4).

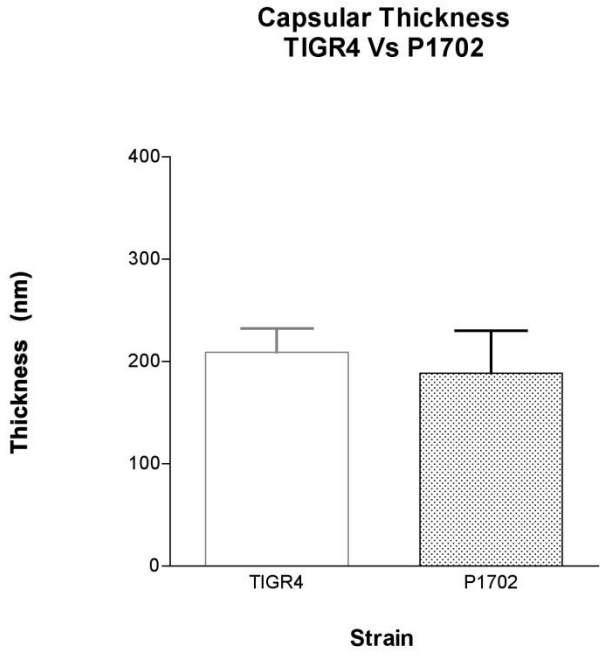


Figure 4.4. Comparison of measurements of capsule thickness in virulent type 4 strain TIGR4 and TIGR4 retransformed with serotype 4 capsule P1702. Data plotted as a bar graph. Bars represent the mean ± SEM. Unpaired t-test showed (GraphPad Prism 4.0, USA) that there was no significant difference in capsular thickness in TIGR4 and P1702. (Number of measurements TIGR4=29, P1702= 25)

TIGR4 expressing serotype 2 capsule showed significantly reduced capsule, which was thinner than both capsule donor serotype 2 strain D39 and parent TIGR4. It was also compared with capsule knock out strain P1672 to see whether capsule formation was taking place or not, which showed some capsule formation in TIGR4^2 (Fig 4.5).

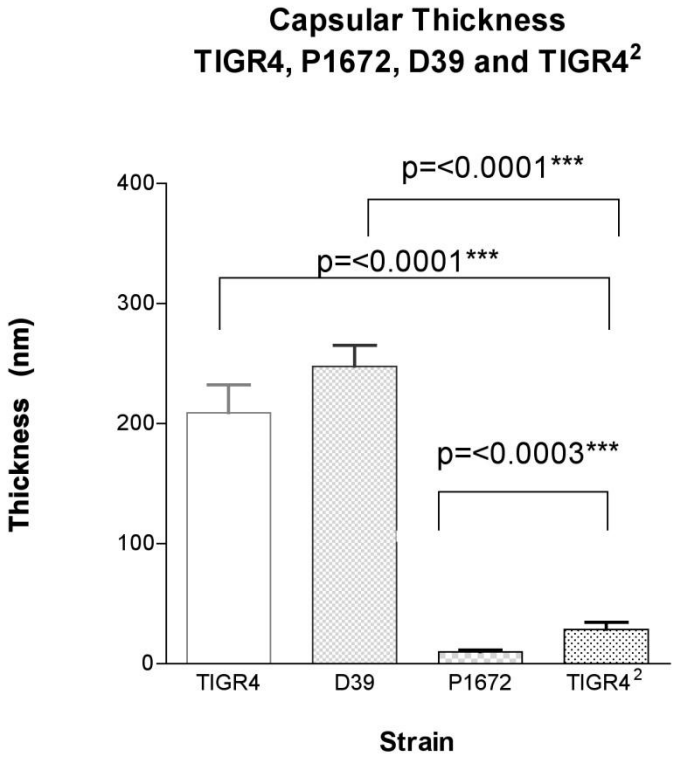


Figure 4.5. Comparison of the measurements of capsular thickness in serotype 4 virulent strain TIGR4, serotype 2 virulent strain D39, Capsule knock-out P1672 and otherwise isogenic TIGR4 expressing serotype 2 capsule TIGR4^2. Data plotted as a bar graph. Bars represent mean ± SEM. Unpaired t-test showed (GraphPad Prism 4.0, USA) that TIGR4^2 capsule was significantly thinner than TIGR4 (p<.0001) and D39 (p<.0001) while was significantly thicker than the unencapsulated strain P1672 (p<.0003). (Number of measurements TIGR4=29, D39=14, P1672=19 TIGR4^2=28)

TIGR4 expressing serotype 3 and serotype 8 capsules also formed reduced capsules which were thinner than both parent and capsule donors (Fig 4.6).

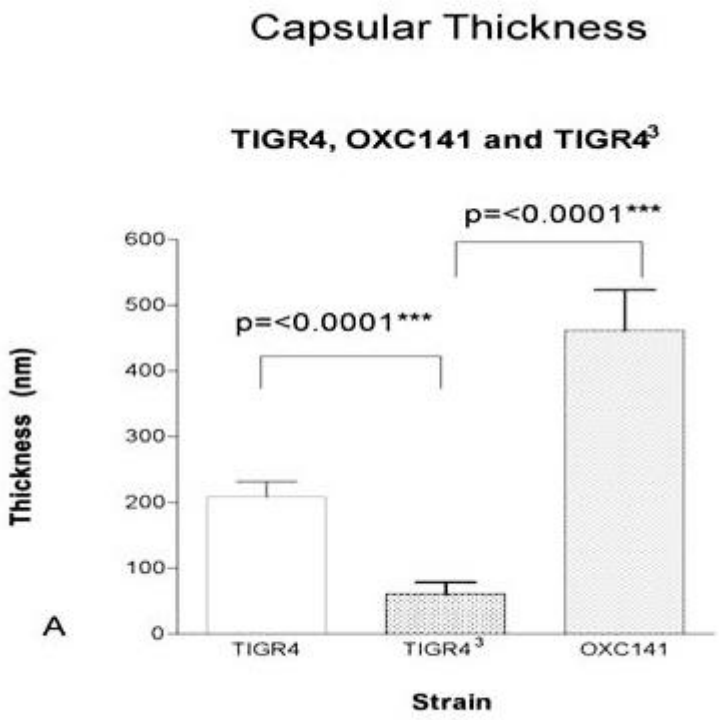


Figure 4.6. (A) Comparison of measurements of capsular thickness in serotype 4 virulent strain TIGR4, capsule donor and otherwise isogenic mutant strains. Comparison of capsular thickness in TIGR4, serotype 3 virulent strain OXC141 and otherwise isogenic TIGR4 expressing serotype 3 capsule TIGR4^3. Unpaired t-test showed (GraphPad Prism 4.0, USA) significant difference (p<.0001) while comparing TIGR4^3 with TIGR4 and TIGR4^3 with OXC141. (Number of measurements TIGR4=29, TIGR4^3=40, OXC141=11)

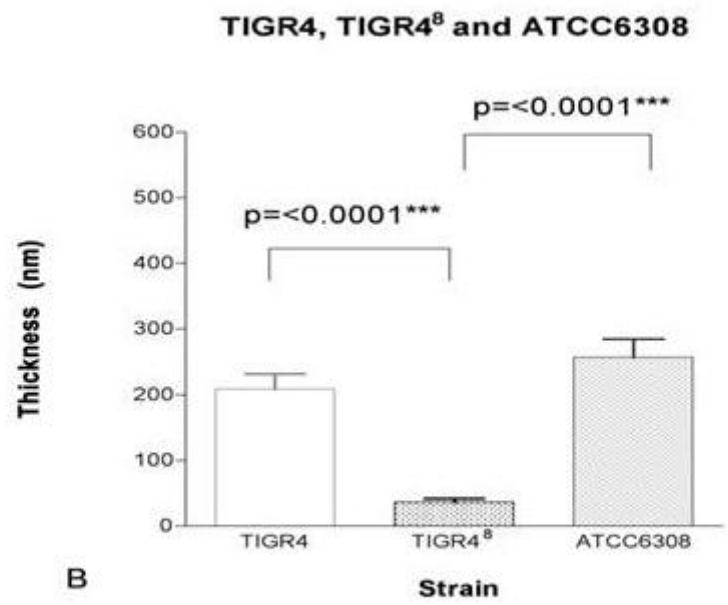


Figure 4.6. (B) Comparison of measurements of capsular thickness in serotype 4 virulent strain TIGR4, capsule donor and otherwise isogenic mutant strains. Comparison of capsular thickness in TIGR4, serotype 8 virulent strain ATCC6308 and otherwise isogenic TIGR4 expressing type 8 capsule TIGR4[8]. Unpaired t-test (GraphPad Prism 4.0, USA) showed significant difference (p<.0001) while comparing TIGR4[8] with TIGR4 and and TIGR4[8] with ATCC6308. Data plotted as bar graphs. Bars represent the mean ± SEM. (Number of measurements TIGR4=29, TIGR4[8]=40, ATCC6308=23)

When type 8 capsule was expressed in a D39 background it was observed that thickness of serotype 8 capsule being expressed in serotype 2 background was same as compared with the capsule donor strain ATCC 6308 (Fig 4.7).

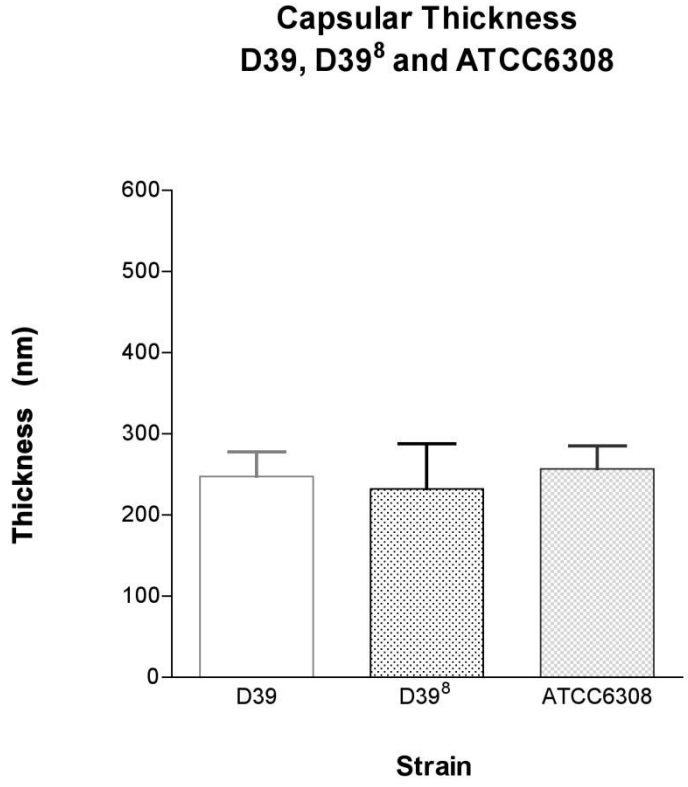


Figure 4.7. Comparison of measurements of capsular thickness in serotype 2 virulent strain D39, serotype 8 virulent strain ATCC6308 and otherwise isogenic D39 expressing serotype 8 capsule D39^8. Unpaired t-test showed (GraphPad Prism 4.0, USA) no significant difference between the capsular thickness of D39^8 when compared with D39 and ATCC6308. Data plotted as bar graphs. Bars represent the mean ± SEM. (Number of measurements TIGR4=29, D39^8=19, ATCC6308=23)

On comparison of thickness of serotype 8 capsule in different backgrounds, it was observed that serotype 8 was thicker in D39 background as compared to TIGR4 background (Fig 4.8).

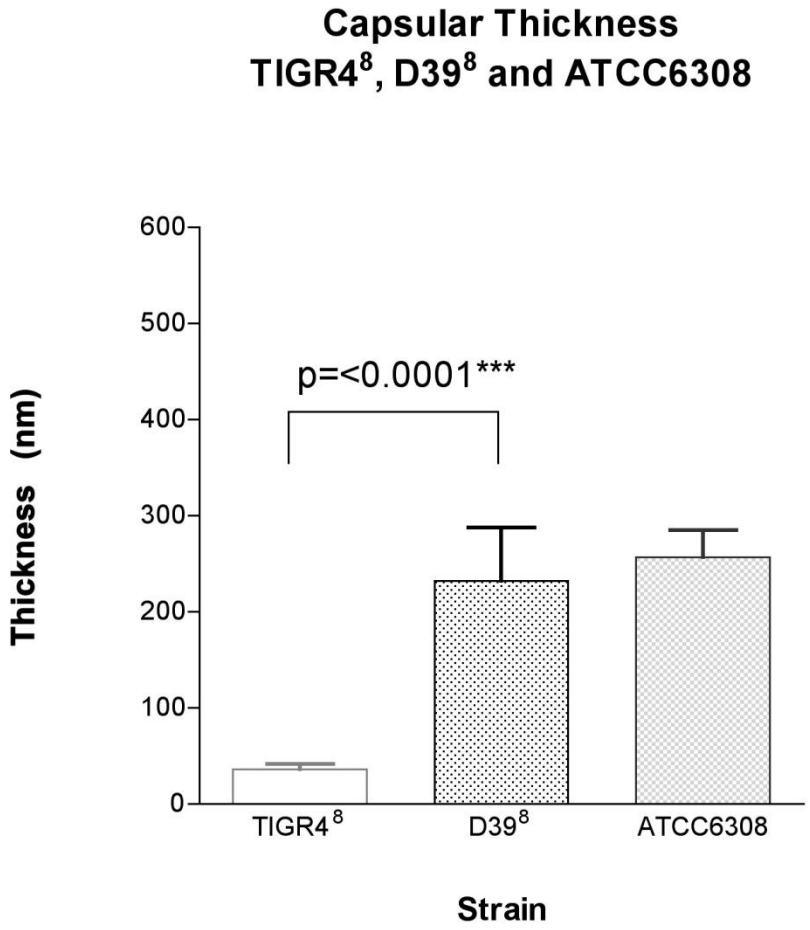


Figure 4.8. Comparison of measurements of capsular thickness in otherwise isogenic TIGR4 expressing serotype 8 capsule TIGR4^8, otherwise isogenic D39 expressing serotype 8 capsule D39^8 and serotype 8 virulent strain ATCC6308. In D39^8, the capsule is much thicker than TIGR4^8. Unpaired t-test showed (GraphPad Prism 4.0, USA) significant difference (p<.0001) between capsule thickness of D39^8 when compared with TIGR4^8. Data plotted as bar graphs. Bars represent the mean ± SEM. (Number of measurements TIGR4^8=40, D39^8=19, ATCC6308=23)

4.3 Capsular polysaccharide in capsule switch strains:

Semi-quantitative Stains-All Assay was performed with Stains-All (Sigma-Aldrich, UK) and relative amount of capsular polysaccharide was measured. Calculated value for 10^9 cfu showed significant differences in capsule formation in different capsule switch strains.

It was observed that there was no difference in TIGR4 and TIGR4 strain replaced with type 4 capsule (Fig 4.9).

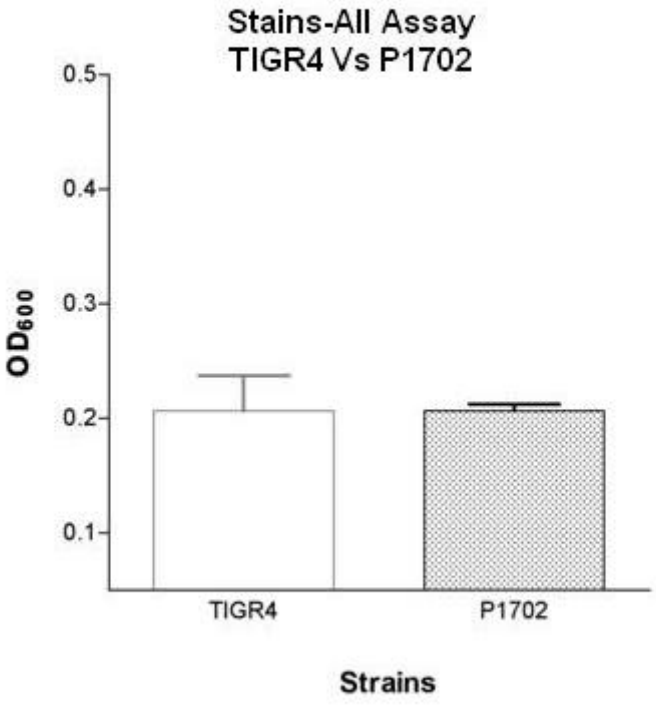


Figure 4.9. Comparison of measurements of capsular polysaccharide for 10^9 cfu by Stains-All Assay of virulent serotype 4 strain TIGR4 and TIGR4 replaced with serotype 4 capsule P1702 using 3 replicate experiments for each. Unpaired t-test showed (GraphPad Prism 4.0, USA) no significant difference between the two strains. Data plotted as bar graphs. Bars represent the mean ± SEM.

There was significant difference between TIGR4 and TIGR4^{2} while no detectable difference was observed in TIGR4 and its mutants expressing serotype 3 and serotype 8 capsules (Fig 4.10).

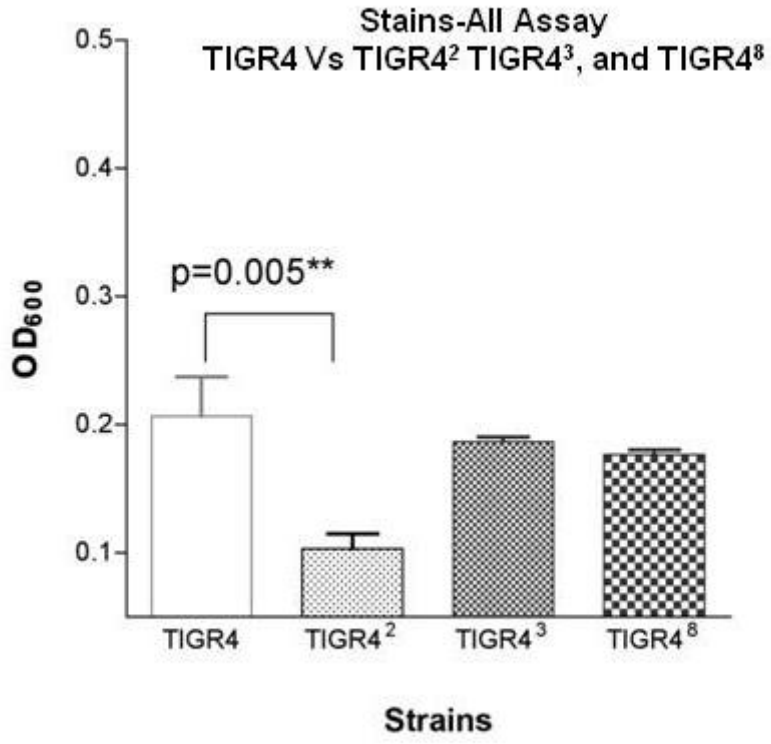


Figure 4.10. Comparison of measurements of capsular polysaccharide for 10^{9} cfu by Stains-All Assay in TIGR4 and capsule switch strains. otherwise isogenic TIGR4 strain expressing serotype 2 capsule TIGR4^{2}, otherwise isogenic TIGR4 strain expressing serotype 3 capsule TIGR4^{3} and otherwise isogenic TIGR4 strain expressing type 8 capsule TIGR4^{8} using 3 replicate experiments for each. Unpaired t-test (GraphPad Prism 4.0, USA) shows significant difference (p<.005) between TIGR4 and TIGR4^{2}. Data plotted as bar graphs. Bars represent mean ± SEM.

On comparison of D39 and D39^{8} it was observed that amount of capsular polysaccharide was significantly higher in D39^{8} as compared to parent strain D39 (Fig 4.11).

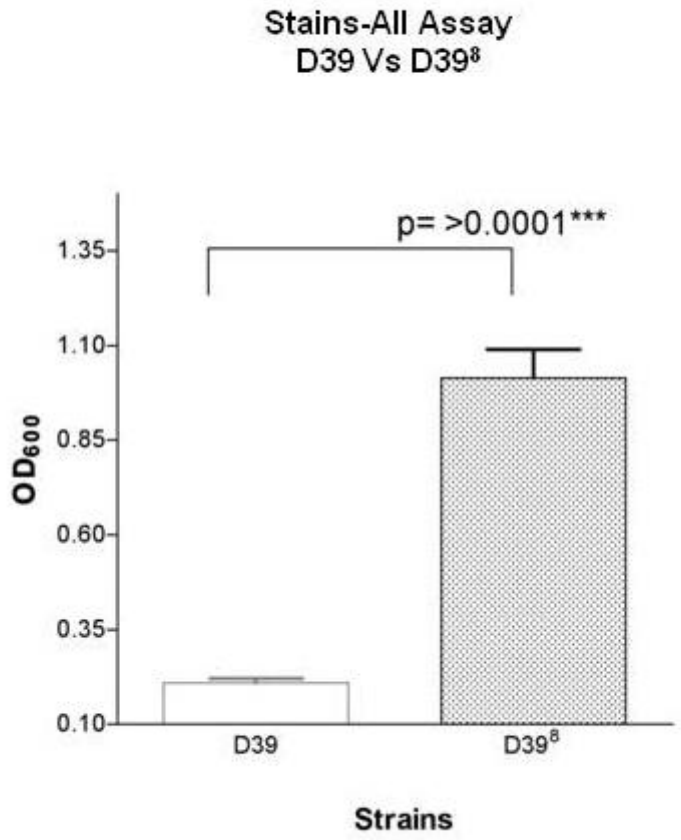
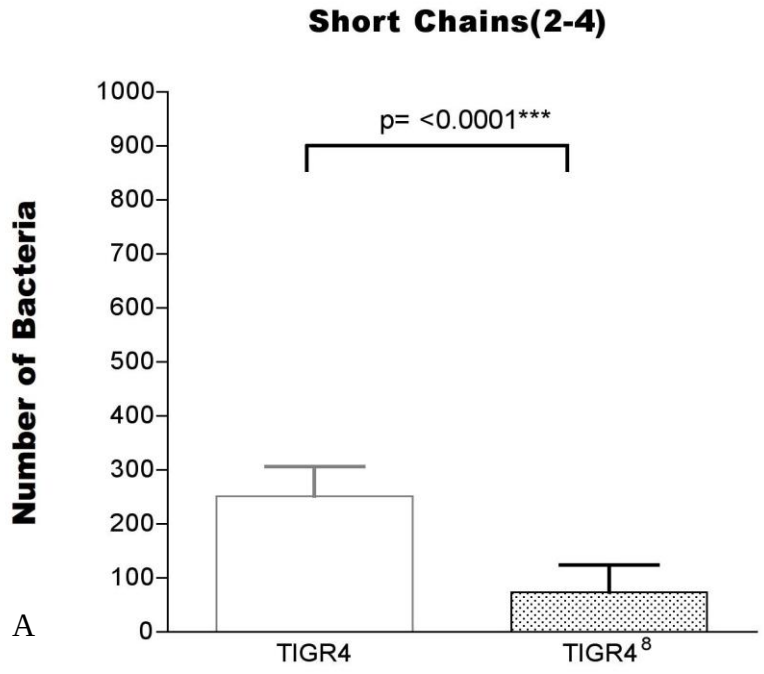


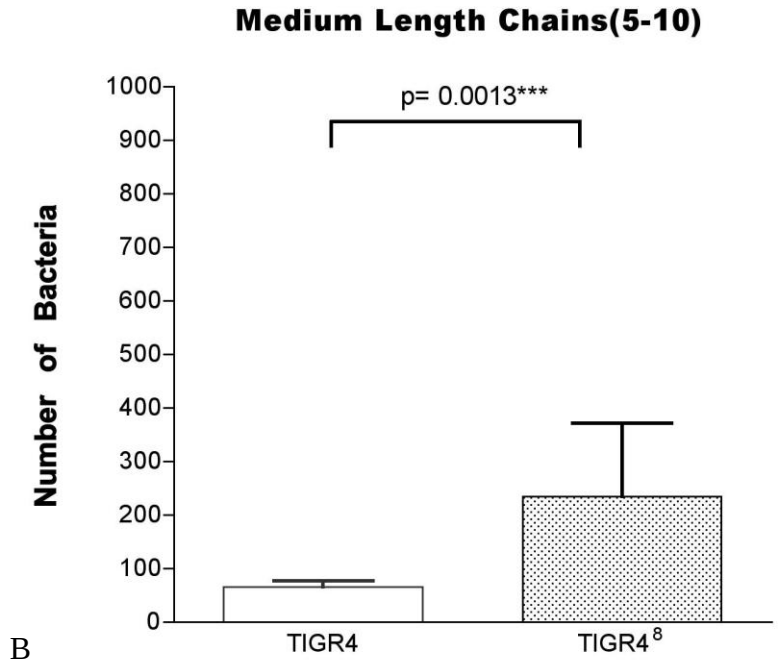
Figure 4.11. Comparison of measurements of capsular polysaccharide for 10^9 cfu by Stains-All Assay of virulent serotype 2 strain D39 and otherwise isogenic D39 expressing serotype 8 capsule $D39^8$ using 3 replicate experiments for each. Unpaired t-test (GraphPad Prism 4.0, USA) shows significant difference between the two strains (p<.0001). Data plotted as bar graphs. Bars represent the mean ± SEM.

4.4 Chain formation:

It has been known for a very long time that *S. pneumoniae* is most commonly found in short chains and pairs. This phenomenon of chain formation has been studied in Group A streptococci and pneumococci for about half a century in the past and certain factors were reported to be affecting their chain length including temperature and pH (Ekstedt & Stollerman, 1960). Some pneumococcal and other streptococcal mutants have also been reported to be forming longer chains (Locke *et al.*, 2007; Sanches-Puelles *et al.*, 1986; Tomasz, 1968). It has also been reported that growth in the presence of immune serum results in increase in chain length in different streptococcal groups (Stewardson-Krieger *et al.*, 1977;Ekstedt & Stollerman, 1960). Relationship of longer chains with pneumcococcal virulence have also been described recently and it was shown that smaller chains are associated with increased virulence (Dalia & Weiser, 2011). In capsule switch mutants it was noted that mutants expressing type 8 capsule in TIGR4 and other backgrounds formed longer chains than TIGR4 itself. This phenomenon was observed in all serotype 8 strains, regardless of their virulence potential (Fig 4.12).

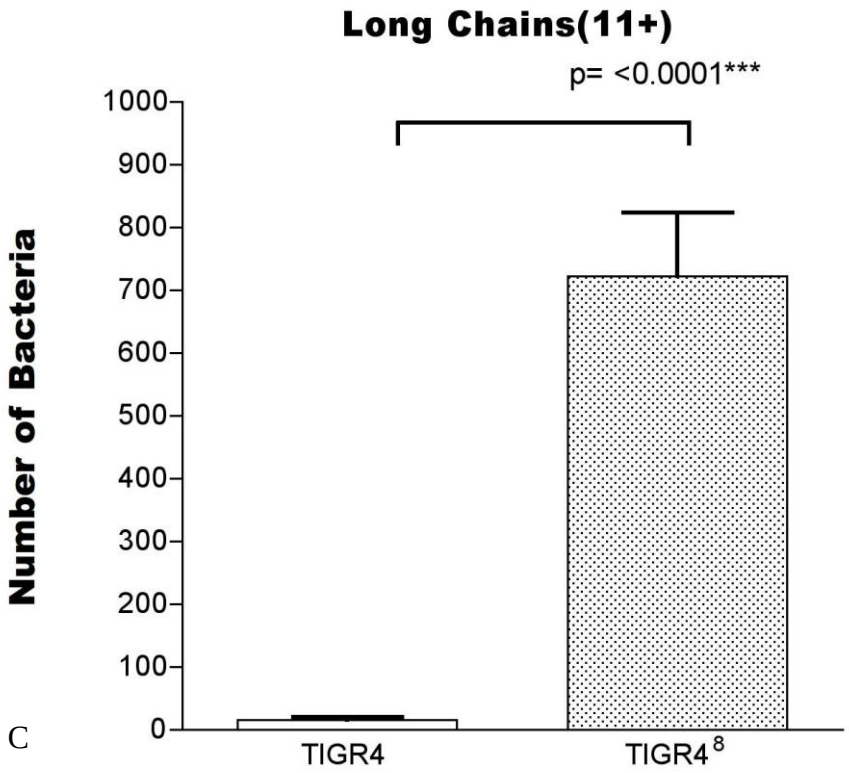


A



B

Figure 4.12. Chain formation in virulent serotype 4 strain TIGR4 and its otherwise isogenic capsule switch mutant expressing serotype 8 capsule TIGR4^{8}. TIGR4 organizes in smaller chains as compared to TIGR4^{8}. Unpaired t-test (GraphPad Prism 4.0, USA) shows (A) The number of bacteria forming short chains is significantly higher in TIGR4 (p<.0001). (B) Number of bacteria forming medium sized chains is significantly higher in TIGR4^{8} (p<.0013).



C

Figure 4.12. Chain formation in virulent serotype 4 strain TIGR4 and its otherwise isogenic capsule switch mutant expressing serotype 8 capsule TIGR4^{8}. TIGR4 organizes in smaller chains as compared to TIGR4^{8}. (C) Number of bacteria associating in long chains is significantly higher in TIGR4^{8} (p<.0001). Data plotted as bar graphs. Bars represent mean ± SEM.

Similar behaviour was observed in D39^{8} as D39^{8} formed longer chains as compared to the parent strain D39, which tend to form short chains (Fig 4.13). 403^{8} also showed the same behaviour (data not shown).

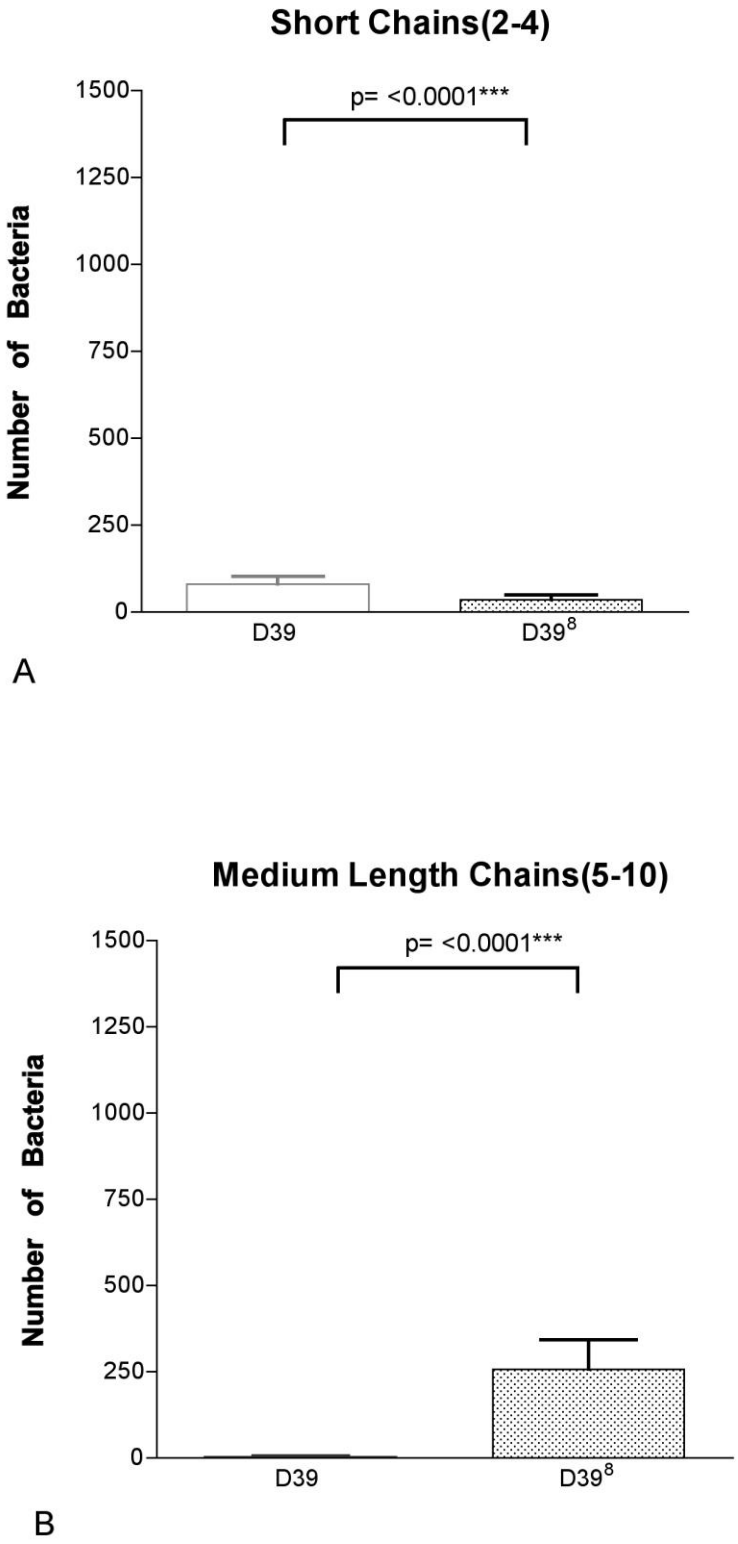


Figure 4.13. Chain formation in serotype 2 virulent strain D39 and its capsule switch mutant expressing serotype 8 capsule D39[8]. D39 organizes in smaller chains as compared to D39[8]. Unpaired t-test (GraphPad Prism 4.0, USA) shows (A) Number of bacteria associating in short chains is significantly higher in D39 (p<.0001). (B) Number of bacteria associating in medium sized chains is significantly higher in D39[8] (p<.0001).

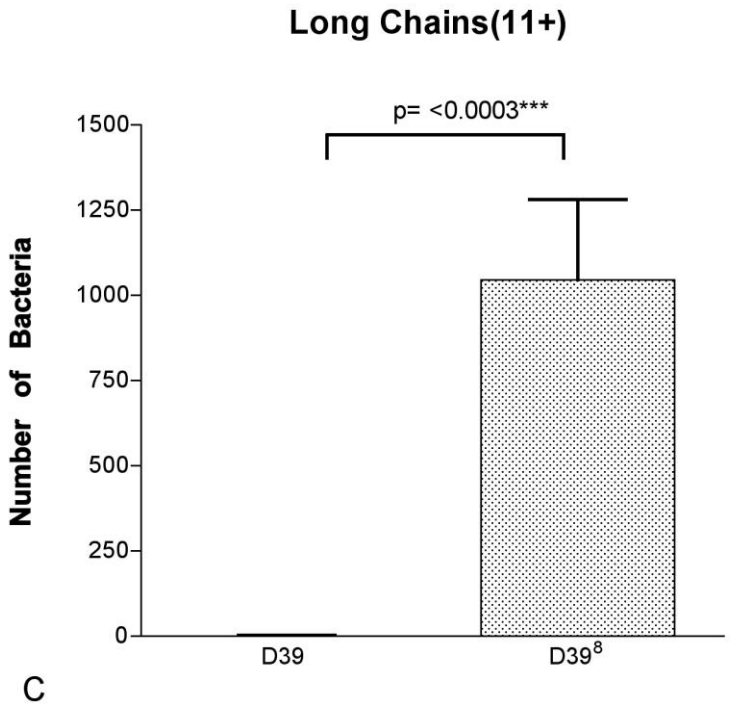


Figure 4.13. Chain formation in serotype 2 virulent strain D39 and its capsule switch mutant expressing serotype 8 capsule D39[8]. D39 organizes in smaller chains as compared to D39[8]. Unpaired t-test (GraphPad Prism 4.0, USA) shows (C) Number of bacteria associating in long chains is significantly higher in D39[8] (p<.0001). Data plotted as bar graphs. Bars represent mean ± SEM.

On studying behaviour of parents it was observed that these strains phenotypically resemble serotype 8 strain ATCC6308, which formed longer chains as compared to shorter chains of D39 and TIGR4 (Fig 4.14).

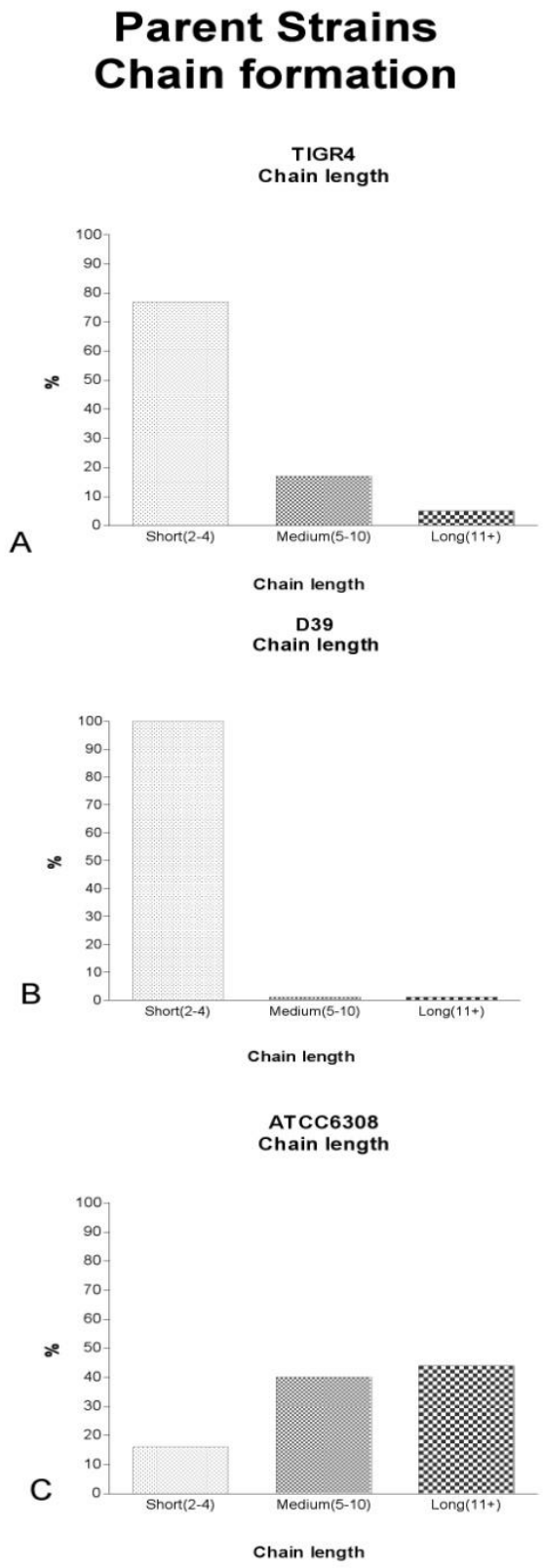


Figure 4.14. Percentage of bacteria organizing in different types of chains in parent strains. (A,B) Serotype 4 virulent strain TIGR4 and seroype 2 virulent D39 organize in smaller chains (C) Serotype 8 virulent strain ATCC6308 associates in medium and longer chains. Data plotted as bar graphs.

4.5 Transformation Efficiency:

Pneumococci are naturally transformable bacteria and this transformability plays an important role in spread of antibiotic resistance, capsular switching and transfer of other important pathogenic factors. Development of competence is a complex phenomenon involving multiple factors (Johnsborg & Havarstein, 2009) and develops for only a very short period during *in vitro* growth (Claverys *et al.*, 2006). Although effect of antibiotics and stress (Johnsborg & Havarstein, 2009; Prudhomme *et al.*, 2006; Claverys *et al.*, 2006) is known to effect competence of pneumococci but no data is available on effect of capsule type or thickness on it though it can be assumed that capsule type, its charge, its thickness or density may have effects on bacterial competence due to physical hindrance.

To study effects of capsular type, TIGR4 and its capsule switch mutants were compared for transformation efficiency using plasmid pVA838 and selecting for erythromycin resistance.

Since capsule switching experiments involve selection for mutants which have been transformable and have picked up capsule genes, there is a chance that subset of population selected after three back-crosses is the most competent one as competence varies among the bacteria (Chen & Morrison, 1987), this method may lead to bias in results if the comparisons are made with parent TIGR4 instead of P1702 (TIGR4 backcrossed with type 4 capsule).

To confirm the above assumption transformation assays were performed in TIGR4 and P1702 and they were found to differ significantly from each other (Fig 4.15).

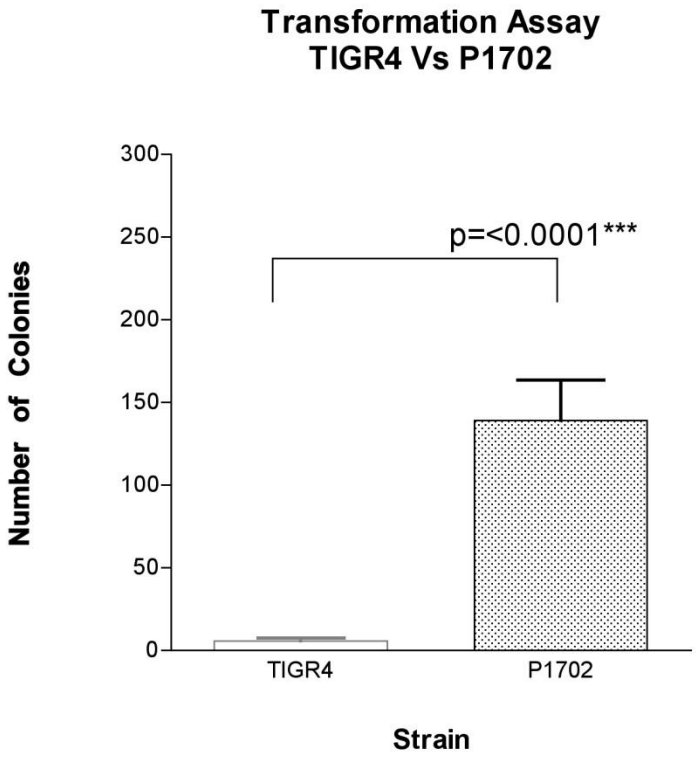


Figure 4.15. Comparison of transformation efficiency between serotype 4 virulent strain TIGR4 and TIGR4 retransformed with serotype 4 capsule P1702. Unpaired t-test (GraphPad Prism 4.0, USA) showed significant difference between the two strains (p<.0001). Data plotted as bar graphs. Bars represent mean ± SEM.

To study effects of capsule on transformation efficiency all comparisons were performed with P1702 strain (Fig 4.16).

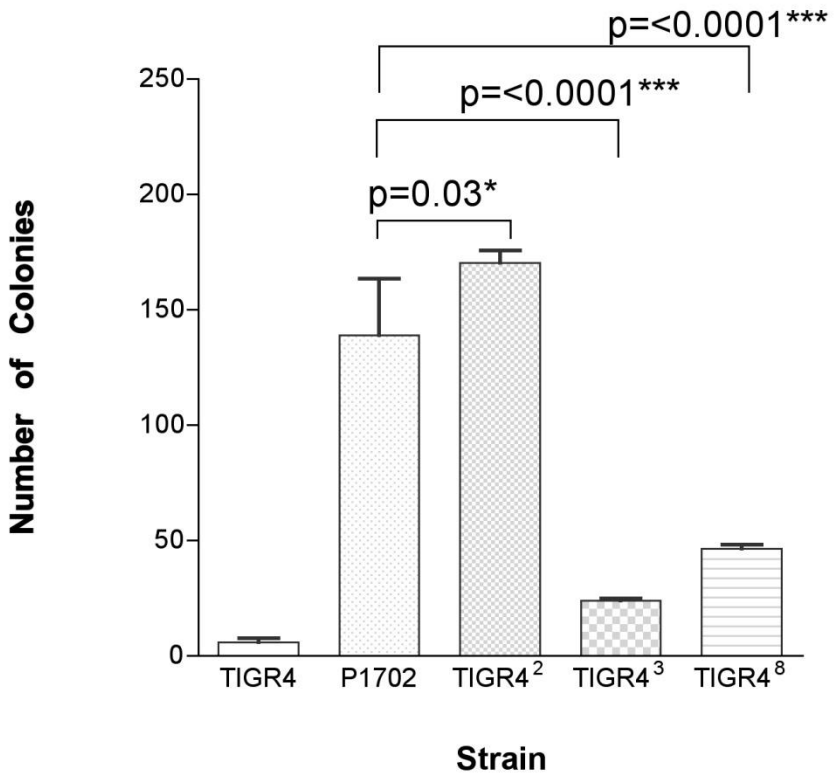


Figure 4.16. Comparison of transformation efficiency between virulent serotype 4 strain TIGR4, TIGR4 retransformed with serotype 4 capsule P1702, otherwise isogenic TIGR4 expressing serotype 2 capsule TIGR4^{2}, otherwise isogenic TIGR4 expressing serotype 3 capsule TIGR4^{3} and otherwise isogenic TIGR4 expressing type 8 capsule TIGR4^{8}. Unpaired t-test (GraphPad Prism 4.0, USA) shows significant difference between P1702 and other capsule switch strains. TIGR4^{2} is significantly more transformable than P1702 (p<.03). While TIGR4^{3} and TIGR4^{8} are significantly less transformable than P1702 (p<.0001). Data plotted as bar graphs. Bars represent the mean ± SEM.

Correlation studies (GraphPad Prism 4.0, USA) showed that transformation efficiency in capsule switch mutants was not correlated with capsule thickness (Pearson's R^2 0.07, p = 0.72) or amount of capsule associated polysaccharide (Pearson's R^2 0.27, p = .48).

4.6 Virulence:

To study virulence of capsule switch strains and to compare it with their parents, parent and capsule donor strains were initially tested in mouse models of intraperitoneal infection. Infection studies with 10^5 cfu showed marked differences between four strains. Median survival time for mice infected with ATCC6308 was 13 hrs, TIGR4 was 21 hrs, OXC141 was 21 hrs and D39 was 23 hrs (Fig 4.17).

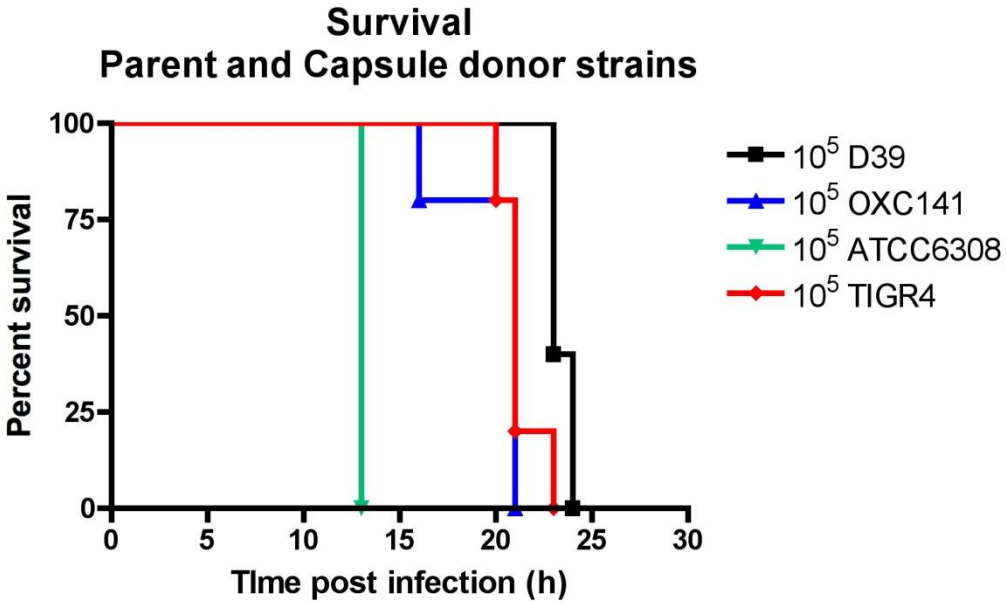


Figure 4.17. Virulence of capsule donor and parent strains. Kaplan-Meier curve showing survival of mice infected intraperitoneally with 10^5 cfu virulent serotype 2 strain D39, virulent serotype 3 strain OXC141, virulent serotype 8 strain ATCC6308 and virulent serotype 4 strain TIGR4, All data plotted as percentage survival as a staircase line with points for all observations against hrs post infection. Five mice used per strain.

To study whether capsule switching has any effect on virulence, low doses of TIGR4 and P1702 were compared against each other, which showed no difference in virulence of the two strains (Fig 4.18).

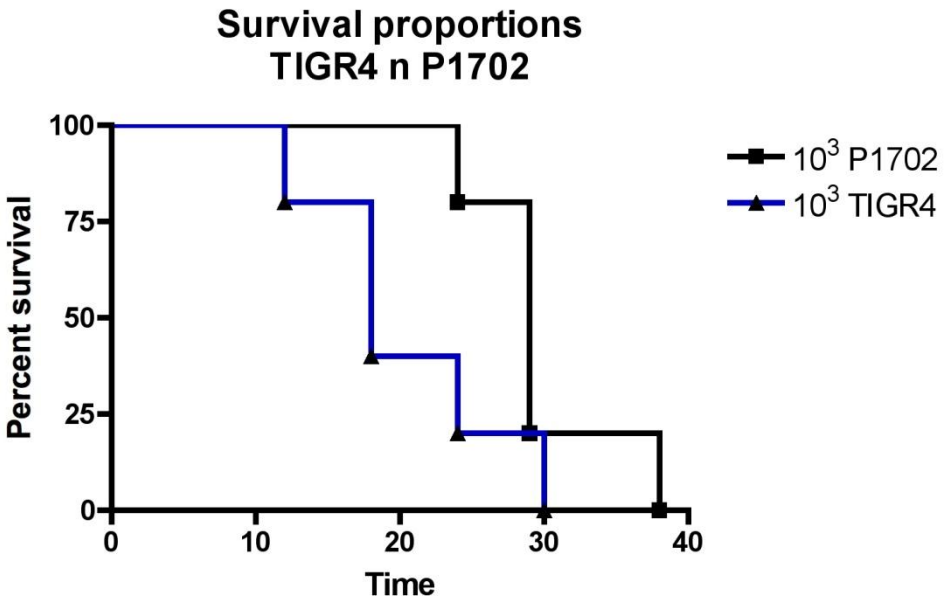


Figure 4.18. Virulence of serotype 4 virulent strain TIGR4 and TIGR4 retransformed with P1702. Kaplan-Meier curve showing survival of mice infected with 10^3 cfu TIGR4 and P1702 intraperitoneally. All data plotted as percentage survival as a staircase line with points for all observations against hrs post infection. Survival analysis showed there was no significant difference in survival time between the two groups by log rank test. All data plotted as percentage survival as a staircase line with points for all observations against hrs post infection. Five mice used per strain.

Mice infected with TIGR4[2] showed no sign of infection and survived till the end of experiment (Fig 4.19).

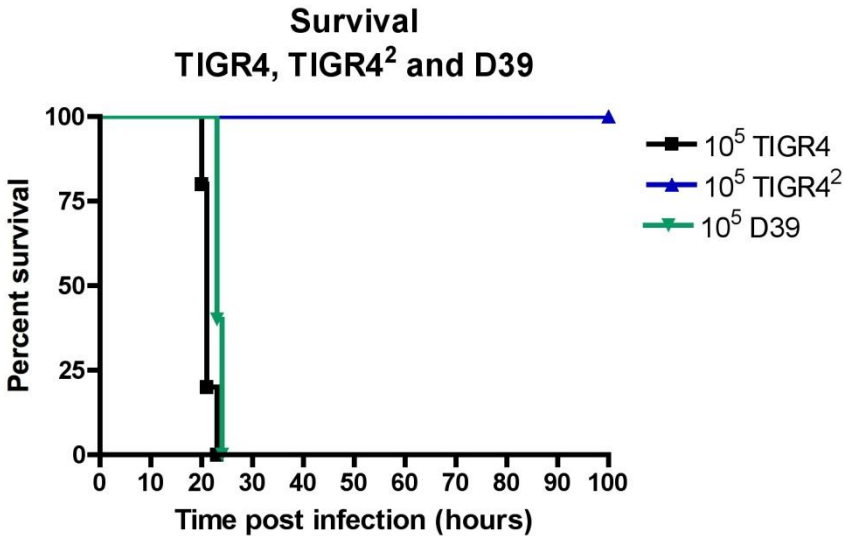


Figure 4.19. Virulence of serotype 4 virulent strain TIGR4, otherwise isogenic TIGR4 expressing serotype 2 capsule TIGR4^2 and serotype 2 virulent strain D39. Kaplan-Meier curve showing survival of mice infected intraperitoneally with 10^5 cfu. All data plotted as percentage survival as a staircase line with points for all observations against hrs post infection. Survival analysis showed there was a significant difference in survival time between the two groups by log rank test. Five mice used per strain.

TIGR4^3 showed marked attenuation (p=.002) as compared to TIGR4 and capsule donor strain OXC141. Median survival time in mice infected with TIGR4^3 was 37 hrs as compared to 21 hrs in those infected with TIGR4 (Fig 4.20).

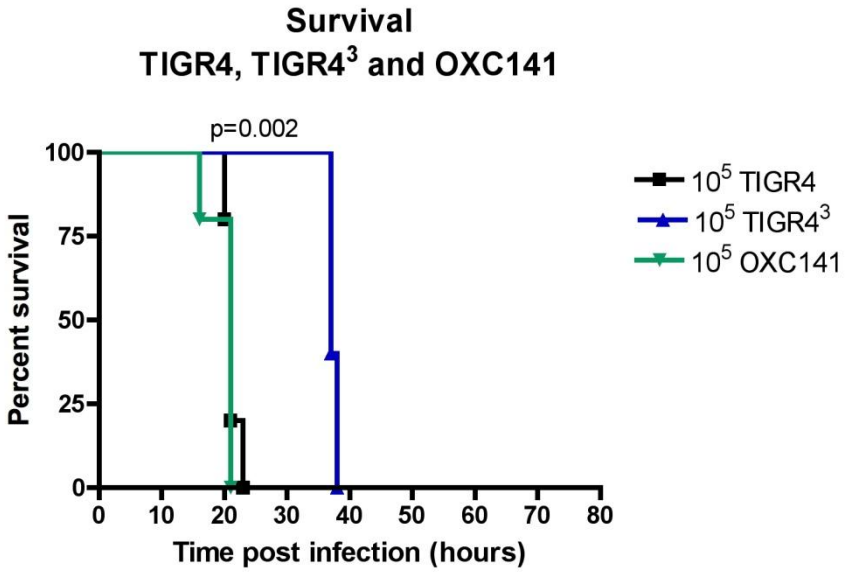


Figure 4.20. Virulence of serotype 4 virulent strain TIGR4, virulent strain OXC141 and otherwise isogenic TIGR4 strain expressing serotype 3 capsule. Kaplan-Meier curve showing survival in mice infected with 10^5 cfu intraperitoneally. Survival curve in mice infected with TIGR4^3 showed marked attenuation as compared to the capsule donor and the parent strain. All data plotted as percentage survival as a staircase line with points for all observations against hrs post infection. Survival analysis showed there was a significant difference in the survival time between the TIGR4 and TIGR4^3 by log rank test (p=.002). Five mice used per strain.

TIGR4^8 also showed marked attenuation (p=.002) as compared to TIGR4 and capsule donor strain ATCC6308. Median survival time in mice infected with TIGR4^8 was 78 hrs as compared to 21 hrs in those infected with TIGR4 (Fig 4.21).

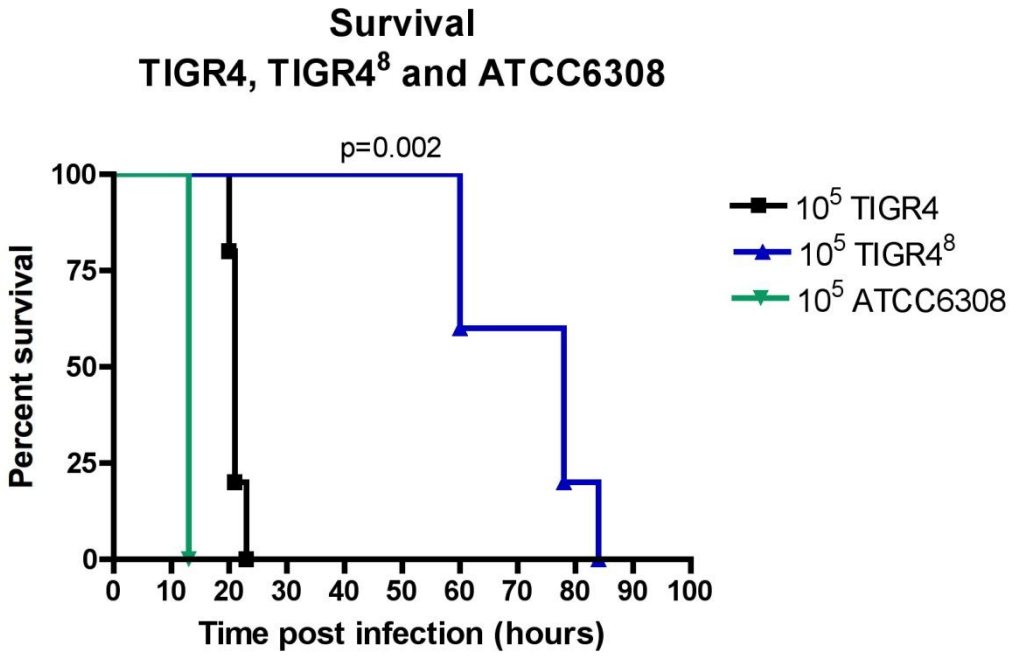


Figure 4.21. Virulence of serotype 4 virulent strain TIGR4, serotype 8 virulent strain ATCC6308 and otherwise isogenic TIGR4 strain expressing serotype 8 capsule TIGR4$^{8.}$ Kaplan-Meier curve showing survival of in mice infected with 10^{5} cfu intraperitoneally. Survival curve of TIGR4^{8} showed marked attenuation as compared to capsule donor and parent strains. All data plotted as percentage survival as a staircase line with points for all observations against hrs post infection. Survival analysis showed there was a significant difference in the survival time between TIGR4 and TIGR4^{8} by log rank test (p=.002). Five mice used per strain.

D39^{8} expressing type 8 capsule in D39 background showed a markedly different behaviour than TIGR4 as D39^{8} showed a very high virulence, which was similar to ATCC6308 as compared to D39 and mice infected with D39^{8} showed same median survival time as those infected with ATCC6308 (13 hrs) as compared to those infected with D39 (23 hrs) (Fig 4.22).

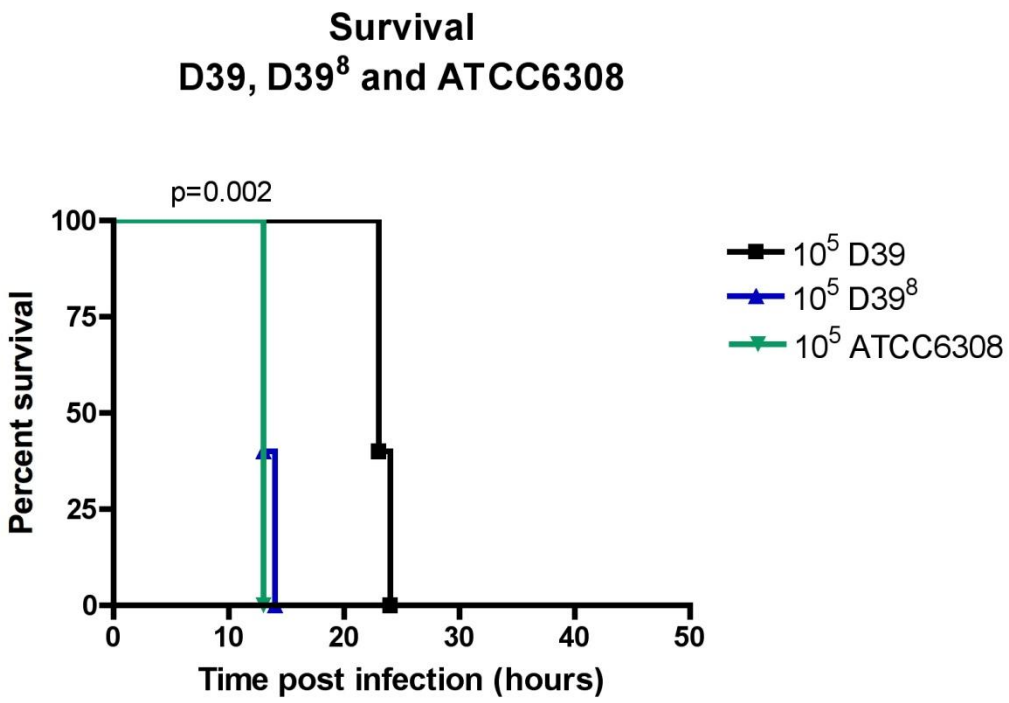


Figure 4.22. Virulence of serotype 2 virulent strain D39, serotype 8 virulent strain ATCC6308 and otherwise isogenic D39 expressing serotype 8 capsule $D39^8$ in mice infected with 10^5 cfu intraperitoneally. Kaplan-Meier curve showing survival of $D39^8$ showed marked increase in the virulence as compared to the parent strain D39. All data plotted as percentage survival as a staircase line with points for all observations against hrs post infection. Survival analysis showed there was a significant difference in the survival time between the mice infected with D39 and those with $D39^8$ by log rank test (p=.002). Five mice used per strain.

4.7 Adaptability of the capsule-switch strains:

It is well-known that animal passage increases virulence of bacteria as passaged bacteria are generally used to infect animal models as standard inoculum (Saladino *et al.*, 1997; Canvin *et al.*, 1995). Bacteria, while infecting animals, have been reported to differentially express their virulence factors including capsular genes (LeMessurier *et al.*, 2006; Ogunniyi *et al.*, 2002; Mahdi *et al.*, 2008). Pneumococcal capsule has also been reported to be differentially regulated during different stages of pathogenesis (Hammerschmidt *et al.*, 2005) and highly encapsulated bacteria were found to be highly virulent (Kim & Weiser, 1998).

To test the adaptability of capsule switch strains, those recovered from animals were reused to infect a new set of animals to see if their virulence can reach the virulence-level of TIGR4 strains. It was observed that there was significant increase in virulence of animal-passaged strains but they still remained less virulent than TIGR4 (Fig 4.23 and 4.24).

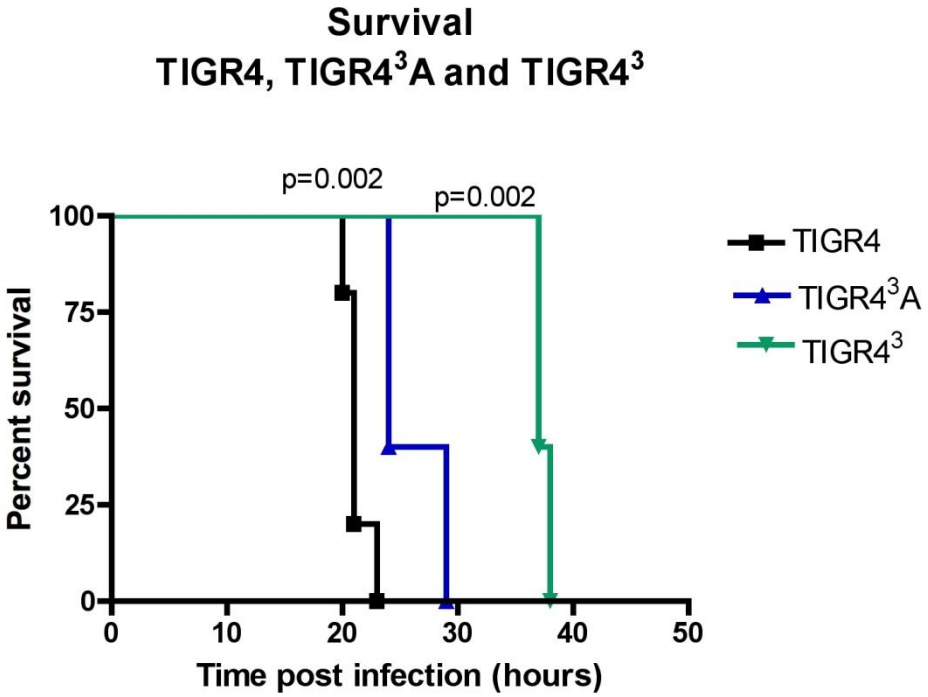


Figure 4.23 Virulence of serotype 4 virulent strain TIGR4, animal passaged otherwise isogenic TIGR4 strain expressing type 3 capsule TIGR4^{3}A and otherwise isogenic TIGR4 strain expressing serotype 3 capsule TIGR4^3. Kaplan-Meier curve showing survival in mice infected with 10^5 cfu intraperitoneally. Survival curve of TIGR4^{3}A showed marked increase in the virulence as compared to TIGR4^3. All data plotted as percentage survival as a staircase line with points for all observations against hrs post infection. Survival analysis showed there was a significant difference in survival time between mice infected with TIGR4^3 and TIGR4^{3}A (p=.002) and those with TIGR4 and TIGR4^{3}A (p=.002) by log rank test. Five mice used per strain.

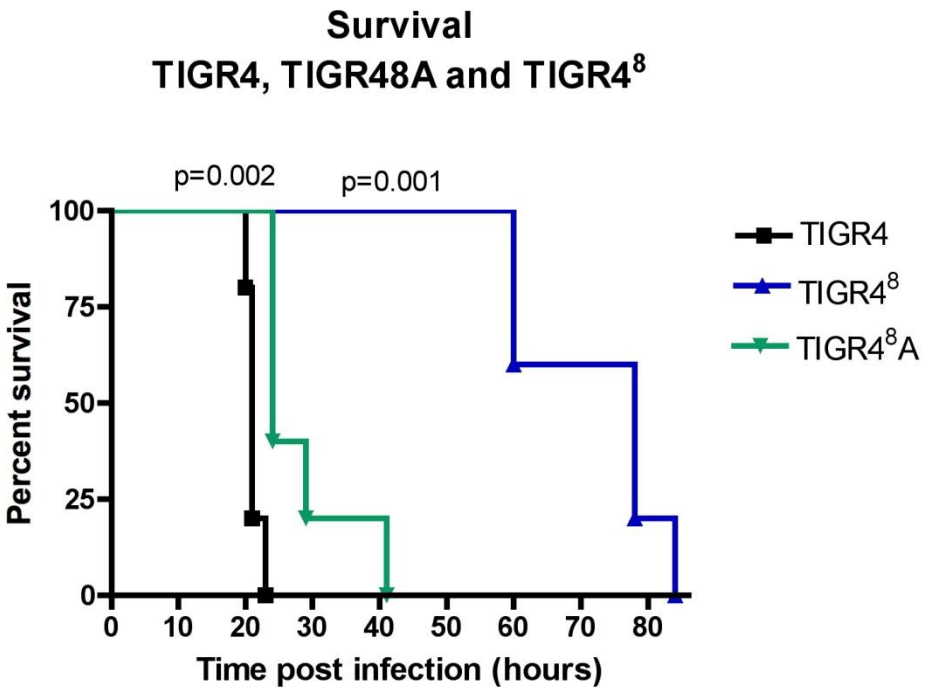


Figure 4.24 Virulence of serotype 4 virulent strain TIGR4, animal passaged otherwise isogenic TIGR4 strain expressing serotype 8 capsule TIGR4^{8}A and otherwise isogenic TIGR4 strain expressing type 8 capsule TIGR4^8 in mice infected with 10^5 cfu intraperitoneally. Kaplan-Meier curve showing survival of TIGR4^{8}A showed marked increase in virulence as compared to TIGR4^8. All data plotted as percentage survival as a staircase line with points for all observations against hrs post infection. Survival analysis showed there was a significant difference in survival time between mice infected with TIGR4^8 and TIGR4^{8}A (p=.001) and those with TIGR4 and TIGR4^{8}A (p=.002) by log rank test. Five mice used per strain.

4.8 Discussion:

Capsule switching is an important evolutionary process in pneumococci, which allows them to change and adapt according to environmental pressures. In the current era of intercontinental travel, pneumococcal vaccines and widely available effective antibiotics, it has become an extremely important tool for them to spread globally as it not only allows them to escape from effects of natural immunity and vaccines but also helps in spread of drug-resistant strains across the globe.

Since different capsules have different chemical composition and sugars needed for their construction have to be supplied by the metabolic and transport machinery of the pneumococcus (Bentley *et al.*, 2006), there is a possibility that pneumococci of all genetic backgrounds cannot express all types of capsular polysaccharides and it is only possible for certain types to accept and express few different capsular types. Capsule switching events are frequent and may occur as a response to environmental stresses (Golubchik *et al.*, 2012; Croucher *et al.*, 2011; Brueggemann *et al.*, 2007) and are mainly facilitated by high homology of up-stream and downstream regions across the capsule, as the capsule locus is located between two genes called *dexB* and *aliA* (Bentley *et al.*,2006). It is already known that there are gross differences in the genetic constitution of capsule locus and biochemical structure of capsular polysaccharide of different serotypes (Bentley *et al.*, 2006). Many studies indicate that these differences may be related to their virulence, their interaction with the host and many *in vitro* and *in vivo* characteristics as the capsule is one of the major determinants of pneumococcal biology (Hyams *et al.*, 2010; Battig & Muhlemann, 2007; Sandgren *et al.*, 2004; Sjostrom *et al.*, 2006; Hausdorff *et al.*, 2000a; Hausdorff *et al.*, 2000b; Kelly *et al.*, 1994). Capsule switching has been reported more frequently in some specific genetic backgrounds, which have altered their capsule type more than once and have spread across the globe whereas in some serotypes switching has not been reported such as serotype 1 (Croucher *et al.*, 2011; Coffey *et al.*, 1998b; Munoz *et al.*, 1991).

All strains used in these experiments have been genome sequenced except ATCC6308, which will be sequenced by our lab in the near future. They were chosen in order to maximize observations on pneumococcal diversity by selecting

strains of different virulence, having different in the size of capsule locus as all of them demonstrate differences in all these categories. These differences can be summarized in Table 4.2:

Table 4.2. Characteristics of Parent and capsule donor strains

Strain	*Cps* Locus size	Median Survival Time of Mice infected with 10^5 IP
TIGR4	20.9 kbp	21 hrs
D39	21.6 kbp	23 hrs
OXC141	10.7 kbp	21 hrs
ATCC6308.	14.4 kbp	13 hrs

The biochemical structure of the capsular polysaccharide of these strains is also different and is summarized in Table 4.3.

Table 4.3. Capsule constitution of parent and capsule donor strains

Strain	Capsule Constitution
TIGR4	Galactose, N-Acetyl mannosamine, N- Acetyl fucosamine, N Acetyl galactosamine
D39	Glucuronic Acid, Glucose and Rhamnose
OXC141	Glucuronic Acid, Glucose
ATCC6308.	Glucuronic Acid, Glucose, Galactose,

A number of studies have previously used capsule switch strains and their findings have significantly contributed to understanding of biology of capsule switching. Kadioglu *et al*. (2002) and Abeyta *et al*. (2003) have used a D39 mutant expressing type 3 capsule. Kadioglu *et al*. (2002) used an otherwise isogenic D39 FP50 expressing serotype 3 capsule, which was PCR amplified from virulent strain A66 and inserted using homologous recombination with kanamycin cassette. They reported that infectivity of strains was affected by both serotype and genetic background in a site-dependent manner as A66 and the mutant behaved similarly in nasopharynx, while the mutant was also cleared easily from lungs as compared to the wild type. Capsule-switch mutant was also found to be

avirulent in case of intranasal infection (Kadioglu *et al.*, 2002). Abeyta *et al.* (2003) used a D39 expressing serotype 3 capsule JD803, which was constructed by transforming D39 with serotype 3 genomic DNA from a strain containing erythromycin resistance marker inserted within capsule locus (Abeyta *et al.*, 2003). They reported that mutant showed increased deposition of C3b as compared to serotype 3 wild type strain, though it was less than D39 (Abeyta *et al.*, 2003). These findings are also supported by recent studies using multiple capsule switch strains of TIGR4 (Melin *et al.*, 2010). D39 expressing serotype 3 mutant was also reported to be similar in virulence as compared to D39 in both intraperitoneal and intravenous infection (Kelly *et al.*, 1994). A similar type 5 mutant expressing type 3 capsule was reported to be avirulent as compared to a type 6 mutant expressing type 3 capsule, whose virulence increased as compared to parent type 6 strain (Kelly *et al.*, 1994). Though it is difficult to compare these mutants as they were not isogenic though it can be inferred that both genetic background and capsule contribute towards virulence, and infectivity may be site-specific. Two studies have also used spontaneously arising capsular switch strains. Nesin *et al.* (1998) using naturally switched 23F strains expressing type 3 capsule reported that virulence was affected by capsule type as these mutants were lethal as compared to parent 23F which were non-lethal even at high doses using intraperitoneal route of infection. Another study using a serotype 14 strain and its naturally switched mutant expressing type 9V capsule did not find any difference in virulence of the two strains using intranasal route of infection (Mizrachi Nebenzahl *et al.*, 2004). During this study, instead of using non-isogenic strains or naturally occurring capsular switch strains, mutant strains were constructed in isogenic background as described earlier (Trzcinski *et al.*, 2003). These strains can be considered more reliable as

the chances of genetic variation are less as compared to naturally occurring capsule switch strains, which might harbour other small mutations.

In this study, using capsule switch mutants, the effect of capsule was studied on a number of biological properties of pneumococcus including growth, chain formation, transformability and virulence.

Since capsule incurs a considerable metabolic cost, it was proposed earlier that the capsule effects pneumococcal growth *in vitro*. In contrast to previous research which indicates that unencapsulated strains grow slower than encapsulated strains (Battig & Muhlemann, 2007) capsule-switch strains may have growth deficiencies (Trzcinski *et al.*, 2003) and more recently that growth is dependent on capsular type (Hathaway *et al.*, 2012), our studies with TIGR4, TIGR4 capsule switch mutants and unencapsulated TIGR4 did not show any detectable growth deficit in our TIGR4, its capsule switch and unencapsulated mutant strains in BHI. We also did not find any growth deficit in unencapsulated TIGR4 strain as compared to wild-type TIGR4. TIGR4, its capsule switch mutants and capsule knock-out Janus intermediate all had similar lag phase length, and similar curve in log phase of growth. We also did not find any shortening in the lag phase in unencapsulated TIGR4 as has been reported for D39. Our findings show that rate of pneumococcal growth in TIGR4 is independent of presence or absence of capsule or capsule type though it is a complex structure and has a long and complicated synthetic pathway. Our findings could be explained by difference of methodology in obtaining readings for plotting growth curves, as in our studies growth curves were plotted by taking a known number of colony forming units as starting inoculum and plotting curve using viable counts in contrast with other studies which used certain quantity from a subculture

containing unknown number of bacteria from overnight growth and plotted curves using optical density, which can be different for same number of bacteria and can vary with the degree of capsulation (Hathaway *et al.*, 2012; Battig *et al.*, 2006; Trzcinski *et al.*, 2003)

Electron microscopy and Stains-All Assay demonstrated differences in capsule formation in parents and capsule switch strains and it was observed that TIGR4^{2} had the thinnest capsule with the least amount of polysaccharide formation. TIGR4^{3} and TIGR4^{8} formed significantly less amount of capsule as compared to TIGR4 and their donor strains. D39^{8} demonstrated differences in formation of capsular polysaccharide as compared to TIGR4^{8} and though capsule thickness of D39, D39^{8} and ATCC6308 was similar, D39^{8} formed much more capsular polysaccharide as compared to parent strain D39. Formation of capsular polysaccharide seems to be related with both the genetic background and capsule constitution. Decrease in formation of capsular polysaccharide could be explained on the basis of biochemical structure of capsules as TIGR4 capsular polysaccharide mainly contains acetylated amino sugars and does not have glucose, galactose, rhamnose and glucuronic acid as its constituents. There is a possibility that an ample supply of some of these sugars cannot be maintained by TIGR4 sugar transport and synthesis mechanisms. The above hypothesis is also supported by the fact that D39 made plenty of type 8 capsule, which contains galactose instead of rhamnose found in D39 capsule, which is a non-house keeping sugar and is uniquely found in type 2 capsule among our capsule switch strains. It has a separate pathway of synthesis with genes *rmlA*, *rmlC*, *rmlB* and *rmlD* located within the type 2 capsule locus, which contains three rhamnose units as its structural constituents (Bentley *et al.*, 2006). These genes are also present in some other pnuemococci such as serogroup 6, serogroup 7, serogroup

17, serogroup 18 and serogroup 19 though they differ in number of repetitive rhamnose subunits. There is a possibility that TIGR4, if spontaneously acquires one of these capsules may be unable to incorporate repetitive units of rhamnose in its capsular polysaccharide structure and may become attenuated due to insufficient capsule information, though TIGR4 capsule switch mutants expressing simpler capsular polysaccharide with just one rhamnose unit have been constructed and reported to be forming capsules with proper thickness (Hyams *et al.*, 2010b). Simplified capsular polysaccharide structures are shown in Fig 4.25.

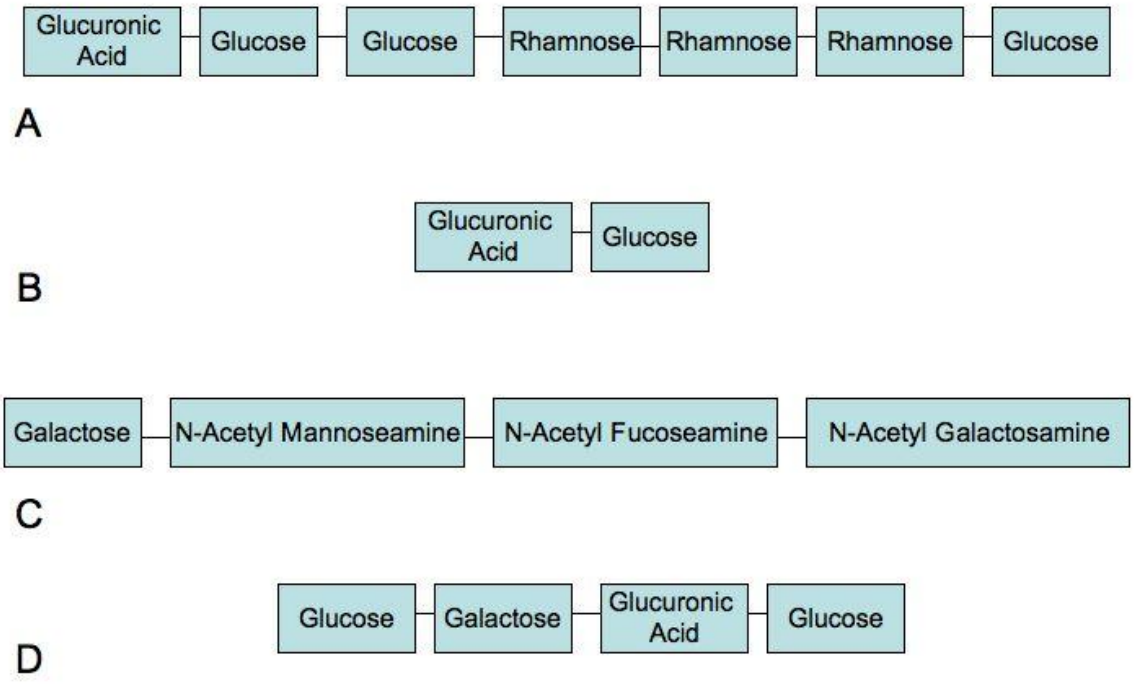


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Figure 4.25. Schematic diagrams of capsular polysachharide structures showing constituent sugars (A) Serotype 2 capsule. (B) Serotype 3 capsule. (C) Serotype 4 capsule (D) Serotype 8 capsule. Redrawn with modification and simplification from Bentley *et al*. (2006).

It might also be inferred that capsule switching may be related to transformability as many pneumococci that are not transformable would not be able to accept and thus switch to newer capsule types. There is a possibility that thickness, density, capsular charge or type of capsule also plays a role in the transformability of pneumococci as they might offer a physical or charge barrier to DNA molecules coming in contact with pneumococcus, though our studies did not establish a statistical correlation between capsular thickness or amount of capsule with transformability of our capsule switch mutants. Since during construction of capsule-switch strains only those colonies are picked which have integrated the capsule genes, there was a chance that after three times back crossing, capsule switch strains might be the most transformable ones and might give a false value if compared with parent TIGR4 strains. The comparison of TIGR4 and capsule switch strain expressing type 4 capsule showed significant difference in transformability of the two strains. To remove the bias all analyses were done by comparing capsule-switch strains with strain P1702, which showed TIGR4^{2} to be significantly more transformable as compared to P1702, TIGR4^{8} was comparatively less transformable while TIGR4^{3} was the least transformable among capsule switch strains. Transformability might be related to biochemical constitution of capsule and there is a possibility that after acquiring certain capsules the population may become more transformable or less transformable depending upon the type of capsule.

Chain formation is a characteristic feature of pneumococci and they tend to associate themselves in variable number of chains. Significance of number of bacteria per chain is not clearly understood but it has been reported in the past that very long chains are associated with decreased virulence (Ekstedt & Stollerman, 1960). More recently, Dalia and Weiser (2011) also showed that

decrease in length of chain is associated with increased virulence and allows evasion of complement, though host immune system responds by agglutinating antibodies to overcome this strategy (Dalia & Weiser, 2011). It has also been reported that some mutant strains show longer chains as compared to normal strains (Barendt *et al.*, 2009; Sanches-Puelles *et al.*, 1986). The results showed that pneumococci expressing type 8 capsule tend to form longer chains, consisting of 12-30 pneumococci. Relationship of chain-length with virulence could not be established as though $D39^{8}$ and ATCC6308 were highly virulent, $TIGR4^{8}$ was attenuated as compared to parent TIGR4 strain, though it showed adaptability and there is a possibility that it might reach to virulence of TIGR4 with repeated animal passage or become more virulent. There is a possibility that longer chains pose difficulty in phagocytosis and are associated with increased virulence contrary to the findings of Dalia and Weiser (2011), similar to the phenomenon observed in *Mycobacterium tuberculosis* (Hunter *et al.*, 2006).

To study the effects of capsule switching parents and capsule switch strains were tested in intraperitoneal mouse model of infection. Results showed that the process of capsule switching itself does not affect virulence as mice infected with TIGR4 and P1702 did not demonstrate any difference in virulence. Results also showed that different capsules have different effects on virulence of a given strain while same capsule can have different effects on strains of different genetic background. These variations might be due to effects of biochemical structure on complement deposition, which is vital for development of host immunity against the pneumococcus (Abeyta *et al.*, 2003; Winkelstein, 1981). These complement proteins are organized in three pathways classical, alternative and mannose-binding lectin pathways, which are serially activated

to opsonize bacteria (Walport, 2001a; Walport, 2001b). Activation of complement results in deposition of cleaved complement protein C3b on the surface of bacteria which can cause opsonophagocytosis by interacting with complement receptors present on neutrophils (Lambris *et al.*, 2008), resulting in death of bacteria within phagolysosome (Standish & Weiser, 2009). Since the pneumococcal capsule resists opsonization by complement by multiple mechanisms including inhibition of IgG and C-reactive protein binding and reduction of degradation of surface bound C3b (Hyams *et al.*, 2010a) variation in its thickness or density may completely uncover or partially expose the surface structures, which can then directly interact with the complement, resulting in subsequent phagocytosis. It has also been shown that susceptibility to complement-mediated killing is serotype dependent (Hyams *et al.*, 2010a) and resistance to complement-mediated lysis is more related to capsular serotype than genetic background (Melin *et al.*, 2010) so a change in serotype might result in variation of virulence.

It was also observed that passage of strains through animals significantly increased virulence of less virulent strains but it was still not comparable to TIGR4. Increase in the pneumococcal virulence with animal passage is a known phenomenon (Chiavolini *et al.*, 2008), it is not clear yet whether this was due to change in expression of capsule genes, increased capsule formation or other changes in the pneumococcal biology. An increase in capsular expression *in vivo* has been reported in several studies (Ogunniyi *et al.*, 2002; Mahdi *et al.*, 2008), thought others did not find any change in capsular expression *in vivo* in mouse models of infection (LeMessurier *et al.*, 2006; Orihuela *et al.*, 2004). Since increase in expression of many virulence factors have been reported with exposure to animals in a number of studies (Mahdi *et al.*, 2008; LeMessurier *et*

al., 2006; Orihuela *et al.*, 2004; Ogunniyi *et al.*, 2002), there is a possibility that exposure to the animal environment might have allowed selection of those strains, which are able to adapt to the environment more readily and repeated passage through animals might render them equally or more virulent as TIGR4.

It can be inferred that capsule switching is not a universal phenomenon and a successful capsule switch is only possible with suitable combination of genetic background and capsule type so there would be limitations in development vaccine escape mutants, which could possibly spread and cause treatment issues.

Having compared effects of capsule switching on pneumococcal biology, the whole genome sequence of avirulent strain 403 was obtained and was compared with genome sequence of virulent TIGR4 strain to find genome-wide changes responsible for differences in virulence. To study the differences in expression of different virulence factors, microarray experiments were performed, comparing 403 gene-expression with that of TIGR4.

5 Comparison of an avirulent strain 403 with virulent strain TIGR4 using whole genome sequencing and microarray.

S. pneumoniae is an extremely diverse organism having a highly variable genomic content due to which different strains vary significantly in terms of virulence and other biological properties. The highly transformable pneumococcal genome easily allows mutations, which may result in an increase or a decrease in pathogenicity of a particular strain. The best way to understand the nature of these mutations and to study their effect is to compare the target strain with a standard strain in terms of genetic content, gene expression and other *in vivo* and *in vitro* characteristics. This approach must involve the complete genome sequence as the first and foremost step as a complete, high quality genome sequence ensures smooth progress during all downstream work, and can explain phenotypic variation among the strains. It also permits identification of genomic regions harboring mutations and enables *in silico* comparison of different genomes using BLAST (Basic Local Alignment Search Tool) or other software.

Microbial genome sequence analysis has been an important approach to understanding the biology of different microorganisms since the complete genome of *Haemophilus influenzae* was published in July 1995 (Fleischmann *et al.*, 1995). It has been utilized for comparison of different streptococci including pneumococcus (Lanie *et al.*, 2007) and other organisms (Ellison *et al.*, 2008; Howard *et al.*, 2006) and has been a useful approach to identify pathogenic factors and to improve the understanding of the mechanism of microbial pathogenicity. Identification of genetic differences between

pathogenic and non pathogenic strains allows definition of phylogenetic relationships between them and may help in identification of potential therapeutic targets for medical interventions, which has been the major goal of bacterial comparative genomics.

Avirulent strain 403 is particularly interesting as it is derived from virulent strain TIGR4, which is opposite to what has been suggested in serogroup 6 strains that virulent pneumococcal strains tend to have evolved from carriage strains (Robinson *et al.*, 2002). In addition to this, since there are serotype specific and tissue specific virulence factors that are affected by transcriptional regulation, we also performed microarray experiments comparing 403 with TIGR4 to identify key genes responsible for altered phenotype in 403, which revealed more differences between the two strains and showed altogether 288 genes to be differentially expressed in 403 as compared to TIGR4 as summarized in Table 5.5.

403 and TIGR4 are both sequence type 205, serotype 4 strains , a serotype which is frequently implicated in cases of invasive pneumococcal disease (IPD) and have been also been observed to cause invasive disease in animal models of pneumococcal infection (Sandgren *et al.*, 2005; Sandgren *et al.*, 2004). Their characteristics are summarized in Table 5.1.

Table 5.1. Comparison of TIGR4 and 403

Strain	Serotype	MLST Type	Virulence
TIGR4	4	205	Virulent
403	4	205	Avirulent

This chapter compares 403 with TIGR4, and discusses the nature of spontaneously arising single nucleotide polymorphisms (SNPs) in 403. In addition, relative transcription patterns determined by microarray analyses, and possible effects of these differences have been discussed.

5.1 Comparison of virulence of 403 and TIGR4:

Strain 403 was evaluated for its virulence potential in mouse model of sepsis and a group of MF1 mice were challenged intraperitoneally with 1 x 10^5 cfu, using the same quantity of virulent TIGR4 strain as control. It was observed that not only were these strains were avirulent but also that they failed to produce any bacteraemia. All the 403 challenged mice survived until the end of experiment as compared to their TIGR4 counterparts, which reached the end point by 30 hrs (Fig 5.1).

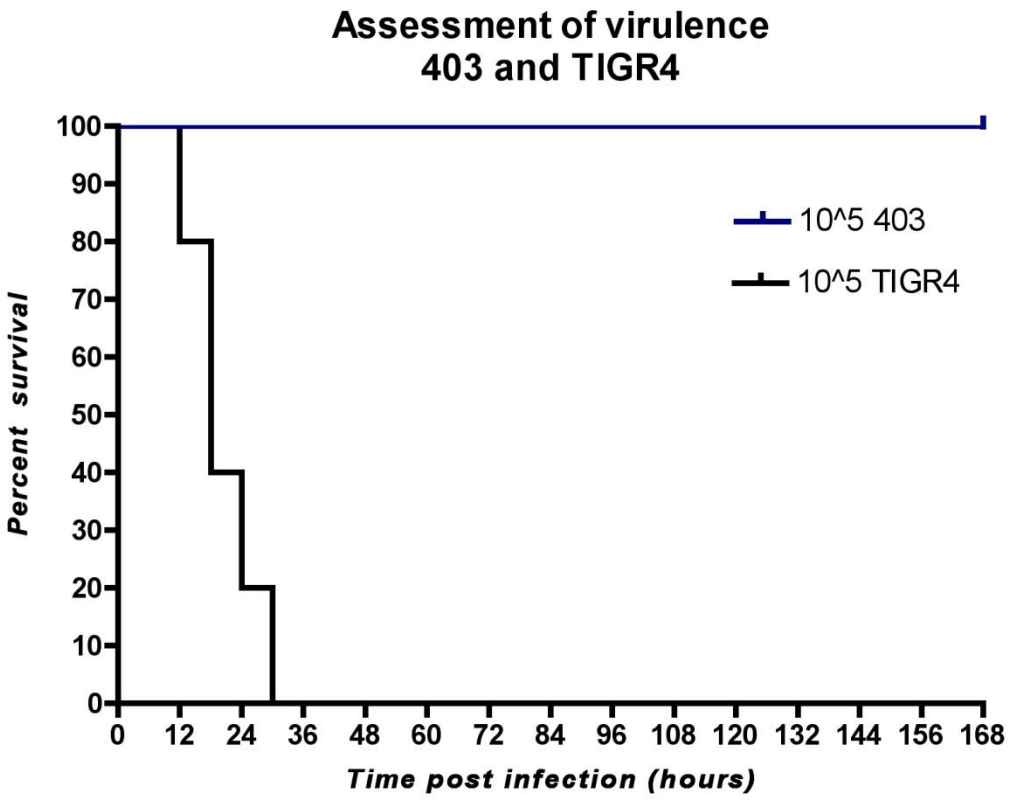


Figure 5.1. Kaplan-Meier curve showing survival of MF1 mice challenged IP with 1 x10^5 cfu/200 µl with *Streptococcus pneunomiae* strains 403 and TIGR4. All data plotted as percentage survival as a staircase line with points for all observations against hrs post infection. Median survival time for TIGR4-challenged mice was 18 hrs as calculated by log rank test (GraphPad Prism 4.0, USA). Five mice were used in each group.

5.2 Genomic comparison of 403 and TIGR4:

Whole genome sequencing of strain 403 was carried out using Illumina® paired end-sequencing. Genomic alignments were performed between complete genome sequence of TIGR4 strain and the consensus sequence, which was generated for strain 403, resulting in an almost complete sequence alignment with only few discrepancies, which are discussed in following sections.

Sequence alignments showed high homology shared by the two strains, without any remarkable chromosomal rearrangements. However, 35 single-nucleotide polymorphisms (SNPs) were identified in strain 403, some of which resulted in altered amino acid sequence in coding regions of annotated genome, while 6 SNPs were present in intergenic regions. These SNPs affected several classes of genes, including biosynthetic pathways of several macromolecules, competence, DNA repair, cell division and fermentation. It can be easily speculated that changes in structure or function of translated products might contribute towards the altered phenotype and pathogenicity of 403.

22 Insertion-deletions and a 12 base pair deletion (1132424-36 of reference strain) were also found in 403, which are being analyzed in our lab as a separate project, and only possible effects of the SNPs will be discussed in this chapter.

5.2.1 SNPs in intergenic region:

On analysis of genome sequencing data, six out of thirty five SNPs were found in intergenic regions. These are summarized in Table 5.2.

Most of the SNPs present in intragenic regions were not associated with any change in expression profile of surrounding genes. SNP present at positions 476404, 730678, 1622058 and 1622059 were not found to be affecting genes present upstream and downstream to them and only two SNPs at positions 463629 and 463630 were associated with expression profile change of their surrounding genes as shown in Table 5.2.

Table 5.2. SNPs in the inrtragenic regions and genes upstream and downstream to the SNPs with expression profile change in two genes.

Consensus	Reference	Reference	Variation	Upstream genes	Downstream Genes
463629	463630	G	A	Hypothetical protein (Sp0482) 1.25+	ABC transporter (Sp0483) 1.14-
463630	463631	A	G	Hypothetical protein (Sp0482)	ABC transporter (Sp0483)
476404	476406	G	T	Na/Pi cotransporter II related protein (Sp496)	Hypothetical protein (Sp0497)
730678	730678	C	T	Petpidyl prolyl cis trans isomerase(Sp0771)	Hypothetical protein (Sp0772)
1622058	1622066	G	A	ABC transporter (Sp1717)	Hypothetical protein (Sp1718)
1622059	1622067	A	G	ABC transporter (Sp1717)	Hypothetical protein (Sp0718)

These two SNPs were found to be associated with altered expression profile of genes present upstream and downstream to them, hypothetical protein gene (Sp0482) located upstream to them was up regulated while the ABC transporter present downstream to them (Sp0483), was down regulated.

5.2.2 Synonymous SNPs:

Seven SNPs resulted in synonymous mutations and did not cause any change in the amino acid sequence of translation product. Though variable effects of synonymous SNPs are not reported for bacteria but they have been shown to be associated with certain disease conditions in humans, (Chen *et al.*, 2010; Ho *et al.*, 2010) hence there remains a possibility of these SNPs may be associated with altered biology of 403 as some of them occur in genes vital for pneumococcal growth and pathogenesis including capsule biosynthesis, DNA replication and repair and competence as shown in Table 5.3.

Table 5.3. Synonymous SNPs in 403 and expression profile change in 403.

Gene	TIGR4[1]	Reference[2]	Variation[3]	Amino Acid Change[4]	Consensus[5]	Reference[6]	Expression Profile change[6]
Capsular polysaccharide	Sp0103	C	T	-	106332	106332	-
DNA polymerase IV	Sp0458	T	G	-	431222	431223	-
Gln-2	Sp2245	G	A	-	807984	807984	-
glyA	Sp1024	G	T	-	967258	967257	1.78+
RecN; DNA repair protein	Sp1202	C	T	-	1135386	1135396	-
Prolyl oligopeptidase family protein	Sp1343	C	G	-	1267209	1267219	-
ComF;competence protein	Sp2207	G	T	-	2127991	2127997	-

[1] Gene annotation in TIGR4
[2] Nucleotide in the reference genome TIGR4
[3] Nucleotide in consensus sequence of 403
[4] Amino acid change in 403
[5] Position of the SNP in 403 consensus sequence
[6] Position of the SNP in the reference sequence
7 Expression profile change.

Microarray analysis did not show any change in expression profile of these genes except *glyA* which codes for a serine hydroxymethyltransferase that is involved in multiple metabolic pathways such as glycine, serine and threonine metabolism, lysine degradation, methane metabolism and cyanoamino acid metabolism and is responsible for reversible interconversion of glycine and serine with tetrahydrofolate, which serves as one-carbon carrier. Expression profile changes are summarized in Table 5.3.

5.2.3 Non synonymous SNPs:

The rest of the 22 mutations were found to be non synonymous and were associated with change in amino acid sequence of genes, though microarray showed only four of these genes to be differentially regulated in 403. This finding does not rule out a malfunctioning translational product, which might contribute towards avirulence of 403. Differentially regulated genes with SNPS are summarized below.

1. Sp1161, which is putative acetoin dehydrogenase complex, E3 component, dihydrolipoamide dehydrogenase, was found to be upregulated along with Sp1162 and Sp1163 which are E1 and E2 components of same enzyme and are involved in multiple metabolic processes including metabolism of alanine, serine, threonine, pyruvate, glycine and aspartate, dergradation of valine, leucine and isoleucine, glycolysis and gluconeogenesis, regulation of NAD/NADH ratio and carbon storage (Xiao & Xu, 2007).

2. Sp1645 (*relA*) which codes for GTP pyrophosphokinase and involved in cellular adaptation to atypical conditions was also found to be upregulated. It has been reported that *relA* mediates uptake and utilization of certain ions under nutritionally stressed condition and does not play much important role when nutrients are abundantly present. It is known to be an important virulence factor which helps bacteria to adapt during disease progression (Kazmierczak *et al.*, 2009). Since it is known to cause repression of rRNA synthesis and affect patterns of gene transcription, protein synthesis and enzyme activation (Wolz *et al.*, 2010), there is a possibility that mutation causing amino acid change from valine to phenylalanine in *relA* product along with a 1.5 increase in expression might be the reason for generalized increase in aminoacid acquisition and decreased synthesis of ribosomal proteins in 403.

3. Sp1003, which codes for conserved hypothetical protein and Sp0272, which is ribosomal protein S7 gene were found to be down regulated.

Table 5.4. SNPs present in 403.

Gene	TIGR4 Annotation	Reference	Allele Var	Reference	Consensus	Amino Acid Change
Cell Envelope:						
Capsular polysaccharide biosynthesis protein Cps4E	Sp0350	G	A	324506	324505	Asp173Asn
Cofactors, Prosthetic groups and Carrier Biosynthesis						
gor; Glutathione reductase	Sp0784	A	G	737108	737108	Cys382Arg
Cell Division:						
Cell division protein FtsW, putative	Sp1067	T	G	1001704	1001704	Phe143Val
Fermentation:						
Acetoin dehydrogenase complex, E3 component,	Sp1161	T	G	1096817	1096817	Asn395Thr
MATE efflux family protein	Sp1164	C	G	1101585	1101585	Arg405Pro
MATE efflux family protein	Sp1164	G	C	1101586	1101586	Arg405Gly
Carbohydrate and Energy Metabolism:						
*lacD:*Tagatose 1,6-diphosphate aldolase	Sp1190	C	G	1127020	1127021	Val300Leu
*lacD;*Tagatose 1,6-diphosphate aldolase	Sp1190	G	C	1127021	1127022	Cys299Trp
recP-1	Sp1615	G	A	1517039	1517030	Ala173Val
Degradation of proteins:						
Prolyl oligopeptidase family protein	Sp1343	G	C	1267220	1267210	Thr120Arg
DNA metabolism:						
Type II DNA modification methyltransferase, putative	Sp1431	G	T	1350621	1350612	Glu291Stp

Gene	TIGR4 Annotation	Reference	Allele Var	Reference	Consensus	Amino Acid Change
Adaptations to Atypical conditions:						
relA; GTP pyrophosphokinase	Sp1645	C	A	1543556	1543547	Val140Phe
Transcription:						
rpoC; DNA-directed RNA polymerase subunit beta	Sp1960	C	T	1862985	1862977	Gly1113Glu
DNA Interactions:						
bglG family transcriptional regulator	Sp2131	G	A	2042324	2042318	Thr647Ile
Nucleoside/Nucleotide metabolism						
adk; Adenylate kinase	Sp0231	C	T	204679	204679	Ala40Val
Transport						
ABC transporter, substrate-binding protein	Sp0145	G	C	146055	146055	Leu181Phe
ABC transporter, ATP-binding protein	Sp1918	G	T	1828206	1828198	His218Gln
Phosphate ABC transporter, ATP-binding protein, putative	Sp1397	C	A	1320529	1320520	Asp244Tyr
Hypothetical proteins:						
Hypothetical protein	Sp1003	G	C	947167	947168	Val59Leu
Hypothetical protein	Sp1334	G	T	1257186	1257176	Pro58Gln
Hypothetical protein	Sp1715	A	G	1618223	1618214	Glu130Gly
Ribosomal Proteins:						
30S ribosomal protein S7	Sp0272	A	G	247805	247805	Lys90Glu

Other genes harboring SNPs having important biological functions are:

1. Sp0350: Also known as *cps4E* encodes a glycosyl-phosphotransferase that transfers glucose-1-phosphate units to growing undecaprenyl phosphate glycolipid (Cartee *et al.*, 2005). It has been reported that disruption of this gene does not affect the viability of bacteria (Xayarath & Yother, 2007;Hava & Camilli, 2002) though *cps4E* mutants were found to be capsule deficient but good biofilm formers. Capsule deficient mutants were also found to be good at biofilm production (Munoz-Elias *et al.*, 2008).

2. Sp0231 or *adk,* which codes for an adenylate kinase and is required for proper maintenance and growth of cell, was reported among essential genes by allelic replacement mutagenesis (Song *et al.*, 2005).

3. SP1960 or *rpoC,* which is a DNA dependent RNA polymerase was also reported to be among essential genes by allelic replacement mutagenesis (Song *et al.*, 2005).

4. Sp1190 or *lacD* which codes for tagatose 1,6-diphosphate aldolase and is an important enzyme in galactose metabolic pathway, where it converts D-tagatose 1,6-bisphosphate to glycerone phosphate and D-glyceraldehyde 3-phosphate. Though *lacD* itself did not show decreased expression but a number of other genes related to galactose metabolism showed lesser levels of expression as compared to TIGR4.

All above genes have important biological functions and it can be predicted that functional disruption of any one of these genes alone or in combination might result in loss of pathogenicity as some of these mutations were also found to be affecting expression of genes that play important roles in pneumococcal metabolism, its regulation and virulence.

A microarray analysis revealed even more difference between the two strains and showed altogether 288 genes to be differentially expressed in 403 as compared to TIGR4 as summarized in Table 5.5 and discussed below.

5.3 Expression patterns in 403:

5.3.1 Surface Structures and virulence factors:

Pneumococcal surface is composed of three major components which include polysaccharide capsule, peptidoglycan cell wall and cell membrane. These layers are also embedded with surface proteins which are very important for pneumococcal virulence and interaction with host cells and their expression levels can affect virulence of any strain. In our microarray studies we observed altered expression levels of a number of genes related to surface structures in 403 and most of these showed decreased expression levels. Important surface related genes with altered expression levels are discussed below.

1. *strH* is an exoglycosidase, which resists neutrophilic opsonophagocytic killing in the human body and act in conjugation with two other virulence factors, a neuraminidase *NanA, and* a beta-galactosidase *BgaA* (Dalia *et al.*, 2010). *BgaA* did not show altered expression in 403, while *NanA* is known to be non-functional in TIGR4 due to a frame-shift mutation

(Pericone *et al.*, 2002). It has been shown that they reduced deposition of C3 component of the complement on surface of pneumococci, which provides an escape route against phagocytic killing. As pneumococcus encounters a number of glycoconjugates on host epithelial surface including membrane associate glycoproteins and mucin (Lodish *et al.*, 2000), and these glycosidases modify them to assist in colonization and subsequent invasion of cells (Burnaugh *et al.*, 2008), a 1.5 fold decreased expression of *strH* along with absence of neuraminidase A might contribute towards attenuation of 403.

2. *pulA* is another downregulated gene that codes for a putative pullulanase present on pneumococcal surface, which codes for a protein called SpuA. It was shown to play a role in binding to a number of complex carbohydrates including submaxillary mucin, asialofetuin and fetuin and exhibiting strepadhesin activity in Group A streptococci (Hytonen *et al.*, 2003). Pullulanase deficient mutants have also been observed to be deficient in cell adhesion (Hytonen *et al.*, 2006).

3. *MurB* and *MurF* are other two enzymes less expressed in 403, which are critical for cell wall synthesis and important drug targets. Their decreased expression may be related to defects in cell wall synthesis (Smith, 2006; El Zoeiby *et al.*, 2003).

4. *CpoA* is a downregulated gene that encodes for a glycosyl transferase and mutations in *cpoA* have been reported to affect pneumococcal competence along with beta-lactam resistance (Grebe *et al.*, 1997).

Some other genes coding for hypothetical proteins and proteins of unknown functions were also noted to have lower expression in 403 as compared to TIGR4, while only one notable gene Sp0378 coding for choline binding protein J involved in adherence, was found to be expressing highly in 403 as compared to TIGR4.

Reduced expression of these genes may contribute towards lack of pathogenicity of 403 as proteins coded by these enzymes are vital for invasion of tissues and expression profile of surface factors favors a colonization phenotype as compared to an invasive one.

5.3.2 Genes related to metabolic pathways:

5.3.2.1 Carbohydrate metabolism

Pneumococcus can metabolize a few sugars such as glucose, sucrose, raffinose, lactose, trehalose, inulin and maltose as sources of energy (Hava & Camilli, 2002). A number of genes coding for enzymes participating in galactose metabolism such as *galM, glmS, galK, galT, aga, nagB* and fructose metabolism such as *fruB* were down-regulated though genes coding for enzymes related to glucose metabolism were upregulated along with those coding for enzymes of gluconeogenesis such as acetoin dehydrogenase complex, which indicates preferential utilization of glucose over other resources in 403.

Genes *adhE* and *adh* are iron and zinc containing alcohol dehydrogenases involved in anaerobic metabolism, which were observed to have lower expression levels in 403 as compared to TIGR4.

5.3.2.2 Amino acid and protein metabolism:

Analysis of data over amino acid synthesis showed that genes coding for enzymes related to metabolism of basic amino acids including arginine and lysine were downregulated (*argF, arcA and proC*) though those related to synthesis of aromatic amino acids including phenylalanine, tyrosine and tryptophan biosynthesis (*pheA, tyrA, aroC, trpA, trpB, trpC, trpD and , trpG*) and hydrophobic amino acids were up-regulated. Another important gene *nspC* coding for carboxynorspermidine decarboxylase was found to be down-regulated, which is known to participate in a number of metabolic processes including 1 and 2 methylnaphthalene degradation, 3-chloroacrylic acid degradation, alkaloid biosynthesis, benzoate degradation, phenylpropanoid biosynthesis, purine metabolism, pyruvate, tryptophan and tyrosine metabolism.

A number of ribosomal proteins were also observed to have decreased expression including *rpsD, rpsJ, rplC, rplB, rpsN, rpmD, rpsG, rplM, rplA, rpsP, rplT,pepF, rpmA, rplL, rpsR , rpsB* and *tsf*.

5.3.2.3 Transport proteins:

A number of transporters related to various metabolic pathways were found downregulated in 403 which include *fruA* (fructose specific), Sp1682 (Sugar ABC transporter, permease protein), Sp1683 (Sugar ABC transporter, sugar-binding protein), Sp0063 (PTS system, IID component), Sp1684 (PTS system, IIBC components), *rafF* (Sugar ABC transporter, permease protein), *rafE* (Sugar ABC transporter, sugar-binding protein), *malX* (Maltose/maltodextrin ABC transporter, while choline transporters *proWX* and *proV* and genes related to

phosphate ABC transport system *pstC*, *pstA*, *pstB* and *phoU* were found to be expressing highly in 403.

Differences in expression profiles of the two strains are summarized in Table 5.5.

Table 5.5. Differentially regulated genes in strain 403.

Differentially regulated Genes in 403	Annotation in TIGR4	Expression in 403	Expression in TIGR4	Fold Change in 403
Virulence determinants				
pulA; Putative alkaline amylopullulanase	Sp0268	0.251	0.678	2.7-
xseA; Exodeoxyribonuclease VII, large subunit	Sp1207	0.767	0.987	1.29-
nanA; Neuraminidase A, authentic frameshift	Sp1693	0.279	0.953	3.42-
Cell Envelope				
strH; Beta-N-acetylhexosaminidase	Sp0057	0.715	1.073	1.5-
Lipoprotein	Sp0149	0.836	0.999	1.19-
cps4G; Capsular polysaccharide biosynthesis protein cps4,	Sp0352	0.744	0.989	1.33-
Membrane protein	Sp0637	0.654	0.96	1.47-
Conserved hypothetical proteins:	Sp1851	0.251	0.727	2.9-
cpoA; Glycosyl transferase CpoA	Sp1075	0.852	0.986	1.16-
*murB;*UDP-N-acetylenolpyruvoylglucosamine reductase	Sp1390	0.762	1.025	1.35-
murF;UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-	Sp1670	0.792	1.044	1.32-
Cell wall surface anchor family protein	Sp1992	0.639	1.033	1.62-
Stress related				
General stress related protein 24, putative	Sp1804	0.572	0.958	1.67-
DNA repair, recombination and modification				
MutT/nudix family protein	Sp0119	0.708	1.192	1.68-
dnaG; DNA primase	Sp1072	0.693	0.945	1.36-

Differentially regulated Genes in 403	Annotation in TIGR4	Expression in 403	Expression in TIGR4	Fold Change in 403
DNA repair, recombination and modification				
ligA; DNA ligase, NAD-dependent	Sp1117	0.711	1.06	1.49-
*rexA;*Exonuclease RexA	Sp1152	0.904	1.053	1.16-
MutT/nudix family protein	Sp1669	0.849	1.014	1.19-
cbf1; Cmp-binding-factor 1	Sp1980	0.699	1.017	1.45-
Carbohydrate and Energy Metabolism				
galM; Aldose 1-epimerase	Sp0066	0.576	0.992	1.72-
glmS; Glucosamine--fructose-6-phosphate aminotransferase	Sp0266	0.576	0.989	1.72-
fruB; 1-phosphofructokinase, putative	Sp0876	0.173	0.692	4-
nagB; Glucosamine-6-phosphate isomerase	Sp1415	0.552	0.931	1.69-
npl; Putative N-acetylneuraminate lyase	Sp1676	0.427	0.918	2.15-
galK; Galactokinase	Sp1853	0.311	0.757	2.43-
galT; Galactose-1-phosphate uridylyltransferase	Sp1852	0.291	0.669	2.3-
Putative dextran glucosidase DexS	Sp1883	0.164	0.616	3.76-
aga; Galactose metabolism	Sp1898	0.182	0.567	3.12-
rpe; Ribulose-phosphate 3-epimerase	Sp1983	0.773	0.97	1.25-
adhE; Alcohol dehydrogenase, iron-containing	Sp2026	0.148	0.764	5.16-
adh; Alcohol dehydrogenase, zinc-containing	Sp2055	0.376	0.944	2.51-
malP; Glycogen phosphorylase family protein	Sp2106	0.403	0.883	2.19-
malQ; 4-alpha-glucanotransferase	Sp2107	0.38	0.815	2.14-
fcsK; Putative L-fuculose kinase fucK	Sp2167	0.367	0.762	2.08-
Aminoacid metabolism and Acquisition				
cad; Lysine decarboxylase	Sp0916	0.447	0.938	2.1-
speE; Spermidine synthase	Sp0918	0.431	0.916	2.13-

Differentially regulated Genes in 403	Annotation in TIGR4	Expression in 403	Expression in TIGR4	Fold Change in 403
Aminoacid metabolism and Acquisition				
proC; Pyrroline-5-carboxylate ,Amino acid biosynthesis:	Sp0933	1.018	1.161	1.14-
arcA; Arginine deiminase	Sp2148	0.296	0.846	2.86-
argF; Ornithine carbamoyltransferase	Sp2150	0.415	0.853	2.06-
Nucleoside/Nucleotide metabolism				
purF; Amidophosphoribosyltransferase	Sp0046	0.546	0.934	1.71-
purH: Phosphoribosylaminoimidazolecarboxamide	Sp0050	0.542	1.049	1.94-
pyrG; CTP synthase	Sp0494	0.794	1.031	1.3-
nspC; Carboxynorspermidine decarboxylase	Sp0920	0.53	1.079	2.04-
mtf; 5-methylthioadenosine/S-adenosylhomocysteine	Sp0991	0.905	1.032	1.14-
tdk; Thymidine kinase	Sp1018	0.935	1.104	1.18-
udk ; Uridine kinase	Sp1208	0.798	1.167	1.46-
guaA	Sp2072	0.167	1.074	6.43-
carB; Carbamoyl-phosphate synthase, large subunit	Sp1275	0.529	1.017	1.92-
apt; Adenine phosphoribosyltransferase	Sp1577	0.636	0.995	1.5-
Cofactor, Prosthetic Group and Carrier Metabolism				
ispA; Geranyltranstransferase	Sp1205	0.819	1.075	1.31-
mvaS; Hydroxymethylglutaryl-CoA synthase	Sp1727	0.805	0.948	1.18-
Thiamine pyrophosphokinase	Sp 1982	0.778	1.001	1.29-
Reguatory Functions:				
lacR; Lactose phosphotransferase system repressor	Sp0875	0.169	0.647	3.83-

Differentially regulated Genes in 403	Annotation in TIGR4	Expression in 403	Expression in TIGR4	Fold Change in 403
Reguatory Functions:				
Serine/threonine protein phosphatase	Sp1201	0.745	0.922	1.24-
Putative transcriptional repressor	Sp1203	0.784	1.044	1.33-
Putative fucose operon repressor	Sp2168	0.487	0.932	1.91-
Transcription				
rpoE; Putative DNA-directed RNA polymerase, delta subunit	Sp0493	0.692	0.94	1.36-
rnc; Ribonuclease III	Sp1248	0.99	1.111	1.12-
aspB; GTP-sensing transcriptional pleiotropic repressor CodY	Sp1584	0.618	1.055	1.71-
Translation and Protein Synthesis				
rpsD; Ribosomal protein S4	Sp0085	0.648	1.047	1.62-
trmU; 5-methylaminomethyl-2-thiouridylate-Methyltransferase	Sp0118	0.834	1.011	1.21-
rpsJ; Ribosomal protein S10	Sp0208	0.634	0.98	1.55-
rplC; Ribosomal protein L3	Sp0209	0.781	1.026	1.31-
rplB; Ribosomal protein L2	Sp0212	0.754	1.084	1.44-
*rpsN ;*Ribosomal protein S14	Sp0222	0.724	1.085	1.5-
rpmD; Ribosomal protein L30	Sp0228	0.649	0.995	1.53-
rpsG; Ribosomal protein S7	Sp0272	0.667	1.024	1.54-
rplM; Ribosomal protein L13	Sp0294	0.704	1.023	1.45-
RNA methyltransferase, TrmH family	Sp0486	0.622	1.124	1.81-
rplA; Ribosomal protein L1	Sp0631	0.606	1.074	1.77-
rpsP; Ribosomal protein S16	Sp0775	0.576	0.958	1.66-
rplT; Ribosomal protein L20	Sp0961	0.615	0.97	1.58-
pepF; Oligoendopeptidase F	Sp0979	0.74	1.044	1.41-

Differentially regulated genes in 403	Annotation in TIGR4	Expression in 403	Expression in TIGR4	Fold Change in 403
Translation and Protein Synthesis				
Protease maturation protein, putative	Sp0981	0.936	1.104	1.18-
Ribosomal large subunit pseudouridine synthase	Sp1099	0.435	0.878	2.02-
rpmA; Ribosomal protein L27	Sp1107	0.66	0.968	1.47-
rplL; Ribosomal protein L7/L12	Sp1354	0.635	0.939	1.48-
rpsR; Ribosomal protein S18	Sp1539	0.644	0.925	1.44-
rplM; Ribosomal protein L13	Sp0294	0.704	1.023	1.45-
rplA; Ribosomal protein L1	Sp0631	0.606	1.074	1.77-
rpsP; Ribosomal protein S16	Sp0775	0.576	0.958	1.66-
rplT; Ribosomal protein L20	Sp0961	0.615	0.97	1.58-
pepF; Oligoendopeptidase F	Sp0979	0.74	1.044	1.41-
Protease maturation protein, putative	Sp0981	0.936	1.104	1.18-
Ribosomal large subunit pseudouridine synthase	Sp1099	0.435	0.878	2.02-
rpmA; Ribosomal protein L27	Sp1107	0.66	0.968	1.47-
rplL; Ribosomal protein L7/L12	Sp1354	0.635	0.939	1.48-
rpsR; Ribosomal protein S18	Sp1539	0.644	0.925	1.44-
ksgA; Dimethyladenosine transferase	Sp1985	0.815	1.099	1.35-
rpsB; Ribosomal protein S2	Sp2215	0.618	1.028	1.66-
tsf; Translation elongation factor Ts	Sp2214	0.602	0.995	1.65-
htrA; Serine protease	Sp2239	0.844	1.077	1.28-
Unknown functions				
GTP-binding protein	Sp0004	0.754	0.998	1.32-
Hypothetical protein	Sp0099	0.561	0.941	1.68-
Conserved hypothetical protein	Sp0100	0.611	1.155	1.89-
Hypothetical protein	Sp0223	0.783	1.056	1.35-
Hypothetical protein	Sp0270	0.757	1.029	1.36-

Differentially regulated genes in 403	Annotation in TIGR4	Expression in 403	Expression in TIGR4	Fold Change in 403
Unknown functions				
Conserved hypothetical protein	Sp0409	0.25	1.004	4.02-
Hypothetical protein	Sp0703	0.402	0.974	2.42-
Hypothetical protein	Sp0704	0.472	0.968	2.05-
Conserved hypothetical protein	Sp0742	0.703	0.987	1.4-
KH domain protein	Sp0776	0.508	0.899	1.77-
Conserved hypothetical protein	Sp0783	0.63	1.108	1.76-
Hydrolase, haloacid dehalogenase-like family	Sp0805	0.677	0.985	1.45-
Conserved hypothetical protein	Sp0919	0.488	0.975	2-
Hypothetical protein	Sp0987	0.825	0.988	1.2-
Conserved hypothetical protein	Sp1003	0.605	1.031	1.7-
Conserved hypothetical protein	Sp1004	0.362	0.912	2.52-
Hypothetical protein	Sp1142	0.888	1.086	1.22-
Conserved domain protein	Sp1174	0.588	1.07	1.82-
Conserved hypothetical protein	Sp1384	0.787	0.986	1.25-
Oxidoreductase, putative	Sp1472	0.428	0.917	2.14-
Hypothetical protein	Sp1476	0.705	1.045	1.48-
Hypothetical protein	Sp1477	0.755	0.971	1.29-
Conserved domain protein, authentic frameshift	Sp1532	0.668	1.006	1.51-
Conserved domain protein	Sp1533	0.654	0.988	1.51-
Isochorismatase family protein	Sp1583	0.637	0.933	1.46-
Conserved hypothetical protein	Sp1685	0.428	0.893	2.09-
Conserved hypothetical protein	Sp1801	0.687	1.23	1.79-
Hypothetical protein	Sp1802	0.564	0.996	1.77-
Hypothetical protein	Sp2071	0.545	0.986	1.81-
Hypothetical protein	Sp2105	0.646	0.958	1.48-
Hypothetical protein	Sp2182	0.369	0.801	2.17-

Differentially regulated genes in 403	Annotation in TIGR4	Expression in 403	Expression in TIGR4	Fold Change in 403
Tranporters				
PTS system, IID component	Sp0063	0.541	1.087	2.01-
secY; Preprotein translocase, SecY subunit	Sp0230	0.81	1.025	1.27-
ABC transporter, ATP-binding protein	Sp0483	0.884	1.012	1.14-
Sodium:alanine symporter family protein	Sp0408	0.591	1.091	1.85-
ABC transporter, ATP-binding protein	Sp0707	0.462	0.93	2.01-
Amino acid ABC transporter, ATP-binding protein	Sp0709	0.491	0.889	1.81-
fruA; PTS system, fructose specific IIABC components	Sp0877	0.259	0.711	2.75-
Amino acid permease family protein	Sp1001	0.591	0.884	1.5-
Putative transporter, FNT family	Sp1215	0.406	0.92	2.27-
uraA; uracil permease	Sp1286	0.486	1.138	2.34-
Sugar ABC transporter, permease protein	Sp1682	0.32	0.783	2.45-
Sugar ABC transporter, sugar-binding protein	Sp1683	0.308	0.843	2.74-
PTS system, IIBC components	Sp1684	0.305	0.837	2.74-
ABC transporter, permease protein	Sp1688	0.212	0.725	3.42-
ABC transporter, permease protein	Sp1689	0.337	0.718	2.13-
ABC transporter, substrate-binding protein	Sp1690	0.282	0.678	2.4-
rafF; Sugar ABC transporter, permease protein	Sp1896	0.255	0.599	2.35-
rafE; Sugar ABC transporter, sugar-binding protein	Sp1897	0.223	0.597	2.68-
secE; Putative preprotein translocase, SecE subunit	Sp2008	0.751	0.978	1.3-
malX; Maltose/maltodextrin ABC transporter	Sp2108	0.374	0.833	2.23-
Transposase				
IS1167, transposase, degenerate	Sp0572	0.687	0.941	1.37-
IS66 family element, Orf3, degenerate, This gene has an N-	Sp0644	0.751	0.98	1.3-
Transposase	Sp1064	0.329	0.871	2.65-
Transposase	Sp1582	0.748	1.013	1.35-

Differentially regulated genes in 403	Annotation in TIGR4	Expression in 403	Expression in TIGR4	Fold Change in 403
Transposase				
Transposase, IS200 family	Sp1622	0.333	0.892	2.68-
Transposase	Sp1905	0.712	1.022	1.44-
Signal Transduction				
*hk07;*putative sensor histidine kinase	Sp0155	0.545	0.925	1.7-
treP; Trehalose PTS system, IIABC components	Sp1884	0.127	0.588	4.63-
Upregulated Genes in 403				
Virulence				
Choline binding protein J	Sp0378	1.097	1.023	1.07+
Putative immunity protein	Sp1988	1.308	1.035	1.26+
Membrane proteins				
Putative membrane protein	Sp0858	1.424	1.01	1.41+
Membrane protein	Sp0859	1.488	1.086	1.37+
DNA repair, recombination and modification				
Putative type I restriction-modification system, S subunit, DNA	Sp0505	2.165	1.002	2.16+
MutT/nudix family protein	Sp0794	1.487	0.96	1.55+
DNA polymerase III, epsilon subunit/ATP-dependent helicase	Sp0802	1.259	0.965	1.3+
Putative endonuclease	Sp1251	1.873	0.981	1.91+
nth; endonuclease III	Sp1279	1.348	0.954	1.41+
Carbohydrate and Energy Metabolism				
PEP-utilizing enzymes family protein	Sp0795	1.528	1.027	1.49+

Differentially regulated genes in 403	Annotation in TIGR4	Expression in 403	Expression in TIGR4	Fold Change in 403
Carbohydrate and Energy Metabolism				
gapN; Glyceraldehyde-3-phosphate dehydrogenase	Sp1119	2.587	1.033	2.5+
Acetoin dehydrogenase complex, E3 component	Sp1161	1.668	1.006	1.66+
Acetoin dehydrogenase complex, E2 component	Sp1162	1.52	0.958	1.59+
Putative acetoin dehydrogenase, E1 component	Sp1163	1.508	0.939	1.61+
fer; Ferredoxin	Sp1605	1.507	1.021	1.48+
recP-2; Transketolase	Sp2030	1.488	0.939	1.58+
gpsA; Glycerol-3-phosphate dehydrogenase (NAD(P)+)	Sp2091	1.154	0.928	1.24+
Aminoacid biosynthesis and Acquisition				
IlvN; Acetolactate synthase, small subunit,Putative	Sp0446	2.558	1.246	2.05+
Transulfuration enzyme family protein, authentic point	Sp1214	1.8	0.987	1.82+
leuB; 3-isopropylmalate dehydrogenase, authentic point	Sp1257	1.391	0.965	1.44+
glyA; Serine hydroxymethyltransferase	Sp1024	1.9	1.067	1.78+
Integrase/recombinase, phage integrase family	Sp1159	1.279	0.995	1.29+
Putative chorismate mutase	Sp1296	2.334	1.049	2.22+
pheA; Prephenate dehydratase	Sp1369	1.822	1.046	1.74+
aroA; 3-phosphoshikimate 1-carboxyvinyltransferase	Sp1371	1.614	1.014	1.59+
tyrA; Chorismate synthase	Sp1373	1.471	1.09	1.35+
aroC; Chorismate synthase	Sp1374	1.206	0.986	1.22+
lysA; Diaminopimelate decarboxylase	Sp1978	1.635	1.015	1.61+
trpA; Tryptophan synthase, alpha subunit	Sp1811	2.731	0.965	2.83+
trpB; Tryptophan synthase, beta subunit	Sp1812	3.245	1.167	2.78+
trpC; Indole-3-glycerol phosphate synthase	Sp1814	3.317	0.957	3.47+
trpD; Anthranilate phosphoribosyltransferase	Sp1815	3.12	1.046	2.98+
trpG; Anthranilate synthase component II	Sp1816	3.079	1.122	2.74+
asnA; Aspartate-ammonia ligase	Sp1970	1.616	0.997	1.62+

Differentially regulated genes in 403	Annotation in TIGR4	Expression in 403	Expression in TIGR4	Fold Change in 403
Nucleoside/Nucleotide metabolism				
Phosphorylase, Pnp/Udp family	Sp0075	2.498	1.027	2.43+
Integrase/recombinase, phage integrase family	Sp0506	1.494	0.964	1.55+
pyrC; Dihydroorotase, multifunctional complex type	Sp1167	1.27	1.039	1.22+
ung; Uracil-DNA glycosylase	Sp1169	1.394	1.039	1.34+
Putative type II restriction endonuclease	Sp1222	1.164	1.045	1.11+
mutY; A/G-specific adenine glycosylase	Sp1228	1.422	1.009	1.41+
relA; GTP pyrophosphokinase	Sp1645	1.456	0.99	1.47+
aroF; Phospho-2-dehydro-3-deoxyheptonate aldolase	Sp1700	1.236	0.963	1.28+
Cofactor Metabolism				
ribC; Riboflavin synthase, alpha subunit	Sp0177	2.468	1.14	2.16+
pdxK; Phosphomethylpyrimidine kinase,putative	Sp1598	2.031	1.081	1.88+
5-formyltetrahydrofolate cyclo-ligase family protein	Sp2095	1.695	1.017	1.67+
Fatty Acid Metabolism				
fabG; Oxidoreductase, short chain dehydrogenase/reductase	Sp0793	1.614	1.03	1.57+
Transcription				
Putative transcriptional regulator	Sp0461	2.013	0.971	2.07+
Transcriptional regulator	Sp1130	1.243	0.971	1.28+
PolyA polymerase family protein	Sp1554	1.35	1.017	1.33+
Translation				
truA; tRNA pseudouridine synthase A	Sp1599	2.057	1.065	1.93+
tyrS; tyrosyl-tRNA synthetase	Sp2100	1.534	1.09	1.41+

Differentially regulated genes in 403	Annotation in TIGR4	Expression in 403	Expression in TIGR4	Fold Change in 403
Post translational protein alterations				
pepT; Peptidase t	Sp1008	2.048	0.947	2.16+
lplA; Lipoate-protein ligaseputative	Sp1160	1.841	1.002	1.84+
Serine/threonine protein kinase	Sp1732	1.213	0.975	1.24+
Unknown functions				
Conserved hypothetical proteins	Sp0024	1.878	1.023	1.84+
Hypothetical protein	Sp0133	1.219	1.098	1.1+
Conserved hypothetical protein	Sp0239	1.879	0.97	1.96+
Hypothetical protein	Sp0448	1.98	1.005	1.97+
Hypothetical protein	Sp0449	2.127	0.999	2.13+
Conserved hypothetical protein	Sp0482	1.278	1.019	1.25+
Conserved hypothetical protein	Sp0785	1.527	1.067	1.43+
Hypothetical protein	Sp0792	1.604	1.016	1.58+
Hypothetical protein	Sp0901	1.348	0.998	1.35+
*hemK;*HemK protein	Sp1021	1.722	1.039	1.66+
Sua5/YciO/YrdC family protein	Sp1022	1.867	1.018	1.83+
Acetyltransferase, GNAT family	Sp1023	1.824	1.042	1.75+
Hydrolase, haloacid dehalogenase-like family	Sp1171	1.506	0.997	1.34+
Conserved domain protein	Sp1175	1.276	1.077	1.18+
crcB protein	Sp1294	1.895	0.984	1.93+
*crcB2;*crcB protein	Sp1295	2.211	1.11	1.99+
DHH subfamily 1 protein	Sp1298	1.43	0.921	1.55+
Amidohydrolase family protein	Sp1356	3.178	1.048	3.03+
Psr protein	Sp1368	1.745	1.089	1.6+
Conserved hypothetical protein	Sp1393	1.611	1.003	1.61+
Conserved hypothetical protein	Sp1462	1.853	0.989	1.87+

Differentially regulated genes in 403	Annotation in TIGR4	Expression in 403	Expression in TIGR4	Fold Change in 403
Unknown functions				
Acetyltransferase, GNAT family	Sp1464	1.931	1.058	1.83+
Hypothetical protein	Sp1465	1.791	0.948	1.9+
ATP-dependent RNA helicase, DEAD/DEAH box family	Sp1483	1.195	0.997	1.2+
Hypothetical protein	Sp1493	1.623	0.933	1.74+
Conserved hypothetical protein	Sp1564	1.3	1.015	1.28+
Conserved hypothetical protein	Sp1565	1.454	1.091	1.33+
Rrf2 family protein	Sp1636	1.194	0.971	1.23+
Metallo-beta-lactamase superfamily protein	Sp1646	1.489	0.984	1.51+
Hypothetical protein	Sp1862	1.558	1.002	1.55+
Conserved hypothetical protein	Sp1922	1.493	1.016	1.47+
Hypothetical protein	Sp1925	1.538	0.967	1.59+
Conserved hypothetical protein	Sp1967	1.163	0.937	1.24+
Hypothetical protein	Sp2004	2.669	1.17	2.28+
Hypothetical protein	Sp2005	2.857	0.789	3.62+
Conserved hypothetical protein	Sp2143	1.969	0.949	2.07+
SPFH domain/Band 7 family	Sp2156	1.585	0.933	1.7+
Transport				
Xanthine/uracil permease family protein	Sp0287	1.567	1.053	1.49+
ABC transporter, ATP-binding protein	Sp0720	1.326	1.049	1.26+
Potassium uptake protein, Trk family	Sp0480	1.249	0.943	1.32+
livH; Branched-chain amino acid ABC transporter	Sp0750	1.577	1.008	1.56+
ABC transporter, ATP-binding protein	Sp0786	1.296	0.956	1.36+
DNA primase, DNA metabolism: DNA replication,	Sp1071	1.907	1.038	1.84+
bta; Bacterocin transport accessory protein	Sp1499	1.31	0.925	1.42+
ntpC; v-type sodium ATP synthase, subunit C	Sp1319	1.599	0.787	2.03+

Differentially regulated genes in 403	Annotation in TIGR4	Expression in 403	Expression in TIGR4	Fold Change in 403
Transport				
ABC transporter, permease/ATP-binding protein	Sp1357	3.205	1.093	2.93+
ABC transporter, permease/ATP-binding protein	Sp1358	2.842	1.079	2.63+
aatB; Amino acid ABC transporter, amino acid-binding protein	Sp1500	2.671	1.05	2.54+
Amino acid ABC transporter, ATP-binding protein	Sp1501	2.813	1.065	2.64+
Amino acid ABC transporter, permease protein	Sp1502	3.036	1.122	2.71+
Cation efflux family protein,	Sp1552	1.479	1.071	1.38+
Oxalate:formate antiporter	Sp1587	1.982	1.057	1.88+
Sodium/dicarboxylate symporter family protein, authentic	Sp1753	1.401	1.06	1.32+
proWX: Choline transporter	Sp1860	1.697	0.967	1.75+
proV; Choline transporter	Sp1861	1.69	0.984	1.72+
amiC; Oligopeptide ABC transporter, permease protein AmiC	Sp1890	1.164	1	1.164+
ABC transporter, ATP-binding protein	Sp2003	3.335	1.059	3.15+
ABC transporter, permease/ATP-binding protein	Sp2073	1.86	1.002	1.86+
ABC transporter, permease/ATP-binding protein	Sp2075	1.747	1.013	1.72+
pstS; Phosphate ABC transporter, phosphate-binding protein	Sp2084	3.742	1.086	3.45+
pstC; Phosphate ABC transporter, permease protein	Sp2085	3.417	0.93	3.67+
pstA: Phosphate ABC transporter, permease protein	Sp2086	3.729	1.001	3.73+
pstB: Phosphate ABC transporter, ATP-binding protein	Sp2087	3.589	0.867	4.14+
phoU; Phosphate transport system regulatory protein PhoU	Sp2088	3.919	0.924	4.24+
Transposons				
IS66 family element, Orf2, interruption	Sp1313	1.314	1.017	1.29+
Putative IS1239, transposase, degenerate	Sp1515	1.363	1.086	1.26+
Transposase OrfA	Sp1927	1.161	0.909	1.28+

Differentially regulated genes in 403	Annotation in TIGR4	Expression in 403	Expression in TIGR4	Fold Change in 403
Transposons				
Transposase OrfB	Sp1928	1.072	0.969	1.11+
Transposase, IS116/IS110/IS902 family, degenerate	Sp2074	1.211	0.962	1.26+
IS1381, transposase OrfB	Sp2079	1.095	0.931	1.18+
Signal Transduction				
DNA-binding response regulator	Sp2000	2.227	0.999	2.23+
hk11; Putative sensor histidine kinase	Sp2001	2.881	1.216	2.37+

5.4 Discussion

S. pneumoniae is an organism which is found globally and continues to cause significant morbidity and mortality across the continents. The ubiquitous nature of pneumococcal carriage and infections was only possible due to very high carriage rate in human population that results from high plasticity of pneumococcal genome and its capability to adapt to a number of environments which vary in terms of population dynamics, antibiotic use, vaccination coverage and competition with other colonizers. This high plasticity of pneumococcal genome results in variation of pathogenicity of different strains and might result in a mutation, which can render a strain completely non-pathogenic. The two strains can be compared in several ways but the most appropriate one is complete genome sequencing as it has the advantage of being extensive and it can identify minor differences in the two genomes with precision.

403 is a strain, which was isolated in our lab and was found to be completely non-pathogenic in mouse models of sepsis. Since the records suggested that this strain evolved from TIGR4, we decided to sequence it to get the complete genome sequence of this strain, which would provide a concrete basis for the future investigations and will ensure quality of downstream research work in the lab.

Genome comparison of the two pneumococcal strains was not very complicated as both the strains have remarkable similarity. Complete assembly of 403 sequences against TIGR4 revealed only a few differences between the two strains and only 35 SNPs were present in 403, which might be responsible for

phenotypic difference between the two strains. Though six of these SNPs were in intergenic regions, and other seven mutations were synonymous and were not associated with any amino acid change, the rest of them were located in functionally important genes which could contribute towards decreased virulence of strain 403.

Microarray analysis showed differential regulation of 288 genes, some of which are critical for pneumococcal virulence and have been reported as being essential for pneumococcal growth. The list also includes a very large number of ABC transporters and proteins of unknown function, whose contribution in 403 phenotype cannot be assessed.

It was observed that a number of genes related to carbohydrate metabolism were differentially regulated in 403 and among sugars (glucose, sucrose, raffinose, lactose, trehalose, inulin, maltose and galactose) utilized by 403 (Hava & Camilli, 2002), genes related to fructose metabolism (*fruB*, *glmS)* and transport (*fruA*), galactose metabolism (*galM, , galK, galT, aga, nagB), trehalose transport (treP), maltose/maltodextrin transport (MalX) and other sugar transporters(raff, rafE,* Sp0063, Sp1682, Sp1683 and Sp1684*)* were down-regulated though genes coding for enzymes related to glucose metabolism(*gapN, gpsA* and Sp0795) were upregulated along with those of gluconeogenesis such as acetoin dehydrogenase complex, which indicates preferential utilization of glucose over other resources in 403. There is a possibility that downregulation of metabolism and transport of other sugars in 403 is due to preferred glucose utilization, which has a known suppressive effect upon the use of other sugars called carbon catabolite repression or CCR. This repressive effect of glucose

over other sugars has been known for more than half a century and was first described by Jacques Monod in 1942 (Gorke & Stulke, 2008). It is defined as:

"….a regulatory phenomenon by which the expression of functions for the use of secondary carbon sources and the activities of the corresponding enzymes are reduced in the presence of a preferred carbon source……. (Gorke & Stulke, 2008)."

Also known as "diauxie," it has been observed in a number of organisms and studied in detail and has been extensively reviewed by Gorke and Stulke, and Duetcher in 2008 and Vinuselvi in 2012 (Vinuselvi *et al.*, 2012; Deutscher, 2008; Gorke & Stulke, 2008). In *S. pneumoniae* a gene *CcpA* related to CCR has been identified, which is known to affect expression of a number of virulence factors according to changes in nutrient availability. *CcpA* expression was not altered in 403, but the role of one or more genes coding for "proteins of unknown functions" causing the same effect cannot be excluded.

Iron and zinc containing alcohol dehydrogenases *adhE* and *adh* were also observed to have lower levels of expression in 403 as compared to TIGR4, which may compromise the organism's ability to effectively colonize and invade host tissues as it would need a fully functional anaerobic metabolism for adaptation to low-oxygen environments within the host since it relies heavily on fermentation and is a facultative anaerobe. Gene *adh* was also reported to be among essential genes for pneumococcal virulence (Hava & Camilli, 2002).

It was also observed that genes coding for enzymes related to metabolism of basic amino acids including arginine and lysine were downregulated (*argF, arcA and proC*) though those related to synthesis of aromatic amino acids including

phenylalanine, tyrosine and tryptophan biosynthesis (*pheA, tyrA, aroC, trpA, trpB, trpC, trpD and , trpG*) and hydrophobic amino acids were up-regulated. Decreased synthesis of arginine appears related to reduced production of spermidine (as indicated by decreased expression of *speE*, spermidine synthase), which is among polyamines and has an important role in cellular growth, proliferation and stabilization and maintenance of subcellular organelles, and membrane systems (Murray *et al.*, 2003). Another downregulated gene *nspC* coding for carboxynorspermidine decarboxylase might be an important contributor responsible for 403 phenotype as it participates in a number of metabolic processes including 1 and 2 methylnaphthalene degradation, 3-chloroacrylic acid degradation, alkaloid biosynthesis, benzoate degradation, phenylpropanoid biosynthesis, purine metabolism, pyruvate, tryptophan and tyrosine metabolism.

Decrease in expression of ribosomal proteins including *rpsD, rpsJ, rplC, rplB, rpsN, rpmD, rpsG, rplM, rplA, rpsP, rplT,pepF, rpmA, rplL, rpsR , rpsB* and *tsf* might be related to over-expression of *relA/Sp1645* (Table 5.5) as it has been reported to have important role in *E. coli* and other gram-negative organisms, where it decreases RNA synthesis and ribosomal proteins, increases synthesis of amino acids, and alters transcription pattern (Dennis & Nomura, 1974). Though its role in *S. pneumoniae* is not detrimental in presence of abundant nutrients but it is mainly required in nutritional stress (Kazmierczak *et al.*, 2009), its up-regulation along with decreased ribosomal protein expression indicates that 403 probably grows under stressful conditions and there is a possibility that it is unable to adapt to eukaryotic host environment as adaption is not only required during nutritional scarcity and stress but is also required to develop a symbiotic relationship with the host, which is the first step towards invasion.

Among the transport proteins, genes related to phosphate ABC transport system *pstS*, *pstC, pstA, pstB* and *phoU* were found up-regulated. It has been reported for gene *pstS* that its expression levels increase during infection and they are responsive to availability of phosphate (Orihuela *et al.*, 2004). More recently, the role of the pst sytem in phosphate transport was described in *Streptococcus mutans* and it was reported that this system is also important for biofilm formation and proper growth (Luz *et al.*, 2012). Genetic regulation of this system is complex and involves a regulon called Pho regulon that is under the influence of a two component regulatory system (Lamarche *et al.*, 2008). Though reasons of up-regulation of the whole operon along with *phoU*, which is the repressor, cannot be explained on the basis of available literature, and are unclear, it can still be assumed that it has some contribution towards the phenotype of 403.

Though exact reasons for altered biology of 403 remains unclear, the complete genome sequence and array data has provided the picture of an evolutionary point in the phylogenetic history of this organism. There is a possibility that differences in sugar and protein metabolism and changes in surface proteins might lead towards a phenotype, which is unable invade the host body and cause disease.

Strain 403 was also tested as a live attenuated vaccine to see if it was protective against challenge with the same as well as different serotypes. Since its complete genome sequence is known and its biological behaviour was studied in detail, it was thought that once it is proven immunogenic, it can be easily altered to increase immunogenicity and any changes in its behaviour could also

be predicted and analyzed on the basis of genome sequence and array data. It can also be made untransformable to prevent further changes in its genome.

6 Avirulent pneumococci as live cell vaccines

Different types of vaccines have been utilized for more than two centuries against infectious diseases. They have been a major weapon against micro organisms and their use has significantly reduced morbidity and mortality associated with infectious diseases (Plotkin, 2005). Different strategies being used for vaccination against infectious diseases include use of killed whole cell vaccine, live cell attenuated vaccines, toxoids, capsular polysaccharides, polysaccharides conjugated with proteins and recombinant proteins (Plotkin, 2005). Among these, polysaccharides and protein-conjugated polysaccharide vaccines have been major vaccination strategies against pneumococcal diseases. Due to the availability of these vaccines along with antibiotics as easier alternatives, other vaccination strategies were never given due importance in pneumococcal research until recently as the human efforts were mainly directed towards development of newer and better antibiotics and improvement of existing vaccines. Because of certain limitations associated with these vaccines, the last few years have seen a surge in research directed towards development of alternative vaccine strategies (Barocchi *et al.*, 2007), which include development of protein based vaccines (Swiatlo & Ware, 2003), use of whole-cell killed vaccines (Malley & Anderson, 2012; Lu *et al.*, 2010; Malley *et al.*, 2004; Malley *et al.*, 2001) and live cell attenuated vaccines (Kim *et al.*, 2012; Roche *et al.*, 2007).

A number of pneumococcal proteins have been tried in different formulations as it has been observed that protection provided by proteins would be serotype independent (Moffitt & Malley, 2011; Ogunniyi *et al.*, 2007; Tai, 2006; Swiatlo &

Ware, 2003). Whole cell killed vaccines and live attenuated vaccines have also been tried against pneumococcal diseases and both have provided promising results. They simultaneously deliver many antigens, which induces immunity in vaccinated animals. It was observed that when unencapsulated pneumococci were used intranasally as whole cell killed vaccine with cholera toxin as adjuvant, it was not only able to prevent nasopharyngeal colonization but also protected against pulmonary infection by capsulated pneumococci (Malley *et al.*, 2001). Similar vaccine in aluminium derived adjuvant, when given as injection induced both cell mediated and humoral immunity with a 30 fold higher antibody response as compared to the previous approach (Malley & Anderson, 2012). It has also been shown that this immunity was inducible in μ MT −/− mice that are incapable of producing antibodies, while mice lacking T lymphocytes or lacking CD4+ T cell responses (MHC II-deficient mice) were not protected (Malley *et al.*, 2005). On the other hand, live attenuated unencapsulated pneumococcal strains used as intranasal vaccine were shown to induce mucosal as well as systemic protection in mice, which was serotype independent and did not require any adjuvant (Roche *et al.*, 2007). It was also shown that this protection was antibody mediated and also required T cells as a significant increase was noted in the levels of IgG in serum and IgA in nasal washes of immunized mice. This protection was not seen in μ MT −/− and MHC II-deficient mice (Roche *et al.*, 2007).

Strain 403 was tested as live attenuated vaccine, as it is an avirulent derivative of TIGR4 and was noted to be able to colonize the mouse nasopharynx, though its virulence potential was not reported (Trzcinski *et al.*, 2003). It was found to be avirulent in a chinchilla model (Personal communication with Dr. Stephen Pelton). This strain was selected to be tested as live cell vaccine as it offers

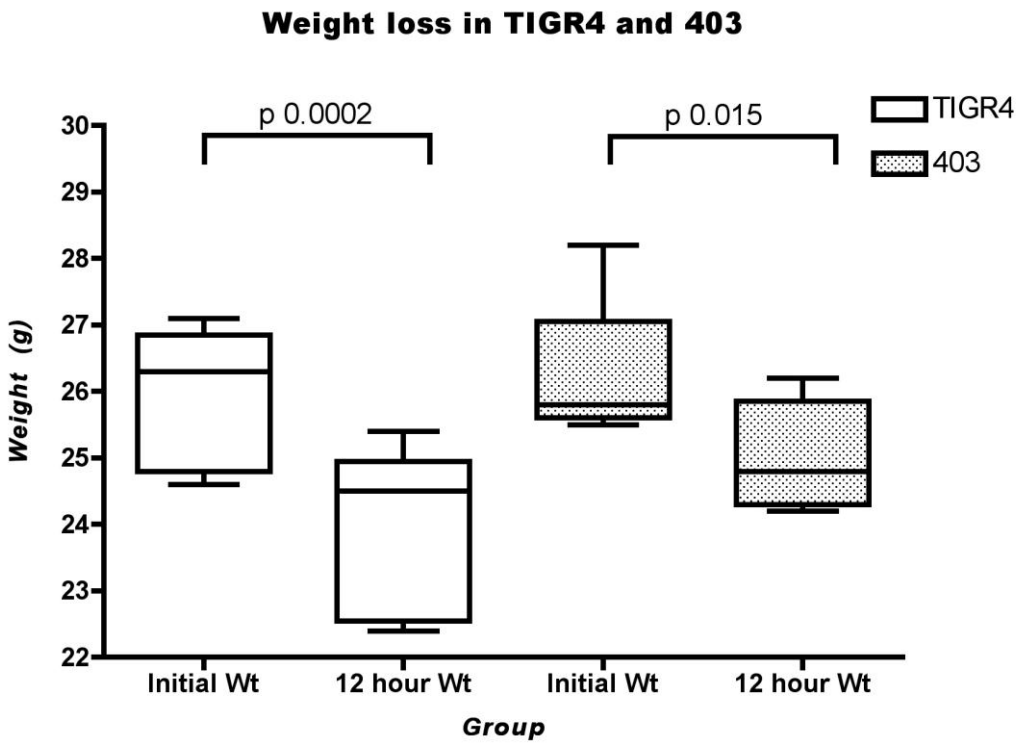
advantage of simultaneous delivery of many immunogens including capsule, which could result in stimulating anticapsular antibodies along with cell mediated response to protein antigens. Its longer presence in the nasopharynx could be translated into a more persistent immune response as it would also mean longer exposure to immunogens. Capsule switch mutants of this strain can also allow simultaneous delivery of different types of capsules which can replace conjugated vaccine as a cheaper alternative and easy to prepare formulation.

6.1 403 as live cell vaccine:

Before using strain 403 as vaccine it was necessary to evaluate its virulence potential in animal models and establish if it is actually harmless to them. MF1 mice were challenged intraperitoneally with 1 x 10^5 cfu/50 µl, using the same quantity of virulent TIGR4 strain as control. It was observed that not only was 403 avirulent but it also failed to produce any bacteraemia.

6.2 Safety of strain 403 as a live-attenuated vaccine:

Strain 403 was also assessed for safety before it could be tested as live attenuated vaccine. It was observed that mice inoculated with 403 lost some weight during the initial 12 hrs post-inoculation but recovered as compared to their counterparts as shown in Fig 6.1.



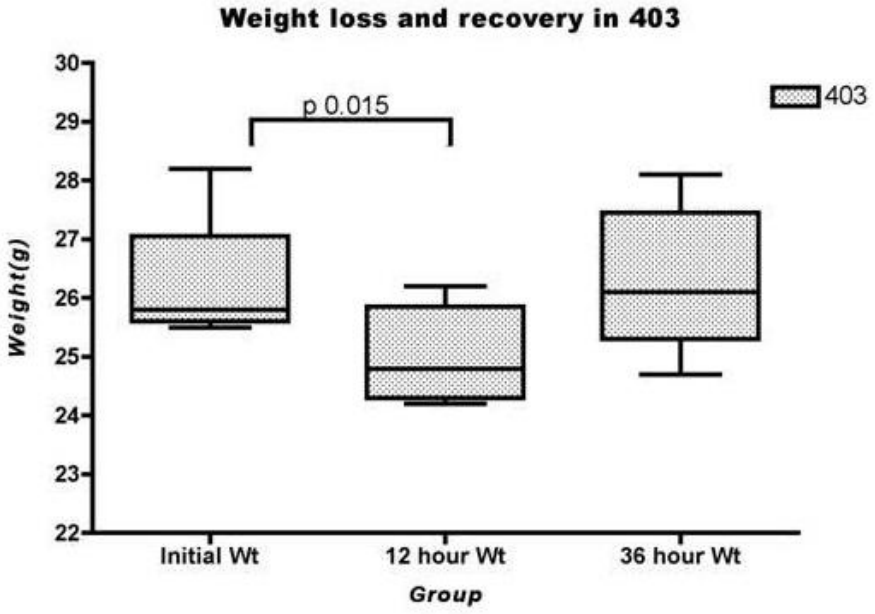


Figure 6.1. Post-inoculation weight loss in MF1 mice inoculated with 10^5 cfu/200 µl strain 403 IP, as compared to mice challenged with 10^5 cfu/200 µl TIGR4 IP.
A. Comparison of weight loss in both the groups during the first 12 hrs. Loss in TIGR4 infected group is more pronounced.
B. Weight loss in mice inoculated with 10^5 cfu/200 µl 403 IP. Mice recovered after 36 hrs as compared to their TIGR4 counterparts which did not survive beyond 30h. Data is plotted as box and whiskers plot with the horizontal line in the box representing the median. Weight changes were compared by paired t-test (GraphPad Prism 4.0, USA).

Some temperature drop was also noticed in mice challenged with 403 during the first 12 hrs of challenge, though it was not statistically significant (Fig 6.2).

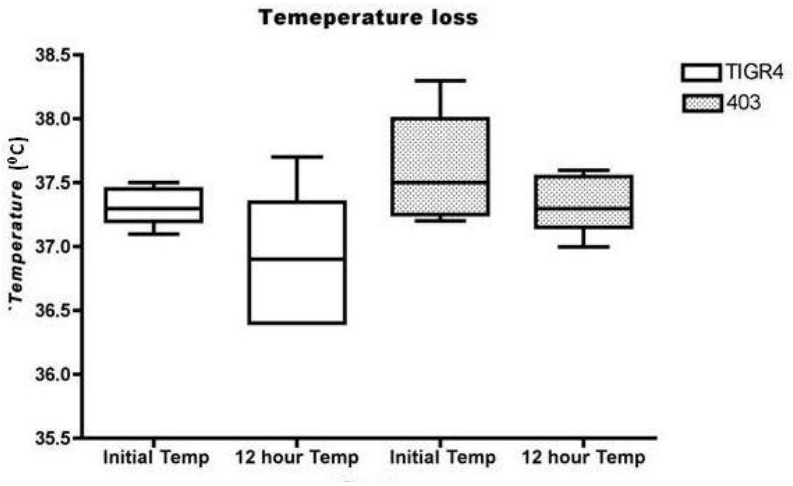


Figure 6.2. Post-inoculation temperature drop during the first 12 hrs in MF1 mice inoculated with 10^5 cfu/200 µl strain 403 IP and mice challenged with 10^5 cfu/200 µl TIGR4 IP. Temperature data is plotted as a box and whiskers plot with horizontal line in the box representing the median. Changes within a group were compared by paired t-test (GraphPad Prism 4.0, USA). Decrease in temperature was more pronounced in mice infected with TIGR4 as compared to their counterparts challenged with 403, which gradually recovered within 24 hrs.

6.3 IP Vaccination with strain 403:

Two groups of out-bred MF1 mice were used to assess potential of 403 strains as live-attenuated vaccine. Mice were vaccinated intraperitoneally with either 5 x 10^6 403 cfu/200µl re-suspended in PBS or PBS alone. Booster doses were given on day 14 and day 28. Mice were left for a month before they were challenged with TIGR4 strains.

On challenging IP with 10^5 cfu/200µl TIGR4 suspended in PBS, vaccinated mice survived significantly longer than their counterparts (p=0.0018) though only 20% of vaccinated mice survived till the end of the experiment. Development of symptoms was also slower in the vaccinated group (Fig 6.3).

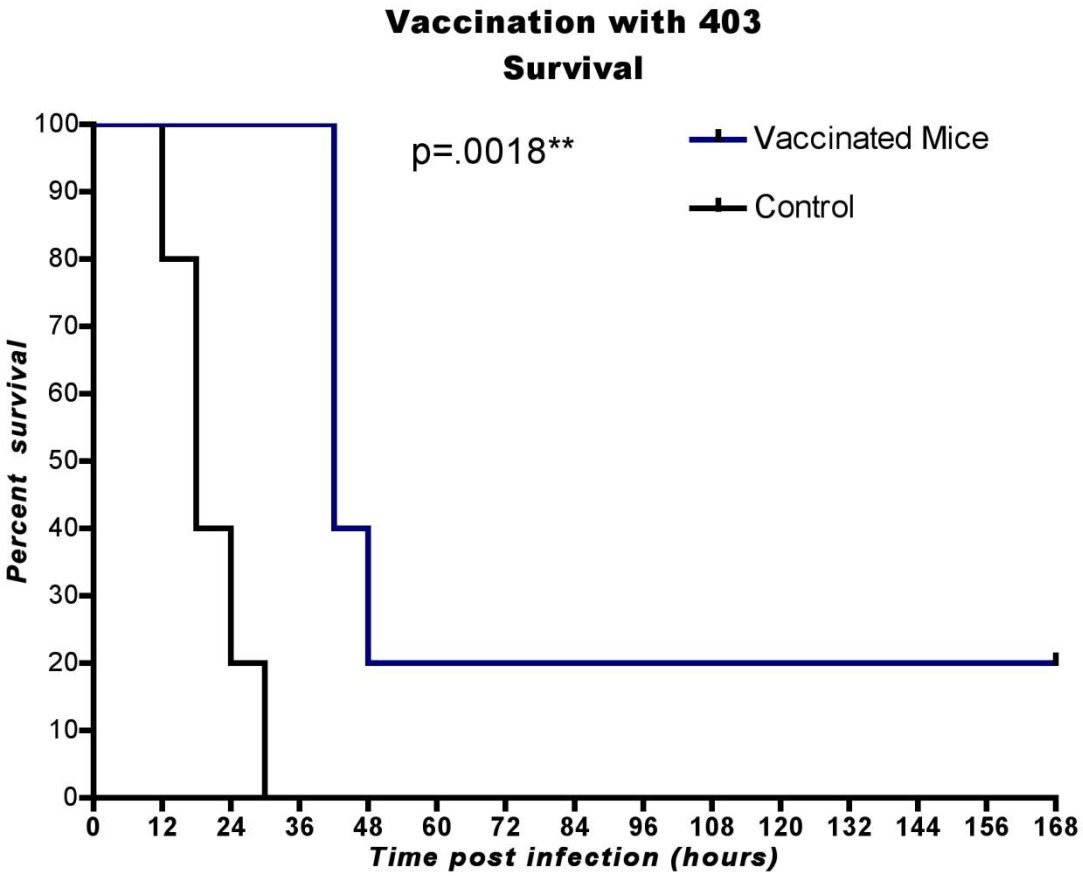
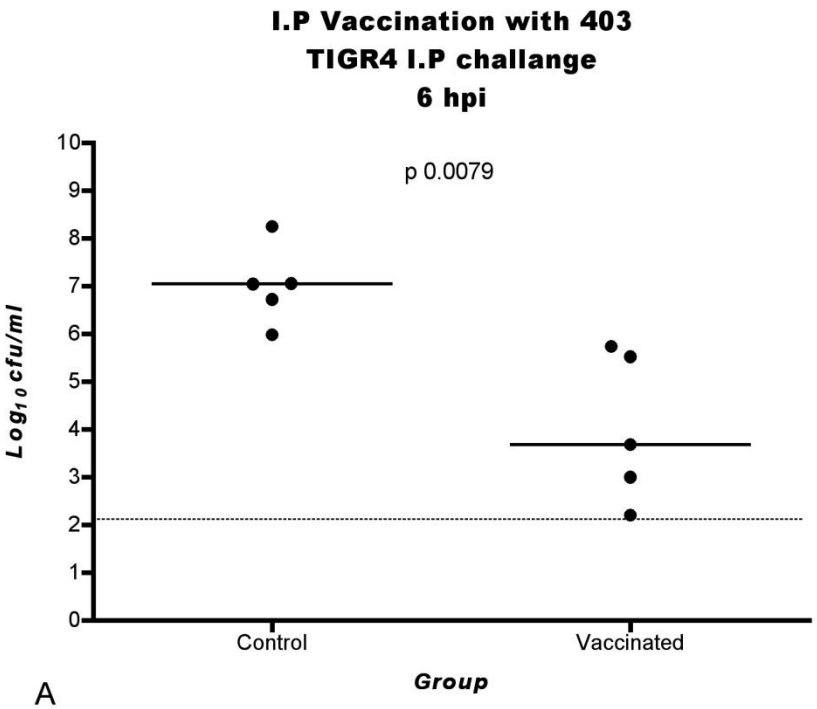


Figure 6.3. Survival curve of mice 3x vaccinated IP with 5 x 10^6 cfu of strain 403/ 200µl PBS or PBS only and challenged with 1x10^5 cfu/200 µl TIGR4 IP. Mice vaccinated with 403 strains survived longer than control mice and 20% survived till end of experiment. Median survival time for vaccinated mice was 42 hrs as compared to that of 18 hrs for control mice. Survival analysis showed there was a significant difference in survival time between the two groups by log rank test (p=0.0018). All data plotted as percentage survival as a staircase line with points for all observations against hrs post infection.

Level of bacterial load in vaccinated mice was also different as compared to non-vaccinated group and former showed decreased bacteraemia as compared to the latter. Levels of bacteraemia were significantly different at the 12 hour time point. All the analyses were performed while all mice were alive unless stated otherwise.

The first mouse from the control group was culled at 12 (hours post infection) hpi and the next two were sacrificed at 18 hpi after they reached moribund state. There was significant difference in bacteraemia levels in the two groups at 6 and 12 hour time points (Fig 6.4-6.5). A continuous drop in bacteraemia was observed in vaccinated group after a peak at 24 hrs, which reached the lowest level at 42 hrs, where mice had to be sacrificed because they had reached moribund state.



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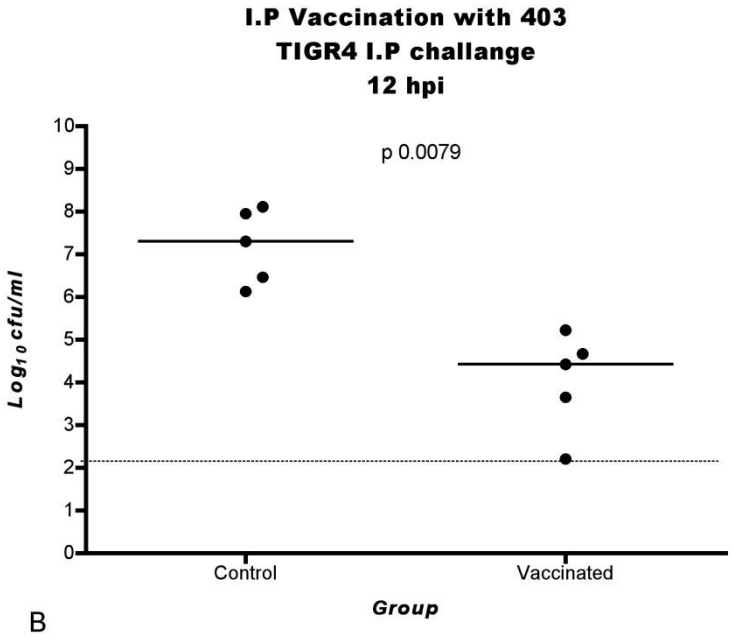


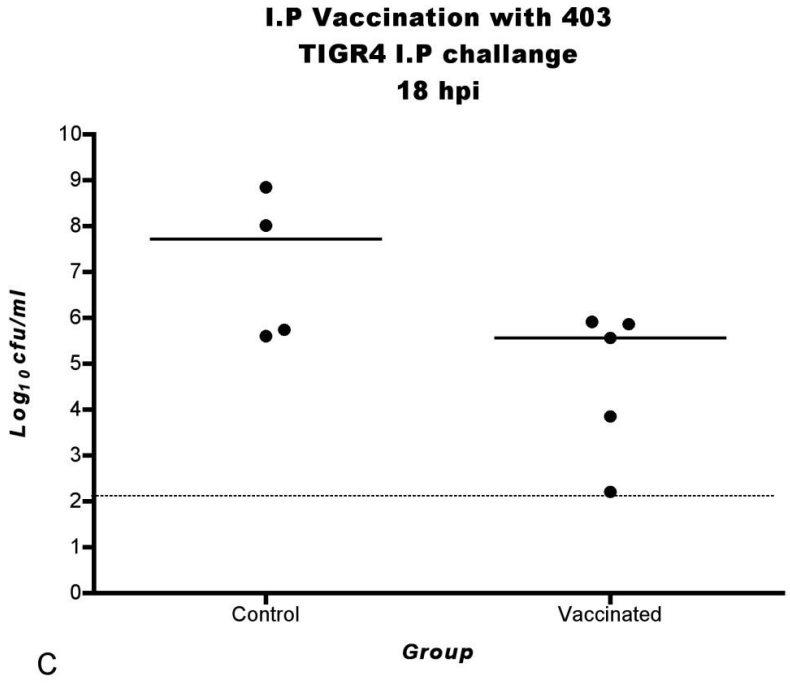


Figure. 6.4. Comparison of bacterial load during 6-18 hrs post-challenge with 1x10^5 cfu/ 200 µl TIGR4 IP in mice 3x vaccinated IP with 5 x 10^6 cfu of strain 403/ 200µl PBS and control group given PBS only. Groups were compared with Mann-Whitney test (GraphPad Prism 4.0, USA). Circles mark individual mice. Horizontal dotted line represents the limit for detection. Horizontal bar represents median. (A) Bacteraemia count at 6 hpi. There was significant difference between PBS control and vaccinated mice (p=-0079) (B) Bacteraemia count at 12 hpi. There was a significant difference between PBS control and vaccinated mice (p=-0079) (C) Bacteraemia count at 18 hpi. Statistical analysis was not performed for this time point as one of the control mice had been sacrificed at 12 hpi.

Mann Whitney's test showed significant difference (p=.0079) in the bacterial load between the two groups at 6 and 12 hrs (GraphPad Prism 4.0, USA) as shown in Fig 6.5.

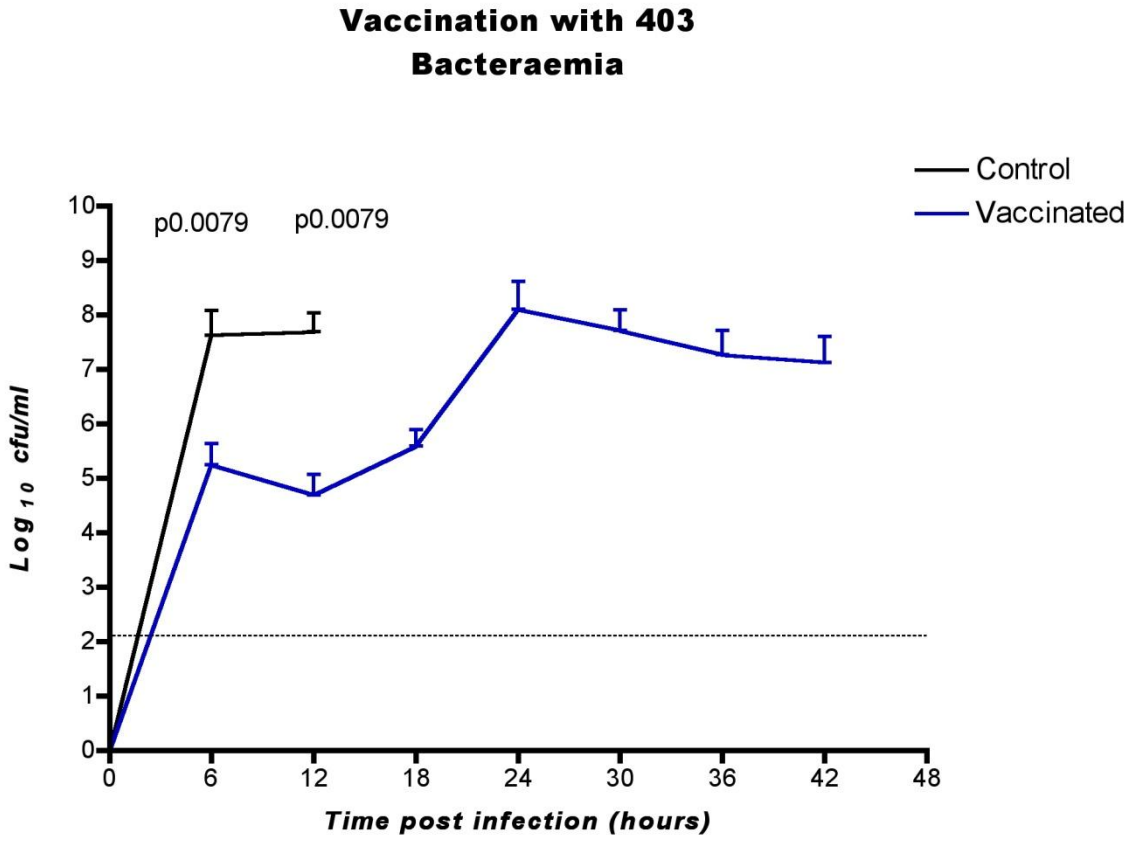


Figure 6.5. Comparison of bacterial load at different time points in mice challenged with 1x10^5 cfu/ 200 µl TIGR4 IP in groups vaccinated IP with 5 x 10^6 cfu of strain 403/ 200µl PBS and 200 µl PBS only. Mann Whitney's test showed significantly different bacterial load at 6 and 12 hour time points (GraphPad Prism 4.0, USA). Statistical analyses were not performed beyond 12 hrs. First mouse in the control group died at 12 hrs, while first mouse in the vaccinated group died at 42 hrs. Dotted line represents the limit for detection.

On monitoring and scoring for hunching, lethargy and piloerection clinically, vaccinated mice showed delay in the appearance of symptoms as compared to the control group, and clinical scores were significantly different at 12 hour time point (Fig 6.6).

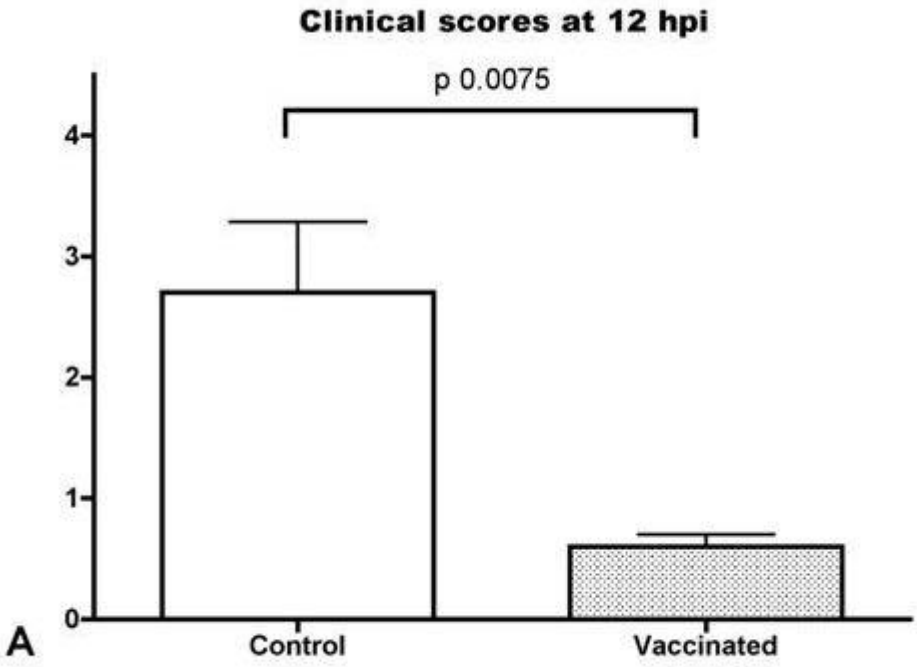


Figure 6.6. Clinical score comparison at 12 hour time point of control group inoculated IP with 200 µl PBS and mice 3x vaccinated with 5 x 10^6 cfu of strain 403/ 200µl PBS and challenged with 1x10^5 cfu/ 200 µl TIGR4 IP. Data plotted as a bar graph. Bars represent mean ± SEM. Unpaired t-test showed (GraphPad Prism 4.0, USA) that here was a significant difference in clinical scores at 12 hour time point between the two groups.

6.4 Vaccination develops reactivity in mouse sera:

To confirm if mice developed serological reactivity against TIGR4 strain, western blots were performed using mouse sera as a source of primary antibodies against TIGR4 cell lysate (Fig 6.7).

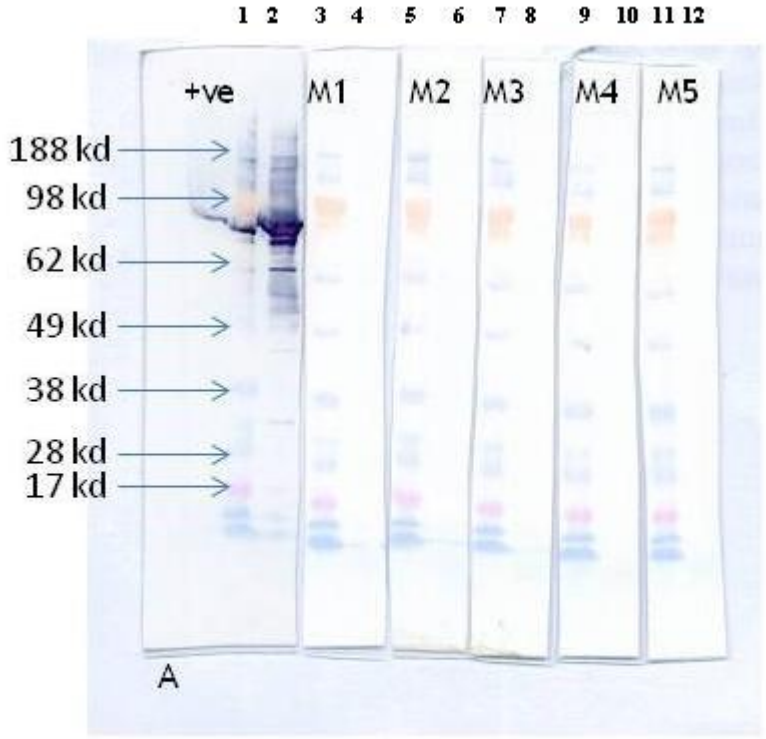


Figure 6.7. Western blot using pre-immune and post-immune sera from vaccinated mice as source of primary antibodies against TIGR4 cell lysate. 10 µl of ladder and sample was loaded in lanes. Molecular weights of major bands are indicated with the help of arrows. Serum was used in 1/100 while secondary anti-mouse antibodies were used at 1:1000 dilution.
(A) Pre-immune Sera.
Lane 1: Protein ladder.
Lane 2: Positive control using TIGR4 lysate in a previously immunized mouse.
Lane 3: Protein ladder
Lane 4: Pre-immunization serum from Mouse M1
Lane 5: Protein ladder
Lane 6: Pre-immunization serum from Mouse M2
Lane 7: Protein ladder
Lane 8: Pre-immunization serum from Mouse M3
Lane 9: Protein ladder
Lane 10: Pre-immunization serum from Mouse M4
Lane 11: Protein ladder
Lane 12: Pre-immunization serum from Mouse M5

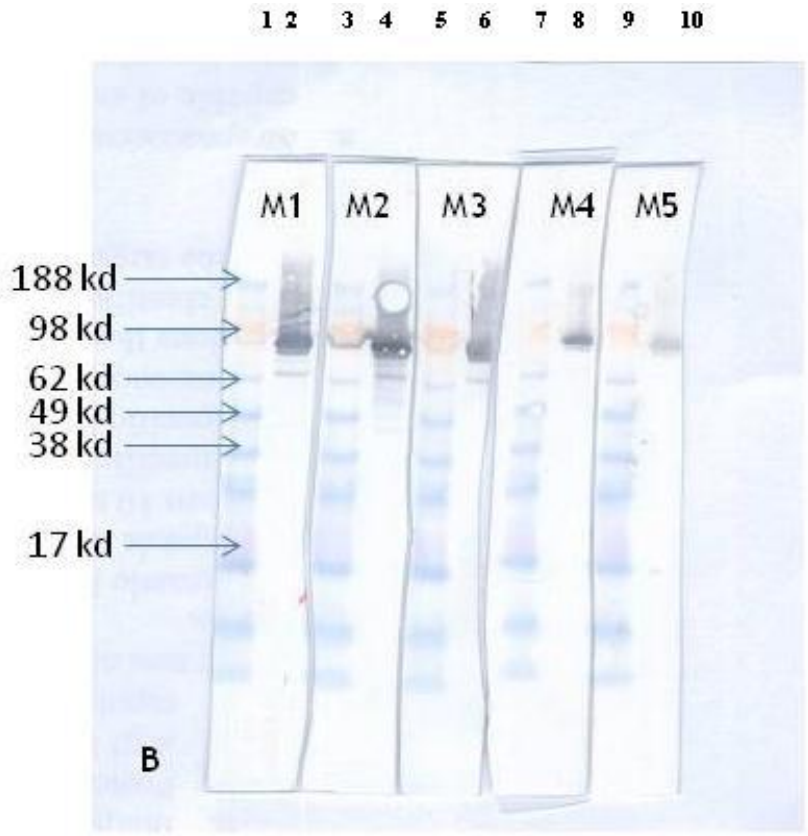


Figure 6.7. Western blot using pre-immune and post-immune sera from vaccinated mice as source of primary antibodies against TIGR4 cell lysate. 10 µl of ladder and sample was loaded in lanes. Molecular weights of major bands are indicated with the help of arrows. Serum was used in 1/100 while secondary anti-mouse antibodies were used at 1:1000 dilution.
(B) Post immune sera.
Lane 1: Protein ladder
Lane 2: Post-immunization serum from Mouse M1
Lane 3: Protein ladder
Lane 4: Post immunization serum Mouse M2
Lane 5: Protein ladder
Lane 6: Post immunization serum Mouse M3
Lane 7: Protein ladder
Lane 8: Post immunization serum Mouse M4
Lane 9: Protein ladder
Lane 10: Post immunization serum Mouse M5.

Western blot showed that antibodies had developed in mice after vaccination against TIGR4 proteins and serum after vaccination of mice was reactive against them.

6.4.1.1 Mouse serum also reacts with the type 4 capsular antigens:

Quellung reactions are an important tool to show if anticapsular antibodies had developed in vaccinated mice as they are considered important defence mechanism and help in providing protection against pneumococcal infection. Quellung reactions performed using sera from vaccinated mice were positive against TIGR4 as well as 403.

Further experiments were performed to see effects of vaccine administration through IN route, effect of vaccination on challenge with lesser number of bacteria and protection against different serotypes

6.4.2 Intraperitoneal immunization and low dose challenge:

10 MF1 mice were divided into two groups, and were given PBS or vaccinated 3x with strain 403 as described previously in Section 1.3.

Mice were challenged intraperitoneally with 5 x 10^3 cfu/ 200µl and were bled 6 hourly and monitored for development of clinical symptoms during the course of the experiment.

As compared to previous experiment, median survival time of both groups increased by thirty hrs. There was not statistical difference between the two groups. There was one survivor in the vaccinated group which was able to clear

the infection after reaching a relatively high level of bacteraemia but two of the vaccinated mice had to be sacrificed earlier in experiment (Fig 6.8).

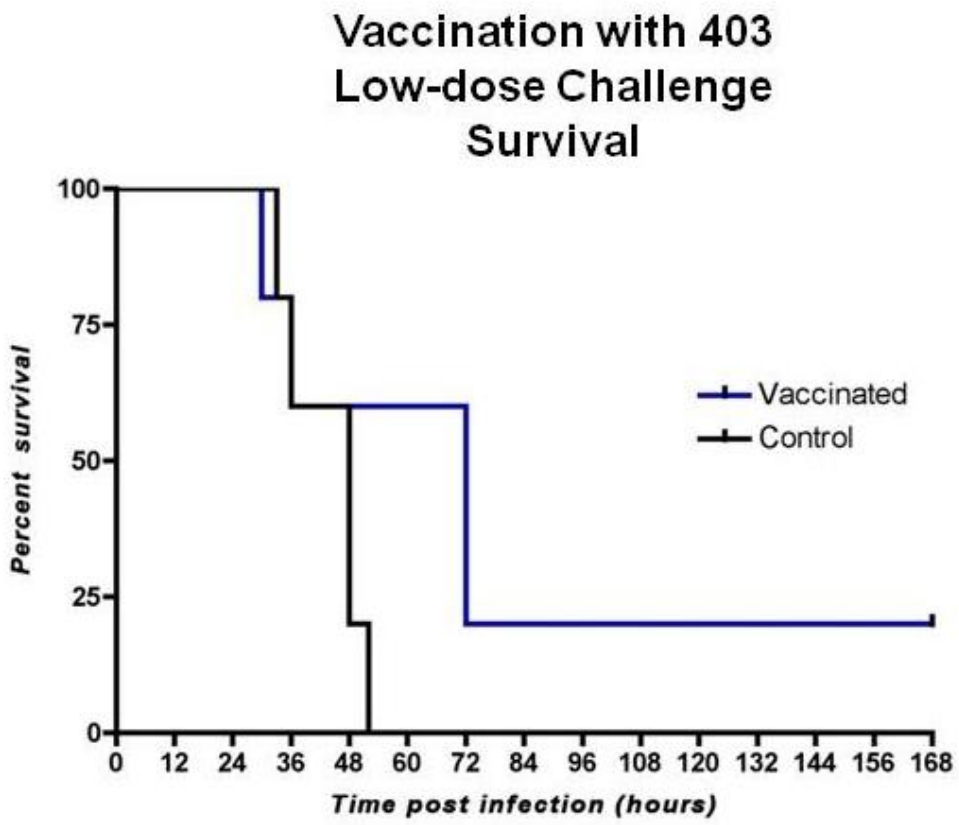


Figure 6.8. Survival curve of mice challenged with 5 x 10^3 cfu/ 200 µl TIGR4 IP in groups 3x vaccinated IP with 5 x 10^6 cfu of strain 403/ 200µl PBS and 200 µl PBS only. Mice vaccinated with 403 strains survived longer than unvaccinated mice and 20% survived till the end of experiment though survival analysis did not show any statistically significant difference in survival time between the two groups. Median survival time for vaccinated mice was 72 hrs as compared to 48 hrs for control mice (GraphPad Prism 4.0, USA). All data plotted as percentage survival as a staircase line with points for all observations against hrs post infection. Five mice used in each group.

There was no statistical difference in the bacteraemia in the two groups. In vaccinated mice, after a very high level of bacterial load of 10^8 cfu/ml at 24

hour time point, the count dropped back to 10^6 cfu/ml at 30 hrs though only 20% survived beyond 72 hrs (Fig 6.9).

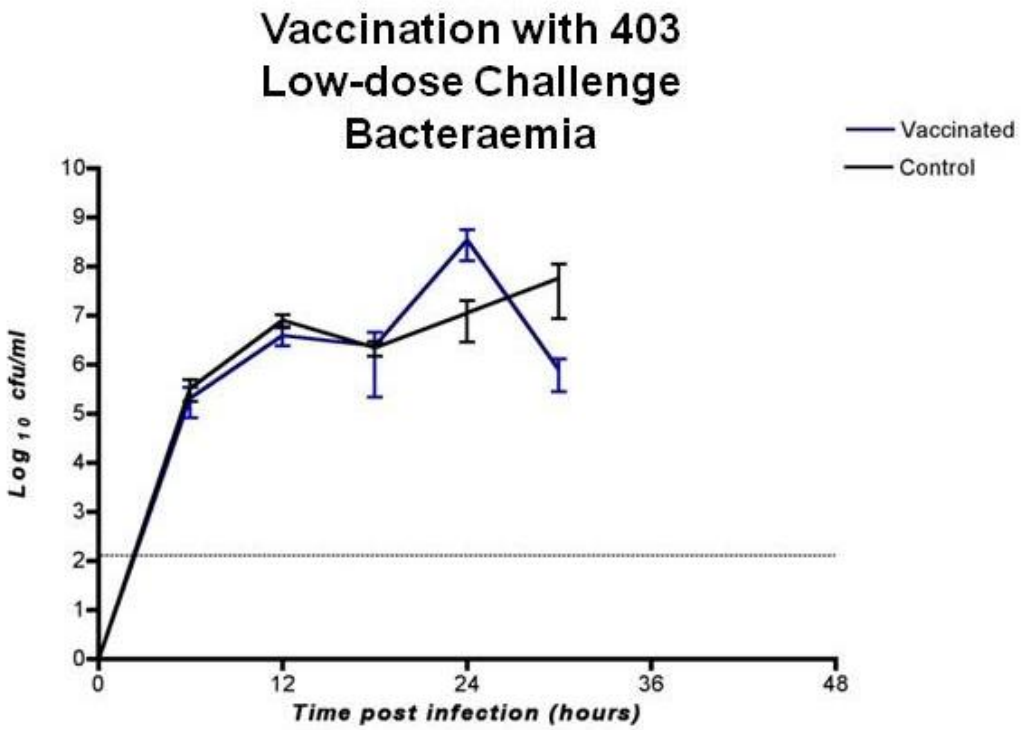


Figure 6.9. Comparison of the bacterial load in mice challenged with 5 x10^3 cfu/ 200 µl TIGR4 IP in groups 3x vaccinated IP with 5 x 10^6 cfu of strain 403/ 200µl PBS and 200 µl PBS only. Graph shows no notable difference in bacterial load in two groups. Dotted line represents limit for detection.

Individual mouse-data is shown in Fig 6.10.

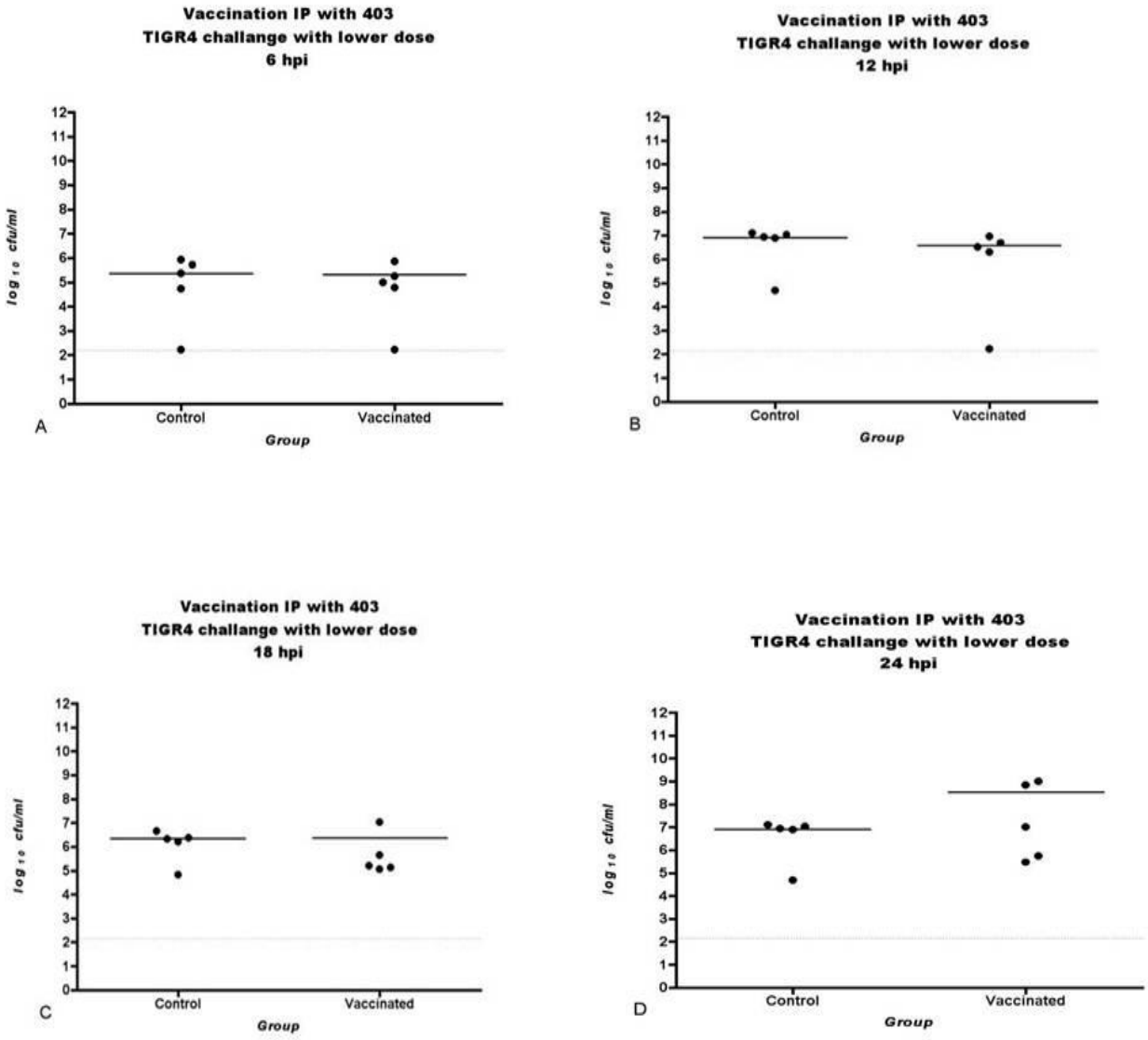
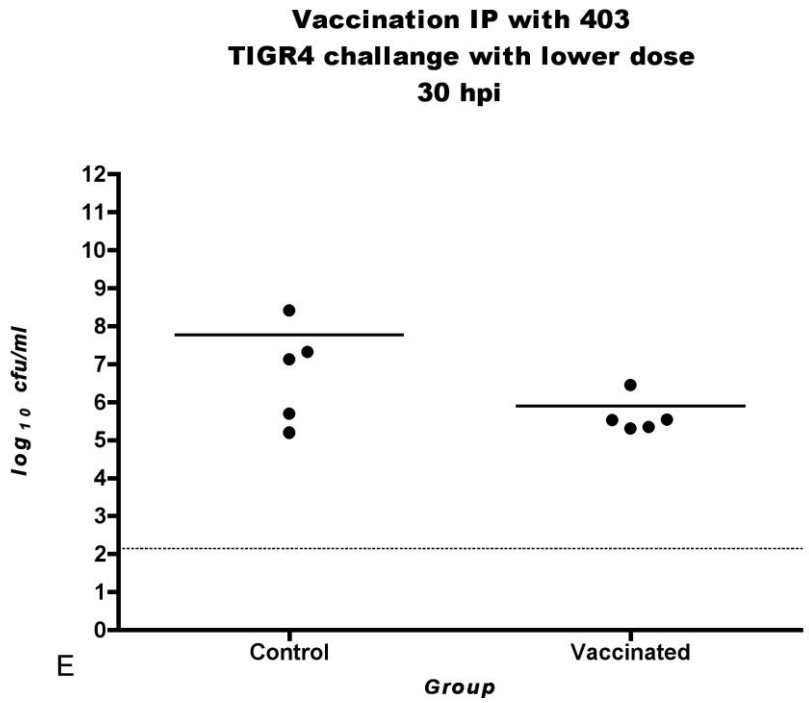
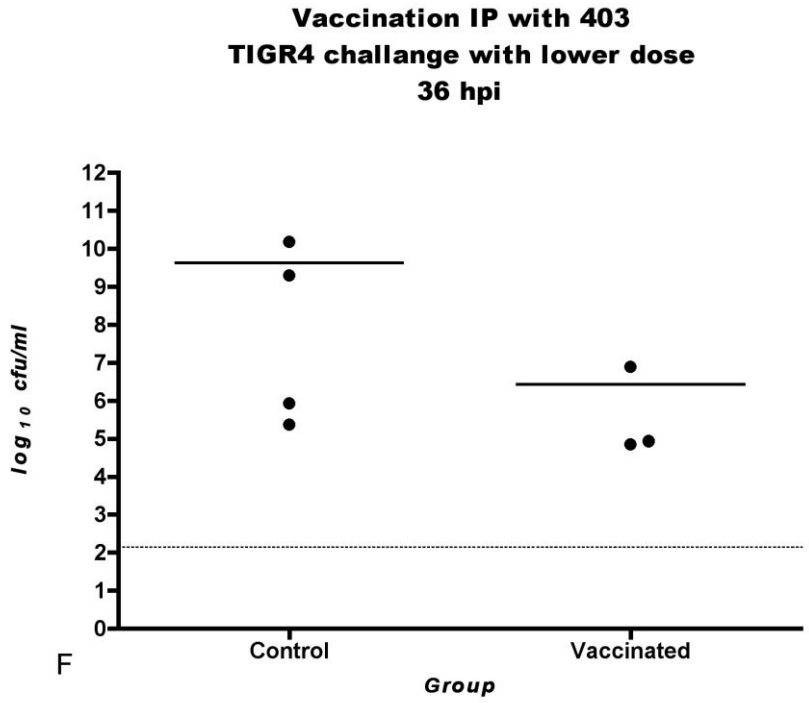


Figure 6.10. Comparison of bacterial load during 6-36 hrs post-challenge with $5x10^3$ cfu/ 200 µl TIGR4 IP in mice vaccinated 3x IP with 5×10^6 cfu of strain 403/ 200µl PBS and control group given PBS only. Groups were compared with Mann-Whitney test (GraphPad Prism 4.0, USA). Circles mark individual mice. Horizontal dotted line represents the limit for detection. Horizontal bar represents the median. (A) Bacteraemia count at 6 hpi. (B) Bacteraemia count at 12 hpi. (C) Bacteraemia count at 18 hpi. (D) Bacteraemia count at 24 hpi.



E



F

Figure 6.10. Comparison of bacterial load during 6-36 hrs post-challenge with $5x10^3$ cfu/ 200 µl TIGR4 IP in mice vaccinated 3x IP with 5×10^6 cfu of strain 403/ 200µl PBS and control group given PBS only. Groups were compared with Mann-Whitney test (GraphPad Prism 4.0, USA). (E) Bacteraemia count at 30 hpi. (F) Bacteraemia count at 36 hpi.

Though both groups did not show much difference in levels of attained bacterial load there was significant difference in clinical symptoms between the two groups (p= 0.0317)((GraphPad Prism 4.0, USA). The vaccinated group lost less weight as compared to the control group though it was not statistically significant (Fig 6.11).

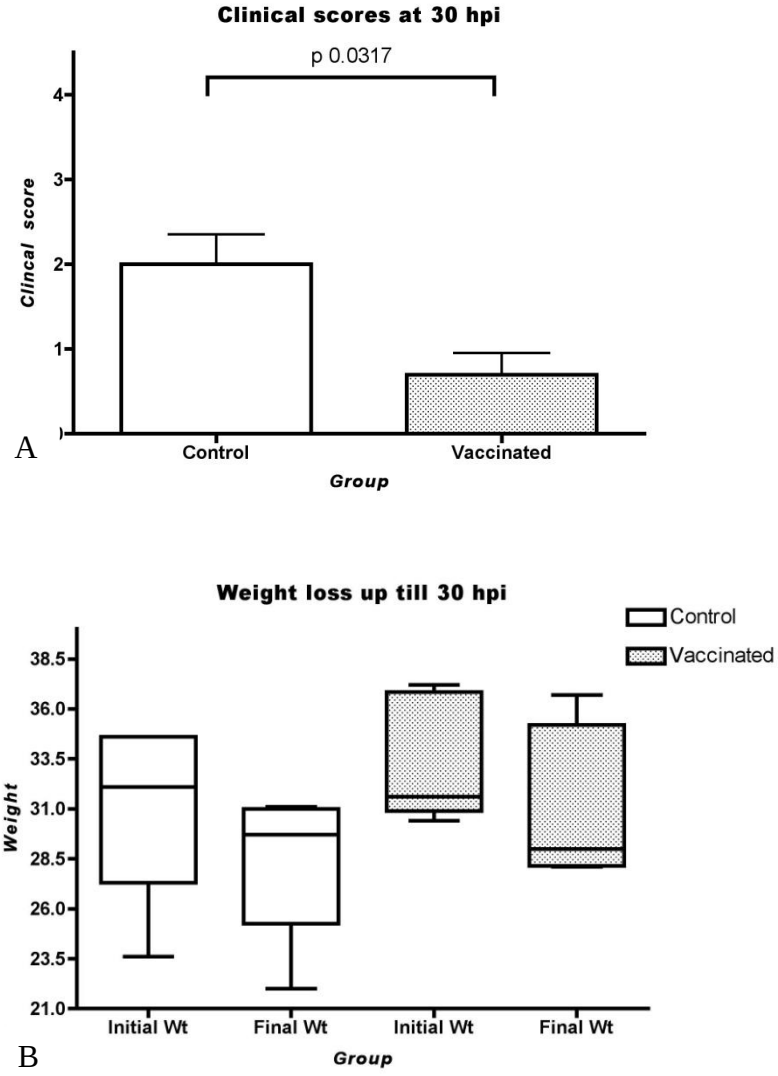


Figure 6.11. Comparison of weight and clinical symptoms at 30 hrs time points in mice challenged with 5 x 10^3 cfu/ 200 µl TIGR4 IP. Vaccinated group was 3x inoculated IP with 5 x 10^6 cfu of strain 403/ 200µl in PBS and control group was given PBS only. (A) Comparison of clinical scores. Unpaired t-test showed significant difference in clinical symptoms (GraphPad Prism 4.0, USA). Bars represent the mean ± SEM (B) Comparison of weight-loss. Weight changes are plotted as a box and whiskers plot with horizontal line in the box representing median.

6.4.3 Intranasal Immunization of mice:

Thirty MF1 mice were divided into two groups. The first group was vaccinated intranasally with 5 x 10^7 cfu/10µl under general anaesthesia, while control group was given 10 µl PBS. Intranasal booster doses were given on day 14 and 28 and sample bleeds were taken before each vaccination and on day 42.

6.4.3.1 Intranasal challenge of mice with TIGR4 shows weak trends:

Five mice were taken from each group and were challenged intranasally with 5x10^6 cfu TIGR4/50µl. Twenty percent of vaccinated mice survived and cleared infection as compared to none surviving in the control group (Fig 6.12).

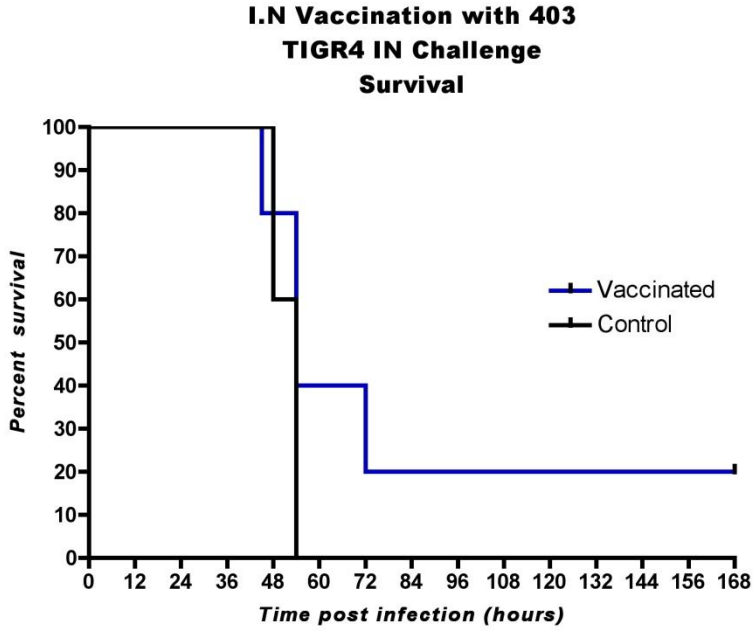


Figure 6.12. Survival curve of mice challenged with 5x10^6 cfu/ 50 µl TIGR4 IN. Vaccinated group was vaccinated IN with 5 x 10^7 cfu of strain 403/ 10µl in PBS and control group was given 10 µl PBS only. 20% survival was seen in vaccinated group. All data plotted as percentage survival as a staircase line with points for all observations against hrs post infection. Log rank test did not show any significant difference between the two groups. The median survival time for both the groups was 54 hrs. Five mice were used in each group.

There was no significant difference in levels of bacteraemia the two groups (Fig 6.13).

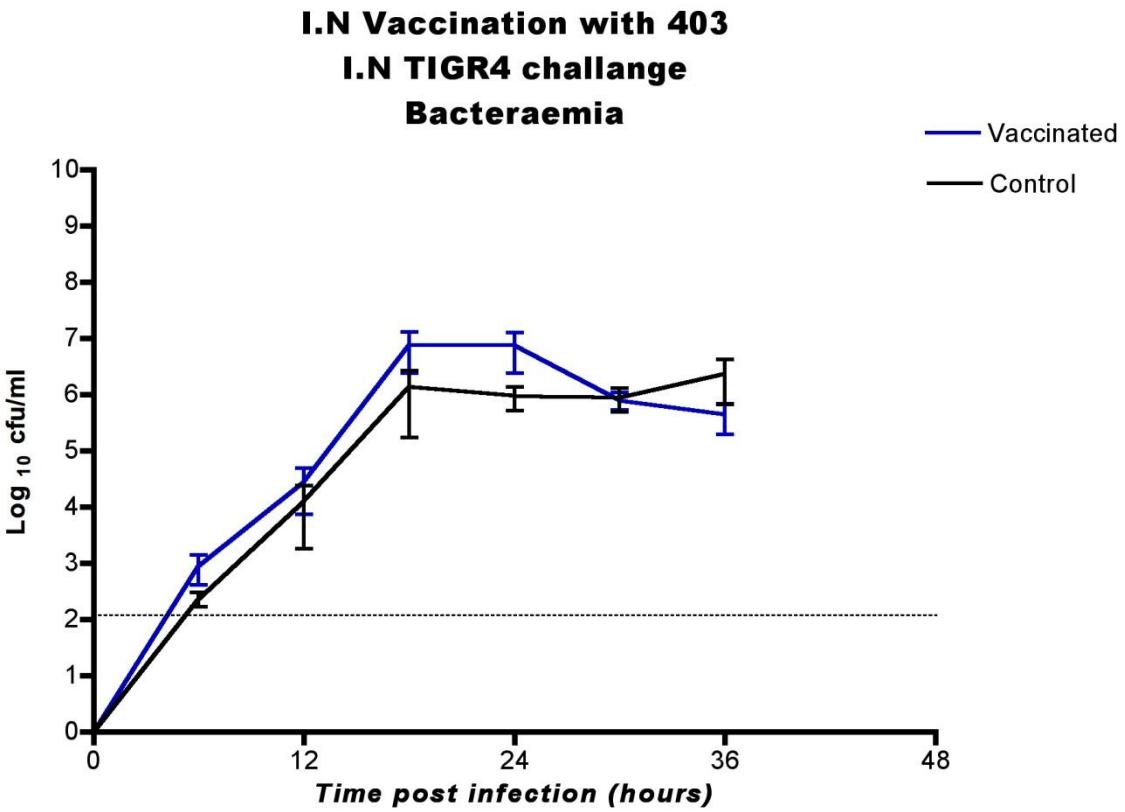


Figure 6.13. Bacteraemia during 36 hrs post IN infection in mice challenged with $5x10^6$ cfu/ 50 µl TIGR4. Vaccinated group was 3x vaccinated IN with 5 x 10^7 cfu of strain 403/ 10µl in PBS and control group was given 10 µl PBS only. There was no significant difference in level of bacteraemia between the two groups. Five mice were used in each group.

The first vaccinated mouse died at 45 hrs before any of control group mouse, though the last one to be culled at 72 hrs also belonged to vaccinated group. One of the mice from vaccinated group cleared infection and survived till the end of experiment.

6.4.3.2 Intranasal challenge of mice with A66:

Five vaccinated and five control mice were challenged intranasally with bioluminescent serotype 3 strain A66.1 using 5 x 10^6 cfu/50 µl dose. The results did not show any significant differences between the two groups. There were no survivors in any group, though 40% mice from vaccinated group reached 96 hour time point as compared to 20% from the control group (Fig 6.14).

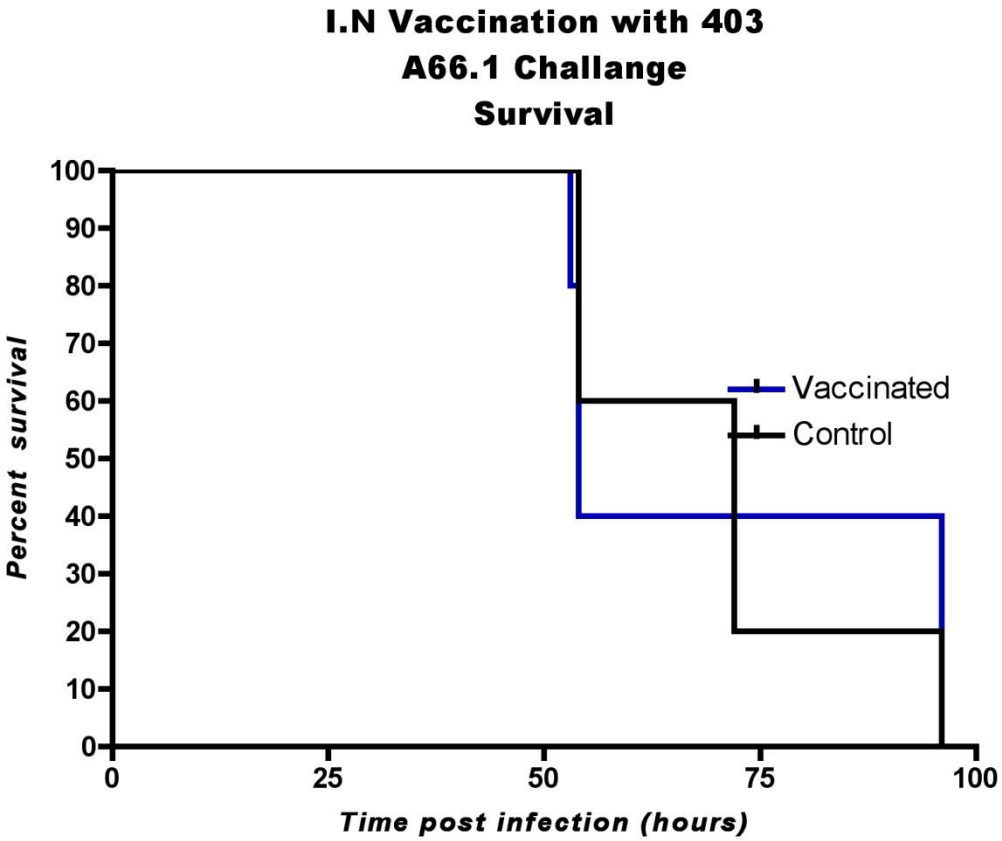


Figure 6.14. Survival curve of mice challenged with 5x10^6 cfu/ 50 µl bioluminescent strain A66.1 IN. Vaccinated group was 3x vaccinated IN with 5 x 10^7 cfu of strain 403/ 10µl in PBS and control group was given 10 µl PBS only. There was no significant difference in the two groups. All data plotted as percentage survival as a staircase line with points for all observations against hrs post infection. . Five mice were used in each group.

Group analysis did not show significant differences in bacteraemia in vaccinated group as compared to control group, on excluding vaccinated mouse M5 as it

showed exceptionally high bacteraemia levels, some difference could be seen in the two groups though it did not reach statistically significant level (Fig 6.15).

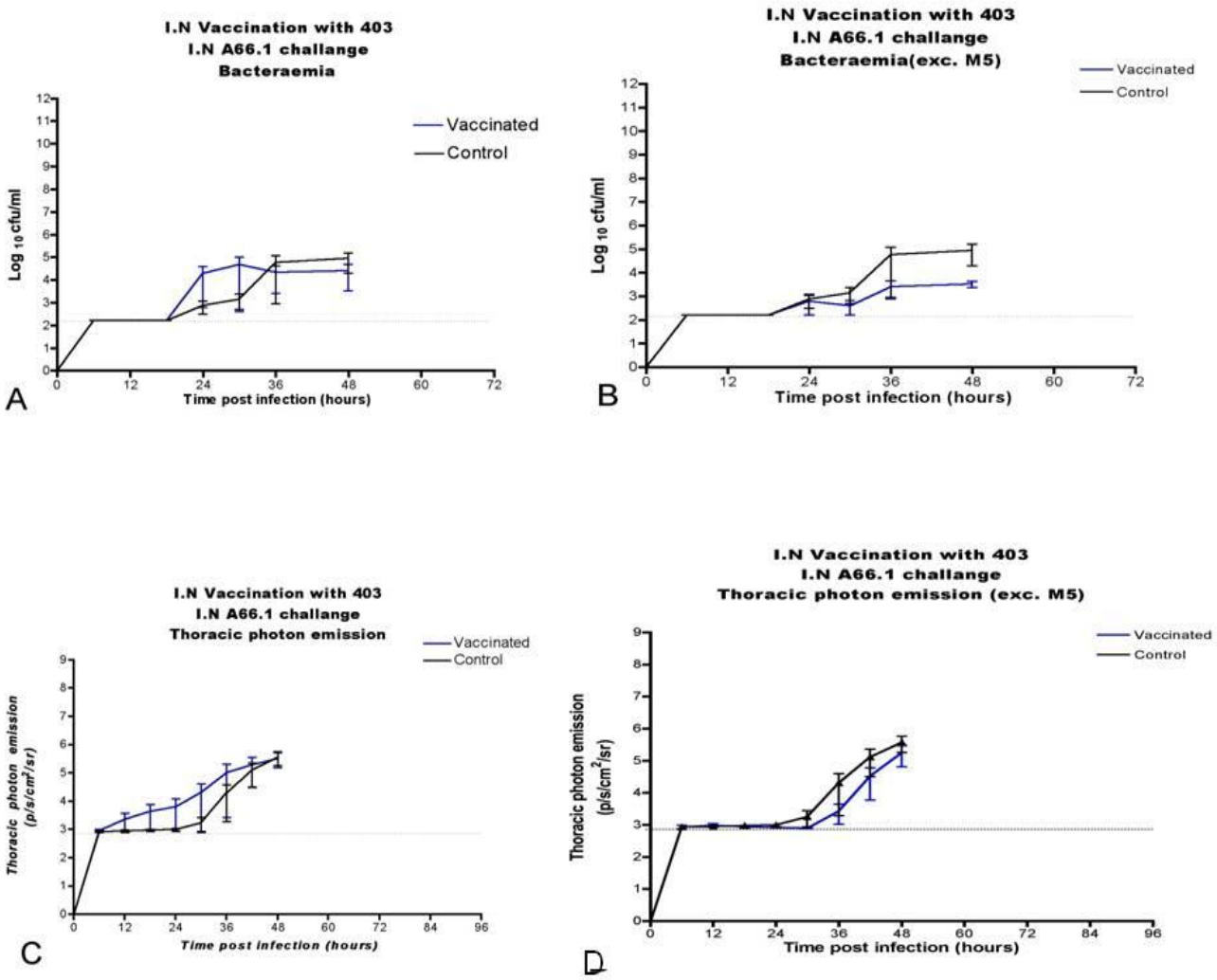
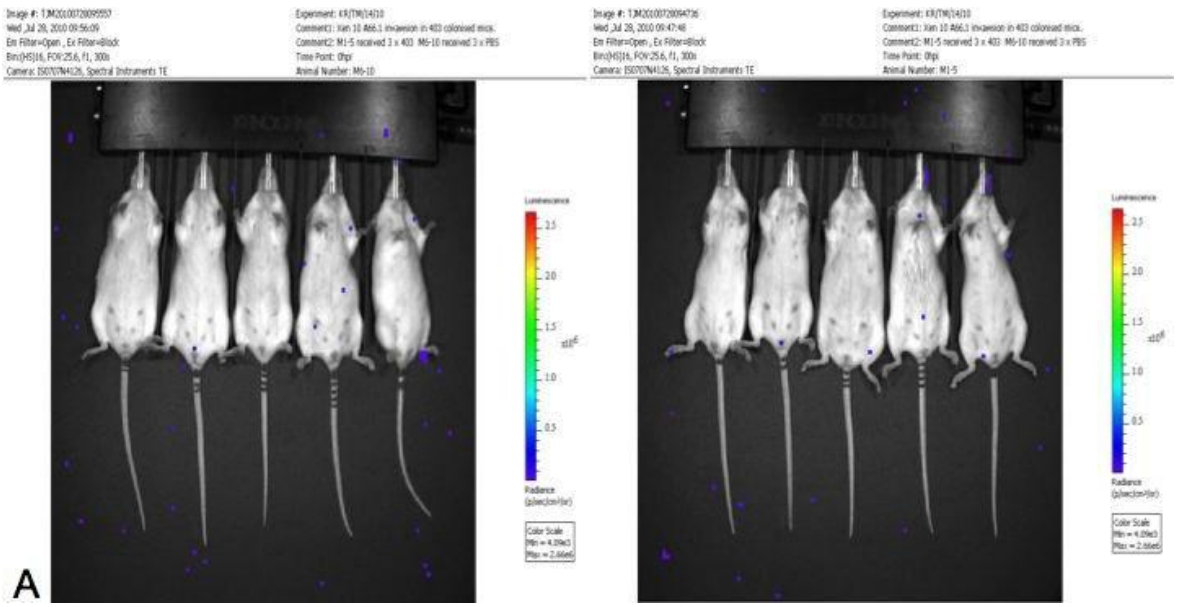


Figure 6.15. Bacteraemia and thoracic photon emission in mice challenged with $5x10^6$ cfu/ 50 µl bioluminescent strain A66.1 IN. The vaccinated group was 3x vaccinated IN with 5 x 10^7 cfu of strain 403/ 10µl in PBS and control group was given 10 µl PBS only. (A) Bacteraemia from 0-48 hrs in the two groups. (B) Bacteraemia from 0-48 hrs excluding outlier M5. (C) Thoracic photon emission from 0-48 hrs. (D) Thoracic photon emission from 0-48 hrs excluding M5. Five mice used for each group.

Bacteraemia results were consistent with images when compared with Thoracic Photon Emission recorded by the IVIS imaging system (IVIS: Caliper Life Sciences, UK), which is an optical imaging system for non-invasive monitoring of disease progression without killing the animals at each stage. It records photon emission

from diseased animal and uses this data with 3D reconstruction of these images to localise source of bioluminescence, allowing visualisation of the spread of infection within the body of living animal (Fig 6.16).



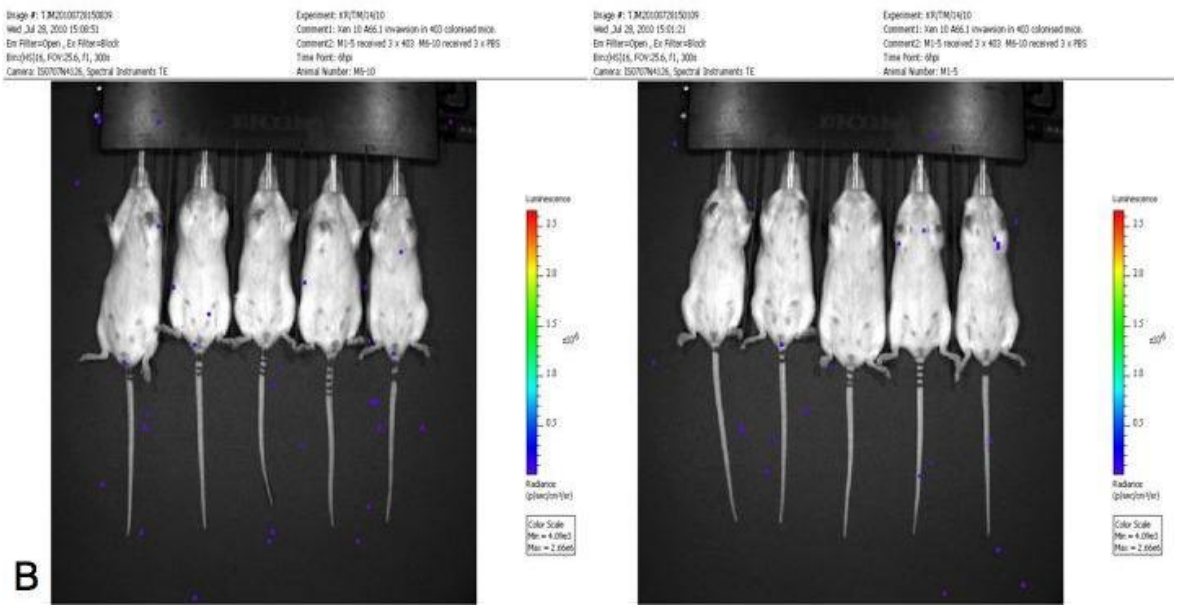


Figure 6.16. IVIS images showing photon emission in mice challenged with 5x10^6 cfu/ 50 µl bioluminescent strain A66.1 IN. Vaccinated group was 3x vaccinated IN with 5 x 10^7 cfu of strain 403/ 10µl in PBS and control group was given 10 µl PBS only. Control mice are on left (M6-M10) and vaccinated mice on the right (M1-M5).
(A) 0 hr time point. (B) 6 hr time point. Infection developing in M5.

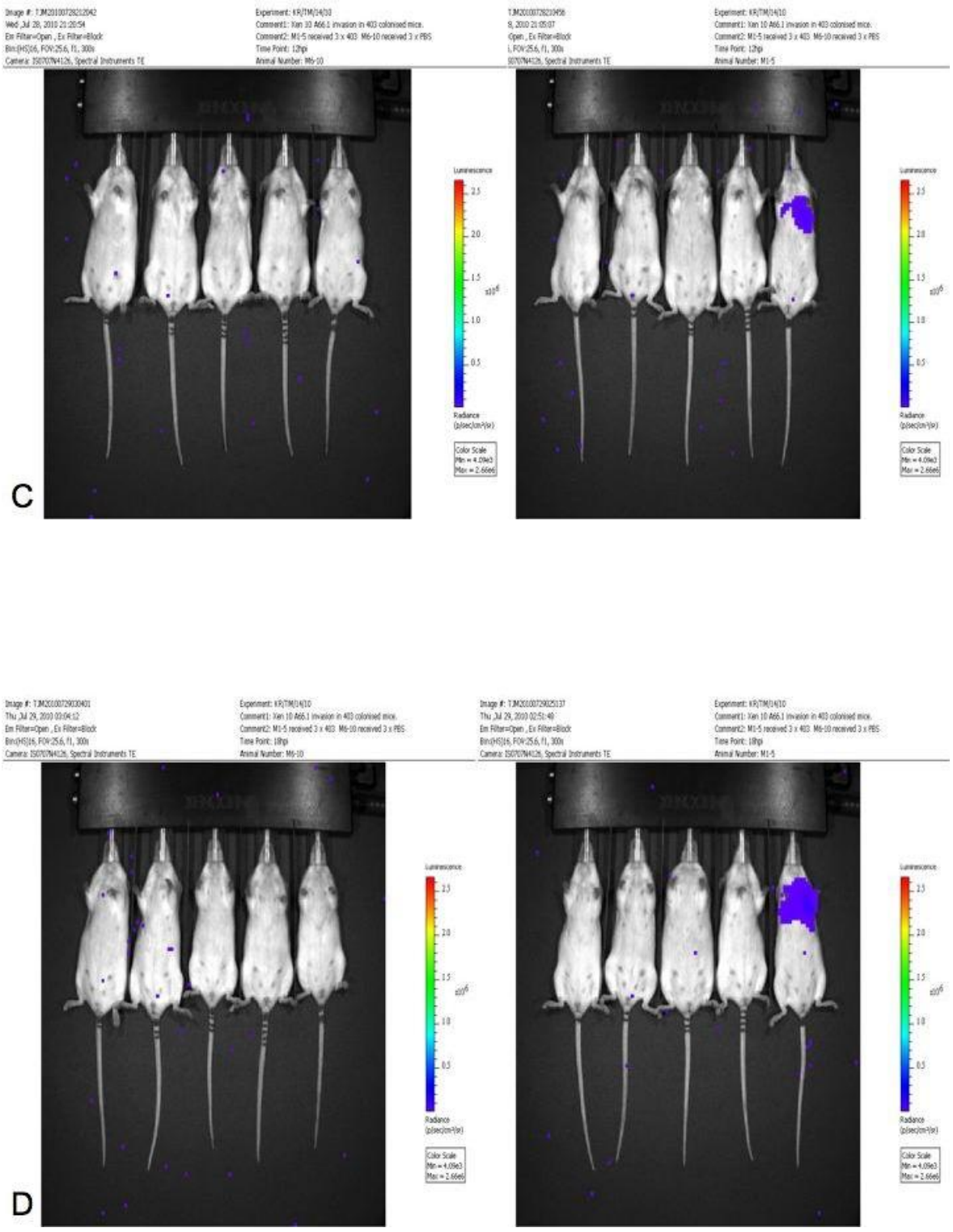
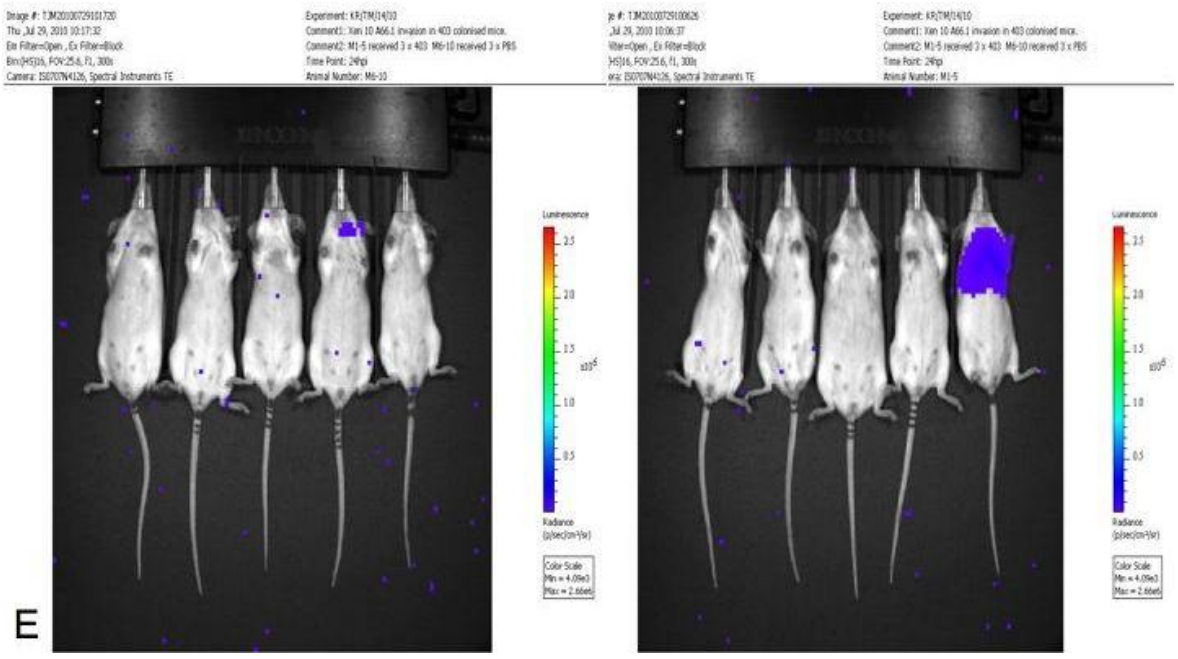


Figure 6.16. IVIS images showing photon emission in mice challenged with 5x10^6 cfu/ 50 µl bioluminescent strain A66.1 IN. Vaccinated group was 3x vaccinated IN with 5 x 10^7 cfu of strain 403/ 10µl in PBS and control group was given 10 µl PBS only. Control mice are on left (M6-M10) and vaccinated mice on the right (M1-M5). (C) 12 hr time point. Infection developed in M5. (D) 18 hr time point. Progression of infection in M5.



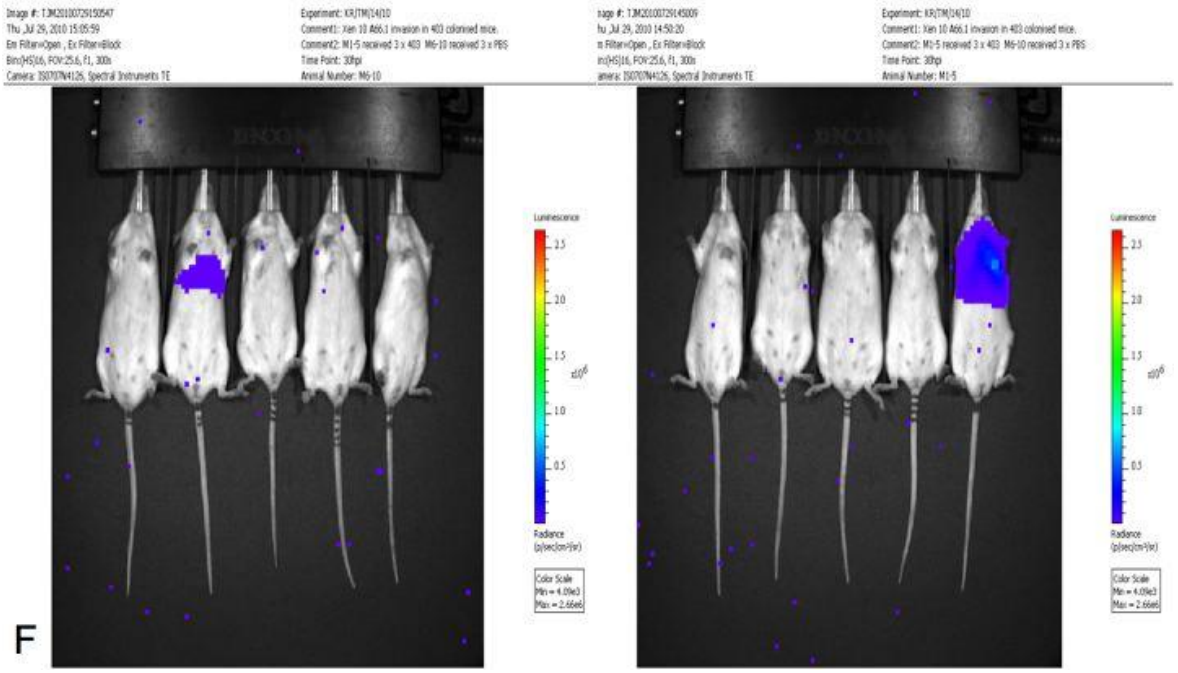
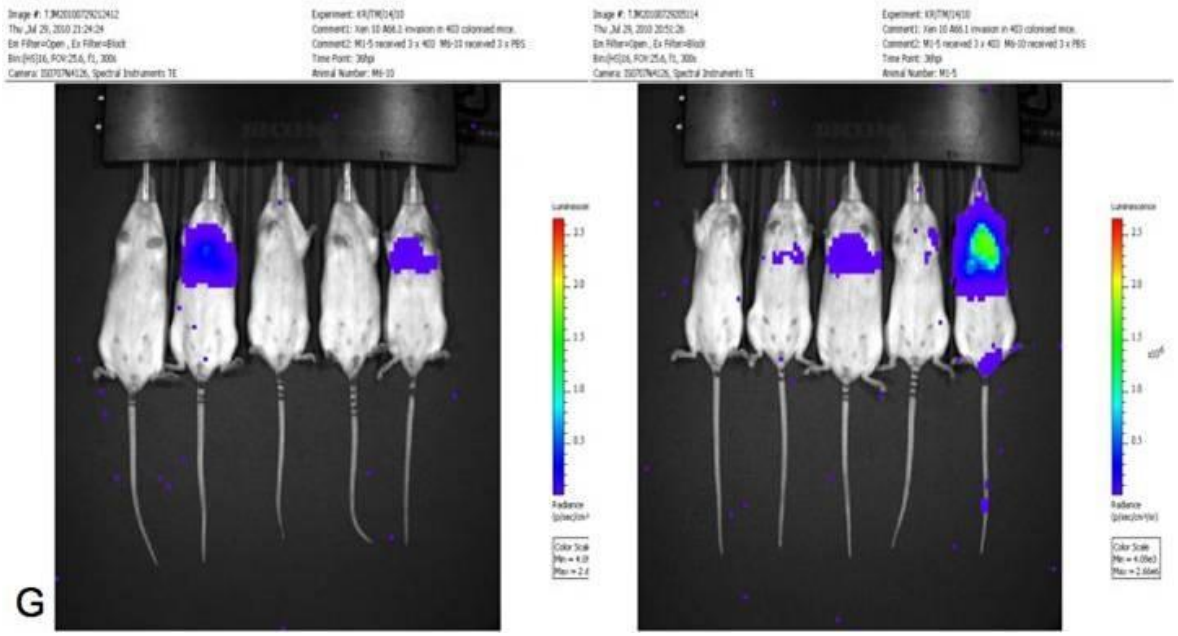


Figure 6.16. IVIS images showing photon emission in mice challenged with 5x10^6 cfu/ 50 µl bioluminescent strain A66.1 IN. Vaccinated group was 3x vaccinated IN with 5 x 10^7 cfu of strain 403/ 10µl in PBS and control group was given 10 µl PBS only. Control mice are on left (M6-M10) and vaccinated mice on the right (M1-M5). (E) 24 hr time point. Infection has also appeared in M9. (F) 30 hr time point. Infection has developed in several mice in both the groups.



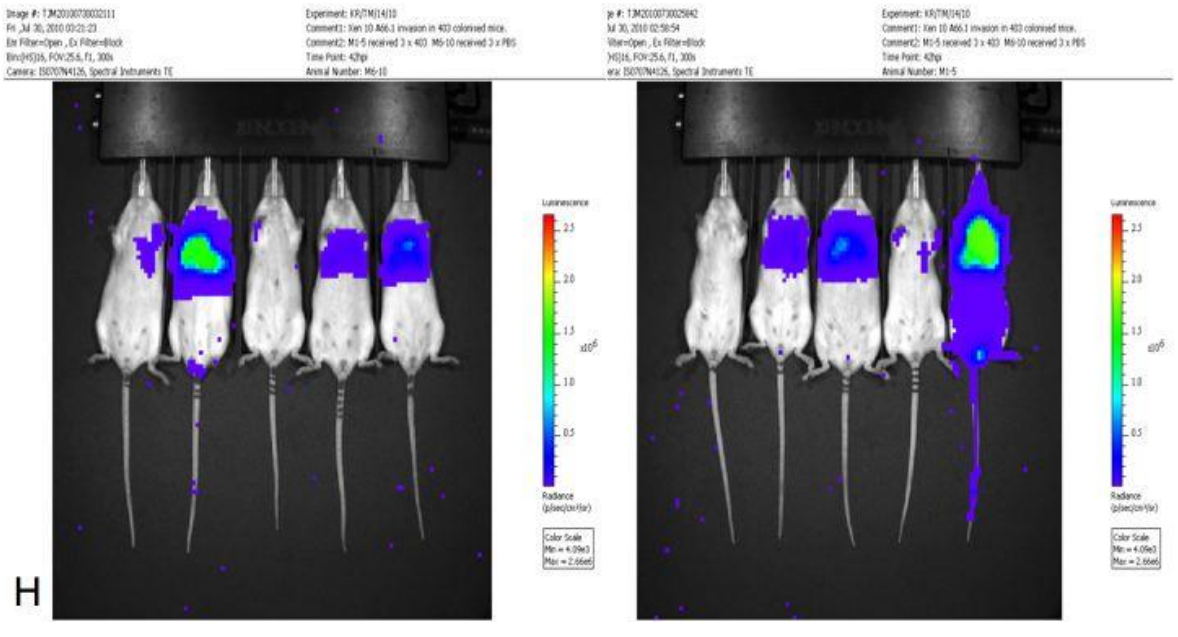


Figure 6.16. IVIS images showing photon emission in mice challenged with 5x10^6 cfu/ 50 µl bioluminescent strain A66.1 IN. Vaccinated group was 3x vaccinated IN with 5 x 10^7 cfu of strain 403/ 10µl in PBS and control group was given 10 µl PBS only. Control mice are on left (M6-M10) and vaccinated mice on the right (M1-M5). (G) 36 hr time point. (H) 42 hr time point. Infection can be seen particularly around the lung areas in heavily infected mice.

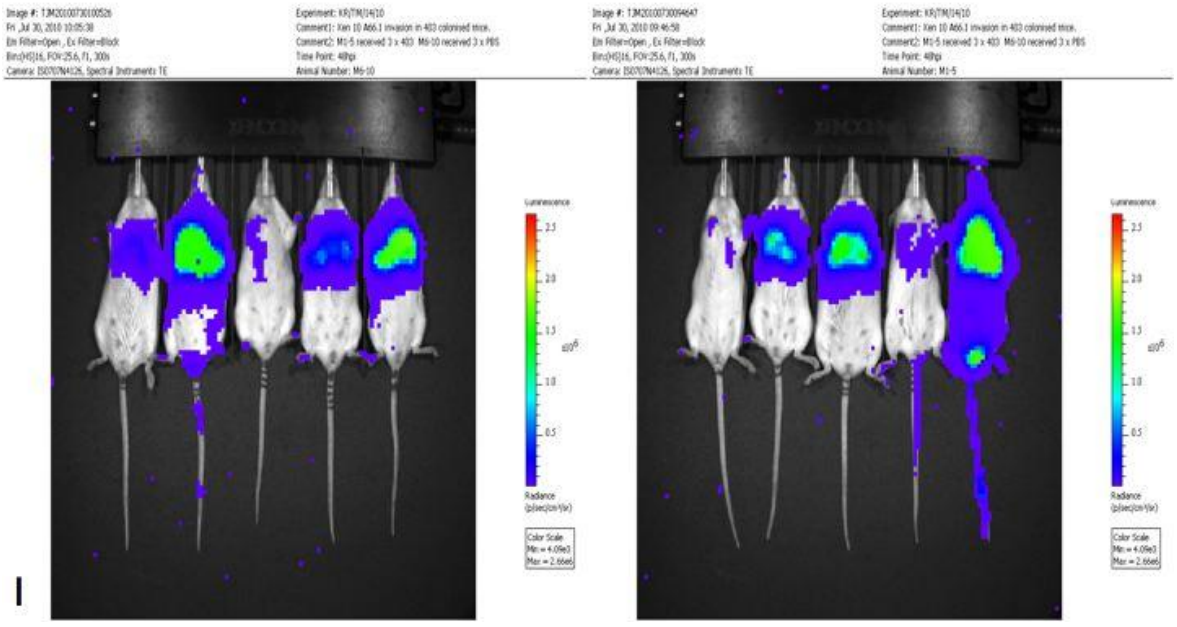


Figure 6.16. IVIS images showing photon emission in mice challenged with 5x10^6 cfu/ 50 µl bioluminescent strain A66.1 IN. Vaccinated group was 3x vaccinated IN with 5 x 10^7 cfu of strain 403/ 10µl in PBS and control group was given 10 µl PBS only. Control mice are on left (M6-M10) and vaccinated mice on the right (M1-M5). (G) 36 hr time point. (I) 48 hr time point. Full blown infection in M5, M7, M9 and M10

IVIS data did not show any significant difference in progression of infection in two groups.

6.4.3.3 Intranasal challenge with ATCC6308:

Both the groups were challenged with highly virulent type 8 strain ATCC6308 using 5x 10^3 cfu/50 µl dose. One of the unvaccinated mice (M7) did not develop any bacteraemia, so it was excluded from all analyses, while a vaccinated mouse (M4) developed very high bacteraemia and became sick before all the others. The analysis was performed with and without M4.

There were no survivors in any of the group, and there was no difference in survival.

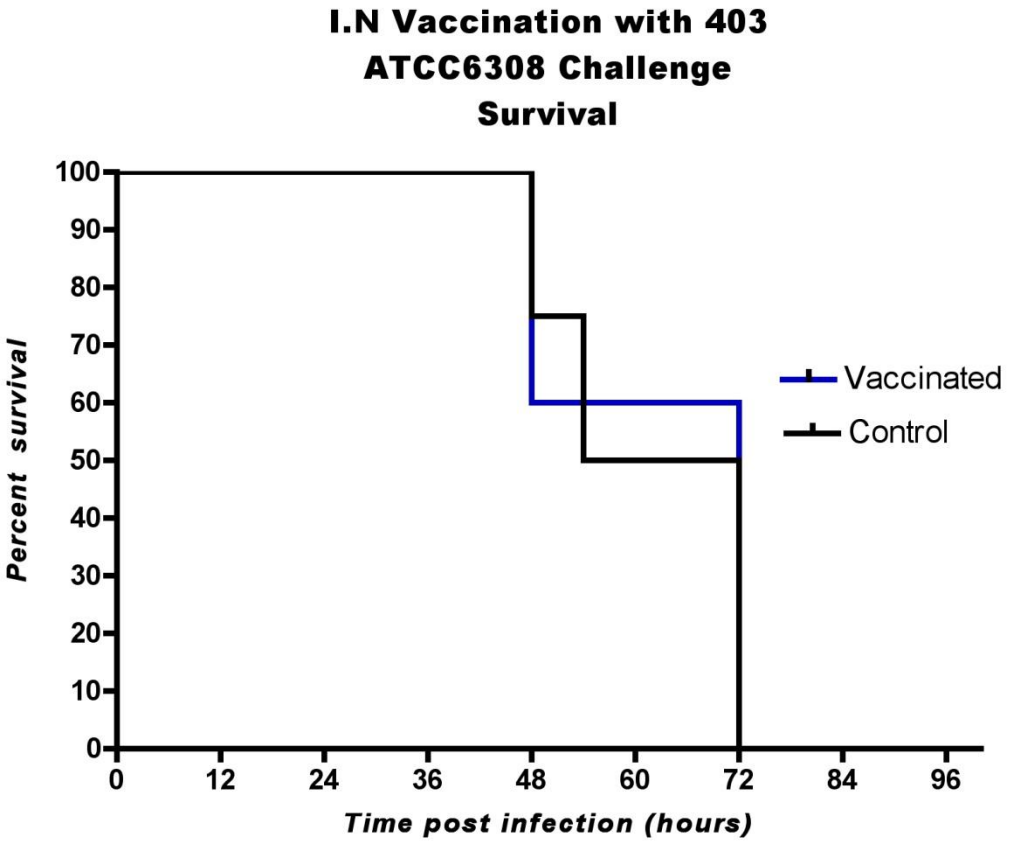
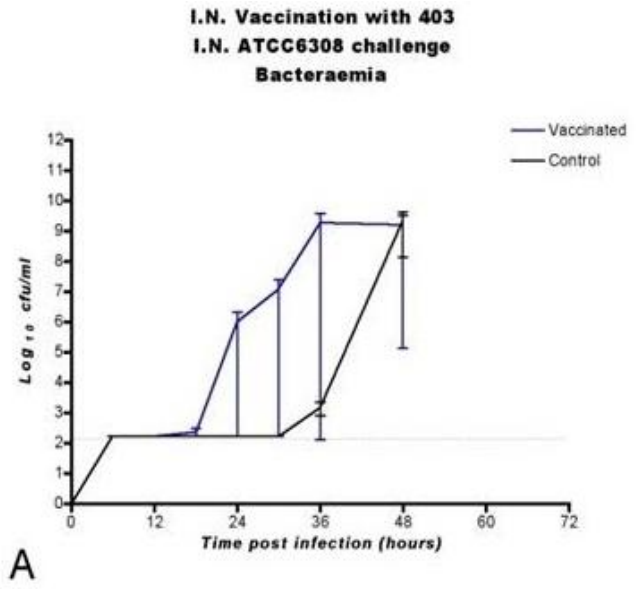


Figure 6.17. Survival curve of mice challenged IN with $5x10^3$ cfu/ 50 µl strain ATCC6308. Vaccinated group was 3x vaccinated IN with 5×10^7 cfu of strain 403/ 10µl in PBS and control group was given 10 µl PBS only. There was no difference in survival of two groups. All data plotted as percentage survival as a staircase line with points for all observations against hrs post infection.

Initial group analysis showed that the level of bacteraemia was higher in vaccinated mice as compared to control mice. On analyzing results by excluding outlying mouse M4, vaccinated mice showed to have developed lower level of bacteraemia as compared to controls (Fig6.18).



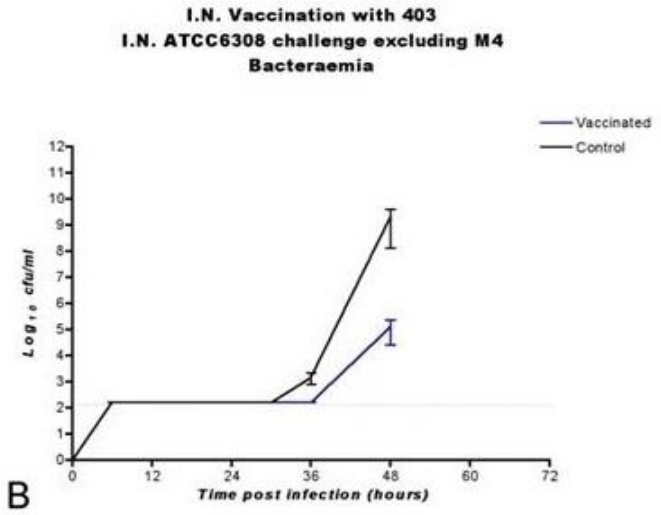


Figure 6.18. Bacterial load of mice challenged IN with $5x10^3$ cfu/ 50 µl strain ATCC6308. Vaccinated group was vaccinated IN with 5×10^7 cfu of strain 403/ 10µl in PBS and control group was given 10 µl PBS only. (A) Bacteraemia including vaccinated outlier M4. (B) Bacteraemia excluding vaccinated outlier M4.

The analysis of bacteraemia at different time points was performed including and excluding outlier M4. Though difference of level of bacteraemia did not

reach statistically significant levels there were clear trends of decreased bacteraemia in vaccinated group as compared to control group after exclusion of M4 (Fig 6.19).

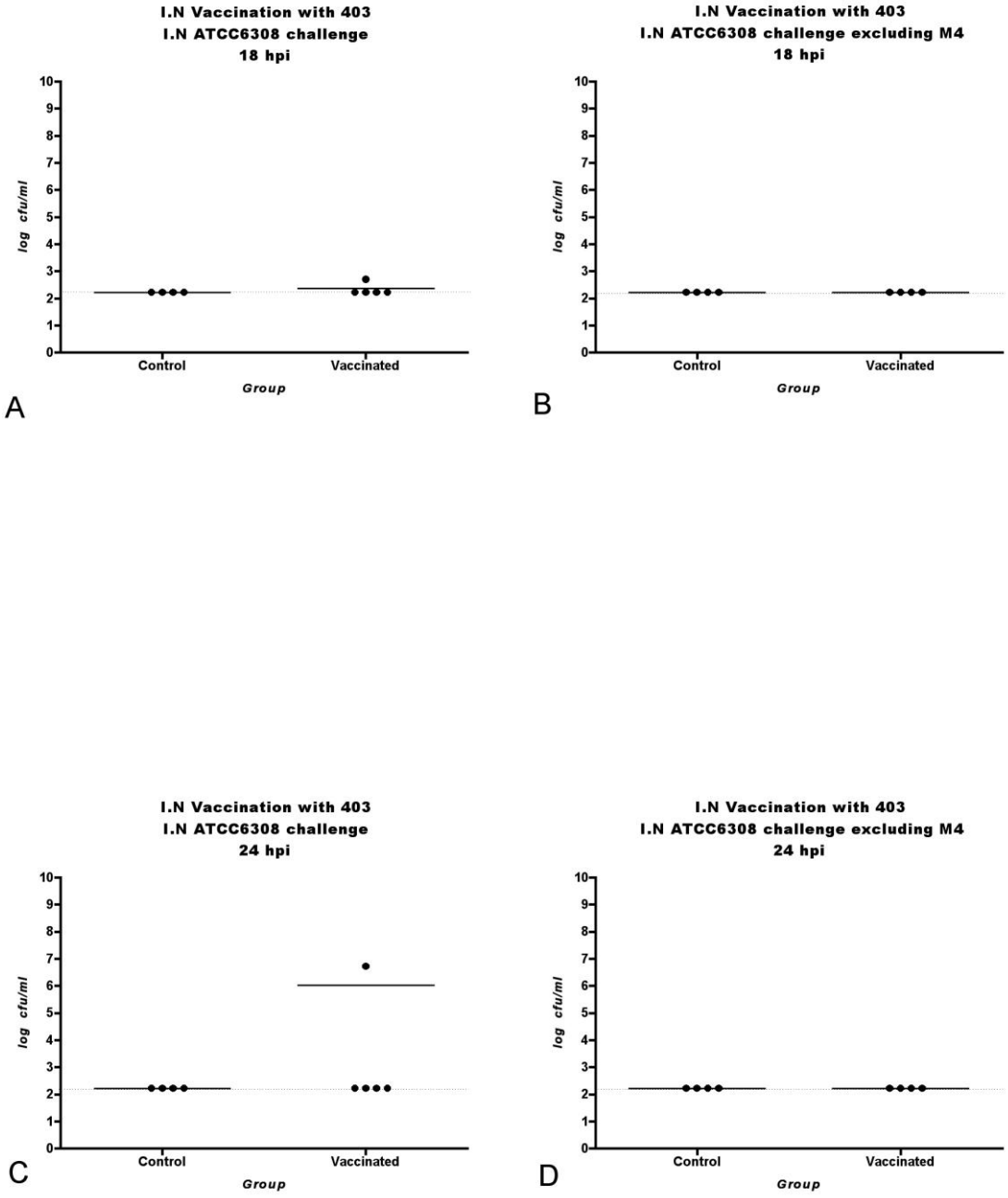


Figure 6.19. Comparison of bacterial load during 18-48 hrs post-challenge IN with $5x10^3$ cfu/ 50 µl strain ATCC6308. The vaccinated group was 3x vaccinated IN with 5×10^7 cfu of strain 403/ 10µl in PBS and control group was given 3x 10 µl PBS only. Groups were compared with Mann-Whitney test (GraphPad Prism 4.0, USA). Circles mark individual mice. Horizontal dotted line represents the limit for detection. Horizontal bar represents the median. (A) Bacteraemia count at 18 hpi. (B) Bacteraemia count at 18 hpi excluding M4

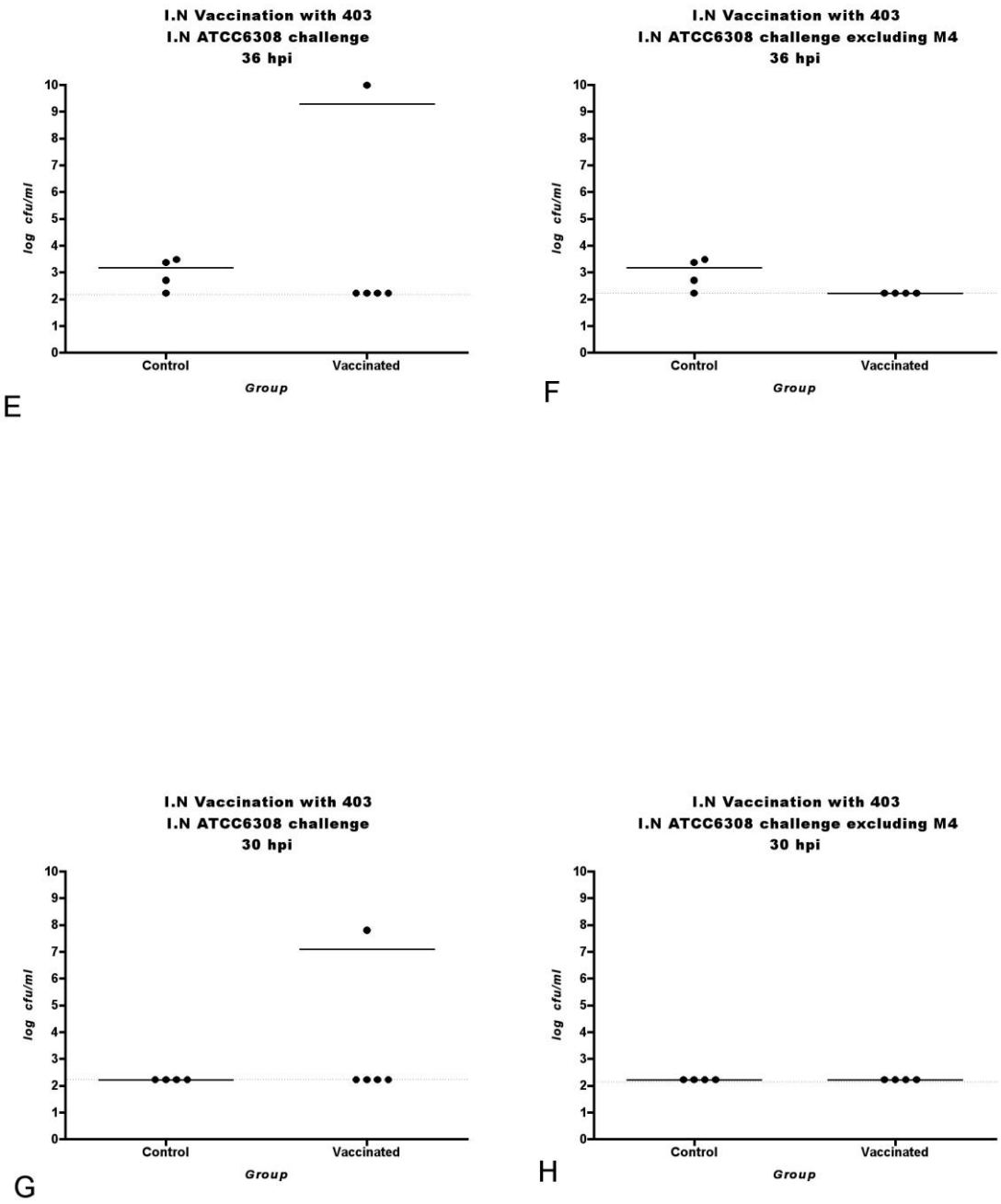


Figure 6.19. Comparison of bacterial load during 18-48 hrs post-challenge IN with $5x10^3$ cfu/ 50 µl strain ATCC6308. The vaccinated group was 3x vaccinated IN with 5×10^7 cfu of strain 403/ 10µl in PBS and control group was given 3x 10 µl PBS only. Groups were compared with Mann-Whitney test (GraphPad Prism 4.0, USA). Circles mark individual mice. Horizontal dotted line represents the limit for detection. Horizontal bar represents the median. (E) Bacteraemia count at 30 hpi. (F) Bacteraemia count at 30 hpi excluding M4. (G) Bacteraemia count at 36 hpi. (H) Bacteraemia count at 36 hpi excluding M4.

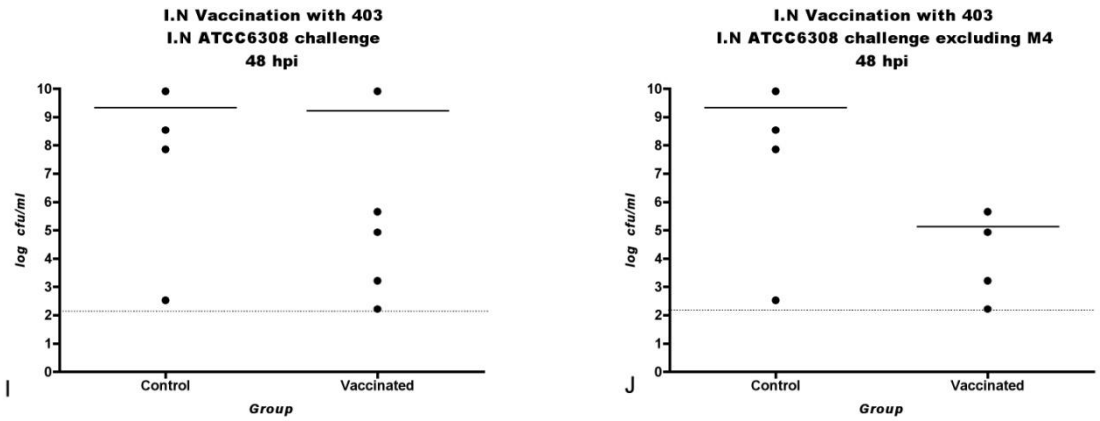


Figure 6.19. Comparison of bacterial load during 18-48 hrs post-challenge IN with $5x10^3$ cfu/ 50 µl strain ATCC6308. The vaccinated group was 3x vaccinated IN with 5×10^7 cfu of strain 403/ 10µl in PBS and control group was given 3x 10 µl PBS only. Groups were compared with Mann-Whitney test (GraphPad Prism 4.0, USA). Circles mark individual mice. Horizontal dotted line represents the limit for detection. Horizontal bar represents the median. (I) Bacteraemia count at 48 hpi. (J) Bacteraemia count at 48 hpi excluding M4

Though data obtained from this experiment was spread but conclusion can still be drawn that IN vaccination with 403 was not effective in protecting against ATCC6308 challenge.

In summary, intranasal vaccinations not only failed to provide complete protection against the same serotype which was used for vaccination but also there was almost no protection against serotypes other than one used to vaccinate mice, though weak trends of decreased bacteraemia were observed in some mice for homologous strain.

6.5 Discussion:

The quest for new vaccine targets is intense than ever as pneumococcal diseases are rising at an alarming rate. The current vaccines, which use different types of pneumococcal capsular polysaccharide (CPS) and induce antibodies that are

serotype-specific, are capable of activating and fixing the complement and promote opsonisation and phagocytosis (Plotkin, 2008). There have been consistent efforts for discovery of newer pneumococcal vaccines targeted against proteins that could act against all strains regardless of serotype.

Whole cell killed vaccines have been used in a number of studies as it can induce immunity by delivering bulk of pneumococcal antigens and induce serotype independent immunity by humoral as well as cell mediated mechanism (Malley & Anderson, 2012). The observation that intranasal whole cell killed vaccine containing unencapsulated pneumococci prevents colonization as well as pneumonia by capsulated pneumococci when administered with cholera toxin (Malley *et al.*, 2001) and induces both cell mediated and humoral immunity when used with aluminium derived adjuvants (Malley & Anderson, 2012) makes whole cell killed vaccines a promising approach.

Live attenuated unencapsulated pneumococcal strains used without adjuvant as intranasal vaccine have also been shown to induce mucosal as well as systemic serotype independent protection in mice (Roche *et al.*, 2007). This protection was also shown to involve both humoral and cell mediated immunity (Roche *et al.*, 2007).

As a combination of the above two approaches, we tested serotype 4 avirulent strain 403 intraperitoneally and than intranasally to observe the magnitude of protection in vaccinated mice. The idea behind using live attenuated strain was to stimulate multiple immune mechanisms by delivering many immunogens at one time along with longer exposure as nasopharyngeal colonization would expose antigens other than capsule. Since the capsule gets down-regulated in the nasopharynx (Weiser *et al.*, 1996; Weiser *et al.*, 1994) there was a possibility

that other pneumococcal proteins will also get longer exposure in nasopharynx along with the capsule, which could result in stimulation of both arms of immune system and induce capsule-specific antibodies as well as CD4+ cell mediated immunity. Results of vaccination by intraperitoneal route showed only partial protection against homologous strain though there were significant differences in levels of bacteraemia, survival, weight and temperature losses, while heterologous strains were not tested. On the other hand, intranasal vaccination and challenge with homologous strains showed trends of decreased bacteraemia though it did not reach statistically significant levels at most time points. It also did not provide any protection against heterologous strains.

It is quite clear that strain 403 on its own, if used as a vaccine, is not strongly immunogenic and is not able to provide complete protection against infection, especially through intranasal route. The data obtained from these experiments is quite variable though some of its fluctuation can be attributed to use of out-bred MF1 mice. Another reason for variation in data could be route of administration as it might affect the efficacy of vaccine as differences in magnitude of immune response related to route of administration have been reported in the literature. Hirabayashi and co-workers (1990) have reported superiority of intranasal route over other routes for hemagglutinin vaccine used in conjugation with cholera toxin against influenza A virus (Hirabayashi *et al.*, 1990), similar observations were reported by Tamura and co-workers (1992) while comparing intranasal vaccination route with subcutaneous route of vaccination for the same vaccine (Tamura *et al.*, 1992). Though Gai and co-workers (2008) reported superiority of IP vaccination over IN route while vaccinating against SARS coronavirus with inactivated virus (Gai *et al.*, 2008), Meitin *et al.* (1991) while vaccinating against H1N1 with inactivated H1N1 have

also reported superiority of Intraperitoneal route (Meitin *et al.*, 1991). In case of pneumococcal vaccination, Malley and Anderson (2012) have reported that intraperitoneal immunization with whole cell killed vaccine administered with aluminium based adjuvants produced >30 fold superior antibody response as compared to the intranasal route (Malley & Anderson, 2012). These observations show that route of administration may affect efficacy of vaccine and an efficacious vaccine might become inefficient due to change in the site of delivery.

Though strain 403 does not provide complete protection against pneumococcal infection as live attenuated vaccine but it was noted to have some effect through intraperitoneal route. Immunized mice not only showed decreased bacteraemia levels but also survived significantly longer than control mice. It is tolerated well, and does not produce any bacteraemia on its own. Moreover, sera from them were reactive against pneumococcal proteins ranging in size from 62-188 kd that might include pspA, pspC, IgA proteases, Srt, Pht and other proteins of unknown function, which are among surface associate proteins and have been reported to be involved in development of immunity in humans (Giefing *et al.*, 2008). It was also observed that sera from immunized mice were able to agglutinate serotype 4 pneumococci. Considering the above facts, there is a possibility that 403, if combined with a suitable adjuvant can provide better protection than used alone as this approach have been shown to work with avirulent shigella strains as it was observed when avirulent shigella strains were given with cholera toxin and heat labile enterotoxin and their mutated versions, enhanced the immunogenicity of live attenuated shigella strains was observed (Hartman *et al.*, 1999). If immunogenic potential of 403 can be enhanced, it will prove to be a safe vaccine, which would be cost-effective, easy to produce and

administer. The strain 403 also offers and added advantage that its capsule-switch mutants can be produced, (Some constructed and discussed in chapter 3), and used in the same way as a polysaccharide conjugate vaccine as a number of 403 mutants expressing different capsular types can be administered together in live or killed form. This can also help in dealing with vaccine escape phenomenon as in case of emergence of a vaccine escape serotype, 403 expressing that particular serotype causing infections can be added to the regimen to produce protection for newly emerging serotype.

Based on the information gathered from the work presented in this chapter, a number of experiments can be done to further investigate the potential of 403 as live attenuated vaccine and enhance its immunogenicity. Intraperitoneal immunizations can be performed by supplementing the vaccination mixture with appropriate quantity of suitable adjuvants to see if this could enhance immunogenicity of the vaccine. In case of positive results, vaccination process can be repeated with capsule switch mutants to enhance options of vaccination.

7 Concluding thoughts and final discussion:

The aim of this thesis was to study the effect of genetic variation on pneumococcal biology. As a part of this project capsule switch mutants were constructed in three genetic backgrounds and were compared for effect of capsular switching on their biological characteristics. EM and capsule polysaccharide measurements showed differences in capsule formation in capsule switch mutants and it was seen that amount of capsular polysaccharide and capsular thickness was different in different mutants as compared to each other and with the parent, though expression of capsular genes could not be investigated due to time constraints. It was also observed that capsular switching is not always beneficial for the organism and it can actually decrease the virulence as the same capsule was observed to be having different effects on different genetic backgrounds. This might be the reason that capsule switch mutants of only certain MLST types have been reported and switching of certain capsular serotypes (such as serotype 1) have not been observed. Our findings also suggest that capsular switching might not be a universally favorable phenomenon despite high homology of the flanking regions (Bentley., 2006) and a successful capsule switch would only be possible if it is supported by a suitable genetic background. This information can be considered encouraging as it can be assumed that vaccine escape phenomenon will have its limitations and only a few types of vaccine escape mutants will emerge in the future and magnitude of this problem will be smaller than it is generally thought. These findings might influence future vaccine design as if the possible emergent vaccine escape mutants can be predicted in a population by studying the population dynamics and serotype distribution, it would be possible to alter the vaccine coverage to

cover the expected emerging serotypes and create herd immunity in the population.

We observed that presence or absence of the capsule does not have an effect on any phase of the growth of TIGR4 and D39 strains in BHI, though it has major effects on transformability and tendency of pneumococci to associate in chains. Further investigations such as microarrays could reveal the effect of capsule switching on expression of genes elsewhere in the pneumococcal genome, which might increase our knowledge about the relationship of the capsule to the rest of the genome and the dynamics of capsule switching and explain the relationship of the capsule genes with the genetic background.

The effect of chain formation on pneumococcal virulence needs to be studied in detail using animal models. Our findings have shown that capsule type may result in formation of longer or shorter chains, but conclusions could not be drawn about the effects of chain length on bacterial virulence. Further studies observing the chain formation in the infected animal might provide a conclusive answer.

We studied the avirulent strain 403, which is a derivative of highly virulent and well-studied strain TIGR4 for investigation of genetic basis for its lack of virulence. Both the strains were compared by whole genome sequencing and microarray analysis. Genome analysis revealed only a few mutations in strain 403, but microarray showed 288 genes to be expressed differently in this strain. Though exact reasons of the non-virulent nature of strain 403 remains unknown but it was observed that several genes related to key metabolic pathways had different expression profiles as compared to TIGR4. There is a possibility that differences in expression of genes related to carbohydrate and protein

metabolism or transport systems in the two strains might be responsible for the non-virulent nature of 403. Though there are limitations of microarray analysis and this approach requires further confirmation and validation of findings by RT-PCR, it has documented the genes that can be further investigated. In addition, if the exact genetic cause of the avirulence of 403 can be found, it can serve as an interesting drug target. Any drug that can target a particular gene or its product, can result in the loss of virulence of highly virulent invasive strains and can serve as a treatment modality for the invasive pneumococcal disease.

Strain 403 was also tested as a potential live attenuated vaccine as it was thought to function as a vehicle capable of delivering the complete set of pneumococcal antigens. Since it can colonize the nasopharynx it was also thought that longer exposure will aid to the development of better immune response and would be able to induce serotype independent immunity by humoral as well as cell mediated mechanisms. It was further theorized that if 403 could provide sufficient protection, its capsule switch mutants could also be used as a vaccine, combining several serotypes in a single dose, without conjugating to any adjuvant. This approach could particularly be useful as it would only require the construction of capsule switch mutants of every serotype, their conversion to non-transformable forms by the knock-out of competence genes and administration according to the requirement of each population along with a suitable adjuvant.

Though trial of 403 was not very successful as it failed to provide any protection through intranasal route but partial protection was observed through intraperitoneal route and significant differences were noted in levels of bacteraemia, survival, weight and temperature losses against the homologous

strain. There is still a possibility that 403 as a live attenuated vaccine can be improved by addition of a suitable adjuvant, which if successful, could provide a cheaper alternative for present vaccines. It has the potential to serve as a comobination of live attenuated and whole cell killed vaccines as it would provide the antigen exposure for prolonged period of time resulting in the development of immunity which would be directed agasint the capsular polysaccharides as well as other proteins. Another most important feature of the proposed vaccine is cost effectiveness, as once constructed and prepared, bacterial strains can be stored and supplied in unlimited quantity for unlimited period of time. If immunogenicity can be improved, 403 along with its capsule switch mutants have the potential to become an easy to use and cost-effective vaccine, which can be easily produced in bulk and altered when needed.

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Conference participation and presentations:

Poster Presentations:

1. Genetic Variation and Virulence of *Streptococcus pneumoniae*. Noori, M.Y. and Mitchell, T.J. Internal Poster presentations at University of Glasgow in 2009 and 2010.

Oral Presentations:

1. Genetic variation and virulence of *Streptococcus pneumoniae* at 11th Europneumo 2011 in Amsterdam, The Netherlands in June 2011.

2. Genetic variation and virulence of *Streptococcus pneumoniae* at the Internal Seminar, Institute of Infection, Immunity and Inflammation, University of Glasgow May, 2011.