University of Glasgow | Institute of Infection, Immunity & Inflammation

Stress Response and Pathogenicity in *Streptococcus pneumoniae*

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

Doctor of Philosophy

By

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B.Sc. (Hons), M.Res (Infection & Immunity)

2013

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Acknowledgment

I owe it all to Almighty who is worthy of all praise for granting me the strength to undertake this research task.

First of all, I am really grateful to my supervisor Professor Tim Mitchell, for his knowledge, overwhelming support and guidance throughout this project. It was a great opportunity for me to do my master and doctoral projects under his supervision and to learn from his research expertise. I am also thankful to my assessors Dr. Gillian Douce and Dr. Donal Wall for their co-operation.

I express my gratitude to all members of "TJM group" Dr. Andrea Mitchell, Dr. Ashleigh Holmes, Dr. Carol McInally, Catherine Dalziel, Dr. Jiang Tao Ma, Dr. Jenny Herbert, Dr. Kirsy Ross, Dr. Matt Horsham, Dr. Muhammad Kashif, Dr. Yahya Noori during all these years as they have been kind enough to extend their friendship and help at various phases of this research, whenever I needed them. A special thanks to Denise Chandlish, June Irvine and Ryan Ritchie for their valuable help with scientific techniques. In addition, great thanks to Dr. Andrea Mitchell for helping in bioinformatic analysis and to show me how genome comparison is performed. I would also thank Dr. Herbert for the technical assistance in the microarray experiments and Dr. Noura Algamdi for her contoinous and honest side-by-side suggestions and support during this project.

Mostly, my sincere gratitude to the Ministry of Higher Education and Taibah University in Saudi Arabia for granting me the scholarship and financial support to pursue my Ph.D at the University of Glasgow. I am very much indebted to Dr Abdellah Akhkha who recommended University of Glasgow to me and for his continuous assistance throughout this study. I would like to thank my friends in Saudi Arabia and here in Glasgow, who encouraged me and helped me at every stage of my personal and academic life, and standing by my side in good and bad times to see me achieving my goals. Most of all, my deepest thanks to my mother, to whom I would have not completed this work without her overwhelming support day by day since 2007. Also to my brothers, sisters, my lovely wife and my kids Faisal, Joud, Maha and Hoor for their continuous willing throughout the study.

Abstract

The pathogen *Streptococcus pneumoniae* encounters different levels of oxygen during the infection cycle including colonisation, pneumonia, bateraemia and meningitis. These different anatomical niches require high levels of genome changes to sense and respond to those external environmental stimuli. The bacterial gene expression is known to be affected by oxygen, and it must react properly for survival and for developing invasive pneumococcal desiseases (IPDs).

Microarray techniques have allowed scanning the whole pneumococcal genome during growth in different tensions of oxygen mimicking *in vivo* conditions. It was found that oxygenated growth conditions have significantly elevated several key virulence genes. This was further confirmed with qRT-PCR for a selection of genes implicated in pathogenicity. Moreover, post-transcriptional stages have been also investigated such as protein production, biofilm formation, biological activities and adherence assays for several virulence factors performed under the effect of presence or absence of oxygen.

The data illustrate that 420 out of 2,236 genes (17 % of the entire TIGR4 genome) were differentially expressed in the presence of oxygen compared to its absence. 262 genes (11 %) were over-expressed when pneumococci were grown in oxygenated conditions relative to transcriptional profile in anaerobic growth conditions, indicating the magnitude of roles played by oxygen on pneumococcal gene expression. Anaerobic growth of TIGR4 showed down-regulation of 158 genes (7 %). Oxygen modulates induction of *ply*, *pspC* and other seven genes involved in pili structuring subunits (*rlrA*, *rrgA*, *rrgB* and *rrgC*) and assembling enzymes (*srtB*, *srtC* and *srtD*). This may suggest that the pneumococcal population grown under atmospheric environment is equipped with greater capability to progress IPDs compared to anaerobically grown bacteria. In addition to this, pneumococcal adhesion *in vitro* for TIGR4 grown in oxygenated or anaerobic growth conditions revealed a significant increase in those grown in oxygenated growth conditions, indicating that oxygen may play a key role in bacterial-host attachment. Interestingly, ablation of *pspC* has resulted in similar adhesion percentages of TIGR4 grown under both conditions, oxygenated and anaerobic.

Furthermore, several genes involved in metabolism were up-regulated in oxygenated environment, particularly transporters, which are considered highly important for a bacterium that lacks an electron transport chain, catalase and tricarboxylic acid. Additionally, the results showed phenotypic characterisation and changes in cells morphology from pneumococcal growth curves for several strainswith different genome backgrounds grown under different levels of oxygen concentrations. Further investigation of the pathogen biology revealed differences in pneumolysin production and activity.

These findings highlight that virulence genes expression is induced once the micro-organism is exposed to oxygenated environment, and data analysis has demonstrated potential links between pneumococcal metabolism and their ability to cause diseases.

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Author's declaration

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

Abbreviation

AE	Aerobic
AhpD	Alkyl Hydroperoxidase
AN	Anaerobic
AOM	Acute Otitis Media
AP	Acetyl-Phosphate
ATP	Adenosine Tri-Phosphate
BA	Blood Agar
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool
Blp	Bacteriocins-Like Peptide
Bp	Base pair
CAP	Community Acquired Pneumonia
CbpA	Choline-Binding Protein A
CbpD	Choline-Binding Protein D
CbpF	Choline-Binding Protein F
CbpJ	Choline-Binding Protein J
CBPs	Choline-Binding Proteins
CDM	Chemically Defined Medium
cDNA	Complementary Deoxyribonucleic acid
CNS	Central Nervous System
CFU	Colony Forming Unit
CGH	Comparative Genomic Hybridization
ClpC	Caseinolytic Protease C
CMR	Comprehensive Microbial Resource
CO_2	Carbon Dioxide
CPS	Capsular Polysaccharide
CRP	C-Reactive Protein
CSF	Cerebro-Spinal Fluid
CSP	Competence-Stimulating Peptide
dATP	2'-deoxyadenine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dGTP	2'-deoxyguanosine 5'-triphosphate
DNA	Deoxyribonucleic acid

dNTP	Deoxynucleoside triphosphate
DTT	Dithiothritol
dTTP	2’-deoxythymidine 5’-triphosphate
eBURST	electronic Based Upon Related Sequence Types
ECC	Epithelial Cell Contact
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron Transport Chain
FBS	Fetal Bovine Serum
GAS	Group A Streptococci
GBS	Group B Streptococci
gDNA	Genomic DNA
GT	Generation Time
HK	Histidine Kinase
HIV	Human Immunodeficiency Virus
HPA	Health Protection Agency
HPI	Hour post infection/inoculation
hr	Hour
HRP	Horse Radish Peroxidase
HtrA	High-Temperature Requirement A
Hyl	Hyaluronidase
IFN	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL-8	Interleukin 8
IP	Intraperitoneal
IPD	Invasive Pneumococcal Disease
JCVI	J. Craig Venter Institute
kDa	Kilodalton
LB	Luria-Bertani
LDH	Lactate Dehydrogenise
LRS	Lower Respiratory System
min	Minute(s)
MLST	Multi-Locus Sequence Typing
mM	Millimolar
NA	Not annotated

NanA	Neuraminidase A
NanB	Neuraminidase B
NCBI	National Center for Biotechnology Information
ND	Not determined
NETs	Nutrophilic extracellular traps
N-IPD	Non-Invasive Pneumococcal Diseases
NK	Natural Killer
NS	Not sequenced
Nox	NADH Oxidase
NP	Nasopharynx
NRT	No Reverse Transcriptase
ºC	Degrees Celsius
OD	Optical Density
OXy	Oxygenated
PBS	Phosphate Buffered Saline
P-Cho	Phosphorylcholine
PCR	Polymerase Chain Reaction
PCV	Pneumococcal Conjugate Vaccine
Pht	Pneumococcal Hisitidine Triad
Pia	Pneumococcal Iron Acquisition
Ply	Pneumolysin
PPV	Pneumococcal Polysaccharide Vaccine
PsaA	Pneumococcal Surface Adhesion A
PspC	Pneumococcal Surface Protein C
PTS	Phosphotransferase System
qRT-PCR	Quantitative Reverse Transcriptase
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
rpm	Revolutions per minute
RR	Response Regulator
RT	Room Temperature (~25ºC)
SDS	Sodium Dodecyl Sulphate
sec	Second(s)
SEM	Standard Error of the Mean
SNPs	Single Nucleotide Polymorphisms

SpxB	pyruvate oxidase
ST	Sequence Type
StkP	Serine/Threonine Protein Kinase
TCA	Tricarboxylic Acid Cycle
TCS	Two-Component System
TE	Tris EDTA buffer
TIGR	The Institute for Genomic Research
TLR4	Toll-Like Receptors 4
TNF	Tumor Necrosis Factor
TNF-α	Tumor Necrosis Factor alpha
URS	Upper Respiratory System
URT	Upper Respiratory Tract
UP	Unpublished data and
VC	Viable Count
WHO	World Health Organisation
WT	Wild Type
Zmp	Zinc Metalloprotease

1.Introduction

1.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae, also known as pneumococcus, is an extracellular pathogen considered to be one of the most serious life-threatening causes of infectious disease in the world (Hammerschmidt 2006; Martner *et al.,* 2008). The bacterium is a facultative anaerobe microorganism enveloped within a polysaccharide capsule, which plays an essential role in the bacterial pathogenicity. Currently, there are 93 recognised variant capsular polysaccharide (CPS) structures, called serotypes, which have mostly been identified microscopically as a swelling, resulting from a specific reaction of antibodies, known as Quellung reaction, against the pneumococcal capsule. However, its high technical cost has led to different typing systems such as PCR-based serotyping (Jin *et al.,* 2009; Park *et al.,* 2007; Song *et al.,* 2012).

The pneumococcus is implicated as the most common cause of respiratory infection in the United Kingdom, and the situation is worse in developing countries (Kadioglu *et al.,* 2008). Despite the availability of antibiotics and the successful introduction of vaccines (Azzari *et al.,* 2008), the pneumococcus is responsible for mortality of an estimated 2 million people per year, and the majority have been reported principally in developing countries (Trappetti *et al.*, 2011a; Williams *et al.*, 2012). It is possible that inadequate diagnosis probably results in underestimating the true pneumococcal disease burden (Paterson *et al.,* 2006). Furthermore, the pneumococcus has been reported to cause significant morbidity and mortality in developed countries. For instance, in the United States, there were 50,000 pneumonia cases, 7 million cases of otitis media and 3,000 cases of meningitis per year (Paterson *et al.,* 2006).

Additionally, in 2012, an updated electronic source presented by the health protection agency (HPA) showed 40,000 GP consultations of pneumonia resulted in hospitalisations due to *S. pneumoniae*; and similarly in England and Wales 63,000 cases of acute otitis media (AOM) were caused by the pathogen *S.*

pneumoniae

(http://www.hpa.org.uk/web/HPAweb&HPAwebStandard/HPAweb_C/120300886402).

1.2 Characteristics and genotyping of *S. pneumoniae*

S. pneumoniae can be identified using several specific tests which include bile solubility of broth culture, α-haemolytic activity on a blood agar plate, sensitivity to discs soaked with 5mg ethyl hydrocupereine (optochin), catalase negativity (Greiner *et al.*, 2001; Hendley *et al.*, 1975; Taniai *et al.*, 2008; Wessels *et al.*, 2012), and the technique "swelling reaction" (Park *et al.*, 2007). Moreover, genotypic means that microarray, genome sequencing and Multi Locus Sequence Typing (MLST) can accurately differentiate pneumococcus from other closely related microbes (Hanage *et al.*, 2005).

1.2.1 Multi Locus Sequence Typing (MLST)

In 1998, Enright and Spratt developed the Multi Locus Sequence Typing (MLST) technique for genotyping using a sequence of seven highly conserved housekeeping genes (*aroE, gdh, gki, recP, spi, xpt, ddl*), with allelic variants of each gene allocated a number (Enright *et al.*, 1998). This has facilitated access and comparison between laboratories through the available website (MLST.com) for worldwide studies (Obert *et al.*, 2007). The high sensitivity of this technique can detect bacterial genotype variations through DNA sequencing. For example, in *S. pneumoniae*, *Streptococcus pyogenes*, *Neisseria meningitidis*, *Staphylococcus aureus*, *Enterococcus faecium* and *Salmonella enterica* (Lemee *et al.*, 2004).

There are over 4,000 MLSTs reflecting the high genome plasticity among pneumococci in responding to the environmental conditions. The organism is highly transformable representing a massive level of genomic variation (Leung *et al.*, 2011). For example, examination of the pneumococcal genome by microarray has revealed 8-10% of genes are absent/divergent in any of the medical strains in comparison to TIGR4 as a reference strain (Silva *et al.*, 2006).

Unlike serotyping, MLST is performed using unencapsulated pnaumococci, and also the technique is beneficial after PCR of an extracted DNA from non-viable or clinically isolated viable pneumococcal pathogens (Hanage *et al.*, 2005). Aplications of clinical MLST are reviewed with reporting their advantages and disadvantages (Sullivan *et al.*, 2005).

1.2.2 Microarray

Microarrays are utilised in studies to investigate the whole bacterial genome (Conway *et al.*, 2003) either by looking at presence/absence gene using Comparative Genome Hybridisation (CGH) for bacterial comparison including *Escherichia coli, Salmonella and Shigella* (Chizhikov *et al.*, 2001), or with the use of fluorescently labelled cDNA to study levels of gene expression as generally explaned in Figure 1-1.

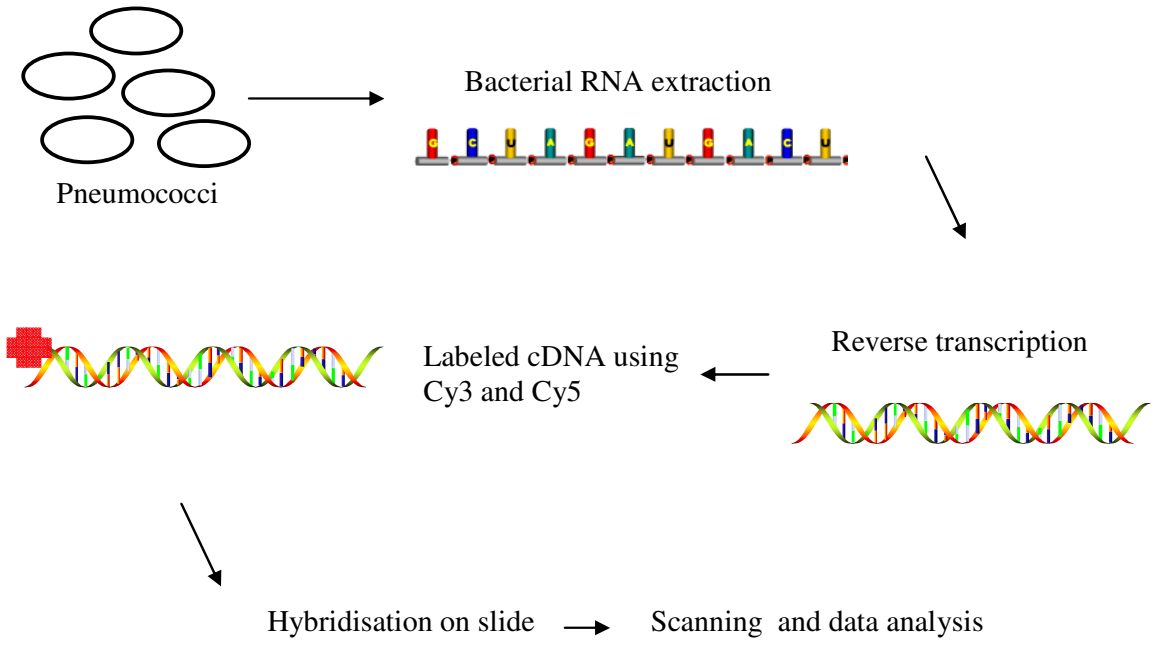


Figure 1-1 Basic microarray procedure

Four steps reflecting the main procedure to perform microarray. After RNA extraction from bacteria, reverse transcription is achieved in order to prepare cDNA before labelling the DNA with the dyes (Cy3 and Cy5). Labelled cDNA then is hybridised on a prepared slide before scanning to obtain expression data.

An example of this can be seen in a study that dissected *Haemophilus* gene expression under different *in vitro* host-mimicking environments (Melnikow *et al.,* 2005).

In the CGH technique, genes are assessed and analysed only for their presence or absence which are practically performed after labelling and hybridising DNA samples against an array glass slide on which the bacterial genome has been prepared (Chizhikov *et al.,* 2001). However, it does not detect levels of expression. Microarray experiments using cDNA have solved this. cDNA is prepared to be hybridised onto the slide to study levels of gene expression that represent the amount of RNA expressed under a specific condition (Bryant *et al.,* 2004).

The microarray technique has been used in several aspects of pneumococcal biology including examination of pneumococcal stress responses to an antibiotic, vancomycin (Haas *et al.,* 2005), studying effects of variant host-mimicking temperatures on gene expression (Pandya *et al.,* 2005) and an *in vivo* investigation of the entire pneumococcal genome collected from different anatomical niches in animal models (Orihuela *et al.,* 2004a).

1.3 Pneumococci and carriage

Streptococcus pneumoniae is carried in the upper respiratory tract (URT) in healthy individuals (Hammerschmidt *et al.,* 2005; Nelson *et al.,* 2007; Price *et al.,* 2012). Mehr and Wood (2012) reported that the carriage percentage of pneumococcal serotypes is 40-60% in young children (Mehr *et al.,* 2012). Interestingly, pneumococcal carriage seemed to be serotype-dependent. Studies with Australian communities have shown that serotype 1 has been rarely isolated from healthy people (Harvey *et al.,* 2011b). However, other similar studies in Kenya undertook culturing several interval swabs from children during months and revealed that type 1 pneumococci were detected for almost 9 days after which the pathogens could not be found again from same childeren (Abdullahi *et al.,* 2012). In fact, pneumococcal colonisation of human nasopharynx is a key

step as it might progress to cause both invasive and non-invasive pneumococcal diseases.

Some individuals are considered to be at high-risk from pneumococcal diseases and predisposed to pneumococcal infections, e.g., patients who have developed viral infections such as influenza (Diavatopoulos *et al.,* 2010), human immunodeficiency virus (HIV), immuno-compromised people and elderly people (Anthony *et al.,* 2012; Li *et al.,* 2012). In addition to these, there are a number of risk factors that can contribute to paving the way for bacterial colonisation, such as smoking (Bakhshaee *et al.,* 2012).

Once the pathogens colonise the host mucosal surfaces and tissues, there will be competition between pneumococci and other organisms for the same ecological niche and nutrients. These bacteria include *Moraxella catarrhalis, Haemophilus influenzae* and *N. meningitidis* (Kais et al., 2006). Therefore, S. pneumoniae has to compete using several competition factors. An example includes production of hydrogen peroxide (H_2O_2) that is released as a by-product of the pathogen´s aerobic metabolism during growth. The peroxide carries out a number of biological functions such as inhibition of growth of other bacteria (Pericone et al., 2000; Regev-Yochay et al., 2007; Selva et al., 2009), interrupts the ciliary beating system leading to epithelial cells damage (Feldman et al., 2002; Hirst et al., 2000a; Regev-Yochay et al., 2007) and mediates pneumococcal-induced apoptosis (Regev-Yochay et al., 2007). Moreover, sialidase, which can modify surfaces of other competitors in the same environmental niche, exposes some competitors to the host's immune system (Burnaugh et al., 2008; Shakhnovich et al., 2002). In addition, pneumolysin, which will be explained in more detail later, is another multi-functional pneumococcal protein which is known to form pores in host cells allowing fragments from nearby H. influenzae to enter epithelial cells that consequently trigger the host immune response against this competitor; therefore, Ply in this case indirectly promotes the immune system toward others (Ratner et al., 2007). Further, the pneumococcus produces bacteriocins to kill other competing bacteria (Dawid et al., 2007; Dawid et al., 2009). However, under certain circumstances, pneumococci can cause serious diseases as they may propagate from the upper respiratory tract (URT) to the

sterile parts of the LRT, resulting in invasive pneumococcal disease (IPD) including pneumonia, septicaemia, meningitis (Cartwright 2002; O'Brien et al., 2009), and N-IPDs such as AOM, sinusitis and bronchitis (Bogaert et al., 2004).

1.4 Diseases caused by *S. pneumoniae*

1.4.1 Acute Otitis Media (AOM)

Pneumococcal infiltration and replication within the Eustachian tube results in a host inflammatory immune response determined as a middle ear infection (Mahadevan *et al.,* 2012; Monasta *et al.,* 2012). In comparison to adults, children are more predisposed to AOM (Corbeel 2007), and up to 80% will develop AOM at least once in the first three years of childhood (Monasta *et al.,* 2012). The progress of the infection can lead to fluid accumulation filling the infection site. Consequently, blocking of the Eustachian tube may cause severe pain and potential deafness if it is not cured. This could affect the education skills or language skills of the child (Allegrucci *et al.,* 2006).

It was reported that in one year only, there were 24 million cases of middle ear infection in the United States (McEwen *et al.,* 2003). Zhou and colleagues stated that over 15 million antibiotic prescriptions were issued for medical treatment because of AOM infection cases (Zhou *et al.,* 2008).

1.4.2 Pneumonia

There were 19,000 deaths out of 866,000 cases of pneumoniae in the United States (Huang *et al.,* 2011). However, in African children, there are up to 4 million cases of deaths per year attributed to pneumococcal pneumonia (Scott 2007).

Although the pneumococcal pathogens have been shown to colonise a large proportion of the human population without causing diseases, the mechanism by which they may transmit to the LRS is poorly understood (Frolet *et al.,* 2010). Once in the alveoli the pathogens can replicate extracellularly, leading to the

release of toxic molecules and causing an inflammatory reaction (Kadioglu *et al.,* 2008). This fluid-microorganism mixture in the alveoli might prevent optimum gaseous exchanges that may alter the infection site from an aerobic to an anaerobic environment. This bacterial assembly will possibly facilitate pneumococcal transition to the adjacent areas (Cano *et al.,* 1986) such as the blood circulation system, possibly causing bacteraemia.

There are several different host micro-ecological conditions, which may have an effect on the bacterial pathogenicity. For example, a study has investigated the possible effect of both the nasopharyngeal surface temperature (33ºC) and the body temperature (37ºC) on pneumococcal gene expression (Pandya *et al.,* 2005). They found an induction in a number of genes encoding adhesive proteins at 33ºC, but not 37ºC, indicating that the nasopharyngeal temperature contributed to *S. pneumonia* colonisation. Nevertheless, oxygen concentration also varies in these two host niches.

1.4.3 Bacteraemia

Pneumonia may result in bacteraemia and this outcome is believed to cause death (Laterre *et al.,* 2005; Myers *et al.,* 2007). Myer and Gervaix also showed that in Switzerland, 50% of the IPD cases developed pneumococcal bacteraemia in children below 3 years old and they stated similar episodes in the United States (Myers *et al.,* 2007). Higher rates were recently reported in a hospital-based study among adult patients who had developed pneumococcal infection from 2000 to 2008 in Denmark, where 81% of *S. pneumoniae* bacteraemia (SPB) was reported (Christensen *et al.,* 2012).

Despite the increase in bacterial resistance to antibiotics, particularly pneumococci due to their known classical evolution ability, penicillin is still the first choice in treating SPB (Christensen *et al.,* 2012).

1.4.4 Meningitis

Pneumococcal meningitis is characterised as an inflammation of the protective membranes of the central narvous system (CNS) (Mook-Kanamori *et al.,* 2011). This follows a pneumococcal infection such as pneumococcal pneumonia, bacteraemia and AOM (Ostergaard *et al.,* 2005). However, the microorganisms can also invade directly to the CSF via the olfactory neurons to the central nervous system (CNS) (van Ginkel *et al.,* 2003).

A study published last year spanning over 19 years has revealed that 45% of adults who have been identified with *S. pneumoniae* in their blood had positive cultures of meningitis (Choi *et al.,* 2012). Another recent investigation in Turkey showed an approximately similar percentage of meningitis in newborn infants and found 42.1% of pneumococcal meningitis among 10,186 hospitalised neonates (Bas *et al.,* 2011).

Symptoms of bacterial meningitis are recognised with a number of manifestations including neck stiffening, headaches, seizures and even coma that consequently may cause death or a serious disability (Menaker *et al.,* 2005). To medically treat the disease, available antibiotics are efficacious, especially those which do not cause bacterial lysis that avoid the release of pneumococcal antigens and pneumolysin into the CSF (Mook-Kanamori *et al.,* 2009).

1.5 Virulence Factors

The number of pneumococcal virulence factors that have been studied has increased gradually in the last two decades (Kadioglu *et al.,* 2008; Watson *et al.,* 1995), with techniques such as signature-tagged mutagenesis (Hava *et al.,* 2002; Lau *et al.,* 2001; Mitchell 2000) and genome sequencing for a number of highly conserved genes including those encode pneumolysin, neumaminidase A and capsular polysacharide (Hu *et al.,* 2012; Williams *et al.,* 2012). These determinants allow the bacterium to interact, colonise and cause a disease. Although sequencing of the whole pneumococcal genome has largely contributed

to understanding the pathogenicity (Tettelin *et al.,* 2001), biological gene functions are still not completely understood. A set of 29 virulence-related genes have been identified to be responsible for *S. pneumoniae* pathogenicity (Oggioni *et al.,* 2006). The majority of the pneumococcal virulence factors and their biological functions in pathogenicity are outlined in Table 1-1.

Table 1-1 Pneumococcal virulence factors and their contribution in pathogenicity

Virulence factors	Biological functions
Pneumococcal surface protein A (PspA)	Contributing to colonisation, pneumonia and bacteraemia (Ogunniyi *et al.,* 2007) Binds the key iron transporter protein lactoferrin (Hammerschmidt 2006)
IgA protease	Leads to evasion of the immune system by degrading IgA and then facilitates colonisation (Poulsen *et al.,* 1996)
Hyaluronidase (Hyl)	Hydrolysing hyaluronic acid situated in connective tissues promoting bacterial spread in the host (Paton *et al.,* 1993)
Pyruvate oxidase (SpxB)	Decarboxylates pyruvate to H_2O_2 and CO_2 during pneumococcal aerobic metabolism (Pesakhov *et al.,* 2007; Spellerberg *et al.,* 1996) *In vivo* gene expression has shown to be up-regulated in the nose when compared to its expression in host lung and blood (Mahdi *et al.,* 2008)
Hydrogen peroxide (H_2O_2)	Induced under aerobic incubation conditions (Spellerberg *et al.,* 1996). Plays an essential role against other species in the same niche (Pericone *et al.,* 2000; Regev-Yochay *et al.,* 2007)

	Interrupts the ciliary beating system causing host epithelial damage and cell injury (Feldman *et al.,* 2002) (Hirst *et al.,* 2000a; Regev-Yochay *et al.,* 2007) Mediates pneumococcal-induced apoptosis (Regev-Yochay *et al.,* 2007)
Pneumococcal surface adhesion A (PsaA)	Fundamental for colonisation and *psaA* mutants were significantly less virulent *in vivo* (Berry *et al.,* 1996) Responsible for transporting manganese (Johnston *et al.,* 2004) and is assumed to transfer iron and zinc across the pneumococcal cytoplasmic membrane (Tseng *et al.,* 2002) Loss of *psaA* resulting in hypersensitivity to the oxidative stress (Johnston *et al.,* 2004)
Zinc metalloprotease (Zmp)	Stimulates TNF-α after triggering an inflammatory response in the lower respiratory system in infected mice (Blue *et al.,* 2003b)
Caseinolytic protease C (ClpC) ClpP Clp protease	Required for LytA and Ply release (Ibrahim *et al.,* 2005) Responds to oxidative stress and involved in virulence (Ibrahim *et al.,* 2005) Implicated in degrading damaged proteins by ROS (Gottesman 1996; Gottesman *et al.,* 1998)
Pneumococcal iron acquisition protein	

A (PiaA)	Genetically conserved in *S. pneumoniae* (Whalan *et al.*, 2006) Iron ABC transporter and required for full pneumococcal virulence in animal murine (Whalan *et al.*, 2006)
Pneumococcal Hisitidine Triad (Pht)	Essential for pathogenicity and binds to Factor H (Ogunniyi *et al.*, 2009) Animal experiments have demonstrated protection against most prevalent pneumococci (Adamou *et al.*, 2001)

1.5.1 Capsule

The pneumococcal capsular polysaccharide (CPS) is believed to be a key virulence determinant for the capability of the bacterium to cause disease. It contains several repeats of multiple sugar structuring (Bentley *et al.*, 2006). The CPS gene expression changes during the pneumococcal infection cycle according to the surrounding environmental conditions (Ogunniyi *et al.*, 2002). In the primary stage of colonisation, the bacterial capsule seems to prevent efficient adherence to host epithelial surfaces (Moscoso *et al.*, 2006), as it entirely covers the pneumococcal cell wall (Kadioglu *et al.*, 2008).

The majority of bacterial isolates display phase variation; i) Expression of a thick CPS when they are collected from the blood stream, to avoid opsonisation that results in bacterial engulfment by phagocytes, ii) Expression of a thinner capsule in the URT to allow binding of epithelial cells (Hammerschmidt *et al.*, 2005; Vannapraseuth 2004; Weinberger *et al.*, 2009).

Interestingly, it has been reported that oxygen availability is responsible for down-regulating CPS in *S. pneumoniae* (Weiser *et al.*, 2001), which will be

discussed in greater detail later, as well as opaque/transparent phenotypes and the role of oxygen in phase variation (Section 1.12).

1.5.2 Choline-binding proteins

There are up to 16 choline-binding proteins (CBPs) placed on the bacterial cell surface (Bergmann *et al.*, 2006). Choline is vital for the pneumococcal growth, cell separation, autolysis and pathogenicity (Rock *et al.*, 2001). Examples of CBP are listed in Table 1-2.

Table 1-2 List of some of the choline-binding proteins

According to (Bergmann *et al*., 2006; Gosink *et al*., 2000).

Choline-binding proteins	Abbreviation
Pneumococcal surface protein A	PspA
Choline-binding protein A Pneumococcal surface protein C Secretory pneumococcal surface proteins A	CbpA PspC SspA
Choline-binding protein D	CbpD
Choline-binding protein F	CbpF
Choline-binding protein J	CbpJ
Choline-binding protein G	CbpG
Choline-binding protein E	CbpE
Autolysin A	LytA
Autolysin B	LytB
Autolysin C	LytC

Pneumococcal cell wall is structured with peptidoglycan to which teichoic acid is covalently linked (Eberhardt *et al.*, 2009), whereas lipoteichoic acid is known to attach to the bacterial cell membrane (Dave *et al.*, 2004a; Fischer *et al.*, 1993). CBPs are non-covalently bound to phosphorylcholine (P-Cho) on the cell wall (Bergmann *et al.*, 2006; Dave *et al.*, 2004a; Kwak *et al.*, 2002) exposing them on the bacterial cell surface (Attali *et al.*, 2008; Hammerschmidt 2006; Rock *et al.*, 2001).

PspC (SP-2190) is a major pneumococcal adhesin factor that mediates attachment to host epithelial cells (Ma *et al.,* 2007), binds immunoglobulin A (IgA) (Lu *et al.,* 2003), is able to bind complement 3, Factor H (FH) (Dave *et al.,* 2004b; Quin *et al.,* 2007) and stimulates interleukin 8 (IL-8) (Madsen *et al.,* 2000). It was found to be regulated by TCS06 as deletion of *rr06* completely abolished *pspC* expression and resulted in loss of bacterial adhesion (Standish *et al.,* 2005). In an *in vivo* study, a *pspC*-deficient mutant has shown a reduction of more than 50% in adhering to its known ligands on eukaryotic cells. The carriage of this mutant in animal experiments reduced 100-fold, whereas this high difference disappeared with sepsis model when mice were infected via the intraperitoneal route using the same mutant as compared to parent strains (Rosenow *et al.,* 1997). Mice infected with *pspC* mutant showed significantly longer survival times compared to parent strains (Iannelli *et al.,* 2004). Similarly, *in vitro* findings have revealed statistical significance, up to 85%, lower ($p<0.001$) adherence of PspC mutant to the human A549 lung cells (Standish *et al.,* 2005).

However, these effects were documented to be strain specific as researchers used a number of serotypes including 2, 3, 4 and 19F, and only TIGR4 *pspC* mutants showed a significant reduction in virulence in animal models in comparison to the TIGR4 parental wild-type (Kerr *et al.,* 2006).

CbpD (SP-2190) was reported to be involved in competence-induced cell lysis (Hakenbeck *et al.,* 2009) and plays a role in colonization as well as CbpE (Bergmann *et al.,* 2006). CbpF (SP-0391) inhibits LytC activity (Molina *et al.,* 2009) and CbpG has shown to contribute to sepsis and increased nasopharyngeal adhesion (Gosink *et al.,* 2000). Furthermore, CbpJ (SP-0378) has been illustrated to be able to bind C-reactive proteins (CRP) (Frolet *et al.,* 2010).

To my knowledge, there are no studies in the literature demonstrating the relationship between these key CBPs and oxygen or describing the role of oxygen in controlling the expression of CBPs.

1.5.3 Autolysin

S. pneumoniae undergoes a peculiar spontaneous tendency to carry out a specific covalent bond cleavage in cell wall, resulting in autolysis once the pathogen reaches the stationary growth phase or when it is exposed to antibiotics (Dagkessamanskaia *et al.,* 2004; Ibrahim *et al.,* 2005; Martner *et al.,* 2008; Ramos-Sevillano *et al.,* 2011).

This property is mediated with autolysin and the function of the molecule is to break down the peptide cross-linking of the peptidoglycan in the cell wall which then leads to bacterial lysis (Martner *et al.,* 2008). Moreover, autolysin can be activated under stress conditions such as penicillin treatment or nutritional starvation (Mitchell 2000). Mitchell has also been pointed out that the autolysis process performed with Lyt activity may enable the bacterium to release the key virulence determinant Ply, as well as discharging vast amounts of pneumococcal cell wall fragments. As a result, the larger the number of these fragments, the higher the inflammatory response (Martner *et al.,* 2008; Vannapraseuth 2004). Experiments in mice revealed that these bacterial components strongly stimulated the immune response resulting in pneumonia, otitis media and meningitis (Hoffmann *et al.,* 2007; Moscoso *et al.,* 2005; Tuomanen 1986; Tuomanen 1987).

LytA is a member of a family of proteins that have been shown to be involved in host colonisation and in causing pneumococcal diseases (Ramos-Sevillano *et al.,* 2011). It has been documented that the autolytic enzyme LytA is implicated in pneumococcal virulence (Berry *et al.,* 1989), LytB contributes to daughter cell separation following the bacterial cell division (Martner *et al.,* 2008), LytC leads to the organism's attachment to the nasophyrengeal cells indicating its role in colonisation, the first pneumococcal step prior to invasive disease (Ramos-Sevillano *et al.,* 2011). Additionally, LytC is actevited at 30ºC, which is similar to URT temperature, and the enzyme was shown to promote pneumococcal adhesion to host nasopharynx (Moscoso *et al.,* 2005). It has been highlighted that its role is synergistic with LytB resulting in an increase in the bacterial

biofilm formation, indicating their cooperative role in pneumococcal biofilm and adhesion (Ramos-Sevillano *et al.,* 2011).

It was found that autolysin-negative mutants cleared rapidly from host lungs, showed no pneumococcal pneumonia with an absence of inflammatory response in the lungs of infected animals (Mitchell 2000).

1.5.4 Pneumolysin (Ply)

Pneumolysin is a multifunctional protein (Feldman *et al.,* 2002; Mitchell *et al.,* 1997). The toxin Ply is known as a major virulence factor in *S. pneumoniae* and is conserved virtually in all pneumococci (Berry *et al.,* 1992; Feldman *et al.,* 2002; Mitchell *et al.,* 1997; Price *et al.,* 2012; Yoo *et al.,* 2010). Although some serotypes showed non-haemolytic activities, antibodies generated against wild type pneumolysin were able to cross-react with those dysfunctional toxins (Kirkham *et al.,* 2006).

Tilley and colleagues showed that the protein Ply forms a large ring-shape in the human cell membranes after binding to cholesterol situated on host membrane (Tilley *et al.,* 2005). This creates pores measured as ~400 Å (Price *et al.,* 2012), and the process is thought to be responsible for several biological activities of the toxin (Mitchell *et al.,* 1997).

The protein is produced intracellularly and was shown to be released under the autolysin effect (Berry *et al.,* 1989; Martner *et al.,* 2008; Steinfort *et al.,* 1989). However, it has been suggested that secretion of the protein in WU2 pneumococci is not dependent on pneumococcal autolysis (Balachandran *et al.,* 2001). Moreover, recent studies documented that before cell lysis occurred, Ply could be detected on the pneumococcal cell wall with Western blot in 18 different serotypes to be localised, and it was biologically active (Price *et al.,* 2009).

The cytolytic Ply has been shown to cause a wide range of immunomodulatory effects including stimulation of nitric oxide production in phagocytes (Braun *et*

al., 1999), induction of inflammatory responses with cytokine production such as tumor necrosis factor α (TNF-α) (Malley *et al.,* 2003), interleukin 1ß (IL-1ß) (Houldsworth *et al.,* 1994) and complement activation (Paton *et al.,* 1984). It can inhibit non-specific defences like the respiratory cilial-beating in epithelial cell lining in the URT (Braun *et al.,* 1999; Harvey *et al.,* 2011a; Malley *et al.,* 2003; Martner *et al.,* 2008; Steinfort *et al.,* 1989; Yoo *et al.,* 2010). Therefore, pneumolysin protein has a wide range of activities in the pneumococcal pathogenicity.

Interestingly, recent studies have detected some strains isolated from patients to express non-haemolytic pneumolysin, and these strains showed greater virulence capability relative to pneumococci in which the toxin was deleted, indicating that pneumolysin possesses a further unknown function (Harvey *et al.,* 2011a; Kadioglu *et al.,* 2008).

Pneumolysin is not the only cytolytic factor in pneumococci; hydrogen peroxide (H_2O_2) produced by *S. pneumoniae* lacking Ply was shown to cause haemolytic activity to human ciliated epithelium cells *in vitro* (Feldman *et al.,* 2002; Hirst *et al.,* 2000b). H_2O_2 is mostly produced during the aerobic pneumococcal metabolism (Section 1.7). However, available data reflecting how this major pneumococcal virulence factor can be affected by the presence or absence of oxygen are extremely limited.

1.5.5 Neuraminidase

The colonization of the nasopharynx is an important stage in the development of pneumococcal disease, and *S. pneumoniae* expresses many factors to colonise epithelial surfaces. The enzyme neuraminidase (NA), also called sialidase, is one of them (Uchiyama *et al.,* 2009). It was reported that the function of this virulence determinant is to cleave a terminal sialic acid (N-Acetylneuraminic acid) from glycoconjugates situated on mucosal surfaces, at the cell membrane of epithelial cells, leading to exposing “unmasked” cell receptors for bacterial adherence (Kadioglu *et al.,* 2008; King *et al.,* 2004).

Studies have shown that pneumococci encode up to three NAs proteins: NanA, NanB and NanC (Manco *et al.,* 2006). It was also demonstrated that both NanA and NanB are implicated in cleaving sialic acid, but these two enzymes have two variant pH optima (NanA at ph 6.5 and NanB at pH 4.5), suggesting different roles for these proteins *in vivo* (Manco *et al.,* 2006).

NanA was reported to contribute in colonising the nasopharyngx and the development of otitis media in a chinchilla as well as being involved in pneumococcal biofilm formation (Parker *et al.,* 2009). Both NanA and NanB mutants were unable to cause sepsis in mice (Manco *et al.,* 2006). In addition, Oggioni and colleagues stated that gene expression during host blood infection was characterized by a decrease in these neuraminidases, A and B, relative to their expression in the lungs (Oggioni *et al.,* 2006).

Further work on *S. pneumoniae* NanA shows its capacity to modify the surface of other organisms competing with pneumococci in the same environmental niche (Burnaugh *et al.,* 2008). For example, a study has pointed out that the enzyme which breaks down sialic acid from both *N. meningitidis* and *H. influenzae* cell surface (Shakhnovich *et al.,* 2002). They also suggested that this cleaving exposes the competitor to be attacked by the host's immune defences, resulting in pneumococcal exploitation of the microenvironment. Furthermore, a recent investigation described that an attachment to the endothelial barrier was mediated with NanA both in *in vivo* animal models and *ex vitro* human brain endothelial cultures (Uchiyama *et al.,* 2009). Additionally, NanA was recently shown to be involved in virulence by promoting pneumococcal biofilm formation (Parker *et al.,* 2009).

1.5.6 Pili

Bacterial pili have been identified first in Gram-negative micro-organisms, and recently in Gram-positive bacteria. They were documented to fulfil multiple bacteriological functions during the microbial life cycle including colonisation, cell aggregation, biofilm formation, twitching motility, and DNA uptake (Hilleringmann *et al.,* 2009; Proft *et al.,* 2009).

To date, two types of pneumococcal islets encoding pili have been identified, pilus islet 1 (PI-1) and pilus islet 2 (PI-2) (Bagnoli *et al.,* 2008). Type 1 (Figure 1-2, A) is expressed by *S. pneumoniae* TIGR4 (Bagnoli *et al.,* 2008; El Mortaji *et al.,* 2012a; El Mortaji *et al.,* 2012b; LeMieux *et al.,* 2006). Pneumococcal PI-1 is a flexible and filament-like measured as 1µm in length, surface-exposed virulence factor (Hilleringmann *et al.,* 2009). Its expression was shown to result in an increase in the adhesion ability *in vitro* to lung epithelial cells (Barocchi *et al.,* 2006; El Mortaji *et al.,* 2010). The contribution of attachment by palliated pneumococci was significantly increased *in vivo*, and similarly in streptococci such as *S. pyogenes* and *S. agalactiae* (Lauer *et al.,* 2005; LeMieux *et al.,* 2006; Nelson *et al.,* 2007). Nelson and co-researchers have found that *S. pneumoniae* mutants without RrgA, one of the pneumococcal pilins, showed significantly less attachment to respiratory cells compared to the wild-type bacteria (Nelson *et al.,* 2007).

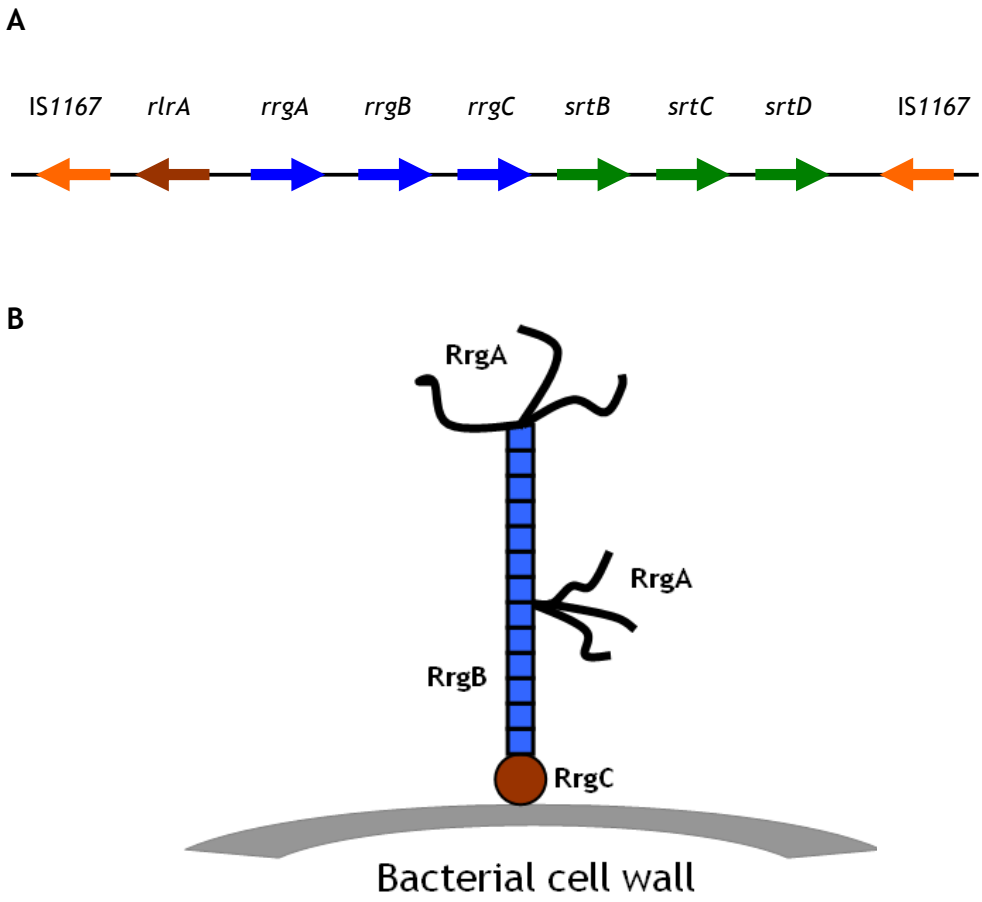


Figure 1-2 Pilus genes and structure in TIGR4 pneumococci

Schematic illustration of PI-1 genes (A) and structure (B). In (A), the brown arrow shows the transcriptional regulator, blue arrows are the structural pilus genes, green indicates the assembling enzymes, and the orange arrows show the flanking sequences, adapted from (Bagnoli *et al.*, 2008). (B) the brown circle represents RrgC protein attached to the gray curve showing the pneumococcal cell wall, blue boxes are RrgB building up the pilus backbone, and RrgA black spikes, modified from (El Mortaji *et al.*, 2012a).

RrgA was reported later in attaching (Bagnoli *et al.*, 2008). Furthermore, studies have illustrated that RrgA and RrgB in TIGR4 PI-1 were shown to bind TLR2, and consequently stimulate an inflammatory response in mice that was associated with the release of TNF-α from macrophages as compared to nonpiliated isogenic mutants (Barocchi *et al.*, 2006; Basset *et al.*, 2013).

Genetically, the pilus 1 proteins have been shown to be expressed by the pneumococcal *rlrA* pathogenicity islet in *S. pneumoniae* TIGR4 (Nelson *et al.*, 2007; Song *et al.*, 2008). Its expression is based on three genes, one major *rrgB*

for the pilus formation and two minor subunits *rrgA* and *rrgC* situated at the pilus proximal shaft anchoring it to the bacterium cell wall (El Mortaji *et al.*, 2012a). All of these are responsible for structuring the bacterial pili, as well as the other three major pilus enzymes (SrtB, C and D) which are responsible for the pilus assembly as shown in Figure 1-1, B (Hilleringmann *et al.*, 2009; LeMieux *et al.*, 2006).

It is worth mentioning that genes responsible for coding *rlrA* islet in *S. pneumoniae* were found to be carried in 27% of clinically isolated pneumococci, which was reported in Portugal from invasive pneumococcal infections (Aguiar *et al.*, 2008). It has been shown that pilus genes are serotype-specific as they were detected in different pneumococcal serotypes. For example, 4, 6A and 35B have pili, whereas types 1, 3, 14, 15A, 5, 7F, and 18C do not possess *rlrA* operon encoding PI-1 proteins (El Mortaji *et al.*, 2012a; Moschioni *et al.*, 2008). However, genome sequencing has recently shown type 2 pilus (PI-2) in the strain INV104 that represents serotype 1 (Bagnoli *et al.*, 2008). It was also revealed that PI-2 contains five genes, *pitA*, *pitB*, *sipA*, *srtG1* and *srtG2* (Figure 1-3).

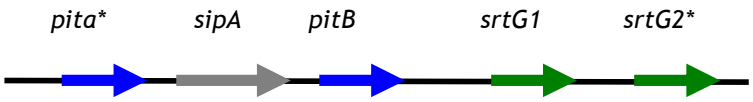


Figure 1-3 Pneumococcal genes of pilus islet 2

Blue arrows are the genes structure coding for pilus proteins, green indicates the genes coding for assembling enzymes, and the grey arrow is a signal peptidase. Stars on *pitA* and *srtG2* indicate that genes contain a frameshift in all for some pneumococcal strains, modified from (Bagnoli *et al.*, 2008).

Analysis of 305 pneumococcal strains showed that PI-2 exists in almost 16% of the strains, and has been associated with pneumococci lacking PI-1 (Serruto *et al.*, 2009). Deficiency of PI-2 has been tested *in vitro* to assess its contribution in adherence. It was found that the PI-2 mutant showed a reduction in its capability to attach different cell lines (Bagnoli *et al.*, 2008).

There is a possibility that *in vivo* oxygen variation controls the expression and synthesis of pilus proteins, but this is yet to be elucidated.

1.5.7 Two-component system (TCS)

It is well known that bacteria are able to sense and respond to the surrounding environmental conditions such as pH and temperature, and accordingly regulate a number of genes. These responses are mediated via the Two-Component Systems (TCS) comprising of two proteins: a histidine kinase (HK) and a response regulator (RR) (Blue *et al.,* 2003a). The HK is anchored to the pneumococcal cell membrane and is involved in sensing external environmental stimuli leading to its phosphorylation (Dutta *et al.,* 1999). The phosphate group is then transferred to the RR, which is situated in the cytoplasm, and possesses a highly conserved DNA binding domain, consequently affecting the protein transcription (Blue *et al.,* 2003a). As a result, a particular bacterial gene expression is displayed in response to external stimuli.

This signalling system has been demonstrated to be involved in a large number of cellular activities including regulating bacterial pathogenicity, osmoregulation, sporulation, photosynthesis, and has been shown to play a major role in the bacterial viability (Lange *et al.,* 1999).

There are 13 HK and RR pairs and an orphan unpaired RR that have been studied in pneumococci. Their roles in gene expression were studied (Lange *et al.,* 1999; Paterson *et al.,* 2006; Throup *et al.,* 2000).

All of these known TCSs are listed in Table 1-3, illustrating their alternative names and their roles in pneumococcal pathogenicity.

Table 1-3 Pneumococcal Two-component systems and their contribution in virulence. quoted and modified from (Paterson *et al.*, 2006).

TCS	Alternative names	Role in virulence
01	480	Yes
02	Vic, MicAB, YycFG and 492	Yes
03	474	No
04	PnpRS and 481	Yes

05	CiaRH and 494	Yes
06	478	Yes
07	539	Yes
08	484	Yes
09	ZmpSR and 488	Yes
10	VncRS and 491	No
11	479	No
12	ComDE and 498	Yes
13	BlbRH and 486	Yes
Orphan RR	RitR and 489	Yes

The majority of these signalling systems are implicated in the pneumococcal virulence and the inactivation of some has revealed variation in the ability to cause diseases in experimental models of infection (Blue *et al.,* 2003a).

The infection site was shown to have an impact on TCSs contribution of virulence as well as the strain background, which all adds to the complexity of analysing and studying such systems (Paterson *et al.,* 2006). An example of this difference has been seen in TCS09 as loss of its function in D39 caused a loss of virulence in animal models. On the other hand, the same defective gene was constructed in another strain 0100993, but in this strain the mutation did not result in attenuation in virulence (Blue *et al.,* 2003a).

In 2004, McCluskey and colleagues reported that mutation of *rr04* in *S. pneumoniae* TIGR4 resulted in attenuation in virulence in comparison to its parent strain. Additionally, McCluskey *et al* also showed that when TCS04 was inactivated in TIGR4, the *psa* operon that is responsible for encoding the manganese ABC transporter system was down-regulated. Furthermore, the operon has been shown to contribute to the pneumococcal virulence and oxidative stress resistance. *rr04* deficiency leads to hypersensitive to hydrogen peroxide killing (McCluskey *et al.,* 2004a).

Additionally, TCS03, 05 and 11 have been reported to be involved in bacterial stress response (Haas *et al.,* 2005), while TCS02 (also called MicAb, VicRK, 492hkrr and YycFG) is essential for pneumococcal survival (Lange *et al.,* 1999).

TCS02 is involved in regulating bacterial cell wall, biosynthesis of fatty acid and expression of the virulence factor PspA (Ma *et al.,* 2007; Mohedano *et al.,* 2005; Ng *et al.,* 2005). Studies have shown that *hk02* mutants were less virulent in causing pneumococcal pneumonia (Kadioglu *et al.,* 2003). Moreover, TCS02 deficiency in *S. mutans* has affected several important processes including growth, adhesion, biofilm formation, and competence (Senadheera *et al.,* 2005).

TCS05 (also called CiaRH) has been documented to affect virulence (Ibrahim *et al.,* 2004a), contribute to nasophryngeal colonisation, biofilm formation and adhesion to host epithelial cells (Trappetti *et al.,* 2011a). It was also shown to play a role in antibiotic resistance, sugar metabolism, competence and regulating the high-temperature requirement A protein (HtrA) (Halfmann *et al.,* 2007; Ibrahim *et al.,* 2004b), HtrA is a key virulence factor, and an oxygen stress responder. HtrA was down-regulated when TCS05 was absent, and the relationship between TCS05 and oxygen has been studied (Echenique *et al.,* 2000).

Furthermore, *S. pneumoniae* expresses TCS13 (*486rr*) which has been implicated in virulence (Throup *et al.,* 2000), quorum-sensing regulon and is known as a bacteriocins-like peptide (Blp) (de Saizieu *et al.,* 2000). Bacteriocins (Blps) are bacterial products that have been shown to inhibit or kill those related competing microorganisms using immunity proteins to protect themselves from their own toxin (Dawid *et al.*, 2007). Dawid and co-researchers also reported that the bacteriocin mutants in 6A serotype background revealed an inability to compete with its parent and serotype 4 pneumococci during nasopharyngeal colonisation.

The orphan regulator RitR (repressor of iron transport, RR489) which also has a role in virulence in animal models (Throup *et al.,* 2000), and was required for lung infection as its deletion led to reduction in pathogenicity (Ulijasz *et al.,* 2004). Furthermore, RitR was described to be involved in iron transportation, a key bacterial growth factor. Most importantly its transcription was associated with oxidative stress response and loss of its function has resulted in an increase

in bacterial H_2O_2 susceptibility (Ulijasz *et al.,* 2004). In addition, *ritR* has been shown recently to be phosphorylated by a serine/threonine protein kinase (StkP) that increases the response regulators affinity to its target DNA (Ulijasz *et al.,* 2009).

Although oxygen availability was shown to control TCS05 (Echenique *et al.,* 2000), how all other TCSs react to oxygen is largely unknown.

1.6 Pneumococcal growth

It is well known that a typical pneumococcal growth curve fallows four main stages: lag, logarithmic (log/exponential), stationary and decline/death phases. Once the pneumococcus is allowed to grow in optimal bacteriological growth conditions or in its host, it will adapt itself enzymatically from the incubation environment that it was grown in previously (Perry *et al.,* 1997). This important adjustment in preparation for the bacterial growth occurs in a new environment, and cells will continue to grow until they synthesise the necessary metabolites and proteins to balance both growth and division to start entering the lag phase (Perry *et al.,* 1997; Swinnen *et al.,* 2004). Additionally, the length of the lag phase was shown to be significantly associated with serotypes; strain with long lag phases are associated with greater invasiveness such as 1, 4, 5, 7F and 14, whereas the ones with short lag phases were linked to carriage, including 6A, 6B, 8, 9V, 15, 18C, 19F, 23F, 33 and 38 (Baettig *et al.,* 2006).

During the logarithmic phase, the number of pneumococcal cells increases exponentially allowing for generation time (GT) calculation. GT was found to be 21 min for serotype 3 in broth cultures (Tauber *et al.,* 1984a). This doubling time, using the same bacteria, rose to 67 min *in vivo* in rabbit tissues (Tauber *et al.,* 1984b). Another study investigating growth curve characteristics in ideal growth conditions for a range of clinically isolated bacteria (11 pneumococci) and showed generation times between 24 to 36 min (Mazzola *et al.,* 2003). The log phase continues until the pathogen faces a number of deleterious factors either in the broth culture or from the host that they grow in. For instance, this

linearised growth phase will be affected by lack of nutrition, pH changes, accumulation of the bacterial waste, and effective responses from the host immune system. All these factors play an essential role in decreasing the pneumococcal population size until they reach the stationary phase (Perry *et al.*, 1997).

In the stationary phase, pneumococcal death rates will be equal to the growth (division), therefore there is no increase in the bacterial population. This is attributed to several effectors such as high metabolite levels (particularly at the end of the phase), accumulation of organic acids and encountering starvation because of nutritional exhaustion. It is worth mentioning that there are several genes known to be expressed only at this phase such as secondary metabolite genes and others involved in survival (*sur*) that are essential for growth during the stationary phase when faced with stress factors (Perry *et al.*, 1997). Bacterial cells will finally enter the decline phase represented by a logarithmic decrease in the death rates.

Pneumococcal growth has been shown to be regulated with several factors such as calcium (Trombe *et al.*, 1992), capsule gene expression (Baettig *et al.*, 2007), different temperatures (Pandya *et al.*, 2005) and variant broth media (Baettig *et al.*, 2006; Slotved *et al.*, 2005). Moreover, pneumococcal growth in the absence of oxygen was reported to be elevated (Baettig *et al.*, 2006). A recent work on *S. mutans* found an impaired streptococcal growth in aerated conditions (Ahn *et al.*, 2007). However, the effect of oxygenic and anaerobic incubation environments has not been investigated in a wide range of pneumococci, and it is well known that bacterial growth under specific conditions may show how they behave biologically.

1.7 Fermentation in *S. pneumoniae* and oxidative stress resistance

S. pneumoniae is known as an aero tolerant anaerobic bacterium and obtains its metabolic energy sufficient to support its growth and cell division from

carbohydrate fermentation. Pneumococcal genome is considered to encode enzymes for converting (oxidation) of carbohydrates to pyruvate via glycolysis (Spellerberg *et al.,* 1996) and the pathways detaies are available on line: http://www.streppneumoniae.com/carbohydrate_utilization.asp , viewed 2013).

The microorganism is believed to frequently encounter variant levels of oxygen during the process of infection (Yesilkaya *et al.,* 2013); for example, oxygen availability have been reported to be 21% in nasopharynx, 10-15% in the alveoli, 5% in the tissues (Burghout *et al.,* 2010) and 1.4% to 1.9% in the human bloodstream (The Clinician's Ultimate Reference, http://www.globalrph.com/martin_4_most2.htm#Oxygen%20Content%20Equation, accessed in March 2013).

However, *S. pneumoniae* has been shown to lack several of the defence mechanisms that other bacteria possess for surviving in an oxidative environment (Hajaj *et al.,* 2012; Pesakhov *et al.,* 2007). Sequencing of the whole pneumococcal genome revealed that the bacterium lacks genes for catalase, which is able to break down hydrogen peroxide into oxygen and water (Taniai *et al.,* 2008), and does not express a large number of genes for aerobic oxidative pathways. For example, *S. pneumoniae* has no genetic material required for haem biosynthesis and even lacks alternative requirements such as cytochromes, and the cytoplasmic proteins involved in aerobic respiration (Auzat *et al.*, 1999; Taniai *et al.*, 2008). Additionally, genes required for a complete electron transport chain (ETC) were not found and none of the known 18 Tricarboxylic acid cycle (TCA) genes considered to play a role in catabolising organic molecules in the presence of oxygen to gather energy for the bacteria to survival (Hoskins *et al.,* 2001) http://www.streppneumoniae.com/carbohydrate_utilization.asp, viewed 2013).

S. pneumoniae is known to obtain the energy needed for growth from metabolising glucose (Carvalho *et al.,* 2013). Under aerobic growth conditions, pneumococci convert glucose into pyruvate (Spellerberg *et al.,* 1996). They also express the pneumococcal enzyme pyruvate oxidase (SpxB), encoded by *spxB* gene, that in turn decarboxylates pyruvate to H_2O_2, carbon dioxide (CO_2) and

acetyl-phosphate (AP) as can be seen in (Figure 1-4, A), this metabolism pathway is also found in other streptococci (Zheng *et al.,* 2011). In addition, activity of SpxB or exogenous supplementation of H_2O_2 was reported to largely elevate mutation rates (Pericone *et al.,* 2002) and decrease pneumococcal growth (Regev-Yochay *et al.,* 2007), indicating less bacterial survival in aerated growth conditions relative to anaerobically grown cells. Battig and colleagues stated that anaerobic growth increased pneumococcal growth (Baettig *et al.,* 2006). Similarly, this was reported with *S. mutans* (Ahn *et al.,* 2007).

Furthermore, under the same oxygen environment, the bacterium expresses NADH oxidase (Nox) which can convert oxygen to water (Auzat *et al.,* 1999). The pneumococcal Nox enzyme is also able to reduce O_2 into H_2O, facilitating pathogen survival in aerobic oxidative stress environments (Pericone *et al.,* 2003).

Pneumococci are known to produce H_2O_2 in aerated incubation conditions during exponential growth phase and the highest levels have been detected at the stationary phase (Taniai *et al.,* 2008). This toxic production was documented to be responsible for the bacterial spontaneous death during the TIGR4 stationary growth phase (Regev-Yochay *et al.,* 2007). It was reported that the elimination of H_2O_2 from the bacterial growth cultures, either with addition of catalase, or growing the pneumococci under anaerobic condition or by deletion of *spxB*, has resulted in significant increase of the pneumococcal stationary phase (Regev-Yochay *et al.,* 2007).

Under anaerobic conditions, carbohydrates will be oxidised into pyruvate which in turn is converted to lactic acid by the enzyme lactate dehydrogenise (LDH) (Figure 1-4). However, group A streptococci (GAS) carry out fermentative growth irrespective of the oxygen availability (McIver *et al.,* 1995), and *spxB* expression was recently detected with Western blot in *S. gordonii* grown aerobically and anaerobically (Zheng *et al.,* 2011).

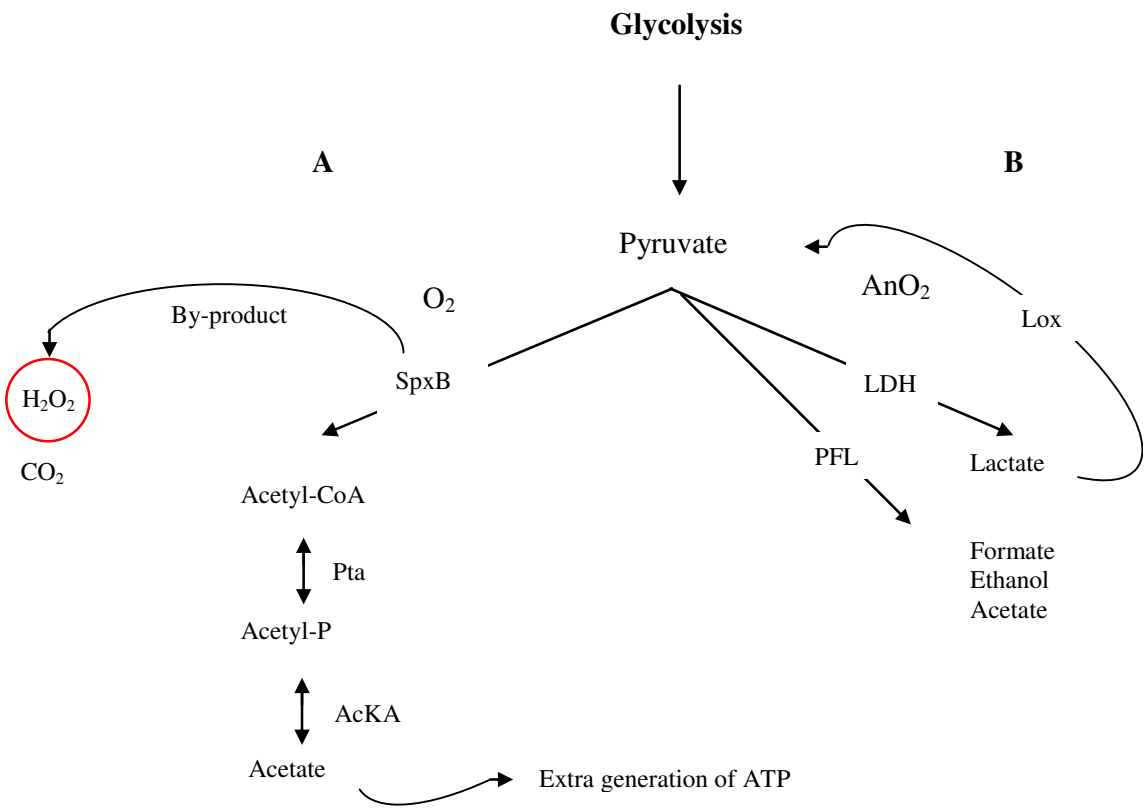


Figure 1-4 Metabolism of pyruvate in pneumococci

Proposed aerobic metabolic pathway (A) and anaerobic (B). Acetyl-P is acetyl phosphate, Lox: lactate oxidase, PFL: pyruvate formate layes, Pta: phosphotransacetylase, AcKA: acetate kinase, LDH: lactate dehydrogenase. Based on (Carvalho *et al.*, 2013; Spellerberg *et al.*, 1996).

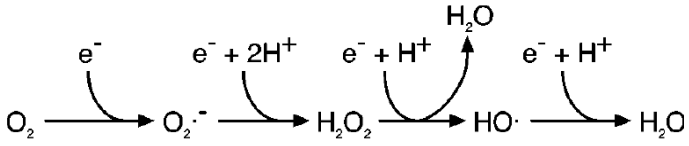
Reduction of pyruvic acid to lactic acid anaerobically results in regenerating most of the NADH (Spellerberg *et al.*, 1996). Moreover, these investigators have also documented that a loss of SpxB function in *S. pneumoniae* mutants has shown pneumococcal disability to grow in a Chemically Defined Medium (CDM) without supplementation with acetate in micro-aerophillic growth conditions. A few years later, Hoskins *et al* and others confirmed this aerobic metabolism pathway and illustrated the acetate outcome from pyruvate (Hoskins *et al.*, 2001; Pericone *et al.*, 2003). Furthermore, the *spxB* mutant was unable to grow with the addition of sub lethal concentrations of H_2O_2 (Regev-Yochay *et al.*, 2007), suggesting that SpxB protects the cells from the effect of H_2O_2. In

addition, in *spxB* mutant, the production of ATP levels was reduced compared to the wild type pneumococci, and SpxB was responsible for producing more than 99% of H_2O_2 when compared with its inactivated pneumococci (Pericone *et al.,* 2000).

It was stated that levels of acetyl-phosphate generated by SpxB activity was induced up to 85% in some of the aerobically grown pneumococci (Pericone *et al.,* 2003). Acetyl-phosphate is considered to have critical biological functions when carbohydrates are aerobically metabolised. For instance, AP is a precursor to acetyl-CoA which is involved as a metabolic constituent for the pneumococcal cell wall, amino acid and biosynthesis of fatty acid (Ramos-Montanez *et al.,* 2010). It would be expected then that any conditions that are able to negatively affect the production of the global intracellular messenger acetyl-phosphate may largely lead to a defect in the bacterial gene expression profiles.

1.8 Sources and effects of oxidative stress molecules

Pneumococci are exposed to two main sources of oxidative stresses; endogenous and exogenous. For the endogenous, the aerobic growth conditions are continuously exposed to reactive oxygen species (ROS), which are known to be deleterious to biomolecules. These reactive intermediates are formed from an incomplete reduction of oxygen to water through respiration as shown in the following chemical reaction according to Fridovich (1998) and Imlay 2003 (Fridovich 1998; Imlay 2003), quoted from Imlay 2003.



These species include hydroxyl radical, superoxide anion and H_2O_2 generate from the metabolic pathways (Zheng *et al.,* 2011), and recently reviewed (Yesilkaya *et al.,* 2013). The effects of these reactive species are well studied as they can damage DNA, proteins and the bacterial cell membrane leading to bacterial

growth inhibition (Imlay 2003; Mostertz *et al.,* 2003; Regev-Yochay *et al.,* 2007). Additionally, pneumococcal organisms have been shown to produce a hydroxyl radical because of Fenton reaction from endogenously produced H_2O_2 (Pericone *et al.*, 2003) Fenton reaction chemically is known to be responsible for generating hydroxyl radical when metal ions are present, iron is a good example. The only ROS that can highly and negativily affect cell contents is hydroxyl radical, particularly DNA resulting in genetic mutation and lipid oxidation leading to plasma membrane damages, whereas superoxide anaion and hydrogen peroxide damage proteins through oxidation (Imlay 2003). This oxidation is known to result in damaging biomolecules through two main mannars; I. oxidation of the plasma membrane, which affects its permeability, nutrient transport through the plasma mebrane and osmosis impalance, and II. Oxidation of nitrogenous bases and then destroying DNA (Imlay 2003).

Exogenous sources of ROS are a further burden for *S. pneumoniae*. Barbuti et al have highlighted the ability of the human immune system to carry out a respiratory burst as a key innate immune defence against bacterial infections (Barbuti *et al.,* 2010). Respiratory burst generates superoxide anion during a phagocytosis process where phagocytes transport extra oxygen to the cell. This transported oxygen is then converted into a superoxide anion followed by diffusion H_2O_2 into the phagosome in order to kill the engulfed bacterium (Martner *et al.,* 2008). An early study has shown that the lack of H_2O_2 production by the immune system affects the infection rates (Klebanoff 1980). He was also stated that this could be noted in patients with chronic granulomatous disease where H_2O_2 is not produced. People diagnosed with this genetic disease are not able to mount the crucial respiratory burst process (Klebanoff 1980). Therefore, although pathogens are phagocytosed, the absence of H_2O_2 leads to insufficient phagositic willing. Nevertheless, pneumococci cannot survive in these patients due to the fact that *S. pneumoniae* cells are known as H_2O_2 producers and this might provide the phagocytes with what they need to perform the pneumococcal killing after engulfment (Klebanoff 1980).

Furthermore, the host neutrophils are able to produce nitric oxide. The reactive nitric oxide also acts as an oxidising antibacterial agent for destroying

microorganisms (Braun *et al.,* 1999). The neutrophils perform the production of nitric oxide in the presence of H_2O_2, which is secreted by the neutrophil itself. Another exogenous source, used as a herbicide, is called paraquat. It is largely known in microbiological laboratories for generating an oxidative stress in bacterial cultures and oxidative assays (Ahn *et al.,* 2007).

1.9 Bacterial defences against oxidative stress

In other bacteria that have developed defences against oxidative stress, there are several bacterial strategies to defeat ROS molecules by detoxification mechanisms. For instance, catalase along with alkyl hydroperoxidase have been reported to play a fundamental role in cooperation to protect *E. coli* against both exogenous and endogenous sources of H_2O_2 (Seaver *et al.,* 2001), while superoxide dismutases (SodA and SodB) react against superoxide anion and have been shown to be crucial survival factors in aerobic growth (Carlioz *et al.,* 1986).

Both of the Gram-negative microbes *Streptococcus typhimurium* and *E. coli* express up to 40 genes controlled by some regulators such as SoxRS and OxyR in response to H_2O_2, and another 40 to paraquat (Demple 1999). Therefore, *E. coli* is believed to be well equipped with a firm global network against oxidative stress (Greenberg *et al.,* 1989).

In addition, *E. coli* possesses two differentially localised types of catalase, periplasmic and cytoplasmic catalases (Heimberger *et al.,* 1988). These are capable of transforming two H_2O_2 molecules to O_2 and H_2O, protecting the organism from exogenous and endogenous sources of H_2O_2 (Fridovich 1998). Furthermore, the bacterium produces RecA protein which can repair the damaged DNA resulting from oxidative stress (Farr *et al.,* 1991).

Gram-positive bacteria have been illustrated to have different mechanisms in their response to oxidative stress as SoxRS and OxyR are not involved in the regulation process. They express the peroxide response regulator (PerR) that is known to be expressed in the soil bacterium *Bacillus subtilis*, but is not present

in pneumococci (Mostertz *et al.,* 2003; Tettelin *et al.,* 2002). PerR was also shown to regulate catalase (*kat)* and alkyle hydroperixidase (*ahp)* in *Staphylococcus aureus* (Horsburgh *et al.,* 2001), which is well known as a catalase positive organism. Additionally, the regulator PerR was reported to be highly important for stress response in group A streptococcus (GAS) as 76 genes were PerR dependent (Grifantini *et al.,* 2011). Furthermore, an investigation has revealed that NADH defective *S. pyogenes* grown in oxygen showed enhanced sensitivity to oxidative stress, and the bacterium was unable to resist its endogenous production of H_2O_2 (Gibson *et al.,* 2000). However, a recent genomic comparison between TIGR4 genome and other streptococci revealed that other streptococci lack some genes known to be involved in oxidative stress response (Yesilkaya *et al.,* 2013). For example, they cited that alkyl hydroperoxidase has not been found genetically in a number of streptococci including *S. pyogenes, S. aglactiae, S. gordonii, Streptococcus suis and Streptococcus sanguinis,* but is present in pneumococci, indicating that pneumococci have developed a specific strategy to react to an oxidative lifestyle compared to other streptococcal pathogens (Yesilkaya *et al.,* 2013).

1.10 Pneumococcal adaptation to oxidative stress

Oxidative stress can be defined as an imbalance occurs between oxygen or its derivatives and the biological systems ability to eliminate toxicity and repairing biomolecules damages. The mechanisms that pneumococci follow to survive in oxidative stress are not completely understood. Pneumococci have to some extent adapted to the stress conditions. An example is some of the pneumococcal genes that have been investigated for their particular responses to stress environments. These putative genes include *nox* codes NADH, *tpx* codes thiol peroxidase, *spxB* codes streptococcus pyruvate oxidase, *sodA* codes superoxide dismutase A, *htrA* codes high-temperature requirement, *ahp* codes alkyl hydroperoxidase and *rgg* codes a regulator (Bortoni *et al.,* 2009; Hajaj *et al.,* 2012; Ibrahim *et al.,* 2004b). In addition to these, a deletion in a pneumococcal operon which was recently found to contain *ccdA* (putative cytochrome), *tlpA* (thioredoxin-like protein) and *msrAB* (methionine sulfoxide

reductase) has resulted in at least 10-fold greater pneumococcal susceptibility to H_2O_2 (Andisi *et al.*, 2012).

SpxB is one of the well known defence mechanisms against ROS. As explained earlier in section 1.7, under aerobic incubation conditions, SpxB reduces O_2 to H_2O_2, which also produces CO_2 and acetyl phosphate. Acetate kinase then transforms this acetyl phosphate into acetate with concomitant ATP production (Auzat *et al.*, 1999). Consequently, the pathogen generates greater amounts of ATP compared to pyruvate conversion to lactate. Pericone and colleagues (2003) have constructed *spxB* mutant and grew them in the presence of oxygen. They detected less ATP production in the *spxB* mutant, which led them to hypothesise that this would affect the DNA repair system resulting in the observed bacterial death. *spxB* mutant was documented to express <1% H_2O_2 production in comparison with the wild-type strain, and the mutants showed an increase in their susceptibility to be killed by H_2O_2 (Pericone *et al.*, 2000). It is worth to mention here that pneumococci have been found to be able to generate more than 2.0 mM of H_2O_2 (Pericone *et al.*, 2002).

Nox is another tool used by pneumococci to face ROS. It reduces oxygen to water; performing four-electron reduction of O_2 to H_2O. A study has constructed *nox*-mutant pneumococci and showed a pleiotropic effects of the bacteria, altering their transformability, pathogenicity, and persistence in murine models of pneumococcal infection and even led to less resistance to oxidative stress compared to the wild type (Auzat *et al.*, 1999; Echenique *et al.*, 2000). Further investigations have demonstrated that *nox* defective pneumococci were attenuated in causing respiratory tract infection (Yu *et al.*, 2001). Recently, ablation of *nox* gene has been reported to result in significantly less pneumococcal adhesion to A549 cells and the mutants pathogenicity was similarly attenuated *in vivo* (Muchnik *et al.*, 2013), indicating possible relationships between *S. pneumoniae* physiology and virulence.

Moreover, *psa*D (also called *tpx*) gene is a part of *psa* operon, and is known to be part of a manganese transporting system. It was shown in a recent study to encode the vital pneumococcal antioxidant, thiol peroxidase (Hajaj *et al.*,

2012). It was also revealed by Hajaj et al that gene expression of *tpx in vitro* was significantly increased in pneumococci grown aerobically relative to their anoxic growth (Hajaj *et al.,* 2012). They also cited that as Tpx is situated on the bacterial cell wall and therefore plays an important role against exogenous peroxides not endogenous.

Yesilkaya and colleagues have also found that when *sodA* was absent in the pneumococcus, the aerobic bacterial growth showed a reduction in virulence after intranasal, but not intravenous infection (Yesilkaya *et al.,* 2000). As mentioned earlier, Sod is known to play a role against superoxide anion and was shown as a crucial survival factor in aerobic growth conditions due to its ability to convert superoxide anion to O_2 and H_2O_2 (Carlioz *et al.,* 1986). Ahp is counted against ROS as well because of the capability of the protein to cleave hydrogen peroxide to alcohol and water (Paterson *et al.,* 2006). In addition to these, NmIR, MerR (Potter *et al.,* 2010) and cysteine desulphurase subfamily protein SufS (Ramos-Montanez *et al.,* 2008) were shown to participate in the pneumococcal defence against hydrogen peroxides.

Furthermore, as mentioned before, *S. pneumoniae* is not only unable to express catalases, but also lacks some of the proteins that are known to be involved in oxidative stress response regulation which are present in other bacteria such as SoxRS, OxyR, NADH peroxidase and PerR (Demple 1999; Pericone *et al.,* 2003), (Section 1.9). However, a study came out during our work reported for the first time a genetic response to oxygen in serotype 2 pneumococci (Bortoni *et al.,* 2009).

Additionally, recent studies on pneumococcal oxidative stress response showed genes (*ccdA, tlpA and msrAB*) involved in H_2O_2 responses (Andisi *et al.,* 2012). Investigation of Tpx which is coded by SP-1651 in TIGR4 (Hajaj *et al.,* 2012) have contributed in increasing our knowledge about relationships between pneumococci and ROS. However, the roles of other enzymes such as glutathione peroxidase (Gpo, SP-1279), which is homologous to Dpr (DNA-binding ferritin-like protein), and glutathione reductase (SP-0784) are still unknown. Potter *et al* have recently reported for the first time that glutathione reductase is utilised by

D39 to resist oxidative stress (Potter *et al.,* 2012), and this is believed to be an orphan study investigating glutathione reductase in *S. pneumoniae*. Therefore, how *S. pneumoniae* responds and survives in an atmospheric condition, which is considered its normal habitat, is still to some extent ambiguous.

1.11 Pneumococcal biofilm formation

Biofilms are developed when pneumococci attach to both biotic and abiotic surfaces (Allegrucci *et al.,* 2006). They can then extrude several extracellular matrixes in which they embed themselves, proliferate and match to each other (Hall-Stoodley *et al.,* 2008). The following definition was described and considered one of the best explanations for biofilm as it generally covers the whole process:

> ''A microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription''.
>
> (Donlan *et al.,* 2002)

Moscoso and co-workers have stated that this attachment ability is responsible for more than 60% of bacterial infections (Moscoso *et al.,* 2006). There are a large number of pathogenic bacteria which can adhere to host cells in the upper respiratory airways, an initial key step to progress to an infection before causing pneumococcal diseases including otitis media (Hall-Stoodley *et al.,* 2008) and sepsis (Tapiainen *et al.,* 2010). There was little available literature on the for biofilm formation by *S. pneumoniae* until recently (del Prado *et al.,* 2010; Moscoso *et al.,* 2006; Tapiainen *et al.,* 2010). It has been documented that pneumococcal biofilms were detected in the middle ear infection, nasopharyngeal colonisation, on the adenoid surface, biopsied mucosal epithelial tissues and among children who have had a tympanostomy tube or implanted medical devices (Allegrucci *et al.,* 2006; del Prado *et al.,* 2010; Tapiainen *et al.,* 2010).

Pneumococcal communities in biofilms are highly structured and they have shown resistance to antibacterial agents, host immune attack and surrounding environmental challenges (del Prado *et al.,* 2010; Tapiainen *et al.,* 2010). It has been reported that there are many phenotypic differences that occur inside this tied pool of microorganisms. For example, large number of genes have found in *S. pneumoniae* to be responsible for both biofilm formation and nasopharyngeal colonisation in mice (Munoz-Elias *et al.,* 2008).

Moreover, several key virulence genes including CBPs are expressed differentially during pneumococcal growth in biofilm relative to other environments (brain, lung and blood) (Oggioni *et al.,* 2006). It was also shown that competence-stimulating peptide (CSP) is required for biofilms and a CSP receptor mutant inhibited the formation of pneumococcal biofilms, and was less virulent to develop pneumonia. Similarly, biofilm formation by *S. mutans* increased 85% in the presence of CSP (Petersen *et al.,* 2004). Furthermore, an investigation of genes involved in the pneumococcal biofilm has shown a decrease in biofilm formation with CBPs deficient mutants in comparison to its parent strain provided (Moscoso *et al.,* 2006).

Trappetti and colleagues have further recent support to these results as they constructed a deletion in both *lic* operon and TCS05. Lic is involved in choline uptake. They found that the mutants showed a reduction in forming biofilms, were not able to translocate from nasopharyngeal colonisation to the lungs, and poorly attached to human alveolar pneumocytes (Trappetti *et al.,* 2011b). The operon *lic* is also present in other respiratory pathogens including *H. influenzae*, *N. meningitidis* and *Pseudomonas aeruginosa*, and known to encode genes responsible for choline uptake and metabolism (Kwak *et al.,* 2002; Rock *et al.,* 2001).

In addition, NanA has been also reported recently to contribute to pneumococcal biofilm formation (Parker *et al.,* 2009). Another study examined the biofilm composition formed by pneumococcal clinical isolates and detected that DNA was present extracellularly in the biofilms (Hall-Stoodley *et al.,* 2008). They confirmed this result by treating the pneumococcal biofilms with DNase

and found there was a significant dose responsive reduction in the biomass of the pneumococcal biofilms. The release of free DNA into the biofilm matrix has been suggested to play an essential role in *S. mutans* biofilms as it was required as a major molecule for initially establishing biofilm formation (Petersen *et al.,* 2004). It is well known that *S. pneumoniae* is a highly transformable bacterium and spontaneously releases DNA under the effect of CSP (Hall-Stoodley *et al.,* 2008; Moscoso *et al.,* 2006). Furthermore, biofilms have been shown to be induced with CSP in several other bacteria including *S. gordonii, S. mutans* and *Streptococcus intermedius* (Oggioni *et al.,* 2006).

Other pneumococcal virulence factors have been shown to affect the bacterial biofilms. Moscoso *et al* undertook a microscopy examination for the bacterial capsule and found that an up-regulation in CPS had a key role against the formation of the biofilm (Moscoso *et al.,* 2006). Moreover, they studied the influence of CPS in pneumococcal biofilms, which revealed that the presence of the capsule has negatively affected the development of the biofilm by more than 60%. A recent work reported that *cpsA,* the first CPS gene in the *S. pneumoniae* capsule operon, was down-regulated during biofilm formation in comparison with the planktonic grown pneumococci (Hall-Stoodley *et al.,* 2008). However, although there is accumulating evidence linking biofilms to virulence, recent studies revealed that biofilms are not essential for virulence, and no correlation was detected between pneumococci collected from PID and N-PID in biofilm formation (Lizcano *et al.,* 2010).

In terms of the oxygen´s effect on biofilm formation, Ahn and co-researchers illustrated that O_2 is involved in regulating *S. mutans* biofilm formation (Ahn *et al.,* 2007), but its role in pneumococcal aggregation and biofilms remains to be investigated. In 2009, a relationship between oxygen and biofilms by *S. pneumoniae* was discovered. Pneumococcal *rgg,* a transcriptional regulator, has been shown to involve in both the bacterial biofilm formation and oxidative stress response (Bortoni *et al.,* 2009).

1.12 Effect of environment on bacterial gene expression

The ability of bacteria to adapt, grow and survive in varied environments and mammalian host anatomical sites requires flexible global gene expression. Therefore, bacterial gene expression, particularly virulence factors, has been shown in various studies to be affected by various environmental conditions. Some examples which are highly related to our topic will be highlighted herein; a high temperature (39ºC) was reported to elevate gene expression of the adhesion factor SspB in *S. gordonii*, an important organism causing dental plaque and endocarditis, whereas changes in osmolarity and high pH decreased *sspB* expression (El-Sabaeny *et al.,* 2000). Recently, environmental influences have resulted in different production of hydrogen peroxide in *S. gordonii* (Zheng *et al.,* 2011). In addition to the above genetic variation, phenotypic differences were also studied in four different bacteria; *Shigella flexneria*, *Bordetella pertussis*, *Yersenia pestis* and *Borrelia burgdorferi*, and most of their virulence genes have shown to involve in responding to temperature variations (Konkel *et al.,* 2000).

It has been recently documented that environmental acidification led to an increase in pilus synthesis and biofilm formation in *S. pyogenes* (Manetti *et al.,* 2010). A selection of *in vitro* growth conditions such as biofilm and planktonic states had an effect on global gene expression of *Moraxella catarrhails*, which is a causative of otitis media and pulmonary diseases (Wang *et al.,* 2007). Sulphide has an effect on gene expression and bacterial growth physiology of *Desulfovibrio vulgaris* (Caffrey *et al.,* 2010). All these have obviously prove that how surrounding environmental changes affect the microbial gene expression.

1.12.1 Environmental factors effecting pneumococcal gene expression

Although there are a large number of studies in the literature focusing on pneumococcal virulence-related genes, regulators and competence (Dagkessamanskaia *et al.,* 2004; Echenique *et al.,* 2001; Hava *et al.,* 2003;

Iannelli *et al.,* 2004; Iannelli *et al.,* 2002; Iannelli *et al.,* 1999; King *et al.,* 2004; Lau *et al.,* 2001; Oggioni *et al.,* 2003), there has been relatively little investigation on the pneumococcal physiology during disease and its behaviour in different environmental conditions. Additionally, several issues remain unclear such as the translocation mechanisms from colonising the host upper respiratory system to the lungs through the blood-brain barrier (Oggioni *et al.,* 2006).

Different *in vivo* environmental conditions have been reported to modulate the pneumococcal pathogenicity. For instance, a scan of the whole genome showed that key virulence genes were regulated differentially based on the body sites that pneumococci collected from including nasopharynx, lungs, blood stream and CSF (Orihuela *et al.,* 2004a). An excellent example of how an *in vivo* niche can affect the bacterial virulence is seen when 10-fold greater adhesion on human epithelial cells was reported with *S. pneumoniae* gathered from peritoneal murine cavities in comparison to those grown in *in vitro* cultures (Orihuela *et al.,* 2000).

Pandya and colleagues have investigated the effect of different host temperatures, such as 21ºC, 29ºC, (representing the environmental temperature) 33ºC (reflecting temperature of nasopharyngeal surface), 37ºC (the body temperature) and 40ºC (as a fever during infection), on the pneumococcal gene expression (Pandya *et al.,* 2005). They have reported phenotypic differences in *S. pneumoniae* and found an induction in genes encoding adhesion proteins at 33ºC that might contribute to nasopharyngeal colonisation. Similar studies have investigated the effect of temperature and pH changes on pneumococci (Mazzola *et al.,* 2003). Furthermore, host-derived compounds have an influence on pneumococcal gene expression. For instance, providing *S. pneumoniae* with mucin in Sicard's defined medium induced *nanA* gene expression (Yesilkaya *et al.,* 2008), and pneumococcal growth in blood cultures affecting production of key proteins (Bae *et al.,* 2006).

Moreover, microarray data, which was validated with qRT-PCR, found that 386 genes were involved in responding to penicillin (Rogers *et al.,* 2007). Host-derived compounds also have been shown to affect mRNA levels of capsular

polysaccharide, pneumolysin and PspC. All these three proteins were elevated in pneumococci exposed to host fluids *in vitro* versus those grown in TYH broth medium (Ogunniyi *et al.,* 2002). Furthermore, growing *S. pneumoniae* (serotypes 6B, 9V, 14 and 23F) in the sugar xylitol has significantly lowered the expression of *cpsB* (Kurola *et al.,* 2009). Streptococci may be able to take and metabolise xylitol through phosphotransferase system (PTS) (Palchaudhuri *et al.,* 2011).

The pneumococcal capsule was also found to be up-regulated in the blood stream *in vivo* versus *in vitro* growth cultures (Ogunniyi *et al.,* 2002), and expression of *nanA* has been changed according to opaque and transparent phase variation (King *et al.,* 2004). They stated that *nanA* had a greater expression and activity in transparent pneumococci than the opaque cells. Transparent variants showed an increase in the nasopharyngeal adhesion ability, and therefore are more efficient in colonisation, while opaque phenotypes have been correlated to resist opsonisation and were linked to causing pneumococcal sepsis (King *et al.,* 2004; Weiser *et al.,* 1994a; Weiser *et al.,* 1994b). In addition, Oggionoi and coinvestigators showed that based on the infected site of the animal models, virulence genes expression showed variation (Oggioni *et al.,* 2006).

All these recent studies have documented the bacterial sensing of external stimuli and accordingly responded by changing its gene expression.

1.12.2 Oxygen availability and pathogenicity

Molecular oxygen can act as a growth substrate, toxic factor, regulator for bacterial gene expression and it is mainly considered as a major regulatory signal of metabolism in facultative anaerobic microorganisms (Unden *et al.,* 1991). Bacterial exposure to stress conditions has been shown to result in switching the expression of the virulence genes, which may lead to increasing the bacterial pathogenicity. For example, it was recently reported that diffusion of oxygen to the villi in the host intestinal tract was associated with virulence in the *Shigella* strain M90T (Marteyn *et al.,* 2010). Moreover, oxygen molecules are required to control the global regulator FNR in *Klebsiella pneumoniae* (Grabbe

et al., 2001; Grabbe *et al.,* 2003). Other bacteria are with FNR O_2-dependence include *E. coli* and *Salmonella* (Unden *et al.,* 1991). Early work has shown that abscess formation by *S. aureus* is enhanced with aeration (Schmidt *et al.,* 1967). Furthermore, growth of *S. mutans* in oxygen resulted in changes virulence gene expression and markedly affected surface protein profiles (Ahn *et al.,* 2007).

Oxygen has been shown in regulating certain bacterial traits such as adhesion factors. For example, invasion assays of the human cell lines (A549) and neuroepithelial cells (PFSK-1) validated with electron micrograph experiments, revealed that there was significantly increase in bacterial counts of intracellular Group B streptococci (GBS) when the pathogen was grown in the presence of oxygen, indicating that oxygen is involved in elevating the bacterial adhesins (Johri *et al.,* 2003). The study also showed that neonatal mice inoculated with GBS grown in oxygen had significantly less survival ($p=0.0007$) than GBS grown anaerobically. This demonstrates that the availability of oxygen is a key environmental stimulus leading to an increase in the bacterial virulence level, and that microbial exposure to oxygenic growth conditions might be a powerful induction for GBS pathogenicity (Johri *et al.,* 2003).

In *S. pneumoniae*, a study shown the first evidence that oxygen can contribute in regulating both pneumococcal transformability and ability to cause disease (Auzat *et al.,* 1999). In addition, the availability of oxygen has been implicated in down-regulation of the pneumococcal CPS (Weiser *et al.,* 2001). A few years later, this important oxygen relationship to phenotypic phase variation was further supported after modulating oxygen concentration during pneumococcal growth in the mid-log phase. The authors' conclusion was as follows:

> ''The variation from anaerobic to aerobic atmosphere during the stationary phase of *S. pneumoniae* cultivation in the bioreactor increased the percentage of the total CPS recovered from the supernatant''.
>
> (Goncalves *et al.,* 2006)

This capsular variation may facilitate the bacterium-host interaction during colonisation, where their adhesins may need to be uncoated, while up-regulation of the capsule genes might be required in the absence of oxygen to avoid the immune response during bacteraemia (Ogunniyi *et al.,* 2002; Weinberger *et al.,* 2009). This phase variation might be also involved in the bacterial transition from being coloniser in the upper airways to invasiveness (Hammerschmidt *et al.,* 2005). It is noteworthy that transparent phenotypes have shown an increased ability to cross the blood-brain barrier (Ring *et al.,* 1998).

During this project, a study has shown global gene expression changes in *S. pneumoniae* in response to air (Bortoni *et al.,* 2009). They reveal that pneumococcal resistance to oxidative stress is associated with virulence, and exposure of *S. pneumoniae* to air was responsible for changes in expression of 69 genes, 54 of which were up-regulated (almost 2.3% of the whole pneumococcal genome); whereas the remaining 15 genes were shown to be down-regulated under the same conditions. Furthermore, interestingly they reported up to an 11-fold transcriptional increase in the regulatory gene *rgg* during growth in aerobic environment. This is the only available published study that scans the whole pneumococcal genome in the presence/absence of oxygen using serotype 2 pneumococci. Hence, there is a need to expand this investigation in order to increase our understanding of how the pneumococcal gene expression is affected by oxygen and responds to different levels of oxygen.

1.13 Genomic variation in *S. pneumoniae*

Several bacterial species show different levels of genomic variation such as *Campylobacter jejuni* (Dorrell *et al.,* 2001) and *Helicobacter pylori* (Bjorkholm *et al.,* 2001), but genomic of *Mycobacterium tuberculosis* seems to be highly conserved (Behr *et al.,* 1999). Pneumococci are historically known as naturally transformable bacteria exhibiting genetic exchange resulting in environmental adaptation. For example, *S. pneumoniae* R6 representing serotype 2 has been fully genome sequenced in 2001 (Hoskins *et al.*, 2001), and similarly the strain

TIGR4 representing serotype 4 in 2001 which was isolated from a patient´s blood (a man aged 30 year) in Norway 1990s (Tettelin *et al.,* 2001). A genomic comparison between these two pneumococcal strains (R6 and TIGR4) revealed that there are 10% variation (Bruckner *et al.,* 2004). Further investigation has been carried out in R6 and TIGR4 pneumococci to explain any genes requirement for invasive disease or carriage (Obert *et al.,* 2006).

Invasive pneumococcal diseases and carriage have been geogrifically associated with some serotypes, for example, common pneumococcal diseases have been reported to be serotype-specific such as type 14, 6B, 5, 6A and 19A in the Middle East, wheras in Europe and the USA, type 1, 19F and 23F cause more IPDs than other serotypes (Mehr *et al.,* 2012). Additionally, some pneumococcal genetic variations have been linked to carriage rather than causing IPDs, an example of this is seen in serotype 3 strains which are usually found in host's nasopharynx with carrying prophages in their genomes (Loeffler *et al.,* 2006). In contrast, type 1 pneumococci are known to be hyper-virulent bacteria that rarely detected in the nasopharyngeal niches (Harvey *et al.,* 2011b; Williams *et al.,* 2012).

Differences in pneumococcal ability to cause a disease are attributed to their genome content. For instance, capsular switching and penicillin resistance are believed to occur as a consecuence of horizontal gene transfere leading to the pneumococcal survival after escaping from a vaccine and acquisition penicillin resistance gene, respectevily (Coffey *et al.,* 1991; Coffey *et al.,* 1998). Furthermore, Weiser et al and other pneumococcal laboratories reported phase variation of pneumococci showing two different phunotypes (transparent and opaque). This was explained earlier in section 1.12.1, and can be considered here as another good example reflecting pneumoccal ability for genomic variation, which can be linked to the bacterial transformatiom capability as pneumococci are know to be naturally competent and equipped with several genes accounted for the pneumococcal competence including *coma, comb, comC, comD and comE* (Pericone *et al.,* 2002). They also showed that oxygen was able to control competence in *S. pneumoniae,* and the spontaneous mutation rates were reduced 10-fold during growth in an anaerobic growth

condition or with addition of catalase where hydrogen peroxide was not detectable. This suggests that oxidative stress occurs in host nasopharyngeal environment due to the presence of oxygen might facilitate gene exchanging interspecies and intraspecies resulting in greater virulence (Pericone *et al.*, 2002).

1.14 Aims of the study

In spite of a number of studies showing the contribution of pneumococcal virulence factors to its pathogenicity, the effects of environmental factors on pneumococcal growth characteristics, gene expression and the bacterial physiology require further investigation. Studying the impact of different environmental conditions might be highly useful and contribute to an understanding of the interaction between the pathogen and the host.

The aims of this study are:

1. To study the effects of O_2 on pneumococcal growth characteristics.

2. Investigate the bacterial gene expression during O_2 exposure.

3. Study possible roles of virulence factors in various levels of oxygen.

4. Evaluate the pneumococcal physiology during both conditions.

This identification could allow us to gain a molecular insight into phenotypic changes during the pneumococcal growth in the presence of oxygen as compared to its absence. To assess these aims, pneumococcal growth characteristics, biofilm formation, and pneumococcal gene expression using microarray, in the precence of oxygen was determined. These were further confirmed with immunoblot for a number of key virulence proteins, adhesion assays and several other experiments including haemolytic assays, peroxide assays and pH assays during pneumococcal growth in the presence/absence of oxygen.

Data from these experiments aim to improve our understanding of how *S. pneumoniae* regulates its virulence determinants in response to oxygen.

Chapter 2 – Materials and Methods

2 Materials and Methods

2.1 Pneumococcal growth

2.1.1 Preparation of media and glycerol stock

Pneumococcal strains used in this study are listed in Table 2-1, and all have been genome sequenced as a collaborative project between our group and the Sanger institute. After preparing ten liters of BHI (Brain-Heart infusion, Oxoid, United Kingdom), each *S. pneumoniae* was streaked on a prepared BAB (Blood Agar Base, Oxoid, United Kingdom) supplemented with 5% horse blood (E&O Laboratories, Scotland, United Kingdom). Plates were then anaerobically incubated overnight in a candle jar at 37ºC to confirm purity using an optochin disc (Mast Diagnostics, United Kingdom); this was prior to a single colony being inoculated in the liquid culture BHI at 37ºC until the bacterial growth reached almost an optical density ~ 0.6 of OD_{600nm}. Each pneumococcal culture has been aliquoted into sterile vials.

Approximately six hundred aliquots were prepared aseptically for each strain so that the same microbial stock can be used in this study and possibly beyond. All were stored, after mixing (bacterial culture and glycerol) properly with a pre-warmed 15% sterile glycerol (Riedel-de Häen, Germany), at –80ºC. For the pathogen *P. aeruginosa*, which was used as a positive control in biofilm assays, the medium Luria-Bertani (LB), (Sigma, United Kingdom) broth was used.

Table 2-1 Pneumococcal strains used in this study

*** *S. pneumoniae* isolated from different sites (Cerepro-Spinal Fluid (CSF) and Blood) but same patient profile. TIGR refers to The Institute of Genomic Research now part of the JCVI – J. Craig Venter Institute, Glasgow refers to the Polyomics Facility, University of Glasgow, and Sanger refers to the The Sanger Institute, Wellcome Trust Genome Campus, Hinxton, Cambridge. UP = unpublished data and NS = not sequenced.**

Accession Number	Strain name/ ID	Genome Sequen-cing facility	Reference	Sero-type
NC_003028	TIGR4	TIGR4 (JCVI)	(Tettelin *et al.*, 2001)	4
UP	TIGR4-403	Glasgow	Unpublished data – due for publication A.M.Mitchell et al	4
NC_021004	03-3038	Glasgow /Sanger	(Donati *et al.*, 2010); Mitchell *et al.*, in preparation	1
NZ_CACF01000000	NCTC7465	Glasgow /Sanger	(Donati *et al.*, 2010); Mitchell *et al.*, in preparation	1
NZ_CACE01000000	P1041	Glasgow /Sanger	(Donati *et al.*, 2010); Mitchell *et al.*, in preparation	1
NC_017591	INV104B	Glasgow /Sanger	(Donati *et al.*, 2010); Mitchell *et al.*, in preparation	1
NC_021005	99-4039 *	Glasgow /Sanger	(Donati *et al.*, 2010); Mitchell *et al.*, in preparation	3
NC_021026	99-4038 *	Glasgow /Sanger	(Donati *et al.*, 2010); Mitchell *et al.*, in preparation	3
NC_017592	OXC141	Glasgow /Sanger	(Donati *et al.*, 2010); Mitchell *et al.*, in preparation	3
NC_018594	A45	Glasgow /Sanger	(Donati *et al.*, 2010); Mitchell *et al.*, in preparation	3
ND	ATCC6308	NS	___	8

For counting pneumococcal population, serial dilution from 10^{-1} to 10^{-7} in a round-bottomed 96-well plate Costar, United (Kingdom) was carried out and viable counts were determined colony forming unit (CFU) by plating out three spots of each dilution on BAB plates. All these spots were allowed to dry at room temperature (20-22°C) for 10-15 min.

BA plates were then incubated overnight anaerobically in a candle jar at 37ºC. In order to calculate the CFU/ml, colonies grown between 10-70 per spot were counted for each pneumococcal strain according to the following equation:

Equation 1-2 for bacterial CFU/ml calculation after serial dilution:

1^{st} spot + 2^{nd} spot + 3^{rd} spot ÷ 3 = average

Average × 50 × dilution factor = average CFU / ml

There were some mutants used in this study for different purposes. These strains and their descriptions are shown in Table 2-2.

Table 2-2 Charecteristics of pneumococcal mutants and *P. aeruginosa* used in this study

NO.	Strain	Description
1	D39Δ*ply*	A positive control used in haemolytic assays, a stop mutant generated by Dr Calum Johnston, a PhD student in our group, 2006.
2	TIGR4Δ*cps*	Used in adhesion assays for comparison with the parent isolate.
3	*P. aeruginosa*	Positive control in biofilm formation assays, kindly provided by Dr Dan Walker's group.

2.1.2 Growth conditions

From the above prepared stock, every vial was thawed rapidly before vortexing; and the start inoculation was ~10^5 CFU/ml, while the incubation temperature was 37ºC. In some experiments, pneumococci were grown in three different levels of oxygen; oxygenated (aerated), anoxygenated (anaerobic) and aerobic (static semi-aerobic) environments. Oxygenated and anoxygenated growth conditions were mainly generated for most experiments.

Experiments were performed in triplicates; the pneumococcal broth tubes had their lids released to allow oxygen exchange and pre-warmed for 24-48 hr before starting an experiment. This pre-warming can also confirm that there was no contamination in the media, which possibly may happen from different sources during preparation.

In the aerobic conditions, 20ml of BHI in 50ml Falcon tubes were incubated routinely in a water bath at 37ºC. After pneumococcal inoculation, optical density (OD_{600nm}) and VC represented as CFU/ml were measured after being vortexed and serially diluted, respectively, at several time intervals. When taking these readings at different time-points, the bacterial culture was mixed properly prior to transferring 1ml into a cuvette (Bio, Rad, United Kingdom) for measuring ODs.

For the anaerobic procedure, bacteria were inoculated similarly into liquid cultures of BHI that had been placed in an anaerobic station to be pre-warmed, removing any unwanted oxygen for 48 hr before an experiment with released lids (miniMACS, Anaerobic Workstation).

Oxygenated conditions were performed by mixing 50ml Falcon tubes containing 20ml BHI medium under shaking at 180 rounds per minute (rpm) for 24 hr.

A related study has performed the three different conditions similarly:

> "streptococci were cultured under low-O_2 conditions in 10-ml broth cultures tightly sealed in 15-ml conical tubes or on agar plates incubated in an anaerobicgas chamber (GasPak; catalogue no. 70304; BBL). High-O_2 conditions were produced when strains were grown in 30-ml broth cultures with vigorous agitation".
>
> (Gibson *et al.,* 2000)

Other studies used bubbling to generate oxygen in the broth media (Bortoni *et al.,* 2009). Oxygen concentration when providing pneumococcal cultures with a stream of filtered bubbles was measured and found to be 20% until mid-log

phase, wheras 0% at the same growth phase was detected in anaerobic growth conditions (Bortoni *et al.*, 2009).

Generation times were calculated according to Cappuccino *et al.*, (2002).

Equation 2-2 for calculation of the bacterial generation time:

GT= t(OD 0.4) – t(OD 0.2).

GT: Generation Time.

t: time in minutes between two points in the log phase.

2.2 Hydrogen peroxide assay

S. pneumoniae TIGR4 was grown under the oxygenated and anaerobic conditions. Their growth rates were taken into account to reach OD_{600nm} ~ 0.6 at the same time. Once the OD in each growth condition was similar, 180µl of culture was placed in triplicate onto a round bottom 96-well plate (Costar, United Kingdom). To this 180µl, 20µl of 3mg/ml ABTS [2,2 azinobis (3-ethylbenzthiazolinesulfonic acid)], and 0.2mg of horseradish peroxide/ml in 0.1 M of sodium phosphate buffer were added and adjusted to pH 7.0.

The reaction then was allowed to develop for 1 hr at room temperature, followed by spinning the 96-well plate for 30 min at 2,000 rpm. One hundred micro-litres then from each well was transformed to a flat 96-well plate (Costar, United Kingdom). To analyse the samples, FLUOstar Optima plate reader (BMG Labtech, United Kingdom) was used at 544nm as described in the literature (Pericone *et al.*, 2003).

Additionally, for comparison with known concentrations, standards of hydrogen peroxide (H_2O_2) were assayed by serially diluting 30% of H_2O_2 in the BHI used for growing the pneumococcus. The broth culture BHI without adding TIGR4 was considered as a negative control.

2.3 pH drop assay

Six universal tubes with 20ml BHI in were pre-warmed overnight. The strain TIGR4 was inoculated in the prepared media and grown under the three conditions: aerobic, anaerobic and oxygenated. From these growth environments, 1ml of bacterial culture was taken for pH measurements hourly in duplicate until pneumococci reached the stationary phase.

All of the statistical analysis for pneumococcal growth curves, hydrogen peroxide assays and pH assays were performed using Graph Pad prism (GraphPad Prism 5, USA 2007).

2.4 Gram staining and microscopic examination

Likewise, the clinical isolate TIGR4 was grown aerobically and anaerobically until approximately the mid-logarithmic phase before smearing 10µl of bacterial culture, grown in each condition, on a clean microscope slide. These films of cultures were exposed to a Bunsen flame for heat-fixing. Each slide was merged for 1 min with crystal violet, rinsed with sterile distilled water after which the slide was flooded for 30 seconds with iodine solution. Crystal violet with iodine is known to form a combination in the bacterial cell wall giving the purple colour. Slides were washed with water followed by gently dropping a few drops of alcohol on before flushing with water again. Safranin was used then to merge the fixed bacterial films for 1 min before washing with water for the final time.

Then, slides were examined under a Zeiss M1 Axioskopp microscope at x40 magnification using Volocity software (PerkinElmer, United Kingdom) for image capturing.

2.5 Screening TIGR4 whole genome

2.5.1 DNA extraction

A single colony of TIGR4 was grown in a normal water bath at 37ºC overnight in 20ml BHI and in order to check its purity, a blood agar (BA) plate was streaked

under aseptic bacteriological conditions; optochin resistance has been confirmed using an optochin disc. The remaining micro-organisms were then centrifuged at 4,000 rpm for 15 mins before discarding the supernatant. The obtained pellet was re-suspended in 1ml of a lysis buffer in an Eppendorf tube (Eppendorf centrifuge 5417C, USA) containing 10µl Tris, 200ml EDTA (from Ambion/Applied Biosystems, United Kingdom), and 50µl SDS (Sodium Dodecyl Sulphate) (Ambion/Applied Biosystems, United Kingdom) prior to incubating at 37ºC for 1hr in a digital dry bath (Accublock™ Digital Dry Bath, Labnet International, United Kingdom).

Five micro-litres of proteinase K, from 20mg/ml stock, (Invitrogen, Scotland) were added to the tube followed by incubation at 50ºC for 3hr. After this, 2µl of RNase A from 10mg/ml stock (Invitrogen, Scotland) was added to the sample. This was incubated at 37ºC for 30 min, and then split into two 1.5 ml Eppendorf tubes (Eppendorf centrifuge 5417C, USA) in order to add an equal volume of phenol: chloroform: isoamyl alcohol (Sigma-Aldich, United Kingdom). Both tubes were inverted several times to mix the samples after which they were centrifuged at 13,000 rpm for 3 min. One-half of a millilitre of the solution upper phase from both tubes was transferred to two fresh 1.5 ml tubes (Eppendorf centrifuge 5417C, USA) before 100µl of 10M ammonium acetate (Sigma-Aldich, United Kingdom) and ~600µl absolute ethanol (Fisher Scientific, United Kingdom, analytical reagent grade) was added to each. The samples were then gently inverted and spun at 13,000 rpm for 30 min in order to pellet the DNA. The supernatant was then carefully removed. Both tubes were opened to air-dry for 15 min, then pellets were re-suspended in 200µl of a TE buffer and incubated at 65ºC for 10 min before storing at -20ºC until required.

The DNA samples were quantified on a Nanodrop ND-1000 spectrophotometer (Agilent Technologies, United Kingdom). To check for quality of the extracted chromosomal DNA, the pneumolysin gene (*ply*) was amplified with primers 52Q (forward) and 52R (reverse) using GoTaq DNA polymerase (Promega, USA), giving a PCR product of 350 bp as expected.

2.5.2 RNA extraction

From the prepared glycerol stock that has been counted and tested for its purity, 30 ml of BHI medium was inoculated with ~10^5 CFU/ml of the strain TIGR4. Pneumococci were growon under the three different levels of oxygen (aerobic, anaerobic and oxygenated) in triplicates until their optical density reached approximately 0.35 OD_{600nm}. Liquid cultures from bacteria grown in each condition were exposed to optochin discs during an overnight incubation at 37ºC on 5% blood agar plates for purity confirmation.

These broth cultures were centrifuged at room temperature for 5 min at 5,000 rpm. Pellets were directly frozen in liquid nitrogen after discarding supernatants from the growth tubes. All samples were stored at -80ºC for RNA extraction.

Before the RNA extraction, a fresh lysozyme TE buffer was prepared, and to make 1 ml of this lysozyme, 10µl 1M Tris HCl at pH 8.0 was made (Ambion/Applied Biosystems, United Kingdom). To this, 2µl of 0.5M EDTA pH 8.0 (Ambion/Applied Biosystems, United Kingdom), 15mg lysozyme (from Sigma-Aldrich, United Kingdom) was added up to 1 ml of nuclease free water (Ambion/Applied Biosystems, United Kingdom).

For extraction, 200µl (15mg/ml) of the lysozyme Tris-EDTA buffer has been added to the pellets followed by vortexing for 10 seconds using a rotmixer (Hook and Tucker Instruments, United Kingdom), before incubating for 15 min at room temperature with vortexing every 2 mins for 10 seconds. For cells lysis, 700µl of RLT buffer was added after which the tubes were vortexed for 10 seconds using a Qiagen RNeasy Mini Kit (Qiagen RNeasy MINI KIT, Qiagen, United Kingdom). These lysates were transferred into a 1.5 ml microcentrifuge tube (Greiner Bio One, Germany) which had been prepared in advance to contain 25-50mg of 100µl glass beads (Sigma-Aldrich, United Kingdom). To facilitate cells disruption, a Hybaid Ribolyser (Hybaid, United Kingdom) was run for 20 seconds three times at a speed of four prior to centrifuging in Eppendorf tubes (Eppendorf centrifuge 5417C, USA) for 10 seconds at 13,000 rpm. After that, 900µl of supernatants was collected and transferred to a new 1.5 ml microcentrifuge tube (Greiner Bio

One, Germany). Five hundred micro-litres of 100% Ethanol (Fisher Scientific, United Kingdom) was added before mixing the supernatants.

From this mixture, 700µl was added in a RNeasy Mini column (Qiagen RNeasy MINI KIT, Qiagen, United Kingdom) followed by centrifuging at room temperature for 30 seconds at 13000 rpm (Eppendorf centrifuge 5417C, USA). The flow-through was discarded and the remaining 700µl was added onto the same RNeasy Mini column before centrifuging at room temperature for 30 seconds at 13,000 rpm. Then, 350µl of RW1 buffer was added to the RNeasy Mini column (Qiagen RNeasy MINI KIT, Qiagen, United Kingdom) prior to similarly centrifuging at the same temperature and speed but for 5 min.

Ten micro-litres of DNase I stock solution was transferred onto 70µl of RDD buffer (Qiagen RNase–free DNase Set, Qiagen, United Kingdom) after which the tube was mixed and gently flicked for collecting liquid from the tube sides. This 80µl was then pipetted into the column directly (RNeasy Mini column silica-gel membrane) before incubating at room temperature for 15 min on the benchtop.

An amount of 350µl RW1 buffer was pipetted to the RNeasy column prior to centrifuging at 13,000 rpm for 30 seconds followed by discarding the flow-through. Again, RW1 was added, but 700µl instead of 350µl, to the RNeasy Mini column and centrifuged for another 30 seconds at 13,000 rpm before discarding the flow-through. Five hundred micro-litres of RPE buffer was added onto the RNeasy Mini column and centrifuged for 30 seconds at 13,000 rpm. Further 500µl of RPE buffer was added after discarding the flow-through to the RNeasy Mini column that was followed by centrifuging for 2 min at 13,000 rpm for drying the membrane of the silica-gel.

In order to elute, 50µl of nuclease free water (Ambion/Applied Biosystems, United Kingdom) was transferred to the silica-gel membrane after the RNeasy Mini column had been pipetted onto a new 1.5 ml collection tube. All tubes were placed for 3 min to stand before centrifuging at 13,000 rpm for 1 min.

Furthermore, possible existence of DNA was treated, for a second time, to be removed by eluting each sample to a nuclease free water (Ambion/Applied Biosystems, United Kingdom) before addition of 1µl Ambion TURBO DNA-free™ (Ambion/Applied Biosystems, United Kingdom). The tubes were then incubated in a block heater for 30 min at 37ºC prior to transferring supernatants containing RNA after centrifuging for 2 min at 10,000 rpm.

To assess the RNA purity, 5µl was kept separately as an aliquot from each sample to be sent for an Agilent 2100 bioanalyser (Agilent Technologies, United Kingdom). RNA integrity was considered above 9 to have a sufficiently high quality. Additionally, Nanodrop ND-100 spectrophotometer (Agilent Technologies, United Kingdom) was used for the RNA quantification. Until required, all remaining samples of this stock were stored at -80ºC.

2.5.3 Polymerase Chain Reaction (PCR)

To confirm the absence of cDNA in the above RNA extractions from the three different conditions (aerobic, anaerobic and oxygenated), the existence of *ply*, as a conserved gene in *S. pneumoniae*, was tested with the primers (Table 2-3) 52Q (forward) and 52R (reverse) using a Techegene thermal cycler (Bibby Scientific, United Kingdom).

Table 2-3 Primers used for PCR amplification of *ply* gene

Primer	Name	DNA sequence 5'-3'	Use
52Q	*ply* forward	ATTTCTGTAACAGCTACCAACGA	Amplification of *ply* gene
52R	*ply* reverse	GAATTCCCTGTCTTTTCAAAGTC	Amplification of *ply* gene

This has been performed according to the following reaction for each of the triplicate extracted RNA samples:

PCR reaction conditions:

- 94ºC for 5 min — 1 cycle
  - 94ºC for 30 s
  - 85ºC for 30 s — 30 cycles
  - 72ºC for 45 s
- 72ºC for 5 min — 1 cycle
- Held at 4ºC

The volume for each sample was 25µl which contained 2µl Go Taq® DNA polymerase (Promega, USA), 5µl Go Taq® buffer (Promega, USA), 3µl dNTPs (Invitrogen, United Kingdom), 0.2µl forward primer, 0.2 µl reverse primer, 0.5µl of *S.pneumoniae* TIGR4 genomic DNA, 0.5µl of an extracted RNA sample and 15.9µl PCR water.

2.5.4 Gel electrophoresis

For demonstrating bands of DNA to assess their quality before using them in microarray experiments, the PCR products were mixed with DNA loading buffer [0.25% bromophenol blue, 40% (w/v) sucrose in dH_2O], and then run in 50 ml of 0.8% agarose gel (Agarose MP, Roche Diagnostics, Germany) in TE with 2µl of SYBERSAFE stain (Invitrogen, United Kingdom), and viewed under ultraviolet light in a UVIpro Gold Gel-doc system (UVItec, United Kingdom) to confirm that the PCR reaction was successful.

2.5.5 Preparation of cDNA

After confirming the high RNA quality, which consists of integrity above 9 on Agilent 2011 Bioanalyser (Agilent Technologies, United Kingdom), 2 µg of the high quality extracted RNA was mixed with 1µl Random primers [3µg/µl (Invitrogen™, United Kingdom)] and up to the total volume of 11µl, nuclease free water was added (Ambion/Applied Biosystems, United Kingdom). To allow primers to bind to the samples, all tubes were heated for 10 min at 70ºC in a Techegene thermal cycler (Bibby Scientific, United Kingdom) before placing them on ice.

Five micro-litres of 5× first strand buffer (Invitrogen™, United Kingdom) was added to each sample, 2.5µl of 100mM (DTT), dithiothritol (Invitrogen™, United Kingdom), 2.3µl of dNTPs containing 5mM dATP, 5mM dGTP, 5mM dTTP and 2mM dCTP) (Invitrogen™, United Kingdom). After this, 2.5µl of SuperScript® II (Invitrogen™, United Kingdom) was added onto each of the extracted RNA samples, whereas 2.5µl of nuclease free water (Ambion/Applied Biosystems, United Kingdom) was added to the sample-associated control instead of the SuperScript® II. All samples then were incubated at 25ºC for 10 min prior to setting the reaction for 90 min at 42ºC.

2.5.6 Microarray protocol

The microarray slides used in this study were obtained from the Bacterial Microarray Group at St. George's Hospital, University of London (BmG@S). The slides have the genes for the entire TIGR4 genome (Tettelin *et al.,* 2001). Additionally, there are 117 genes from the strain R6 genome (Hoskins *et al.*, 2001). All of these have been attached to an aminosilane coated glass slide (Hinds *et al.*, 2002a). The procedures of this bacterial microarray were described previously (Hinds *et al.*, 2002b). Other similar published work used the same technique to study gene expression in *S. aureus* (Witney *et al.*, 2005) and *Campylobacter jejuni* (Dorrell *et al.*, 2005).

The above prepared RNA extracts were used in triplicates in the microarray experiments (as explained in a digram shoing the main steps in the introduction chapter, section 1.2.2) for scanning the whole genome expression of TIGR4 cells grown under different levels of oxygen (aerobic, anaerobic and oxygenated). According to the manufacturer's protocol, 8.75ml of pre-hybridization saline-sodium citrate (20x SSC) (Ambion/Applied Biosciences, United Kingdom) was mixed with 250µl of 20% SDS (Ambion/Applied Biosciences, United Kingdom) and 5ml of 100mg/ml (BSA) Bovine Serum Albumin (Sigma-Aldrich, United Kingdom). The total volume of this mixture was made up to 50ml with double sterile distilled water [sterilisation and filtration using a 0.2µm filter (Millipore, United Kingdom)]. This solution was pre-warmed for 30 min at 65ºC in a Coplin jar (Fisher Scientific, United Kingdom) after being placed in a Techne Hybridizer HB-1D (Bibby Scientific, United Kingdom).

In the array procedure, 9.12µl of nuclease free water (Ambion/Applied Biosciences, United Kingdom) was added to a fresh tube (18 tubes, 9 RNA samples and 9 controls) followed by the addition of a 2µg sample before making the total volume up to 10µl with nuclease free water (Ambion/Applied Biosciences, United Kingdom). One micro-litre of a random primer (Invitrogen, United Kingdom) was pipetted into both groups of tubes sample and controls. All tubes were heated up at 70ºC for 10 min in a Techegene thermal cycler (Bibby Scientific, United Kingdom) before snapping all tubes on ice and exposing them to a short centrifuge. After this, an addition was performed to each sample and control with 5µl 5× first strand buffer (Invitrogen™, United Kingdom), 2.5µl of 100mM DTT (Invitrogen™, United Kingdom), 2.3µl dNTPs, containing 5mM of the following: dATP, dGTP, dTTP and 2mM dCTP (Invitrogen™, United Kingdom), 2.5µl of superscript® II (Invitrogen™, United Kingdom), and dyes [Cy3 and Cy5 (GE Healthcare, United Kingdom)]; 1.7µl of Cy3 was added to each control whereas 1.7µl of Cy5 was used for each sample. The tubes were incubated in a Techgene thermal cycler (Bibby Scientific, United Kingdom) in the dark at 25ºC for 10 min followed by 42ºC for 90 min. To purify Cy3 and Cy5, labelled RNA 3.5µl 2% filtered SDS was added in a new RNA tube and 4.6µl 20x SCC.

The hybridized arrays were washed with 400ml of double distilled water in a Coplin jar (Fisher Scientific, United Kingdom) for 1 min prior to washing again for another 1 min in 400ml of propan-2-ol (VWR International, USA). Each array was put in a falcon centrifuge tube for centrifuging at 1,500 rpm for 5 min before placing in a dust-free box to be ready for hybridization.

cDNA samples were labelled with Cy3 and Cy5 after being combined in a Qiagen Minelute purification column (Qiagen, United Kingdom). Five hundred micro-litres of the PB buffer was transferred to this combination before spinning at room temperature for 1 min at 13,000 rpm (Eppendorf centrifuge 5417C, USA), discarding the flow-through and adding 500µl followed by a similar centrifuging speed and time. The flow-through was again discarded, adding to the same column 250µl PE buffer, centrifuged for 1 min at 13,000 rpm, discarded the flow-through, spun at 13,000 rpm for 1 min without adding the PE buffer to dry before placing the column in a fresh 1.5ml collection tube. This was centrifuged at 13,000 rpm for 1 min and left for 3 min to stand after transferring 15.9µl nuclease free water (Ambion/Applied Biosciences, United Kingdom) onto the membrane centre to elute for hybridization.

The slides then were covered by lifter slips (Erie Scientific Company, USA), and each sample was injected carefully between a cover slip and the array surface, and the arrays were transferred onto hybridization cassettes, sealed and incubated in water at 65ºC for 20 hr in a Techne- Hybridiser HB-1D (Techne, USA) in the dark.

After this hybridization, the arrays were submerged with agitation for 4 min in 20ml of 20× SSC solution and similarly in 1ml of 20% SDS that was made up to 400ml of double sterile distilled water: all of these had been pre-warmed at 65ºC in a Coplin jar (Fisher Scientific, United Kingdom). Microarrays were centrifuged in 50ml centrifuge tubes for 5 min at 1,500 rpm.

2.5.7 Analysis of gene expression

For scanning the slides, one by one they were placed in ScanArray Express™ (Packard Biosciences Biochip Technologies, Perkin Elmer) before capturing TIFF images. These were loaded into Bluefuse (BlueGnome Ltd, Cambridge, United Kingdom) for Microarrays 3.5 © BlueGnome Ltd using Channel 1 for Cy3 labelled image and Channel 2 for Cy5. Gridmap files were purchased from the Bacterial Microarray Group at St. George's Hospital, University of London (BmG@S). Processing analysis of spots was initially involved exclusion of unreliable data because of poor hybridisation. Findings estimated with a confidence of less than 0.1 so have been discarded because of poor hybridization.

Normalization was achieved after importing the Output_fused.xls files into Genespring GX 7.3.1 (Agilent Technologies, USA). Data generated by Genespring GX 7.3.1 (Agilent Technologies, USA) was statistically analyzed using 1-way ANOVA. False discovery rate given %5 false genes as it was set to the p-value 0.05 followed by applying multiple testing correction method using a Benjamini and Hochberg False Discovery Rate.

For comparison, the whole TIGR4 was then transferred to Microsoft Office Excel 2003, (Microsoft, United Kingdom). This gave master list containing the expression of *S. pneumoniae* TIGR4 genes under all of the different levels of oxygen used.

Fold changes for each of the 2,336 pneumococcal genes were then calculated individually; 1) In oxygenated versus anaerobic, 2) Aerobic versus anaerobic and 3) Aerobic versus oxygenated. Furthermore, finding known biological functions for each single gene that was significantly expressed was performed using the CLC (Genomics Workbench programme). Additionally, Comprehensive Microbial Resource (CMR) http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi and Nucleotide BLAST at the National Centre for Biotechnology Information (NCBI) http://www.blast.ncbi.nlm.nih.gov/Blast.cgi were accessed online for gene comparison, functions and even for tracking putative pneumococcal metabolism pathways.

2.6 Quantitative Reverse Transcriptase PCR (qRT-PCR)

It is worth mentioning here that the importance of confirming gene expression for genes of interest through performing qRT-PCR for measuring mRNA levels following what microarray experiments detect is attributed to the fact that arrays may underestimate gene expression. Conway and colleagues concluded that arrays probably cannot reflect the actual ratios for gene induction (Conway *et al.*, 2003).

qRT-PCR Quantitative Real Time PCR experiments were performed in a Techgene thermal cycler (Bibby Scientific, United Kingdom) after the microarray experiments for several genes listed in Table 4-2 in order to confirm their expression.

Table 2-4 Genes selected for qRT-PCR and their primers

Gene NO.	Product	Primer
SP_1227	TCS02	RTF- GGTCGTGAAGCGCTAGAG RTR- TACGAATGGTCTTAGCAACTT
SP_0387	TCS03	RTF- GACCTCCAAGACGATGTAGA RTR- ATTGCTAAGGTCGCGTCA
SP_2082	TCS04	RTF- GTGACAAATGGACGGAAG RTR- ATTCATCACTTTTCGCAGA
SP_2798	TCS05	RTF- GATTTTGCTGGATTTGATGTTGCC RTR- CCGCTCCCAGTTCAAATCCAT
SP_2193	TCS06	RTF- TGAGTGCTCTGGGAGATGAAACT RTR- CGCCAAAGATCCTCTATGACG
SP_0083	TCS08	RTF- GGCAGGTTATCAGGTCTTGG AGGCTGCTCTGGTGATAAGTA
SP_2000	TCS11	RTF- GCTTCAACCGGATGTAGAG RTR- TTCTGCTCGTATCCACTCC
SP_2235	TCS12	RTF- CCAATATCATACAAGACAACG RTR- GAGCCACTTCAAATCCCT
SP_0526	TCS13	RTF- AAAGCACATCATATCATTCC RTR- AGTCCCTTCATCTCTTCATT
SP_0376	TCS orf	RTF- CCAGAAAGAGCAGTATCGG RTR- TCGGCTCAATTTTTCTGC
SP_1219	GyrA	RTF- GCGCGAGCTCTTCCTGATGT RTR- TATGGGGTTTGTCTGGGGTC
SP_1923	PLY	RTF- GATGGCAAATAAAGCAGTAAATGACT RTR- TGATGCCACTTAGCCAACAAATCG
SP_2190	PspC	RTF- GAAAACGAAGGAAGTACCCAAGCA RTR- CTTTAACTTTCACATCGAACTCAGC
SP_0461	RlrA	RTF- CCATCGCAACAGGCTACC RTR- TGTGACCCAATCCATACTTCC
SP_0462	RrgA	RTF- AACCAGTCCAGCGATAGG RTR- CTTCTGTCAAGGTGTATGTCC
SP_0463	RrgB	RTF- ATACACCTGTGAACCACCAAG RTR- CATTCTATCGCTCCAGTTTGC
SP_0464	RrgC	RTF- GTATCTTCTTTGTTATGGCTCTG RTR- ATCATCATAGGAATACGAATCATC
SP_0466	SrtB	RTF- GGTGTCTCGCTTGTATTATCG RTR- TGTCAGCCTCATCCAACG
SP_0467	SrtC	RTF- GTGTCTCGTTATTATTATCGTATTG RTR- CCTCAAGTTCTGCCTTATCC
SP_0468	SrtD	RTF- TCTCGCCTACAATCAACGC RTR- ATAATCTGCTCCCAAATAAACCG
SP_1651	Tpx	RTF- ATGGAAGAAAAAAGATTGGAGT RTR- CGTGTTTGAGTTGAGCAGAT

TIGR4 genomic DNA (gDNA) was used as a positive control, whereas the negative control was nuclease free water (Ambion/Applied Biosciences, United Kingdom). The pneumococcal gene encoding the enzyme DNA gyrase, was used as the reference gene for normalisation. RNA samples which had NRT (No Reverse Transcriptase, SuperScript® II from Invitrogen™, United Kingdom) when preparing cDNA were used as negative controls. Apart from the gDNA controls, all qRT-PCR reactions for genes of interest have been performed in triplicate along with their associated triplicate NRTs controls for the three different levels of oxygen conditions. qRT-PCR plates used for running samples (ABgene, Fisher Scientific, United Kingdom) or 200µl white 8-well strips (ABgene, Fisher Scientific, United Kingdom). The chosen genes, their products and primers used are listed above in Table 2-4.

The total volume for each of the 21 qRT-PCR reactions was 25µl using Mj white plates (Kisker Biotech GmbH, Germany). In each experiment, 12.5µl of 2× Roche SYBR green mastermix (Roche, United Kingdom), 1µl forward primer and 1µl reverse primer, 10µl nuclease free water (Ambion/Applied Biosystems, United Kingdom) and 0.625µl sample were then added to the mastermix.

To choose the best cDNA concentration for the qRT-PCR reactions, *ply* was tested after preparing three serial dilutions; neat, 1:10 and 1:50. The NRTs were similarly diluted before measuring their (samples and controls) concentrations with Qubit fluorometer (Life Technologies, United Kingdom). Working solutions were prepared and standardised according to the Qubit manufacturer´s instructions. Once the best concentration was detected after qRT-PCR, the dilution factor was used to prepare all of other samples, which had been recovered from different pneumococcal growth conditions.

The reaction condition used for these qRT-PCR experiments was as follows:

50ºC 2 mins (1 cycle)
95ºC 10 mins (1 cycle)
95ºC 15 seconds
55ºC 30 seconds (40 cycles)

72°C seconds

72°C 5 mins (1 cycle)

Stored at 4°C

Primer temperatures were changed according to melting temperature.

2.6.1 Testing *ply* qRT-PCR primers

qRT-PCR gene-specific primers for *ply* were designed according to (Ogunniyi *et al.*, 2002) and tested using TIGR4 gDNA with PCR (Figure 2-1) before performing qRT-PCR for all the cDNA prepared samples. The mastermix and PCR reaction was performed as outlined in Section 2.5.3.

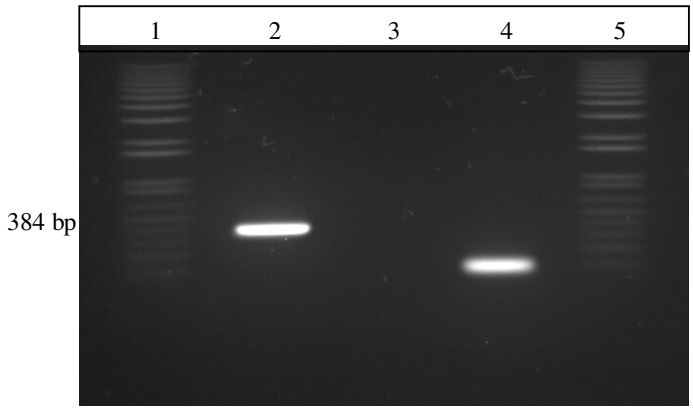


Figure 2-1 Testing *ply* qRT-PCR primers before using in all other cDNA samples

Lanes 1: ladder used, its full size is 1 kb (Life Technologies, United Kingdom), 2: *ply*, 3: negative control (PCR water used), 4: *cps* as positive control and 5: the ladder 1 kb. Annealing temperature was 54°C according to the provider datasheet (Eurofins MWG, United Kingdom). PCR products were mixed with DNA loading buffer 0.25% bromophenol blue, 40% (w/v) sucrose in dH_2O, and then run in 50ml 0.8% agarose gel (Agarose MP, Roche Diagnostics, Germany) in TE with 2µl of SYBERSAFE stain (Invitrogen, United Kingdom) and viewed under ultraviolet light in a UVIpro Gold Gel-doc system (UVItec, United Kingdom).

2.6.2 qRT-PCR analysis

To relatively assess the mRNA levels for each gene of the 21 qRT-PCR experiments, data collected after performing these reactions were analysed using the software Opticon Monitor 3.1.32 (MJ Genework, Bio-Rad Laboratories), and Microsoft Office Excel 2003 used for percentage calculations. With using Excel 2003, a table was made listing all gene expression showing calculated numbers representing up-regulation (above zero) and down-regulation below zero for the conditions used, oxygenated and anaerobic. Statistical analysis was performed in Graph pad (GraphPad Prism 5, USA 2007). Bars showed means of triplicate experiments for each sample plus/minus error bars of standard deviation. A 1-way ANOVA comparing the data cycle threshold (dCT) values (Ct of test gene minus Ct of control genes, *gyrA*).

2.7 Biological activities and virulence factors

2.7.1 Biofilm formation

According to (del Prado *et al.,* 2010) with modification, each pneumococcus listed in Table 2-1 has been grown in triplicate in two different environmental conditions; oxygenated and anaerobic, until the end of its death phase. For the oxygenated biofilm assays, broth culture (BHI medium) in a flat 96-well plate (Costar, United Kingdom) was incubated and shaken at 180 rpm for 24 hr at 37ºC before inoculating 20µl of 2×10^3 CFU/ml pneumococci (10^5 CFU/ml) onto 180µl pre-warmed BHI. The plates were incubated under the same conditions until the decline phase prior to adding 200µl of 1% crystal violet to each well; they were incubated then at 20-22ºC for 10 min. The crystal violet was discarded, washed four times with sterile distilled water prior to all plates dried at 37ºC for 1 hr followed by addition 200µl absolute ethanol to each well.

For the anaerobic biofilms, similar plates were incubated for 24 hr in a static anaerobic station (miniMACS, Anaerobic Workstation) with 180µl of the same batch of the media BHI and inoculated using the same standard bacterial inocula as described above.

To analyse these biofilms, a FLUOstar Optima plate reader (BMG Labtech, United Kingdom) was used at 600_{nm} as described in the literature (Pericone *et al.,* 2003). BHI medium without pneumococcal inoculation was used as a negative control in triplicate, and *P. aeruginosa* was used as a positive control.

2.7.2 Preparation of TIGR4 cell lysates

In order to study the effect of different oxygen levels on several tested proteins, pneumococci were grown until OD_{600nm}: 0.35 under the anaerobic or oxygenated growth conditions. The start inocula were approximately 10^5 CFU/ml in 50ml falcon tubes (Millipore, United Kingdom) of overnight pre-warmed at 37ºC BHI

medium. Then, broth cultures in the tubes were centrifuged at 5,000 rpm for 20 min at 4ºC in Sigma Laboratory centrifuge 4K15 (Philip Harris, United Kingdom).

Pellets were washed and re-suspended in 1ml PBS, cooled on ice in a 1.5ml Eppendorf tube (Eppendorf centrifuge 5417C, USA) with a protease inhibitor (Roche, United Kingdom) before sonication on ice at 10% amplitude for five repeats of 30 seconds with 30 seconds rest using a Soniprep 150 sonicator (MSE, United Kingdom). Pneumococcal cell lysates were also prepared for blotting cytoplasmic and the bacterial cell surface proteins. To isolate the cell surface protein PspC, samples were washed with 2% choline chloride.

All samples were measured for their total protein contents using a Nanodrop ND-1000 spectrophotometer (Agilent Technologies, United Kingdom). Haemolytic assays were then performed prior to storing the rest of the samples at -20ºC for further use.

2.7.3 Toxin activity

The haemolytic activity of the toxin Ply was measured in culture lysates or in purified Ply according to Walker and colleagues (Walker *et al.*, 1987).

Fifty-five micro-litres of Phosphate-Buffered Saline (PBS) was added to all wells in a round-bottom 96-well plate (Costar, United Kingdom), and double diluted 50µl of the samples [centrifuged cell lysates at 13,000 rpm for 10 min in a bench-top centrifuge (Centrifuge 5417C, Eppendorf, United Kingdom)]. Purified Ply of TIGR4 (performed in our group as described by (Mitchell *et al.*, 1989) at a concentration of 0.31mg/ml) was also diluted similarly in volume to be used as a positive control, whereas the negative control was only 50µl of PBS. Additionally, D39 Δ*ply* was tested as another negative control. Then, 50µl of 10mM of freshly prepared DTT (Sigma-Aldrich, United Kingdom) was added to each well, before covering the plate and incubating for 15 min at 37ºC.

A fresh solution of 2% (vol/vol) of horse erythrocytes (E & O Laboratories, Scotland) in PBS was prepared and 50µl was added to each well. The 96-well

plate was incubated at 37ºC for 30 min. Then, 50µl PBS to each well was added before centrifuging the plates at 1000 rpm for 2 min in a 4K15 centrifuge (Sigma-Aldrich, United Kingdom). These haemolytic assays have been repeated at least 3 times for each growth condition.

2.7.4 SDS-PAGE

Pneumococcal cell lysates were diluted 3:1 in NuPAGE loading buffer (Invitrogen, Scotland) before heating up in a digital dry bath (Accublock™ Digital Dry Bath, Labnet International, Inc., United Kingdom) at 72ºC for 10 min. The samples then were loaded onto a pre-cast gel (Invitrogen, Scotland).

The marker used was SeeBlue Plus2 standard marker (Invitrogen, Scotland). A sample volume of 10µl was loaded in each well, and the gel run for 50 min at 200V using MOPS as a running buffer (Invitrogen, Scotland). SDS-PAGE (Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis, Ambion/Applied Biosystems, United Kingdom) gels were stained overnight in Coomassie stain solution (45 ml dH_2O, 45 ml methanol, 10ml acetic acid, 0.25g Coomassie Brilliant blue R250), and destained with 45ml dH_2O, 45ml methanol, and 10ml acetic acid. Descriptions for different running buffers and other solutions; stain and de-stain buffers used are shown in Table 2-5.

Table 2-5 Reagents and buffers used for SDS-PAGE

Buffer		Composition
1	1x NuPAGE MOPS	Proprietary formulation
2	1x NuPAGE MES	Proprietary formulation
3	10x Tricine running	30g Tris,144g Glycine,10g SDS
4	Coomassie R250 stain	1.25g/L coomassie R250,40% EtOH,10% glacial acetic acid
5	Coomassie destain	40% EtOH, 10% glacial acetic acid

2.7.5 Western Blot

Immunoblotting, was done after running proteins of interest in gels (Section 2.7.4) but without staining. Gels were transferred to Hybond-C nitrocellulose membrane (Amersham Biosciences, United Kingdom) to be blotted at 30V for 1 hr using an Xcell II blotting module (Invitrogen, United Kingdom) following the manufacturer´s instruction.

In order to block the membrane, it was kept at 4^{o}C for overnight in 3% skimmed milk (Marvel, United Kingdom) in Tris NaCl, pH 7.4 (10mM Tris, 150mM NaCl, 8mM HCl). After this, the membrane was incubated with a 1/500 dilution of primary anti-body rabbit (anti-pneumolysin) at 37 ^{o}C for 2 hr with shaking at 200 rpm before washing 3 times in 40ml Tris NaCl at pH7.4 for 5 min. The membrane then was incubated at 37^{o}C for 1 hr in 3% skimmed milk in the Tris-NaCl with 1/1000 dilution of secondary anti-body (Anti-Rabbit IgG raised in donkey, GE Healthcare, United Kingdom) prior to washing again 3 times in 40ml of the Tris NaCl for 5 min. The membrane was developed in either 4-chloronapthol or Immobilion Western HRP Chemiluminescent substrate purchased from Millipore (Millipore, United Kingdom). In terms of using a developing buffer, 4-chloronapthol, 30mg 4-chloro-1-naphthol, 10ml Methanol, 40ml Tris NaCl, pH 7.4 and 30µl Hydrogen peroxide were prepared freshly before submerging the membrane for a few minutes at room temperature. This developing method was then stopped with dH_2O.

For the Immobilion Western hybridisation, two reagents were used after protein-membrane blotting. From each reagent, 2ml were mixed immediately before treating the membrane on the mixture. It was developed then in the dark room after being exposed to a photographic film. Antibodies used for developing in the dark room were highly diluted 1:20000 due to the high sensitivity as compared to the dilution factor used when using 4-chloronapthol, which was 1:500.

Immunoblot experiments performed for different proteins, sources of their antibodies, dilution factors used and the development procedures are listed in Table 2-6.

Table 2-6 Antibodies used in Western Blot

Antibody		Source	Dilution factor	Development
1	Anti-Ply	Rabbit	1/500	4-chloronapthol
2	Anti-PspC	Rabbit	1/500	4-chloronapthol
3	Anti-RrgB	Rabbit	1/20000	Dark room

2.7.6 Adhesion assays

Eukaryotic human lung alveolar epithelial carcinoma cells A549 (Human lung epithelial carcinoma cell line, ATCC-CCL-185) (LGC Standards, American Type Culture Collection, United Kingdom) were cultured in an F-12K medium (LGC Standards, American Type Culture Collection, United Kingdom) supplemented with 1% fungizone (Gibco®, Life Technologies, United Kingdom), 1% L-Glutaminase (Sigma-Aldrich, United Kingdom), 10% foetal bovine serum (FBS) (Biosera, United Kingdom), 1% streptomycin and 1% penicillin (Sigma-Aldrich, United Kingdom). Similarly, F-12K was prepared but without adding the anti bacterial agents (streptomycin and penicillin) and used as a bacterial growth culture. The epithelial cells were grown in a $25cm^2$ culturing flask prior to seeding 2ml cell suspension at density of 2×10^5/ml in each of the 24 well. These were incubated for 48 hr at 37ºC in a 5% CO_2 incubator till reaching 70 to 90% confluence.

Pneumococci (TIGR4, TIGR4Δ*cps,* OXC141 and P1041) were grown under anaerobic and oxygenated environments until optical density of OD_{600nm} ~0.35 before inoculating at 1×10^7 CFU/ml onto the culture. Adherence was allowed for 1 hr followed by washing with sterile pre-warmed Dulbeccos PBS (D-PBS, Sigma-

Aldrich, United Kingdom) and treating the host cells using 250µl trypsin (Sigma-Aldrich, United Kingdom) for detaching. Both adherent and non-adherent bacteria were counted after being serially diluted and plated on BA overnight. Data analysis was performed in the programme Graph Pad (GraphPad Prism 5, USA 2007).

2.8 Genome alignments

Each of the pneumococcal genome was downloaded individually from NCBI before isolating the target sequence in a smaller segment out of the whole bacterial genome.

Insert use of Blast and BLAST website link plus CLC package details and general handling of sequence files, use of BLAST within CLC software suite and the use of alignment tools including ClustalAlignment.

Target sequences of candidate regions identified by function, relevance to observed phenotype and from gene expression data were investigated in more detail and genes compared across the available genome sequences of strains included in this project.

All these genome sequences were transulated then to their proteins using CLC programme and compared all pneumococci to each other using TIGR4 as a reference strain for each particular gene including flanks in order to detect mutations; Synonymous SNPs, Non-Synonymous SNPs, deletions and insertions.

Chapter 3 – Pneumococcal Growth

3 Pneumococcal Growth

Bacterial growth under specific conditions usually reflects their growth pattern, and this might be used to show what their behavioural responses in that particular condition are. Accordingly, comparison between several bacterial growth curves can represent their phenotypic differences, the way they behave *in vitro* to respond to the surrounding changes.

The pathogen *S. pneumoniae* is known to encounter different levels of oxygen during the infection cycle including colonising the host upper airway system where oxygen levels are around 21 %. This decreases to 10-15 % in the lower respiratory system into the alveoli, more reduction in human tissues at 5 % (Burghout *et al.,* 2010), and much lower oxygen tension in the middle ear and during pneumonia (Hathaway *et al.,* 2007); whereas the host bloodstream is assumed to be an anaerobic environment (Yesilkaya *et al.,* 2013).

Therefore, this chapter focuses on studying several points including the effects of different environmental factors such as variant levels of oxygen and host-derived fluids on pneumococcal growth. This may enable us to dissect *in vitro* bacterial growth characteristics (phenotypic differences) as a baseline in this topic, which will be used for the rest of the study. This basic step was performed after growing 11 pneumococcal strains with known genome sequences to closely investigate their growth traits under different incubation conditions. All of the strains have been genome sequenced as a collaborative project between our group and the Sanger institute.

There are a number of reasons behind the choice of these pneumococcal strains:

1) They all have been genome sequenced.

2) The most studied clinical strain that has been considerably understood, from a genetic point of view, is TIGR4.

3) Serotypes 1 and 8 are known to be hyper-virulent (Williams *et al.*, 2012), and they have been clinically isolated from patients who were reported with PID.

4) Type 3 pneumococci were selected for slightly different reasons:

I. Two strains were isolated from different sites of a patient.

II. OXC141 strain is mostly found as a host coloniser.

III. A45 is the only equine isolate (in the study) and has never been isolated from a human.

3.1 Aerobic pneumococcal growth

Growth curves of several pneumococcal strains representing the serotypes 1, 3, 4 and 8 (listed in 3-1) were obtained and the standardised start inoculation was ~10^5 CFU/ml at 37ºC.

Table 3-1 The pneumococcal strains used in this project
*** Strains isolated from one patient. MLTS: Multi Locus Sequence Typing.**

Strain Identifier		Host	Serotype	MLST
1	TIGR4	Human	4	205
2	TIGR4-403	Avirulent	4	205
3	03-3038	Human	1	306
4	NCTC7465	Human	1	615
5	P1041	Human	1	217
6	INV104B	Human	1	227
7	99-4039*	Human	3	180
8	99-4038*	Human	3	180
9	OXC141	Human	3	180
10	A45	Equine Disease	3	6934
11	ATCC6308	Human	8	N/A

All pneumococci (Table 3-1) were grown in a standard medium (one batch was prepared and used for all bacterial growth) under the same incubation conditions. Absorbance of OD at 600_{nm} and viability (CFU/ml) were measured at set time-points in triplicate. Each strain was grown at least twice and the mean of the triplicate readings is represented herein. Under the same incubation procedures, the mutants D39Δ*ply* and TIGR4Δ*cps* were grown for different purposes in the study, which will be explained later in a related experiment.

3.1.1 Serotype 4 (TIGR4 and TIGR4-403)

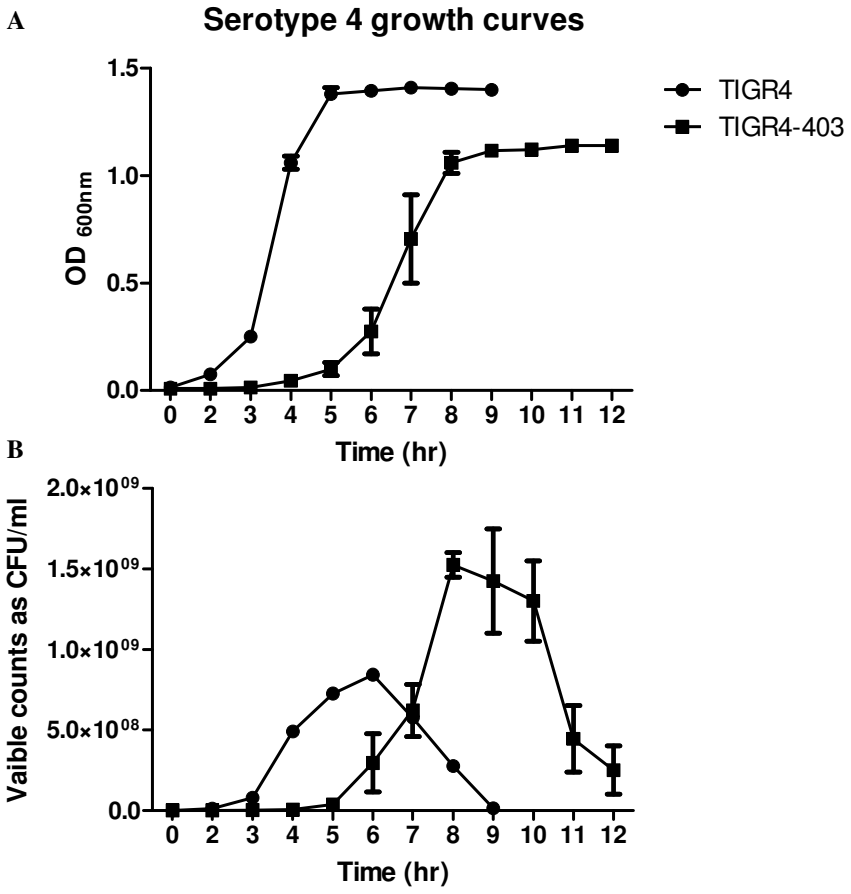


Figure 3-1 TIGR4 and TIGR4-403 growth curves aerobically

Optical density (OD) (A) and viable counts (VC) (B). The log phase started in TIGR4 after 2 hr and the whole pattern of growth lasted approximately 9 hr, whereas TIGR4-403 (the TIGR4 avirulent strain) started the log phase 3 hr until reaching a higher bacterial population unlike TIGR4. OD is the optical density and CFU is the colony forming unit for cells viability. The growth curve was repeated at least three times and each time-point is the mean of three replicates represented as error bars show the standard error of the mean, calculated using Graph Pad (GraphPad Prism 5, USA 2007)

Viable count (VC), which is represented as CFU/ml, of a growth curve typically has four phases: lag, logarithmic (log), stationary and decline phase (also called death phase). Similarly, OD curves have these phases except the decline because cells are counted even after dying, as the spectrophotometer equipment measures both live and dead bacteria. This may explain why the OD curve does not reduce after reaching the highest point during the bacterial

growth, and in terms of the CFU, it will be discussed briefly in all of the following growth curves.

The pathogenic strain TIGR4 took 2 hr before logarithmically reaching the growth peak that lasted for 4 hr. Then, the bacterial cells started to die entering the death phase for a further 3 hr (Figure 3-1, B). The avirulent strain had a higher and longer stationary phase after 4 hr in the exponential phase. Another 4 hr were taken for the cells death. Its population reached around 800 million greater than the clinical isolate TIGR4 (Figure 3-1, B).

The avirulent TIGR4-403 strain slowly grew in its exponential phase resulting in longer generation time, but reaching a higher bacterial population during its growth, about 1.5×10^{9} compared to the growth peak in TIGR4 which was 0.8×10^{9}.

3.1.2 Serotype 1 strains

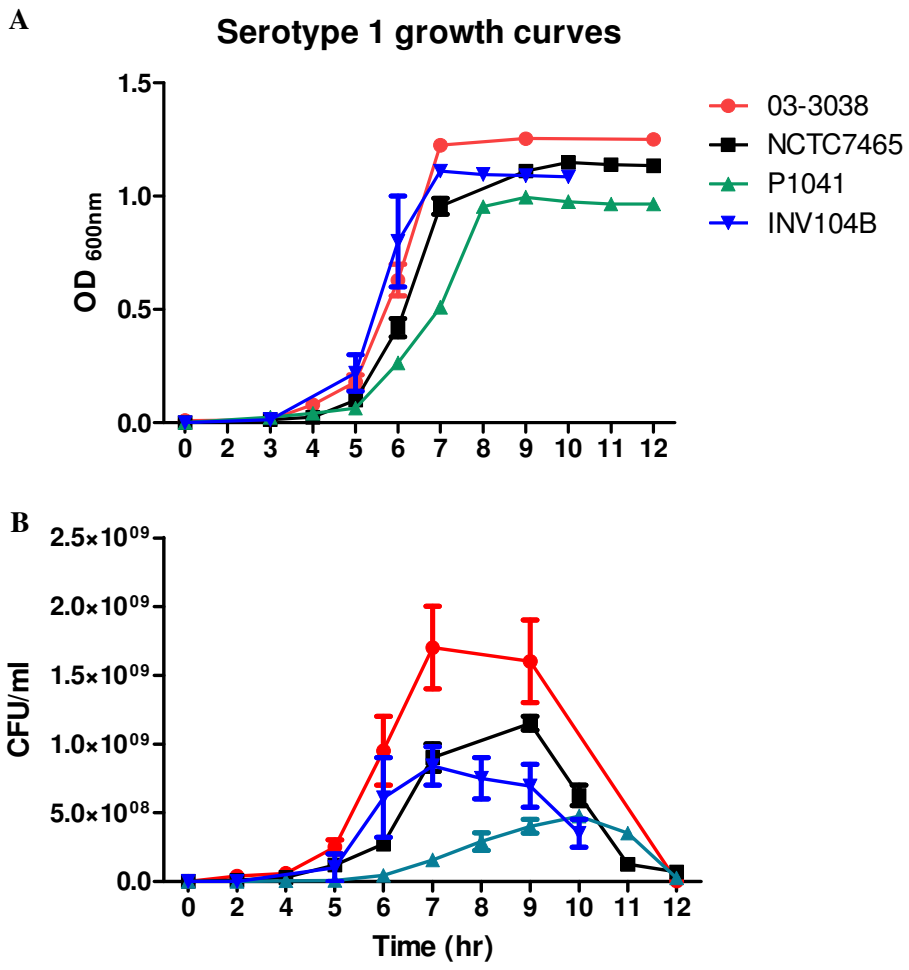


Figure 3-2 aerobic growth curves of serotype 1 pneumococci

Pneumococcal optical densities for serotype 1 growth curves (A) and their viable counts as CFU/ml (B). Four clinical isolates representing serotype 1; 03-3038, NCTC7465, P1041 and INV141. 03-3038 and NCTC7486. They have a generally similar growth pattern, with 03-3038 VC growth showing the highest bacterial population. All survived until about 12 hr. The clinical strain P1041 made a very weak log phase among all of the other grown serotype one representatives, resulting in the slowest recorded generation time. Error bars represent the standard error of the mean using Graph Pad (GraphPad Prism 5, USA 2007)

Growth curves are different between diverse pneumococcal strains. An example can be seen in the growth curve of the isolate 03-3038; the stationary phase took nearly 3 hr after growing exponentially for four hours, the growth then reduced for another 3 hr as shown in Figure 3-2. Regardless of the higher bacterial population and the slight changes in the growth, the isolate NCTC7465

had a similar growth curve to 03-3038 but longer by about an hour in each phase. Both have reached the end of their death phase after almost 12 hr.

With regard to the clinical organism P1041, the graph (Figure 3-2) demonstrates that the life span was also 12 hr resembling 03-3038 and NCTC7486. However, P1041 very slow growth rate its group representatives. As aresult, its doubling time was 63 min, showing the weakest growth rate amongst serotype 1. Moreover, the repeated experiments revealed a high similarity in its growth curves. It seems to be that P1041 has the most reproducible growth curves - when repeated - among all of the clinical strains used in the study.

In addition, the above chart shows that the strain INV104B took approximately 4 hr before starting the sharp logarithmic phase (Figure 3-2). Both of the curves, OD and VC, reached the peak at about 7 hr, and then the viability of the cells gradually decreased for another 2 hr before the growth rate dropped in an approximately parallel direction to the log phase representing the bacterium death phase.

3.1.3 Serotype 3 strains

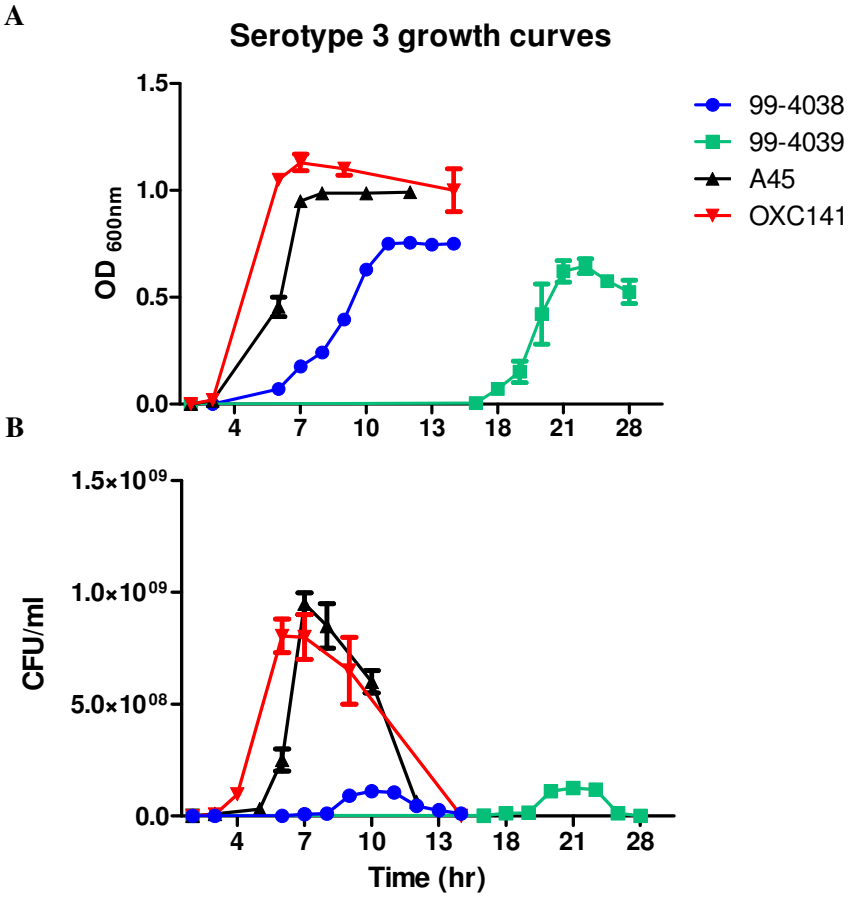


Figure 3-3 Growth curves for serotype 3 pneumococci

Pneumococcal optical densities for serotype 3 growth curves (A) and their viable counts as CFU/ml (B). Four clinical isolates belonging to serotype three; 99-4039 (isolated from CSF in a patient), 99-4038 (isolated from the same patient but from the blood), OXC141 mostly found as a coloniser and the equine isolated strain A45 that has never been seen in humans. Statistical *t-test* analysis using Graph Pad (GraphPad Prism 5, USA 2007)) showed significant differences with p=value 0.0211 between growth curves of 99-4038 and OXC141. Also the p-value for 99-4038 to OXC141 was 0.0421. Error bars represent the standard error of the mean.

The strains 99-4039 and 99-4038 have been isolated from the same patient but different sites, cerebrospinal fluid (CSF) and the patient's blood respectively. The CSF isolate 99-4039 lasted for 16 hr in the lag phase (the longest lag phase period as compared to all of the 11 other pneumococcal strains), followed by the logarithmic growth for 2 hr; 3 hr were taken in the stationary and the death phase started after 23 hr for a few further hours (Figure 3-3, B).

The growth readings showed that the isolate 99-4038 started its log phase at 6 hr before turning to the logarithmic phase. Then it lasted 2 hr, dropped dramatically for 30 min and adjusted to be horizontal-like for another 2 hr before death (Figure 3-3, B). Compared to 99-4039, the whole growth pattern in 99-4038 was shifted 10 hr to the left.

Moreover and in spite of both serotype 3 micro-organisms A45 and OXC141 isolated from different host types, a horse strain (that has never been isolated from humans) and a human coloniser, respectively, both grew *in vitro* in a similar pattern. They all started growing logarithmically at 5 hr for around an hour. Then they died after 13 hr.

However, the A45 growth curve demonstrates a sharper log phase (~45 min) after the first five hours taken in the lag phase (Figure 3-3, B). The pathogen started its decline phase after 6 hr after which it was reduced for another 6 hr. Statistical data analysis revealed significant growth differences (P=0.0211) between 99-4038 and OXC141, and also between 99-4039 and OXC141, respectively, as shown in Figure 3-3, B.

These manifest differences among the same pneumococcal serotypes grown under some condition, reflecting their *in vitro* phenotypic variations.

3.1.4 Serotype 8 (ATCC6308)

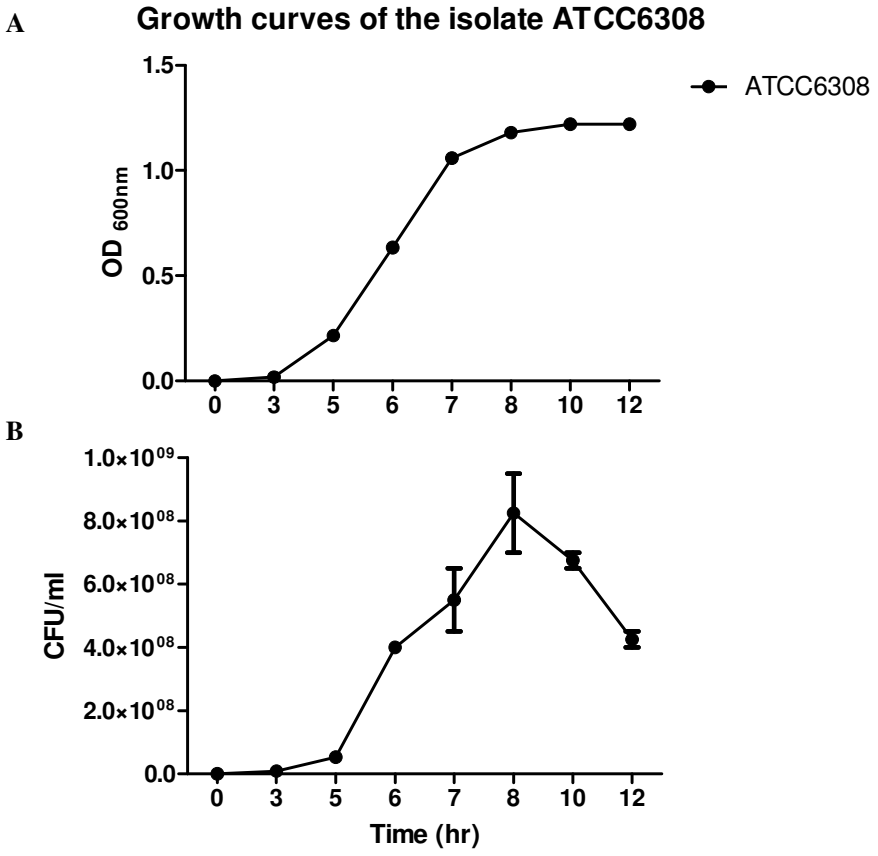


Figure 3-4 Aerobic growth curve for serotype 8 (ATCC6308)

Optical density (A) and viable counts as CFU/ml (B). Error bars represent the standard error of the mean using Graph Pad (GraphPad Prism 5, USA 2007).

In Figure 3-4, the medical isolate ATCC6308 took 3 hr to start the log phase in which 5 hr were taken, and then there was a gradual decrease for almost 6 hr. The approximate time for the whole growth was 15 hr.

3.2 Effect of oxygen on pneumococcal growth

A collection of pneumococci was chosen from different serotypes to be grown under aerobic and anaerobic growth conditions. This was to assess how the availability of oxygen could affect their growth pattern under both incubation condition. The selected bacteria were type 4 (TIGR4), type 1 (P1041) and three isolates representing type 3 (99-4039, 99-4038 and OXC141).

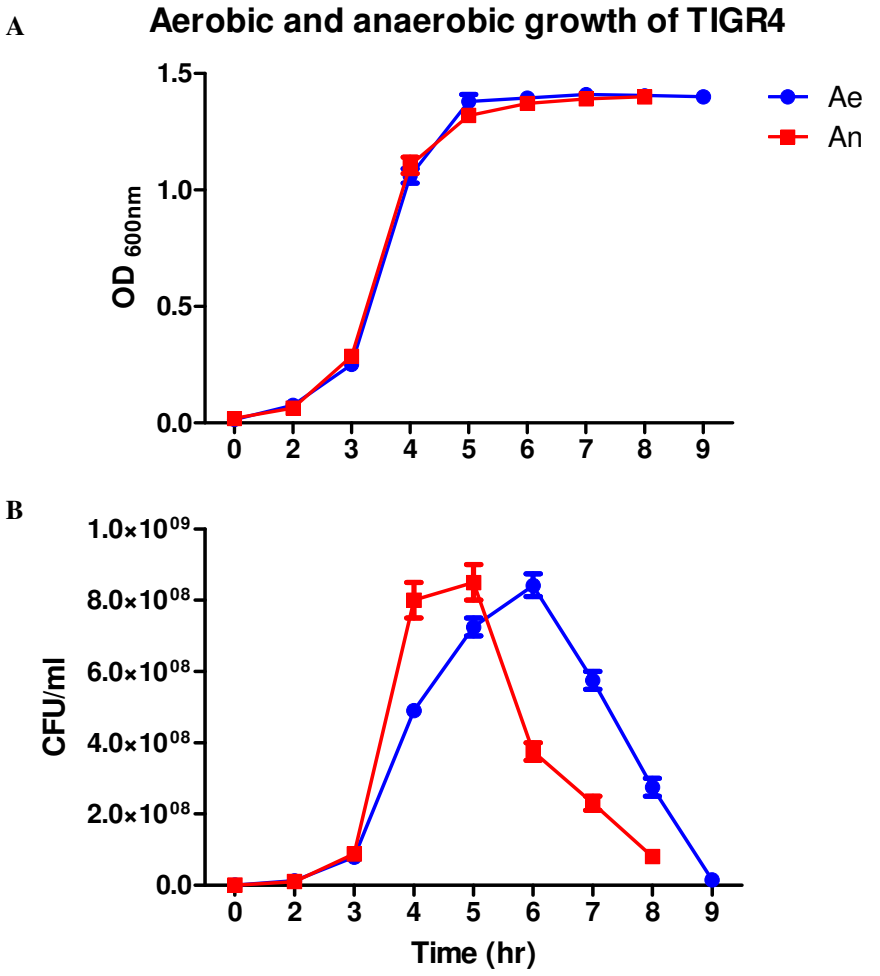


Figure 3-5 TIGR4 growth in aerobic or anaerobic growth conditions

Pneumococcus optical density (A) and viable cells as CFU/ml (B). *S. pneumoniae* TIGR4 grows better anaerobically, sharper log phase and more typical pattern of growth. Error bars represent the standard error of the mean and growth variation is not significant, calculated using Graph Pad (GraphPad Prism 5, USA 2007), and growth curves under both conditions were repeated for at least three independent experiments.

The anaerobic growth conditions of TIGR4 slightly seemed to provide a preferable environment as the bacterium showed a typical growth curve anaerobically as compared to the aerobic. TIGR4 has made an earlier and sharper logarithmic phase under the anaerobic conditions, a typical stationary bacterial phase, and parallel death to the log phase as seen above in Figure 3-5, B.

It is noteworthy that this figure (Figure 3-5) shows difference between the pneumococcal viable counts but not in their OD values. This could be because the spectrophotometer measures absorbance resulting from bacterial cells, their debris and dead organisms when reading a sample, whereas plating on an agar plate will only reflect live pneumococci. Therefore, although ODs suggest that bacterial growth curves are similar, analysis of VCs showed some differences, which are believed to be reflecting true growth states.

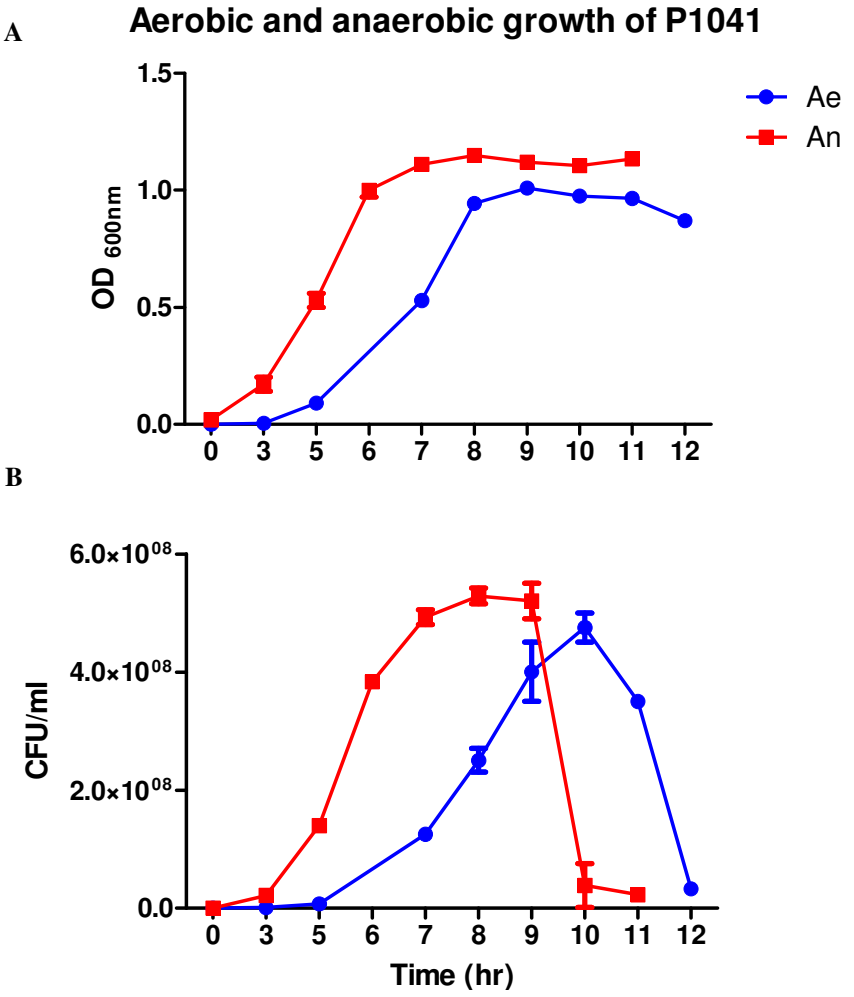


Figure 3-6 P1041 growth in aerobic or anaerobic growth conditions

Bacterial optical densities (A) and CFUs (B). Later log phase in the atmospheric growth environment and the microorganisms had both incubation conditions, similar growth rates and a dramatic sharp death phase. Error bars represent the standard error of the mean and differences between growth curves were not significant using Graph Pad (GraphPad Prism 5, USA 2007).

Although P1041 grew better anaerobically (Figure 3-6, A and B), roughly in the same way as TIGR4, it took a shorter time in the anaerobic growth conditions (only about an hour) during the enzymatic preparation stage (the lag phase). Pneumococcal growth curves in BHI were reported to show enhanced growth rates in anaerobic conditions (Baettig *et al.*, 2006). Affected growth patterns with oxygen molecules were also demonstrated in *S. mutans* (Ahn *et al.*, 2007). The figure shows that there was a prolonged log phase in both of the growth patterns, aerobic and anaerobic. As a result, the doubling time in these two

conditions was similar, as well as their dramatically sharp decline phase, unlike the TIGR4 growth rate.

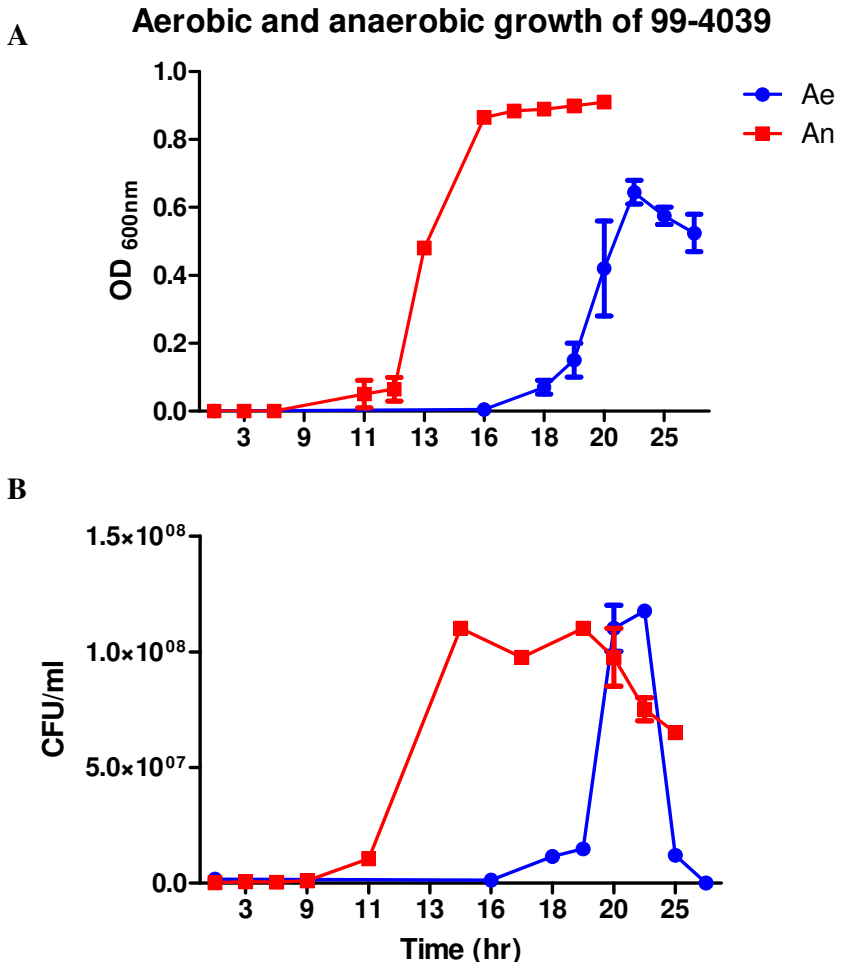


Figure 3-7 99-4039 growth in aerobic or anaerobic growth conditions

Bacterial optical density (A) and colony forming unit (B). Dramatic changes occurred to the CSF strain in the absence of O_2. Seven hours earlier the log phase was reported anaerobically but with similarities in their growth rates. Error bars represent the standard error of the mean using Graph Pad (GraphPad Prism 5, USA 2007).

A clinically isolated microbe 99-4039 isolated from CSF, preferred the environment with limited oxygen. It started the log phase 7 hr earlier anaerobically, and long lag phases in pneumococci have been linked to a number of serotypes associated with virulence (Baettig *et al.*, 2006). The isolate then took a longer time in the stationary phase compared to the aerobic stationary growth phase (Figure 3-7, B). In addition, the anaerobic grown bacteria died slowly compared to the sharp aerobic decline phase.

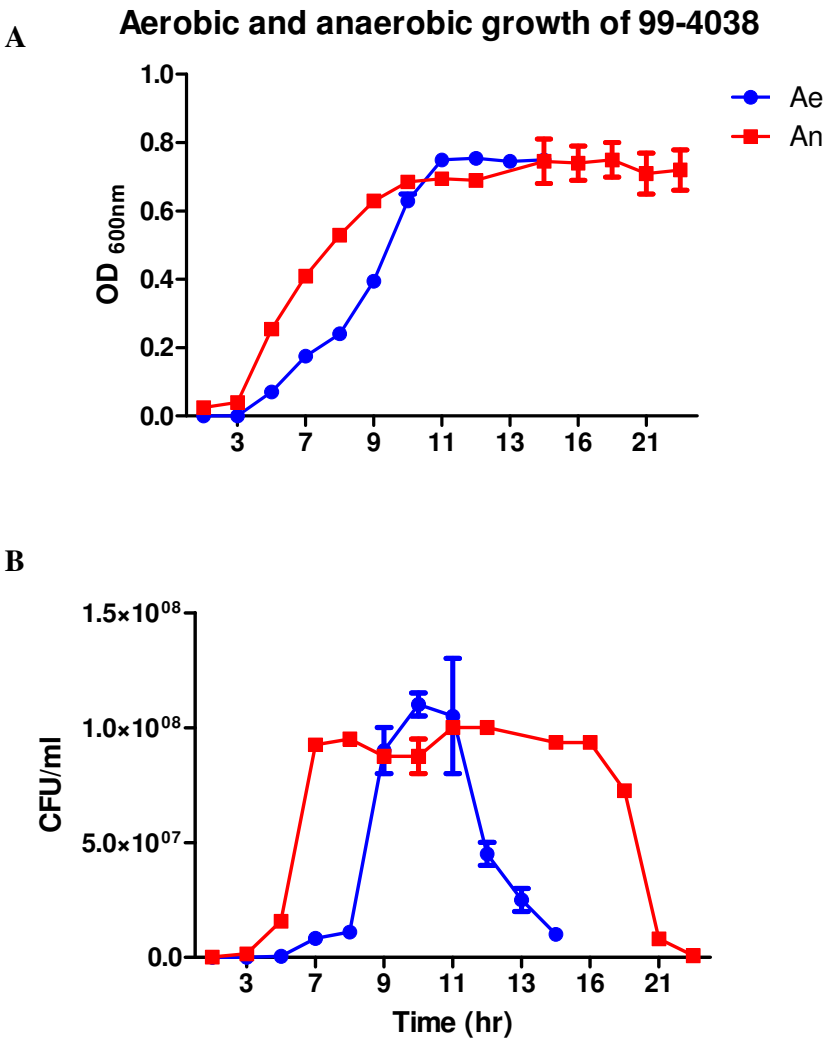


Figure 3-8 99-4038 growth in aerobic or anaerobic growth conditions

Optical density of the grown cells (A) and their CFUs (B). Error bars represent the standard error of the mean using Graph Pad (GraphPad Prism 5, USA 2007).

Serotype 3, 99-4038 (the blood isolated pathogen) showed a predicted faster lag phase anaerobically, resembling the *S. pneumoniae* CSF clinical isolate, before taking 11 hr in the stationary phase (Figure 3-8), showing the longest stationary time taken amongst all of the other tested pneumococci in this project. The whole anaerobic pattern of growth lasted for 24 hr, and that was an enhancement of nearly 10 hr compared to its growth in the presence of oxygen.

Growth curves of pneumococci representing different serotypes showed anaerobic typical growth phases aerobically grown pneumococci (Figure 3-5, 6, 7 and 8). In fact, it has been reported that elimination of H_2O_2 from the growth

cultures has led to a significant prolonged stationary phase (Regev-Yochay *et al.*, 2007).

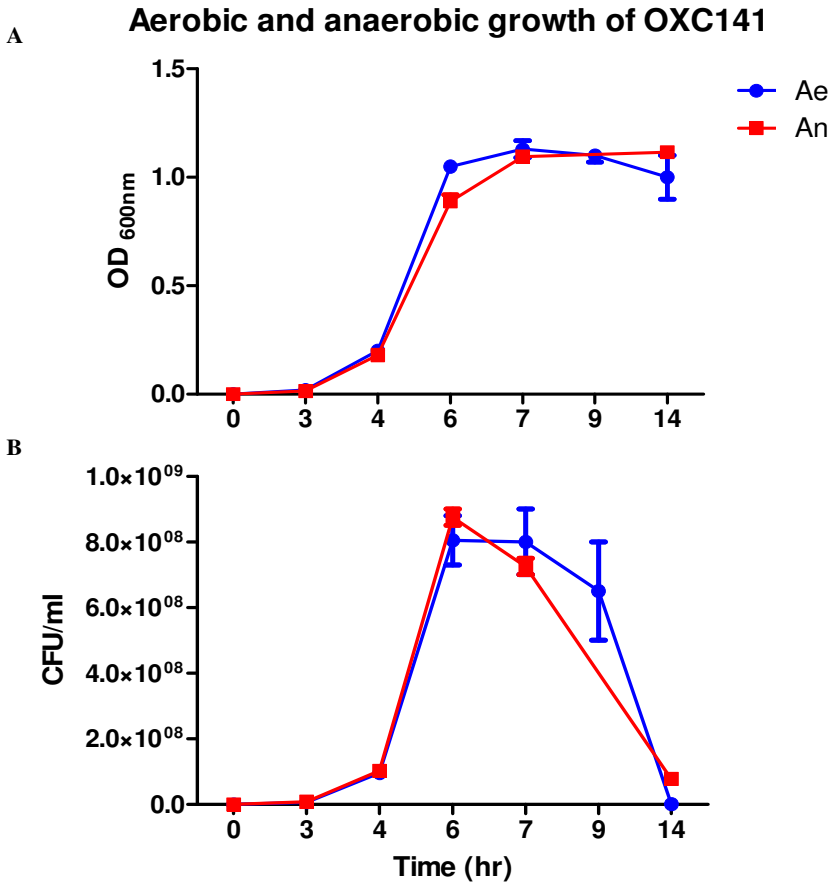


Figure 3-9 OXC141 growth in aerobic or anaerobic growth conditions

The strain OD (A) and viable counts as CFU/ml (B). OXC141 grew dramatically for four hours exponentially after taking about 3 hr in the lag phase. This was followed by a similar sharp death phase. Both growth curves (aerobic and anaerobic) were homologous under both incubation conditions, and it is the only bacterium that grows similarly in aerobic and anaerobic environments compared to other strains tested. Error bars represent the standard error of the mean and growth curves are not significantly different using Graph Pad (GraphPad Prism 5, USA 2007).

The strain OXC141, which is found mostly in carriage (as a coloniser) rather than as a cause of IPD, grew with almost identical and parallel growth patterns in the presence and absence of oxygen (Figure 3-9, B),

All of the above aerobic-anaerobic growth patterns (serotype 4; TIGR4, serotype 1; P1041 and serotype 3; 99-4039 and 99-4038) had, to some extent, earlier log and longer stationary phases anaerobically reflecting generally greater growth rates anaerobically. Dissimilarly, OXC141 viable cells counted during the bacterial growth in both environments (Figure 3-9) have shown that there is a

slightly longer stationary phase under the aerobic conditions in comparison to the absent stationary phase anaerobically. A further confirmation for this outcome was performed.

These pneumococcal phenotypic differences in the aerobic and anaerobic growth conditions could be due to the production of H_2O_2 under oxygen availability compared to its absence as a consequence of pyruvate oxidase activity, hence, peroxide production. Thus, these strains have remarkably different growth patterns that reflect some genetic variation. Differences in 15 pneumococcal growth patterns were studied in BHI and variation was clear between pneumococci (Baettig *et al.*, 2006). It was decided then to perform H_2O_2 assays for TIGR4 growth in oxygenated and anaerobic growth environments.

3.3 Production of H_2O_2 by TIGR4

H_2O_2 assay was performed to compare the produced amount after growing TIGR4 in both oxygenated and anaerobic growth conditions.

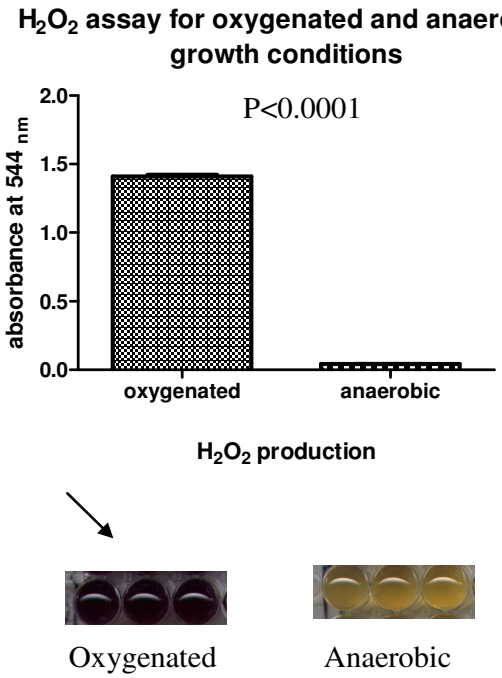


Figure 3-10 Production of H_2O_2 in TIGR4 grown in oxygenated and anaerobic conditions.

The oxygenated growth has led to a massive hydrogen peroxide production compared to the anaerobic growth conditions. The arrow indicates reaction of the oxygenated sample that contained high concentration of H_2O_2 versus anaerobic samples on the right. Each bar shows an average of three readings and error bars represent the standard error of the mean. Statistical analysis was performed using *t-test* in Graph Pad (GraphPad Prism 5, USA 2007).

The oxygenated growth of TIGR4 led to a statistically significant production of H_2O_2 compared to cells grown in the absence of oxygen, indicating proper incubation conditions used (Figure 3-10). Analysis of this assay showed that the amount of peroxide produced by TIGR4 during oxygenated growth conditions was above the range of the calibration range. However, production of peroxide by pneumococci has been determined in some pneumococcal serotypes. For instance, type 9V, 2 and 19 generated approximately 2.7 mM, 2.3 mM, and 5 mM respectively (Pericone *et al.*, 2002; Taniai *et al.*, 2008).

From this and because of the fact that the blood contains the catalase enzyme, the aerobic growth reduction was noticed in several pneumococci, representing

different serotype groups, might be restored if the microbe were grown in the medium but with the addition of blood. As mentioned earlier, supplementing pneumococcal media with catalase to eliminate H_2O_2 has resulted in a significant prolonged stationary phase (Regev-Yochay *et al.*, 2007).

3.4 Pneumococcal growth in blood

It is known that blood contains the catalase enzyme, which breaks hydrogen peroxide down into water and oxygen, suggesting that growing pneumococci, that lack catalase, will lead to greater growth rate in the blood. Therefore, TIGR4 was grown with and without adding 5 % of sheep's blood. Moreover, the clinical blood-isolated strain 99-4038 was also grown similarly to determine its growth in this mixture, BHI-blood medium.

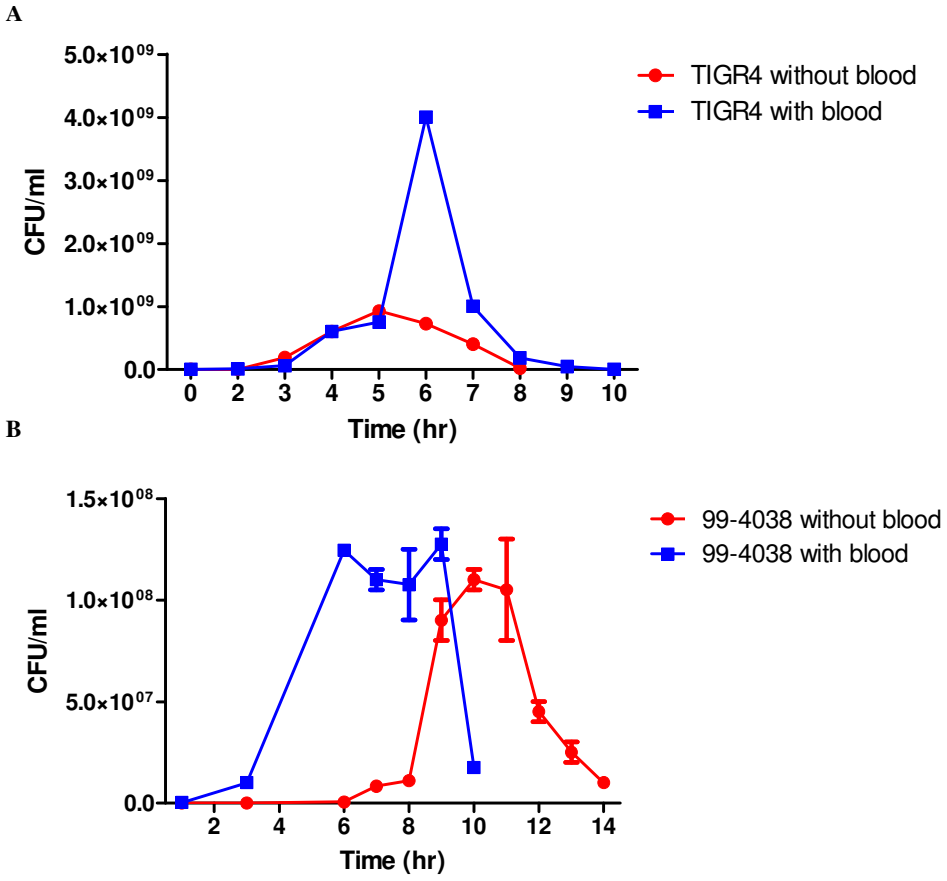


Figure 3-11 Comparison of pneumococcal growth rates with and without blood

Growth curves CFU/ml in BHI without and with 5% blood addition of strains TIGR4 (A) and 99-4038 (B). There was a three times increase in bacterial population of TIGR4 once grown in the BHI provided with 5% blood, whereas the strain 99-4038 had an almost similar growth peak but grew clearly earlier in blood. Error bars represent the standard error of the mean using Graph Pad (GraphPad Prism 5, USA 2007).

S. pneumoniae TIGR4 was able to grow and exceed three-fold in the BHI-blood medium compared to its growth without adding 5 % blood. It took a similar time in the lag phase when growing in blood (Figure 3-11, A).

The growth of the isolate 99-4038 revealed a sharper log phase in the BHI supplemented with 5 % blood and obviously preferred blood containing broth as it grew 6 hr earlier and showed a longer stationary phase relative to its growth without blood addition. This indicates that the pathogen was growing in a less stressful environment compared with its growth without the addition of blood.

Marked differences in bacterial growth curves might be because of the way that the bacterium forms its growth chains. Formation of the chain length could hugely affect the spectrophotometer readings, as this apparatus may count a long chain as one bacterium if a chain randomly faces the sensor vertically. Furthermore, viable counts would be affected as each bacterial chain could form a colony; on the other hand, an individual bacterium will form one colony as well leading to incorrect CFU/ml. Therefore, samples were vortexed before taking readings to avoid a vast number of chains being counted as individual cells, resulting in a false and small final number. It was worth then to clarify this theory by a microscopic examination of TIGR4 growth without vortexing to investigate if oxygen has an effect on the microbial chain morphology during growth; for instance, chain length under variant incubation methods including aerobic, anaerobic and growing in host-derived fluids such as blood.

3.5 Microscopic investigation in different conditions

TIGR4 strain was grown aerobically and anaerobically to microscopically investigate how generally oxygen affects the pathogen morphology. It was grown in both incubation states until their mid-log phase before films were prepared on slides and Gram staining carried out.

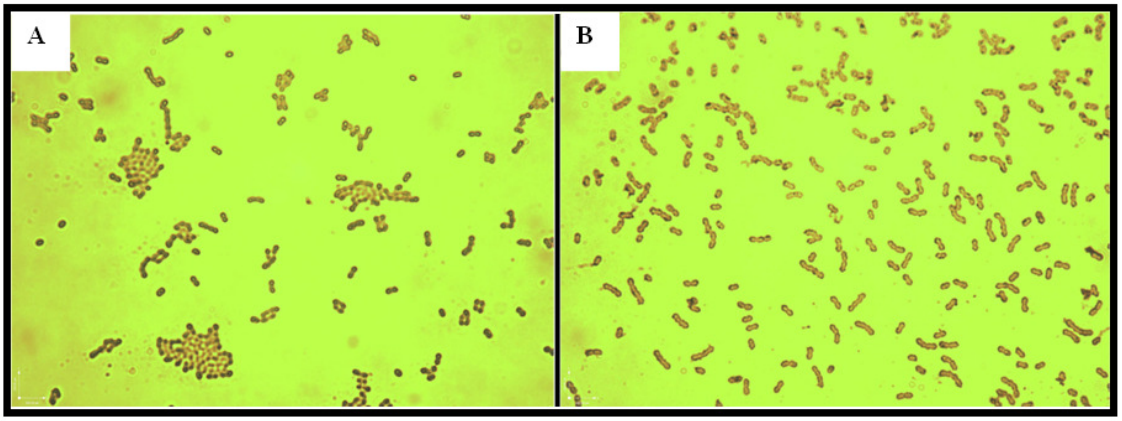


Figure 3-12 Microscopic examination of TIGR4 cells grown aerobically (A) and anaerobically (B)

Examination of TIGR4 un-vortexed growth under these two conditions showed that O_2 does affect the bacterial morphology. TIGR4 demonstrated a tendency in forming clumps A. In contrast, they tended to be in diplococci in the absence of oxygen than grouping together B. Images captured at a magnification of 1×10^3 after Gram staining of bacterial mid-log phase after fixing aerobic and anaerobic grown cells.

The microscopic examination has revealed that the bacterium seems to form clumps, sticking to each other when exposed to the aerobic conditions (Figure 3-12). In contrast, they tend grow separately in the absence of oxygen, forming diplococci or short-chains instead of clumps. However, no changes in chain length were observed.

Furthermore, TIGR4 was also grown aerobically with the addition of 5 % horse blood to the medium used to microscopically assess its growth morphology in the presence of blood-BHI media.

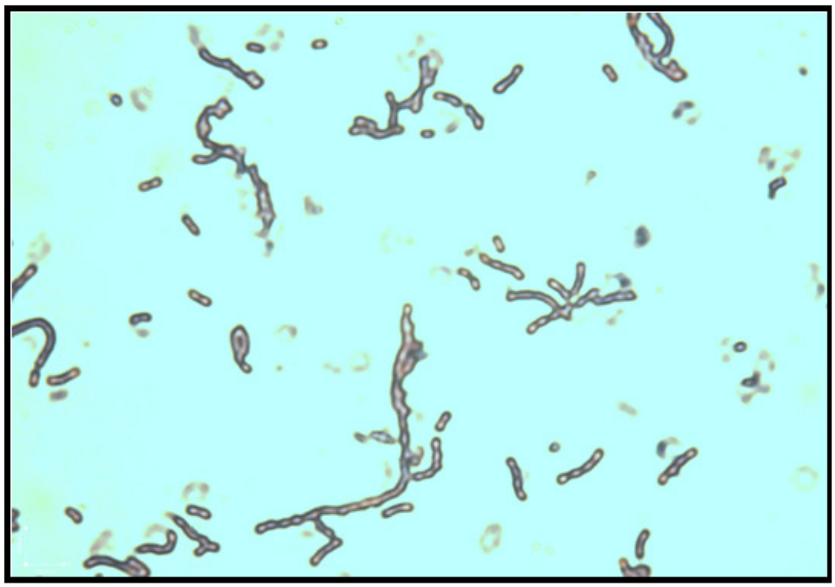


Figure 3-13 *S. pneumoniae* TIGR4 grown in the BHI-blood medium

Microscopically examined TIGR4 in their mid-log growth without vortexing reflects longer bacterial chains compared to their shape under the aerobic/anaerobic growth conditions shown in Figure 3-12. The image was taken at a magnification of 1×10^3 after Gram staining of the bacterial mid-log phase grown in the blood-BHI broth culture.

Figure 3-13 illustrates different growth pattern when cells were grown in the blood-BHI broth culture when compared to their growth with and without oxygen. They formed long chains rather than being in clumps or forming separate diplococci as in the aerobic or anaerobic growth conditions, as seen in Figure 3-12.

In addition to these factors (aerobic, anaerobic and growth in the presense of blood) the acidity levels can strongly affect bacterial growth rates. This may widely vary during growth in different levels of oxygen according to the metabolic pathway employed under each growth condition.

3.6 pH during TIGR4 growth

Pneumococci are well known for producing lactic acid as an outcome of their sugar metabolism. The aim of this experiment is to determine the acidity levels when cells are grown in different levels of oxygen. Therefore, *S. pneumoniae* TIGR4 was grown for 7 hr and the pH was measured in hourly intervals. Cells

were grown under three different levels of oxygen: aerobic, anaerobic and oxygenated conditions.

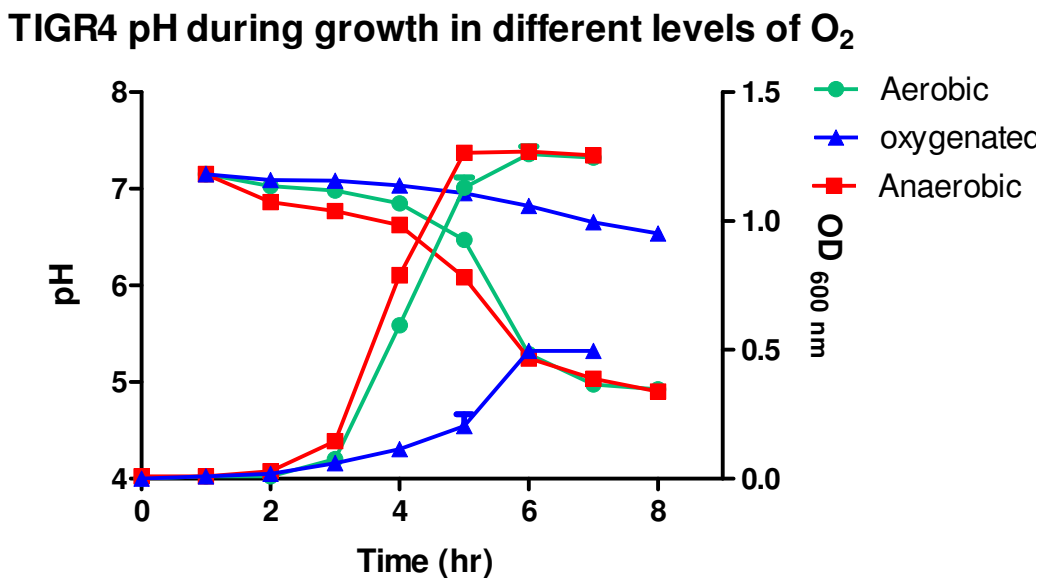


Figure 3-14 pH assay for TIGR4 growth under three different levels of oxygen

Measurement of pH during TIGR4 growth shows that the pH was gradually reduced till it reached around pH 4.3 in both aerobic and anaerobic conditions, but it did not drop to acidic levels in the oxygenated culture as there was not sufficient bacterial growth. Error bars represent the standard error of the mean using Graph Pad (GraphPad Prism 5, USA 2007).

In the pH assays, all of the different growth conditions had pH 7.2 in the beginning. The findings showed a gradual decrease in pH in three different environmental conditions. There was not significant difference between the aerobic and anaerobic growth conditions (Figure 3-14). However, the oxygenated growth did not bring about the same acidity levels found in aerobic/anaerobic growth. This could be due to the very weak pneumococcal growth rate in oxygenated broth culture.

3.7 Pneumococcal phenotypic differences

After gathering several pneumococcal growth characteristics from the above experiments under different growth environments, the period taken in growth pattern and length of lag phase of different strains of *S. pneumoniae* are shown in Table 3-2.

Table 3-2 Characterisation of pneumococcal strains

1) Time taken during the whole growth period. 2) Length of the lag phase. *Strains isolated from 1 patient but 2 sites with the same infection profile, blood and cerebrospinal fluid (CSF). Ae stands for aerobic, Ox is oxygenated, whereas An means anaerobic and NA: not assessed.

Strain	Genome Sequence	Serotype	MLST	Growth time(hr)[1]	Lag phase (hr)[2]
TIGR4	(Tettelin *et al.*, 2001)	4	205	Ae. 10	2
				An. 9	2
				Ox. 10	2
TIGR4-403	Glasgow	4	205	13	4
03-3038	Glasgow /Sanger	1	306	12	3
NCTC7465	Glasgow /Sanger	1	615	12	4
P1041	Glasgow /Sanger	1	217	Ae. 12:30	4.30
				An. 12	1
INV104B	Glasgow /Sanger	1	227	12	3
99-4039 *	Glasgow /Sanger	3	180	Ae. 28	16
				An. 31	11.30
99-4038 *	Glasgow /Sanger	3	180	Ae. 15	6
				An. 24	3
OXC141	Glasgow /Sanger	3	180	Ae. 15	3
				An. 14	3
A45	Glasgow /Sanger	3	6934	13	3
ATCC6308	Glasgow	8	NA	15	5

Table 3-2 shows that the bacterial strains behaved differently in the way they react to oxygen as these pneumococci reflected different phenotypes in terms of their relationship to oxygen availability. For instance, TIGR4 generation times, which are shown in Figure 3-15, illustrated a shorter time anaerobically as compared to its aerobic growth rates indicating that the more oxygen provided to the growth conditions the less generation time the pneumococcus will reveal.

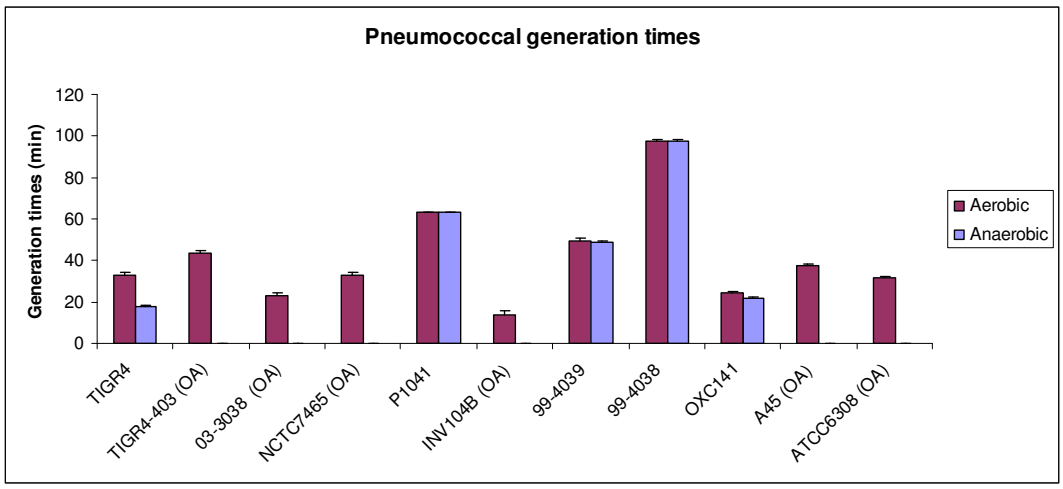


Figure 3-15 Generation times for pneumococci used in this study

TIGR4 generation time was different depending on the oxygen provided to its broth culture. When oxygen is available, the doubling time is decreased. P1041 demonstrates the longest aerobic and anaerobic generation times among all serotype 1 strains. Pneumococci representing serotype 3 had also variant times and similarities in presence/absence of oxygen in the strains 99-4038, 99-4039 and OXC141. (OA) indicates only aerobic as not all pneumococcal strains were grown aerobically and anaerobobaccly, just some representing each serotype used in this study were growth in the two conditions, aerobic and anaerobic. Each coloumn represents three generation times and error bars represent standard error of the mean using Microsoft Office Excel 2003. Statistical analysis using Graph Pad (GraphPad Prism 5, USA 2007) showed no significant differences comparing to TIGR4.

However, Table 3-2 illustrates that P1041 grows similarly under both growth conditions and very slowly, giving the longest generation time among all of the other serotype 1 strains. As a result, its doubling time was the same in the presence or absence of oxygen.

Several questions and ideas can be inferred from these differences in growth characteristics. For example, the variant generation times in the isolates 99-4038 and 99-4039 were interesting. Genome sequencing has shown that their genomes are very closely related with only 3 SNPs that were different, and one of these SNPs was shown recently to alter the expression of PatA/B (pump efflux proteins) which may be responsible for this slow growth rate (Croucher *et al.,* 2013), in press.

It is worth mentioning here that these two strains have been isolated from one patient but from two different sites; 99-4038 was from the patient's blood, whereas 99-4039 from the CSF.

OXC141 also had almost the same doubling time aerobically and anaerobically and grew greater and more rapidly under the available or restricted oxygen compared to P1041. This suggests that they may have particular differences in their genetic expression under aerobic versus anaerobic growth environments; such differences could be related to their metabolic pathways. These differences are investigated in the following experiments.

3.8 Genome comparison

Three key genes can be identified from *S. pneumoniae* because of their involvement in the pneumococcal oxidative stress response. In order to compare these three major genes, several alignments were performed using nine pneumococcal genomes as listed in Table 3-3. Two strains (ATCC6308 and TIGR4-403) from the set used in this study have not been included in the following alignments. The strain ATCC6308 has not been sequenced, and the strain TIGR4-403 has not been published yet. However, the draft genome sequence of TIGR4-403 was compared at these three loci and was found to be 100% identical to parent TIGR4 genomic sequence (A.M.Mitchell, personal communication).

Table 3-3 Gene numbers of *tpx*, *spxB* and *sodA*

Where no gene number is available, contig number has been used instead. NA = not annotated.

Accession Number	Strain	Gene number or contig number		
		***tpx* (thiol peroxidase)**	***sodA* (superoxide dismutase)**	***spxB* (pyruvate oxidase)**
NC_003028	TIGR4	SP_1651	SP_0766	SP_0730
NC_018594	A45	SPNA45_00589	SPNA45_01090	SPNA45_01061
NZ_CACE01000000	P1041	NZ_CACE01000021	NZ_CACE01000002	NZ_CACE01000002
NZ_CACF01000000	SPN7465	NZ_CACF01000008	NZ_CACF01000001	NZ_CACF01000001
NC_017591	INV104B	INV104_14040	INV104_06370	INV104_06090
NC_021004	03-3038	NA	NA	NA
NC_017592	OXC141	SPNOXC_14500	SPNOXC_06930	SPNOXC_06620
NC_021026	SPN994038	SPN994038_14360	SPN994038_06830	SPN994038_06520
NC_021005	SPN994039	SPN994039_14370	SPN994039_06840	SPN994039_06530

Table 2-1 showing nine pneumococcal genomes used in this project. Alignments of relevant loci for these nine strains were performed in order to compare three kay genes; *tpx*, *spxB* and *sodA*. Each of these targeted gene loci with its flanking sequence is shown in Figure 3-16.

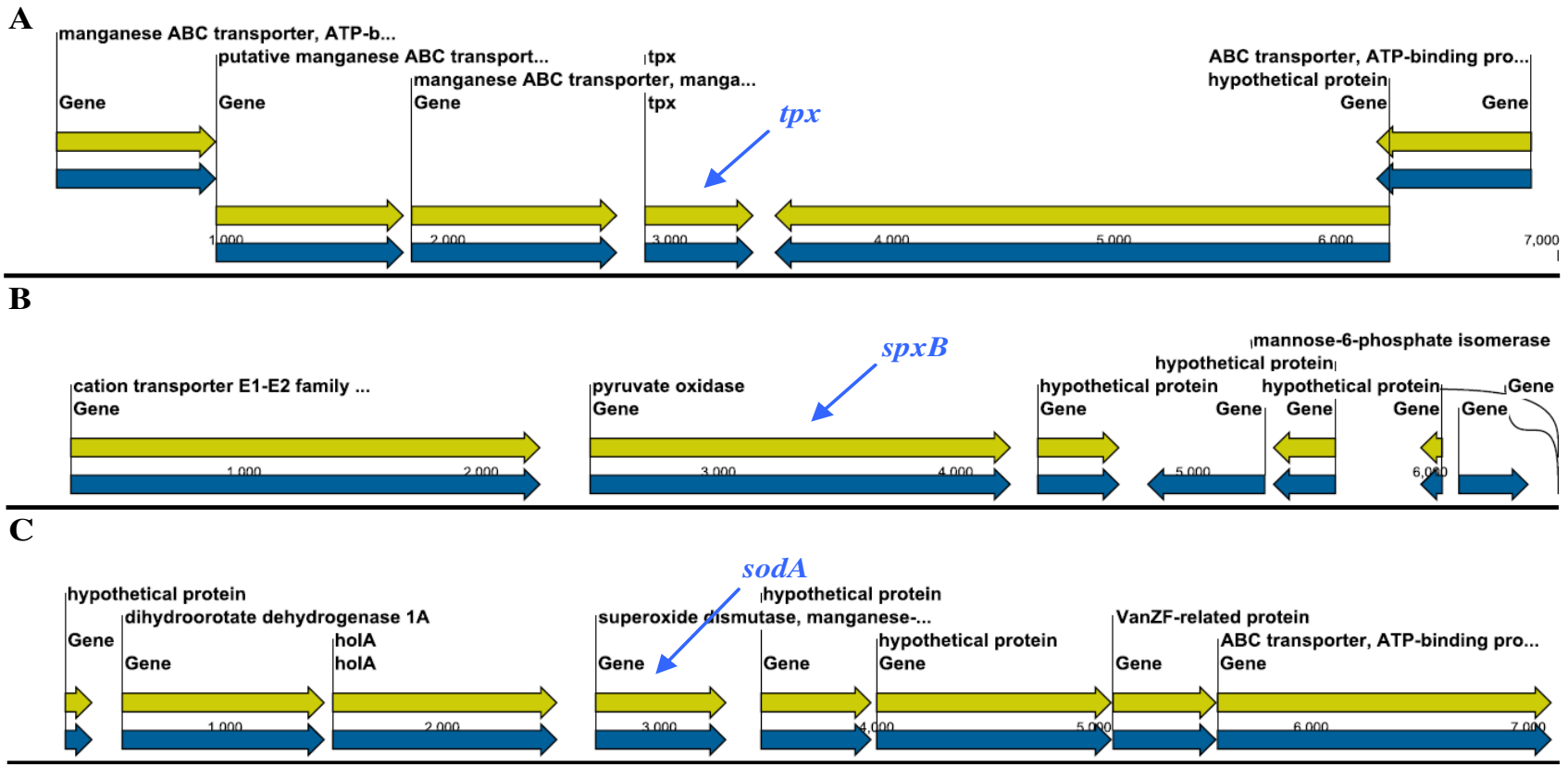


Figure 3-16 Three genes *tpx*, *spxB* and *sodA* with flanking sides

Arrows indicated *tpx* SP_1651 (A), *spxB* SP_0730 (B) and *sodA* SP_0766 (C), as shown in light blue, with at least 2 kb, which were taken into account as flanking regions for each gene.

The three targeted gene sequences were extracted from the TIGR4 genome with at least 2 kb flanking sequence from each side, upstream and downstream. This was followed by a BLAST search against all available pneumococcal genomes to investigate and compare these genes across this subset of strains. Accession numbers are as listed in Table 2-1 (Section 2.1.1).

The TIGR4 gene sequence was used as a reference strain, compared then to the other eight available pneumococcal genomes. It is worth mentioning here that unlike *spxB* and *sodA*, flanking regions for the *tpx* gene (Figure 3-16) were set further away comparing to the rest as *psa* operon was included. Thus, it was worth comparing genomic regions in case of any mutations in the rest of the operon that might affect the Tpx activity. As explained early (Sections 1.5.7 and 1.10), *psa*D (also called *tpx*) is a part of *psa* operon, known as part of a manganese transporting system, and recently shown to encode the vital pneumococcal antioxidant, thiol peroxidase (Hajaj *et al.,* 2012). Manganase cations are known as key microneutrient factors, particularly for pneumococcal pathogenicity (Ogunniyi *et al.,* 2010).

TIGR4 and 403 were studied in this topic for any possible genatic changes in *tpx*, *spxB* and *sodA*, but no SNPs were detected (either Synonymous or Non-Synonynous) between all tested pneumococci. On the other hand, other pneumococci have shown genetic changes in the three tested genes which can be seen in Figure 3-17.

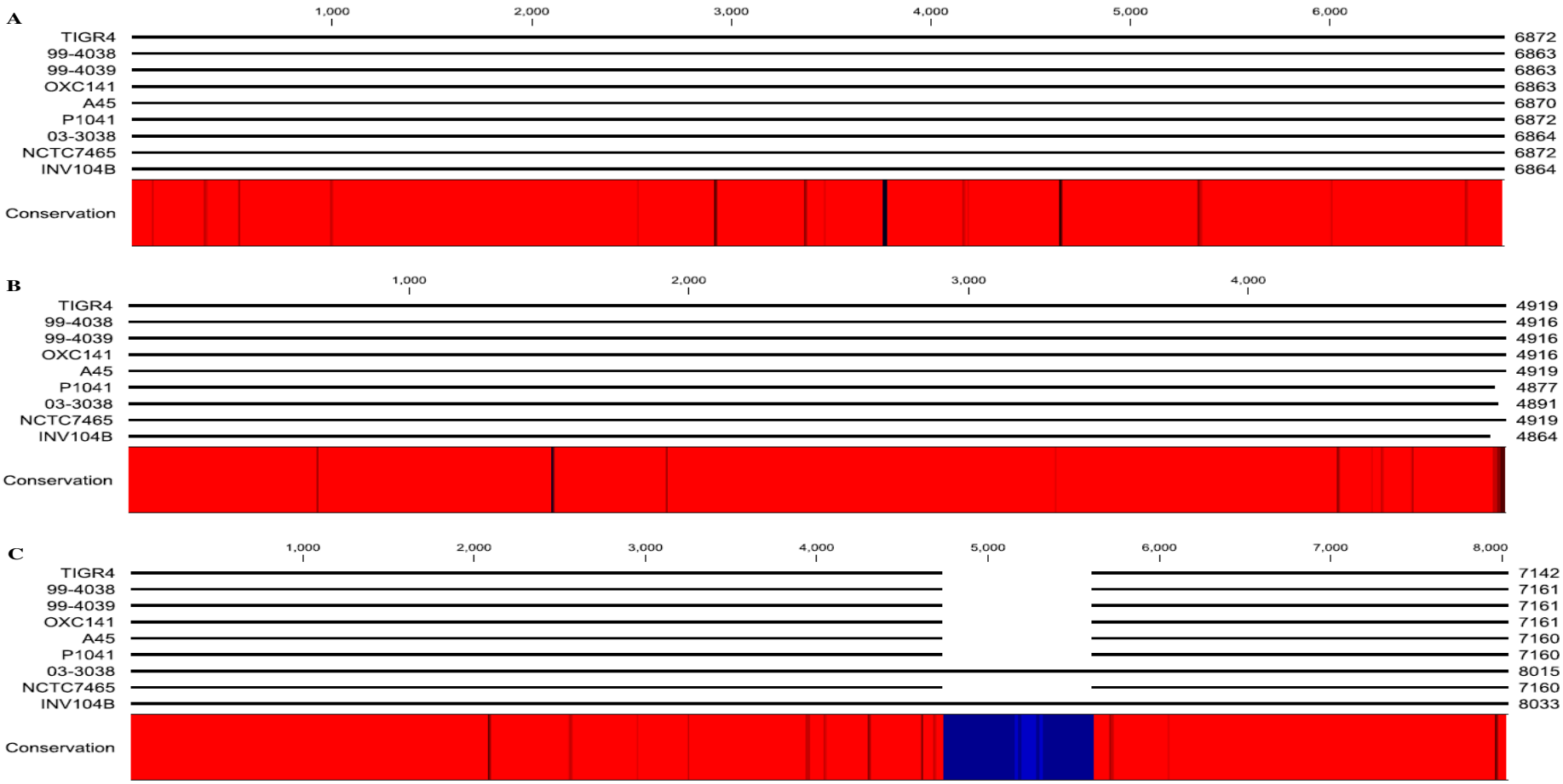


Figure 3-17 Overview of three alignments for *tpx*, *spxB* and *sodA* in nine different pneumococcal strains

Targeted genes *tpx* (A), *spxB* (B) and *sodA* (C) with the flanking regions (at least 2 kb) upstream and downstream. The vertical black lines across the red plot show SNPs detected by CLC programme, and dark navy in (C), *sodA* gene, reflects deletions in which lighter navy represents SNPs. The gene *tpx* is located between the bp number 2885 and 3377, *spxB* from 2456 to 4233 and sodA 2703 to 3321.

Overview panels A, B and C in Figure 3-17 illustate the genetic changes detected in the three tested genes (*tpx*, *spxB* and *sodA*) between pneumococci listed in Table 3-3. The Figure also shows that *spxB* gene is highly conserved across all the genomes displayed. An example of one of the several noticed SNPs was found in serotype 1 (P1041) is shown in the below alignments between the study pneumococci. It revealed a unique mutation (Figure 3-18).

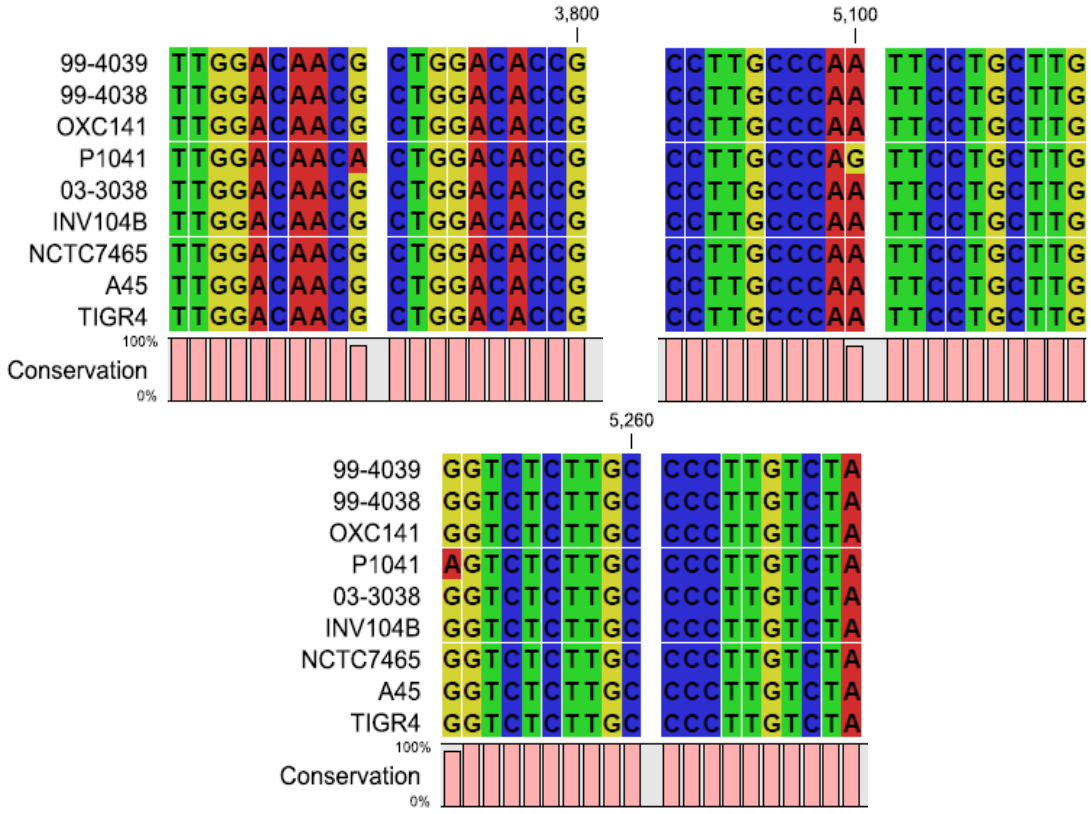


Figure 3-18 Alignment for *tpx* between pneumococci used in the study

These three snapshots alignments display examples of nucleotide changes (positions 3790, 5100 and 5251). These all observed in the strain P1041.

As it was reported early in this chapter (Section 3.1.2), pneumococcal growth charasteristics have showed that P1041, which represents serotype 1, had the slowest log phase among all serotype one strains. For confirmation, its growth was repeated three times (Figure 3-2, B). Consequently, the calculated generation time for P1041 was 63 min, reflecting the weakest growth rate in this serotype group as demonestreted in Figure 3-15. Therefore, it might be that this unique mutation in P1041 is involved in the pneumococcal growth somehow, but this requires further investigation to be assured.

Although that there are a number of detected genomic changes, it is possible that some SNPs are silent, known as Synonymous SNPs, which have different alleles encoding the same amino acid (NCBI), therefore, not any changes will lead to a different protein or altering its biological activity. DNA alighnment and its transulation to proteins revealed that the majority of the SNPs were silent; an example of this is shown in Figure 3-19.

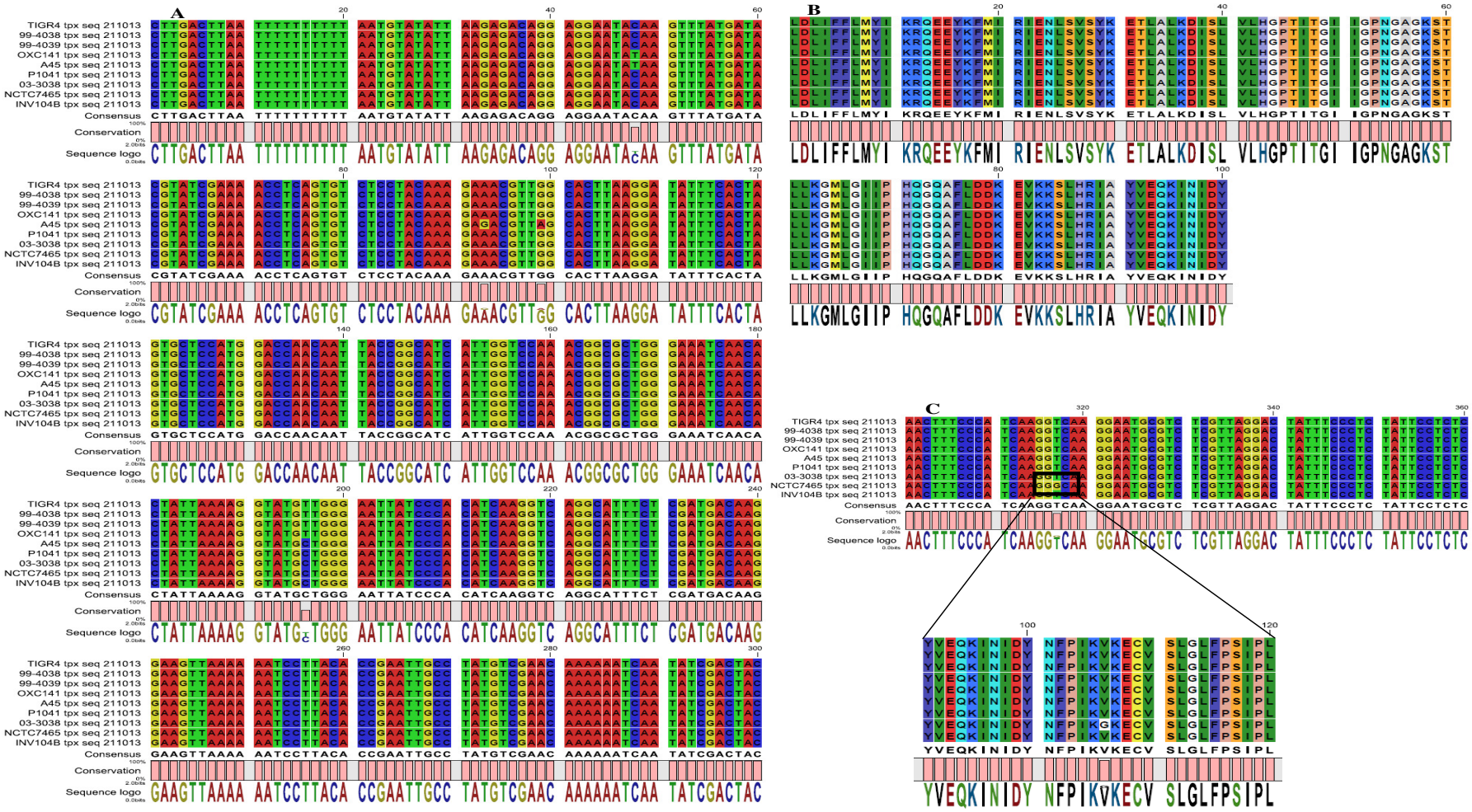


Figure 3-19 Synonymous SNPs and Non-Synonymous SNPs detected in *tpx* gene

Synonymous SNPs: three-hundered bp alignment of *tpx* DNA sequence (A), its 100 amino acid after transulating (B) and a Non-Synonymous SNP with its amino acid change from G (gly) which stands for Glycine to V as Valine (val) (C).

Due to the major number of Synonymous SNPs detected in the three tested genes that will be listed later in this section, Table 3-4 shows the Non-Synonymous SNPs found in each gene.

Table 3-4 List of Non-Synonymous SNPs detected in *tpx*, *spxB* and *sodA*

TIGR4 was used as a refrence strain. Both Non-Synonymous SNPs and Synonymous SNPs were studied in all targeted genes using CLC Genomics Workbench. Amino acids letters are as follow: N stands for asparagine, H: histidine, P: proline, Y: tyrosine, S: serine, A: alanine, K: lysine, D: aspartate, C: cysteine, T: threonine and L: leucine.

Strain	Gene	Refrence (TIGR4)	Amino acid changed	Amino acid position	Conservation
99-4038	*sodA*	N	D	994	67%
	tpx	H	C	1011	89%
	tpx	P	T	1019	56%
	tpx	Y	H	1020	89%
	tpx	P	L	1029	89%
	tpx	S	L	1032	67%
	tpx	A	D	1033	89%
	tpx	K	T	1034	89%
	spxB	N	D	1378	89%
99-4039	*sodA*	N	D	994	67%
	tpx	H	C	1011	89%
	tpx	P	T	1019	56%
	tpx	Y	H	1020	89%
	tpx	P	L	1029	89%
	tpx	S	L	1032	67%
	tpx	A	D	1033	89%
	tpx	K	T	1034	89%
	spxB	N	D	1378	89%
OXC141	*sodA*	N	D	994	67%
	tpx	H	C	1011	89%
	tpx	P	T	1019	56%
	tpx	Y	H	1020	89%
	tpx	P	L	1029	89%
	tpx	S	L	1032	67%
	tpx	A	D	1033	89%
	tpx	K	T	1034	89%
	spxB	N	D	1378	89%
A45	*tpx*	H	C	1011	89%
	tpx	Y	H	1020	89%
	tpx	P	L	1029	89%
	tpx	A	D	1033	89%
	tpx	K	T	1034	89%

	spxB	N	D	1378	89%
P1041	*tpx*	H	C	1011	89%
	tpx	P	T	1019	56%
	tpx	Y	H	1020	89%
	tpx	P	L	1029	89%
	tpx	A	D	1033	89%
	tpx	K	T	1034	89%
	spxB	N	D	1378	89%
03-3038	*sodA*	P	S	906	78%
	spxB	V	I	906	78%
	tpx	H	C	1011	89%
	tpx	Y	H	1020	89%
	tpx	P	L	1029	89%
	tpx	A	D	1033	89%
	tpx	K	T	1034	89%
	spxB	N	D	1378	89%
NCTC7465	*tpx*	H	C	1011	89%
	tpx	Y	H	1020	89%
	tpx	P	L	1029	89%
	tpx	A	D	1033	89%
	tpx	K	T	1034	89%
	spxB	N	D	1378	89%
INV104B	*sodA*	P	S	906	78%
	spxB	V	I	906	78%
	tpx	H	C	1011	89%
	tpx	Y	H	1020	89%
	tpx	P	L	1029	89%
	tpx	A	D	1033	89%
	tpx	K	T	1034	89%
	spxB	N	D	1378	89%

There were deletions and insertions observed in the coding regions of *tpx*, *spxB* and *sodA* and will be stated later in the section, 62 Non-Synonymous SNPs in the three genes were detected (Table 3-4). The most strains found with up to 9 Non-Synonymous SNPs are 99-4039, 99-4038 and OXC141, in contrast to A45 which had only 6 SNPs. Other pneumococci were in between, 7 and 8 Non-Synonymous SNPs. Moreover, although all strains had Non-Synonymous SNPs in *tpx*, *spxB* and *sodA,* A45, P1041 and NCTC7465 had no Non-Synonymous SNPs in *sodA*, only in *tpx* and *spxB*.

Non-Synonymous SNPs were also observed in the flanking regions of each gene with a large number of deletions and insertions. The gene *tpx* is located between the bp 2885 and 3377, *spxB* from 2456 to 4233 and *sodA* from 2703 to 3321, shown in Figure 3-17. Investigating of *tpx* flanks (showed above in Figure 3-16) using CLC programme demonstrated 80 Non-Synonymous SNPs, 136 deletions in the strains 99-4039, 99-4038, OXC141, 03-3038 and INV104B togather, and 153 insertions in the same five pathogens. *spxB* revealed 19 Non-Synonymous SNPs in the flanks only, whereas *sodA* showed 132 Non-Synonymous SNPs, 12 deletions in TIGR4 *sodA*, and for the same gene 6 deletions were noticed in both 03-3038 and INV104B, 536 insertions in 03-3038 and 347 insertions in INV104B as compared to the refernce strain, TIGR4. Hence, several genes are possible candidates for oxidative stress and could be for future work to be studied not only in these three genes (*tpx, spxB* and *sodA*) but also in even much bigger list of genes.

3.9 Discussion

Bacterial growth under specific conditions generally reflects their relationship to that particular environment, and evidence has shown that molecular mechanisms, bacterial fitness and virulence factors are largely changed based on ecological growth conditions (Baettig *et al.,* 2006; Mahdi *et al.,* 2008; Mazzola *et al.,* 2003; Oggioni *et al.,* 2006; Orihuela *et al.,* 2004a).

Pneumococci are known as facultative anaerobe micro-organism preferring anoxygenic growth conditions, but they encounter different oxygen levels during the infection cycle (Burghout *et al.,* 2010; Yesilkaya *et al.,* 2013). The bacterium lacks a large number of proteins known in other bacteria to be required for resisting oxidative stress such as catalase (Pesakhov *et al.,* 2007). This obviously highlights that *S. pneumoniae* possesses unknown factors allowing the bacterium to grow and survive in aerobic growth condition. Therefore, in order to study their growth characteristics in different oxygen concentration, several pneumococcal strains representing different serotypes were investigated. Growth rates of the pneumococci TIGR4, P1041, OXC141, 99-4038 and 99-4039 aerobically and anaerobically showed variations in response to oxygen availability (Figure 3-20).

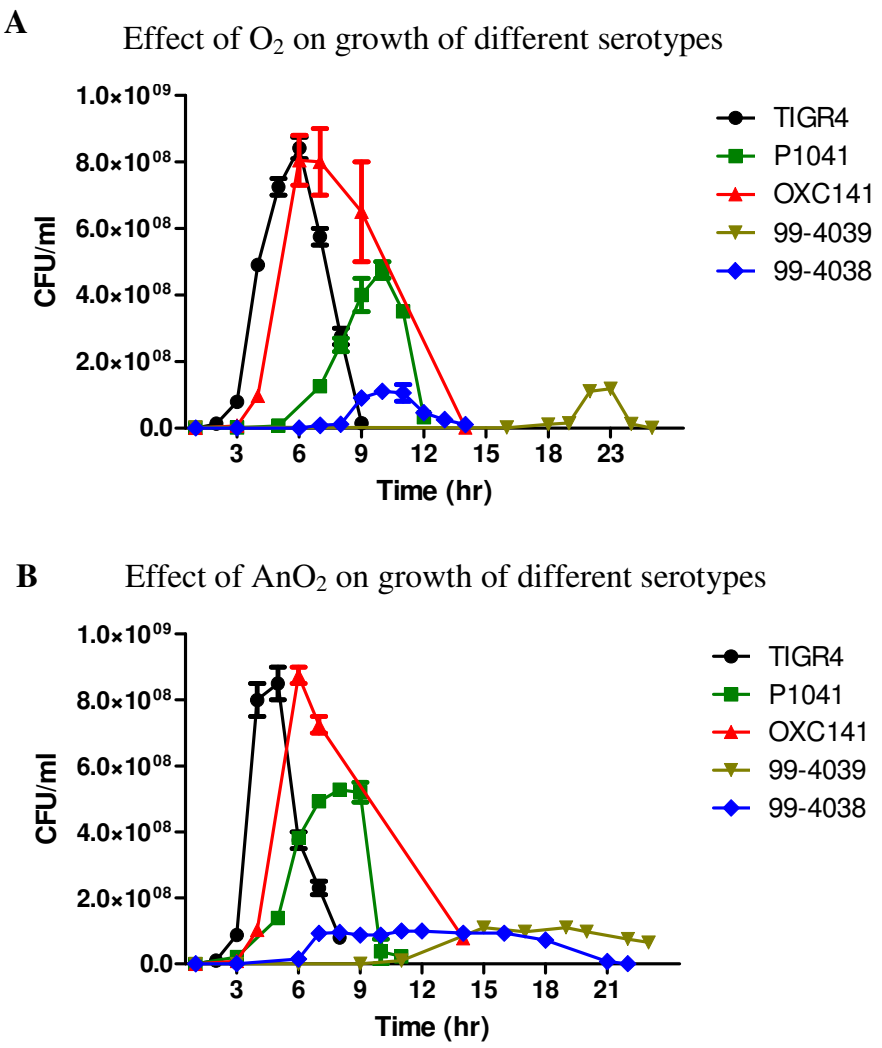


Figure 3-20 Pneumococcal growth under aerobic and anaerobic incubation conditions

Aerobic growth (A) and anaerobic (B). Error bars represent the standard error of the mean using Graph Pad (GraphPad Prism 5, USA 2007).

From the above figure, all serotypes had greater growth rates when oxygen was absent. Pneumococcus has been shown an increase in its growth rate when grown anaerobically (Baettig *et al.*, 2006), and less growth rates were shown with *S. mutans* cells when grown in aerated growth conditions (Ahn *et al.*, 2007). This might relate to effects of H_2O_2 toxicity resulting from the aerobic pneumococcal metabolism (Spellerberg *et al.*, 1996). Taniai and colleagues highlighted that catalase is required in pneumococcal liquid culture to obtain full growth while growing pneumococcus aerobically leads to loss of bacterial viability which can be prevented with the addition of catalase (Taniai *et al.*, 2008). Additionally, it has been stated that exogenous addition or endogenous production of H_2O_2 leads to a decrease in the total growth as chemical

sublimenting of bacterial broths with catalase has prolonged the pneumococcal stationary phase (Regev-Yochay *et al.,* 2007). This was recently supported as serotype 2 pneumococci produced greater levels of H_2O_2 resulting in a reduction in the bacterial growth rate (Carvalho *et al.,* 2013). Furthermore, in other bacteria that have defence mechanisms against oxidative stress such as *E. coli*, catalase along with alkyl hydroperoxidse have been reported to play a complementary role in improving the bacterial growth rates, and protected them from both exogenous and endogenous sources of H_2O_2 (Carvalho *et al.,* 2013). Another possible explanation about these changes in pneumococcal growth patterns could be attributed to their variant genetic content, which may result in bacterial consumption of different carbohydrates and consequently generate different levels of H_2O_2, enabling pneumococci with greater or poorer tolerance.

The figure also demonstrated lag phase differences between pneumococci. For example, the invasive strains 99-4039 and 99-4038 had prolonged lag phases in comparison with a similar serotype OXC141 strain, which is mostly isolated as a coloniser strain. This relationship linking the long lag phase to invasiveness and short phase to colonisation has been reported (Baettig *et al.,* 2006). Moreover, long lag phase has been associated with pneumococcal antibiotic resistance (Mazzola *et al.,* 2003). In line with our results, Mazzola *et al* have also reported that lag phases in pneumococcal clinical isolates generally lasted 2 hr once cells are re-grown in the same media in which their stock inocula have been prepared, and the ranges of their generation times were from 24 to 36 min. Similarly in an earlier study, doubling time was found in serotype 3 pneumococci grown *in vitro* to be 21 min in broth cultures (Tauber *et al.,* 1984a).

Hydrogen peroxide is produced by pneumococci during growth in oxygenated conditions (Spellerberg *et al.,* 1996; Taniai *et al.,* 2008), and lead to their death at the stationary phase as its elimination allows the pneumococcus to carry out a longer stationary phase (Regev-Yochay *et al.,* 2007). H_2O_2 production by *S. pneumoniae* was documented in several pneumococci. Among these is serotype 9V and 2 which produced 2.7 mM and 2.3 mM respectively (Pericone *et al.,* 2002); type 19 (GTC13809 strains) generated approximately 5 mM H_2O_2 during growth in the mid-exponential phase (Taniai *et al.,* 2008).

One of the considerable procedures to scavenge H_2O_2 is to grow the pneumococcus in blood due to the presence of catalase, which is clearly shown in growth curves of isolates representing serotypes 4 and 3, TIGR4 and 99-4038, respectively, as they apparently had greater biomass when BHI cultures were supplemented with blood (Figure 3-11). Consistently, Bae and collaborators compared *S. pneumoniae* gene expression after growing D39 strains with and without blood. They observed global changes in the bacterial protein profile as blood affected up to 414 proteins (Bae *et al.,* 2006). They have stated that this high level of protein variation mainly occurred in the bacterial cellular metabolism such as synthesis of proteins, DNA/RNA metabolism, as well as carbohydrate metabolism. We microscopically examined the pneumococcal morphology during growth with and without blood, and interestingly determined long chains, in 10-20 cell chain formation (Figure 3-11); whereas diplococcal phenotype showed in both aerobic and anaerobic cultures without blood. Early researchers have frequently noticed chain variations in pneumococci (Austrian 1953; Dawson 1934), and GAS chain formation was also found in samples gathered clinically from patients (Ekstedt *et al.,* 1960a; Ekstedt *et al.,* 1960b). Recently, it was reported that long chains facilitated pneumococcal adhesion and colonisation, while short chains were linked to promoting PID (Rodriguez *et al.,* 2012). Consistently, our examination revealed short-chain forms or diplococci under aerobic and anaerobic growth conditions, respectively, but long chains were only noticed during pneumococcal growth in blood.

Pneumococcal growth reduces pH which is counted as one of the possibilities responsible for entering the growth into the death phase, which can result from accumulation of lactic acid from sugar fermentation (Moura Leal *et al.,* 2011). Pneumococcal broth cultures have been shown to decrease the pH causing rapid death when pH reaches below 6.0 (Liberman *et al.,* 2008). This growth cessation is also confirmed with our pH assays that was performed with TIGR4 under three different levels of oxygen. A dramatic drop in growth was obvious once the pH reached 6.0, except in the oxygenated growth cultures where the bacterial population did not grow sufficiently (Figure 3-14). Similarly, *S. aglactiae* was recently found to grow greater at pH 7.0 compared to the growth yield at pH 5.0 (Yang *et al.,* 2012). Moreover, it has been recently stated that a constant pH at 6.5 had a positive effect on pneumococcal growth rate (Carvalho *et al.,* 2013).

Carvalho *et al* detected a 2-fold increase in total biomass of R6 (type 2) pneumococci when pH was under control, but surprisingly D39 showed 64% less growth rate compared to R6 under the same incubation conditions. This growth pH-independence could be attributed to differences in the central metabolism (Carvalho *et al.,* 2013).

Additionally, pH has been linked in a recent investigation to bacterial virulence. The productivity of the pneumococcal key factor CPS was highly increased when compared to bacterial cultivation without pH adjustment (Moura Leal *et al.,* 2011).

Microscopic examination of TIGR4 growing under aerobic and anaerobic environments markedly illustrated bacterial agglutination associated with oxygen availability. In contrast, hypoxic growth environments reflected separate diplococcal cells. This may indicate more pneumococcal ability to form biofilms in oxygen versus its absence. Recently, Bortoni *et al* discovered that Rgg regulator is implicated in both oxidative stress and biofilms. They documented that the loss of its function resulted in pneumococcal sensitivity to oxygen and paraquat, and the ability to form biofilm was largely reduced (Bortoni *et al.,* 2009).

According to Ahn and colleagues, oxygen was negatively involved in regulating biofilms in *S. mutans* (Ahn *et al.,* 2007). In contrast, we detected greater pneumococcal tendency for forming biofilms in oxygen (initial biofilm formation showed as pneumococcal clumps when oxygen is available). If this divergence scenario is true with pneumococci, they may then utilise available oxygen in the nasopharynx to develop biofilms for greater colonisation, which is well known as a major step for IPDs, communication, resisting adverse conditions and elevating growth rate. Another possible hypothesis could be that pneumococci living commensally in the URT exploit oxygen availability to establish biofilms in which the environment is considered to be less oxygenated.

To assess how the pneumococcus TIGR4 genetically reacts to oxygen availability, it was firstly decided to study the effect of oxygen on the whole genome to compare gene expression profiles in the presence and absence of oxygen. This

was through scanning the entire pneumococcal genome by microarrays. This will be discussed in the following chapter (Chapter 4).

Chapter 4 - Effect of Aerobiosis on Pneumococcal Gene Expression

4 Effect of Aerobiosis on Pneumococcal Gene Expression

Most microorganisms including the genera Staphylococci and Enterobacteriaceae are able to defend themselves from oxidative stress; however, S. *pneumoniae* lacks some of the required agents such as catalase, PerR and SoxR to eliminate and regulate ROS generated from pneumococcal aerobic metabolism. Hence, it is still to be completely understood how pneumococci survive and what could be their favoured manipulation strategy during growth in an oxidative environment; colonising human upper airway tracts is a good example.

It is well known that organisms alter their gene expression in response to their surrounding environment, and a comparison of genes expressed under different incubation conditions is highly useful in order to study how genes are regulated in different environments. Consequently, the entire bacterial genome was studied in this chapter to investigate which genes are induced in the presence of oxygen when compared to gene expression during anaerobic environmental growth conditions. Consequently, RNA was collected in the bacterial logarithmic growth phase in both oxygenated and anaerobic growth conditions (Section 2.1.2). Aerated liquid culture was used to mimic the human nasopharynx niche. Therefore, the *in vitro* gene expression might translate the *in vivo* pneumococcal expression when colonising the URT; whereas pneumococcal gene expression in host blood after transmission could be reflected during their growth in oxygen-restricted growth conditions. Moreover, transcription, biological activity, and protein production of the key pneumococcal toxin Ply was evaluated in this chapter using three pneumococcal serotypes, 1, 3 and 4.

4.1 TIGR4 Growth conditions

Three different growth conditions were used primarily for growing S. *pneumoniae*: aerobically, anaerobically and in an oxygenated environment. In the aerobic condition, the liquid cultures used were pre-warmed overnight statically in a water bath (as explained in materials and methods, Section 2.2.1). Similarly, other cultures of the same batch of media were incubated anaerobically, whereas the oxygenated broth cultures were shaken overnight to

expose S. pneumoniae to oxygen in the BHI medium. All these cultures were pre-warmed for 24 hr before starting the experiment, which was considered more than enough time to ensure three targeted points; 1) The medium integrity from any possible source of contamination occurring during preparation. 2) Pre-warming the broth to the pathogen optimal temperature, and 3) Removing the existing oxygen for the anaerobic growth environments. TIGR4 growth patterns under these three different levels of oxygen are shown (Figure 4-1).

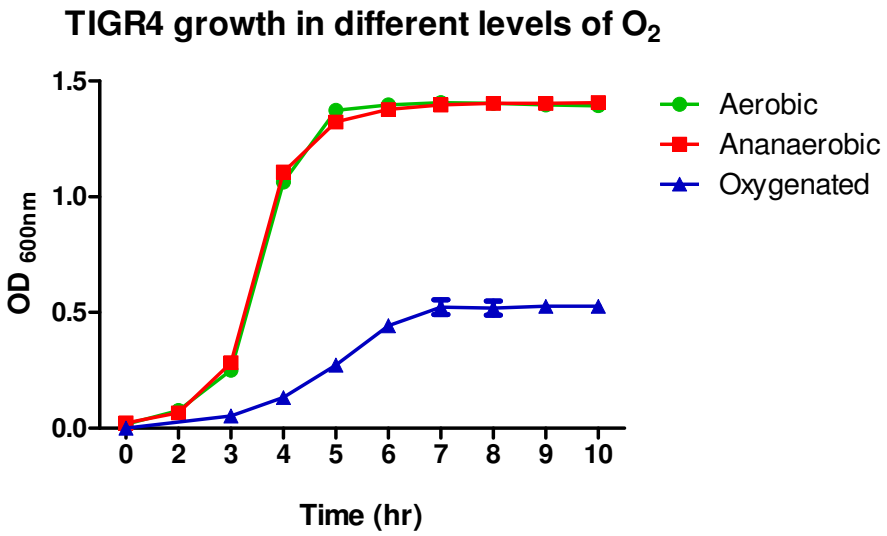


Figure 4-1 TIGR4 growth curves in aerobic, anaerobic and oxygenated environments

Growth curves in three different levels of oxygen are represented by optical density. Under all different growth conditions, the log phase started after three hours. The aerobic and anaerobic environments indicate sharper log phase relative to aerated grown pneumococci which grew significantly weaker (p=0.0454). Averages of at least three independent experiments are presented in each curve. Error bars represent the standard error of the mean. Statistical analysis was performed using a non-parametric Mann-Whitney test in Graph Pad (GraphPad Prism 5, USA 2007).

Growing TIGR4 in the different oxygen concentrations (Figure 4-1) illustrated no significant growth variations between aerobic and anaerobic cultures. In contrast, the oxygenated cells showed a statistically significant reduction (p=0.0454) in the growth rates compared to anaerobic growth. This might reffer to the significant high level of H_2O_2 production during pneumococcal growth in oxygenated states, shown in Chapter 3. It is well known that shaking bacterial growth cultures provides more oxygen concentrations to the medium than incubation in aerobic growth (Baldoni *et al.*, 2010).

These three cultures were grown in order to extract RNA at a specific optical density under all conditions.

4.2 RNA extraction

RNA was extracted from the three conditions used (aerobic, anaerobic and oxygenated) and concentrations were measured by nano-drop, adjusted by dilution to make them all approximately 300 ng/µl; testing the purity of RNA extracts was confirmed using Bioanalyser 2100 (Agilent Technologies, United Kingdom) (Figure 4-2). The figure below illustrates that total RNA extracted under the three different levels of oxygen were sufficient and pure enough for microarray experiments.

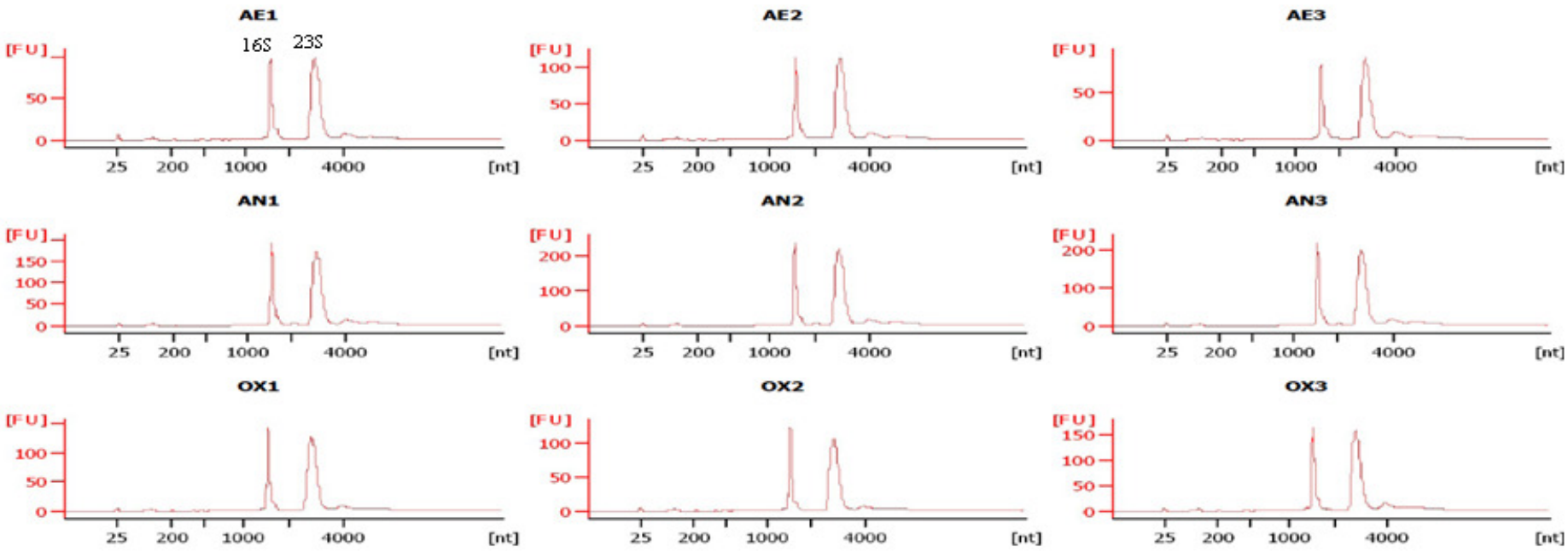


Figure 4-2 Purity of RNA extraction

RNA extraction from aerobic (AE), anaerobic (AN) and oxygenated (OX) cultures have been analysed to nano-drop. FU stands for fluorescent unit, and nt indicates nucleotides. All samples were tested for purity and found sufficient to perform microarrays. 16S and 23S peaks are rRNA, whereas the flat line represents mRNA integrity (Agilent Technologies, United Kingdom).

In light of the fact that the gene for pneumolysin is highly conserved in *S. pneumoniae*, and in order to ensure no DNA was present after extraction of RNA, PCR was carried out to amplify the *ply* gene (Figure 4-3). Lack of a product indicates no DNA was present in any RNA samples.

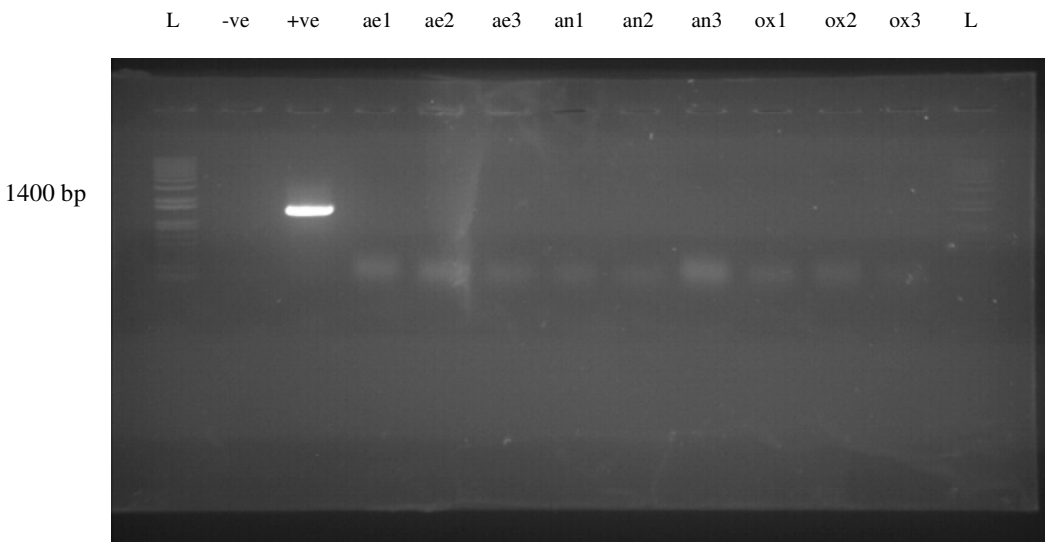


Figure 4-3 PCR of *ply* for confirming absence of DNA

Gel shows a complete absence of DNA in all RNA samples prepared. Positive control is TIGR4 genomic DNA. Negative is the PCR water used in all other PCR samples. ae lanes indicate aerobic, an shows anaerobic and (L) is the DNA size marker in the 1 Kb plus DNA ladder (Life Technologies, United Kingdom).

PCR for all extracts shows - except the positive control – that no *ply* amplification of gene representing pure RNA extracts for microarray experiments.

4.3 Microarray

After extracting RNA from TIGR4 strain, microarray experiments were performed using florescence dyes as explained in materials and methods (Section 2.5.6) in order to label cDNA samples from all conditions, oxygenated, aerobic and anaerobic. Microarray aminosaline slides were prepared by the bacterial microarray group at St. Georges hospital (University of London BμG@S) carrying PCR product for all TIGR4 genes. Hybridisation was then performed on the slides

between probes and microarrays. The slides then were scanned to be visually observed. Moreover, data were normalised due to morphology of the spots. This was achieved using the statistical packages Bluefuse for Microarray 3.5 and Genespring GX 7.3.1.

After normalisation, 420 genes out of 2,336 were found to be differentially expressed under oxygenated conditions compared to anaerobic growth, and 262 genes were shown to be up-regulated during oxygenated pneumococcal growth. Gene expression for aerobic versus anaerobic resulted in 205 differentially expressed genes, whereas oxygenated against aerobic expression profiles showed 67 genes expressed differentially. These are only the significant gene expressions ($p=<0.05$).

4.4 Microarray data analysis

Pneumococcal growth curves showed when comparing oxygenated to anaerobic growth conditions, hence anaerobic and oxygenated conditions were chosen for performing most experiments in the rest of the study. Moreover, gene expression for these two conditions was focused on herein, whereas the other expression profiles (aerobic versus anaerobic and oxygenated versus aerobic) were analysed in more detail in several tables in the appendices.

As explained in materials and methods (Section 2.5.6) the triplicate scanned slides were analysed using the programme Bluefuse for Microarray 3.5 and Genespring GX 7.3.1 giving master list spreadsheets containing expressions of the 2,336 genes under both conditions used (oxygenated and anaerobic). Fold changes were then calculated in these two conditions for each gene and their biological functions were found using CLC Genomics Workbench programme, Comprehensive Microbial Resource (CMR) http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi and Nucleotide BLAST at the National Centre for Biotechnology Information (NCBI). Furthermore, all these genomic analyses were similarly investigated for those genes that expressed differentially during pneumococcal growth in other tested conditions; aerobic/anaerobic and oxygenated/aerobic, as outlined in the appendices. It is worth to mention here

that gene expression less than 2-fold has been reported to be biologically significant (Chaussee *et al.*, 2002).

4.5 Microarray results

A selection of genes that were up-regulated in the presence of oxygenated compared to expression in the absence of oxygen is shown in Table 4-1. Only genes with significant (p=<0.05) changes in expression are listed.

Examples of several key groups were classified in the table and then each section was sorted into ascending order according to gene numbers. The groups include virulence factors, competence, stress and DNA repair, energy metabolism, transporters and amino acid biosynthesis, transcription, translation and hypothetical genes. Hypothetical genes selected in this table are above three-fold over-expression in oxygenated growth environment. A complete gene list is presented in the appendices.

Table 4-1 List of TIGR4 genes significantly expressed in oxygenated versus anaerobic

The table shows gene expression of several functionally classified groups and sorted in ascending order according to the gene numbers; gene expression above 1.5-fold cut-off is listed in the table including virulence factors, competence, stress and DNA repair, energy metabolism, transporters and amino acid biosynthesis, transcription, translation, hypothetical proteins above three-fold over-expression. Genes have been identified by Genespring GX 7.3.1. and their expressions were detected by microarray as either significantly (p=value<0.05) up-regulated (red numbers) or down-regulated (blue numbers) in oxygenic grown cells compared to expression in anaerobic growth conditions. P<0.05 with Benjamini and Hochberg correction applied for multiple testing using 1 way ANOVA test.

Gene	p-value	Fold change in O_2	Annotation
Virulence factors			
SpTIGR4-0532	0.0425	**2.88+**	Bacteriocin, *blpJ*
SpTIGR4-0545	0.0203	**1.93+**	immunity protein, *blpY*
SpTIGR4-0730	0.00752	**3.36+**	pyruvate oxidase,*spxB*
SpTIGR4-1225	0.0179	**1.97+**	vicX protein
SpTIGR4-2190	0.022	**1.50+**	Choline binding protein A (*cbpA/pspC*)
SpTIGR4-2239	0.00704	**2.50+**	serine protease, *htrA*
Competence, stress and DNA repair			
SpTIGR4-0165	0.0402	**1.88+**	Flavoprotein
SpTIGR4-0338	0.00411	**26+**	ATP-dependent Clp protease ATP-binding subunit
SpTIGR4-0515	0.0477	**1.65+**	heat-inducibal transcription repressor, *hrcA*
SpTIGR4-0766	0.0193	**4.17+**	superoxide dismutase, *sodA*

SpTIGR4-0784	0.00411	**3.27+**	glutathione reductase
SpTIGR4-1000	0.00845	**2.37+**	thioredoxin family protein
SpTIGR4-1471	0.03	**3.63-**	oxidoreductase
SpTIGR4-1472	0.01	**4.14-**	oxidoreductase
SpTIGR4-1651	0.00704	**6.96+**	thiol peroxidase, *tpx*
SpTIGR4-1776	0.0365	**2.17+**	Thioredoxin
SpTIGR4-2203	0.0365	**1.63+**	replicative DNA helicase
		Energy metabolism	
SpTIGR4-0324	0.0237	**4+**	PTS system, IIC component
SpTIGR4-0325	0.021	**4.81+**	PTS system, IID component
SpTIGR4-0715	0.0245	**1.71+**	lactate oxidase
SpTIGR4-0875	0.026	**1.77-**	Lactose phosphotransferase system repressor
SpTIGR4-1161	0.0434	**2.66+**	acetoin dehydrogenase complex, E3 component
SpTIGR4-1163	0.00525	**2.29+**	acetoin dehydrogenase, E1 component, beta subunit
SpTIGR4-1164	0.00418	**2.23+**	acetoin dehyrogenase, E1 component alpha subunit
SpTIGR4-1361	0.0425	**1.53+**	homoserine dehydrogenase
SpTIGR4-1415	0.033	**2.41+**	glucosamine-6-phosphate isomerase
SpTIGR4-1587	0.0192	**24+**	antiporter
SpTIGR4-1852	0.00581	**2.47+**	galactose-1-phosphate uridylytransferase
SpTIGR4-1884	0.0265	**2.25+**	trehalose PTS system, IIABC components
SpTIGR4-1894	0.0139	**2.28+**	sucrose phosphorylase
SpTIGR4-2106	0.00562	**3.42+**	glycogen phosphrylase family protein
SpTIGR4-2107	0.0154	**3.50+**	4-alpha-glucanotransferase
SpTIGR4-0199	0.03	**1.82+**	cardiolipin synthetase
		Transporters and amino acid biosynthesis	
SpTIGR4-0044	0.0486	**15.43+**	phosphoribosylaminomidazole-succinocarboxamide synthase
SpTIGR4-0529	0.0327	**1.66+**	BlpC ABC transporter
SpTIGR4-0530	0.0224	**2.14+**	BlpC ABC transporter ATP-binding protein, *blpA*
SpTIGR4-0709	0.0371	**1.51+**	amino acid ABC transporter ATP-binding proten
SpTIGR4-0710	0.0431	**2.73+**	amino acid ABC transporter permease
SpTIGR4-0711	0.0254	**2.89+**	amino acid ABC transporter permease
SpTIGR4-0867	0.00581	**3.87+**	ABC transporter ATP-binding protein
SpTIGR4-0869	0.012	**7.16+**	aminotransferase, class-V
SpTIGR4-1357	0.00562	**1.72+**	ABC transpoter ATP-binding protein/permease
SpTIGR4-1358	0.0344	**1.63+**	ABC transpoter ATP-binding protein/permease
SpTIGR4-1438	0.0431	**2.24+**	ABC transpoter ATP-binding protein
SpTIGR4-1499	0.00451	**2.52+**	bacteriocin transport accessory protein
SpTIGR4-1690	0.0343	**2.20+**	ABC-transporter supstrate-binding protein
SpTIGR4-1717	0.0095	**1.71+**	ABC transpoter ATP-binding protein
SpTIGR4-1860	0.0368	**2.6+**	choline transporter
SpTIGR4-1861	0.0245	**2.82+**	choline transporter
SpTIGR4-1869	0.00664	**2.60+**	iron-compound ABC transporter permease
SpTIGR4-1871	0.0254	**2.43+**	iron-compound ABC transporter ATP-binding protein

SpTIGR4-1872	0.0429	**2.29+**	iron-compound ABC transporter iron-compound-binding protein
SpTIGR4-1895	0.0094	**2.21+**	sugar ABC tranporter permease
SpTIGR4-1896	0.0201	**2.54+**	sugar ABC tranporter permease
SpTIGR4-1897	0.0047	**2.43+**	sugar ABC transporter substrate-binding protein
SpTIGR4-1898	0.00722	**2.51+**	alpha-glactosidase
SpTIGR4-2073	0.0293	**1.58+**	ABC transpoter ATP-binding protein/permease
SpTIGR4-2075	0.00562	**1.76+**	ABC transpoter ATP-binding protein/permease
SpTIGR4-2084	0.014	**1.88+**	phosphate ABC transporter phosphate-binding protein
SpTIGR4-2085	0.0398	**2.08+**	phosphate ABC transporter permease
SpTIGR4-2086	0.0236	**2.09+**	phosphate ABC transporter permease
SpTIGR4-2088	0.0357	**2.10+**	phosphate transporter system regulator protein, *phoU*
Transcription			
SpTIGR4-0046	0.0414	**13.82+**	amidophosphorobosyltransferase
SpTIGR4-0330	0.00704	**1.74+**	suger binding transcriptional regulator, *regR*
SpTIGR4-0716	0.0444	**2.10+**	Transcriptional regulator
SpTIGR4-0798	0.0302	**1.56+**	DNA-binding response regulator, *ciaR*
SpTIGR4-0799	0.0151	**1.60+**	sensor hitidine kinase, *ciaH*
SpTIGR4-1226	0.00581	**2.12+**	sensory box sensor histidine kinase
SpTIGR4-1227	0.0047	**2.21+**	DNA-binding response regulator
SpTIGR4-1809	0.0223	**1.87+**	transcriptional regulator
SpTIGR4-2192	0.0119	**1.65+**	sensor hesitidine kinase
SpTIGR4-2193	0.0119	**1.51+**	DNA-binding response regulator
Hypothetical proteins above three-fold over-expression			
SpTIGR4-0095	0.00688	**36+**	hypothetical protein
SpTIGR4-0868	0.0156	**5.92+**	hypothetical protein
SpTIGR4-1546	0.0119	**4.41+**	hypothetical protein
SpTIGR4-2054	0.0281	**3.90+**	hypothetical protein
SpTIGR4-2017	0.00411	**4.34+**	hypothetical protein
SpTIGR4-2187	0.0363	**10.33+**	hypothetical protein

As can be seen in Table 4-1, a number of key genes were up-regulated significantly in oxygenated conditions compared to anaerobic growth with a p-value less than 0.05. Over-expressed genes included several TCSs that are linked to pneumococcal pathogenicity such as TCS02 and TCS05. Other virulence-related genes were also up-regulated in oxygenated relative to anaerobic growth such as *blpR*, *blpJ*, *blpY*, *blpU*, *vicX*, *htrA* and *spxB*. HtrA and SpxB are involved in oxidative stress response. Expressions of several other *S. pneumoniae* stress related genes such as thiol peroxidase, thioredoxin family protein, thioredoxin reductase, glutathione reductase, flavoprotein and superoxide dismutase have been shown to be induced by oxygenated environment, suggesting that the

incubation condition used in this study were properly established. Thiol peroxidise was significantly elevated, 7-fold higher when oxygenated, compared to anaerobic growth (master lists for all gene expression are shown in the Appendices, Table A-1, A-2, A-3 and A-4).

In *S. mutans*, TCS02 (Vic) loss of function was reported to alter a number of vital streptococcal stages such as growth, adhesion, biofilm formation and development of competence (Senadheera *et al.,* 2005). Additionally, a number of bacteriocins were similarly induced, which may indicate greater pneumococcal ability to compete with other microflora by inhibiting their growth. Pneumococci generating this toxicity have been shown to be protected from the toxin effect (Dawid *et al.,* 2007).

Energy metabolism and related transporters were significantly increased in oxygenated conditions compared to anaerobic grown pneumococci. Among these are several PTSs such as IIC, IIA and IID, amino acid ABC transporter permeases, ABC transporter ATP-binding proteins including iron and sugar transporters. Similarly, a vast number of amino acid ABC transporters were elevated during TIGR4 oxygenated growth. Notably, the gene SP1587 (transporter) was over-expressed 24-fold when oxygenated growth, and the anaerobic array largely showed a significant down-regulation for the same gene (Appendices, Table A-3). It encodes a major pneumococcal antiporter which acts as a transporter facilitator for energy metabolism (Orihuela *et al.,* 2004b), reflecting high levels in the bacterial physiology during growth in oxygen. PTS expression was similarly reported to be induced in oxygenated growth conditions by Bortoni and co-researchers (Bortoni *et al.,* 2009). Bortoni *et al* (2009) also detected an interesting gene with an induction in oxygen compared to anaerobic growth condition known as a regulator called *rgg* (SP2123). Our data did not show this gene, possibly because of different pneumococcal broth cultures with oxygen. Data analysis of our array results revealed that flanking genes were up-regulated in oxygenated growth conditions versus anaerobic (Table A-4, in the Appendices).

Findings of stress response from this study were generally in agreement with Bortoni *et al.,* (2009). Examples of genes induced when pneumococci were grown in oxygenated experiment are seen in several hypothetical genes such as SP0095, SP2017, SP0731, SP0992, and SP1802, a number of ABC transporter ATP-binding proteins, Bgl (Table A-4, in the Appendices), PTS, superoxide dismutase and thiol peroxidise.

Interestingly, several hypothetical genes were significantly over-expressed in oxygenated relative to bacteria grown anaerobically. For example, SP2187, SP2054 and SP0095 were induced 10.33, 3.90 and 36-fold, respectively, indicating their roles in response to oxygen. As determined after accessing and performing genome comparison on web sites such as BLAST at NCBI http://www.blast.ncbi.nlm.nih.gov/Blast.cgi, SP0095 is a transferase, contributing to detoxification in *S. pneumoniae* AP200, 19A and 19F. SP2054 is an antioxidant in 19A. Likewise, analysing the array data for TIGR4 cells grown anaerobically also confirm the same tendency of the expression of three genes as they were significantly under-expressed in the anaerobic growth conditions (Table A-1 and Table A - 3, Appendices).

Comparing data from this study with Bortoni *et al* (2009) for those genes that were under-expressed in oxygenated conditions was also relatively comparable. Among these is *pfl* SP0459 which codes for Pyruvate Formate Lyase (PFL) (Table A – 2, Appendices) and oxidoreductase enzymes such as SP1472 (Table A – 3, Appendices). These oxidureductases were also up-regulated in anaerobic environments which confirmed their detected expression in the oxygenated versus anoxygenated growth conditions (Appendices).

Transcriptional genes reflect an increase in oxygenic conditions such as amidophosphoribosyltransferase, transcriptional regulators and phosphoribosylaminomidazole-succinocarboxamide synthase.

ATP-dependent Clp protease was significantly over-expressed (26-fold) in oxygenated versus anoxic incubation conditions. Additionally, proteases (HtrA is a good example) are generally known as vital factors for degrading damaged

proteins resulting from cells grown in stress conditions (Clarke 1999). Clp family members have been reported to be involved in the pneumococcal response to temperature (heat shock proteins), adhesion *in vitro*, play a role in genetic transformation, and most importantly *clpC*-deficient mutant was not able to express pneumolysin or a number of CBPs including PspC (Charpentier *et al.*, 2000). Charpentier *et al* also showed that the mutants were defective in the nasopharyngeal colonisation and survival in the LRT *in vivo* post-inoculation intranasally. ClpP was recently shown to respond particularly to oxidative stress (Park *et al.*, 2010).

The cell surface protein (choline-binding protein) PspC, two choline transporters (SP1860 and SP1861), and cardiolipin synthetase involved in fatty acid metabolism (found in CLC programme and was reported by Orihuela *et al.*, (2004) that were elevated by oxygenated conditions rather than in their anaerobic expression.

mRNA levels for a number of genes (listed below in Table 4-2) were then measured using qRT-PCR in order to confirm the expression under the oxygenated and anaerobic incubation environments.

4.6 qRT-PCR for a selection of genes

Among the data obtained from the array experiments and because of some of the known key virulence factors, a selection of genes were chosen in order to confirm their gene expression. The selection was only from those which had significant differential expression ($p<0.05$). Moreover, based on the array data listed in Table 4-1, it was decided that due to the fact that main regulatory pathways in pneumococci are believed to be TCSs, it would be interesting to study their gene expression with a more sensitive technique, qRT-PCR. The selection of the genes confirmed by qRT-PCR is listed below in Table 4-2.

Table 4-2 List of genes for qRT-PCR

Genes selected for qRT-PCR to confirm their expression. These include several TCSs, a housekeeping gene, *ply*, *pspC*, PI-1 proteins and *tpx*. All will be shown in more detail next in Chapter 5. P<0.05 with Benjamini and Hochberg correction applied for multiple testing using 1 way ANOVA test.

TIGR4 Gene		Product	Biological function	References
1	SP_1227	TCS02	Key for life, involves in virulence and competence	(Lange *et al.*, 1999)
2	SP_0387	TCS03	Involves in stress response	(Haas *et al.*, 2005; Song *et al.*, 2009)
3	SP_2082	TCS04	Contributes to virulence & involves in oxidative stress	(McCluskey *et al.*, 2004b)
4	SP_0798	TCS05	Linked to HtrA, colonisation, antibiotic and stress response	(Paterson *et al.*, 2006; Sebert *et al.*, 2002)
5	SP_2193	TCS06	Involved in virulence, regulates PspC	(Ma *et al.*, 2007; Standish *et al.*, 2005)
6	SP_0083	TCS08	For survival *in vivo*	(Throup *et al* ., 2000)
7	SP_2000	TCS11	Stress response	(Haas *et al.*, 2005)
8	SP_2235	TCS12	Competence (*ComE*)	(Iannelli *et al.*, 2005)
9	SP_0526	TCS13	Bacteriocin-like peptide (*blpR*)	(de Saizieu *et al.*, 2000)
10	SP_0376	rr orf	Iron repressor (*ritR*)	(de Saizieu *et al.*, 2000)
11	SP_1219	GyrA	Housekeeping gene	(Johnson *et al.*, 2005)
12	SP_1923	Ply	Multi-functional toxin	(Berry *et al.*, 1989; Steinfort *et al.*, 1989)
13	SP_2190	PspC	Adhesion to host cells	(Ogunniyi *et al.*, 2007)
14	SP_0461	RlrA	Pilus islet	(Basset *et al.*, 2011)
15	SP_0462	RrgA	Pilus adhesion	(Kreikemeyer *et al.*, 2011)
16	SP_0463	RrgB	Pilus backbone	(Kreikemeyer *et al.*, 2011)
17	SP_0464	RrgC	Pilus structuring	(Kreikemeyer *et al.*, 2011)
18	SP_0466	SrtB	Pilus assembling	(Kreikemeyer *et al.*, 2011)
19	SP_0467	SrtC	Pilus assembling	(Kreikemeyer *et al.*, 2011)
20	SP_0468	SrtD	Pilus assembling	(Kreikemeyer *et al.*, 2011)
21	SP_1651	Tpx	Anti-oxidant	(Hajaj *et al.*, 2012)

mRNA levels for the qRT-PCR selection shown in the above table (21 genes) will be discussed in the next chapter (Chapter 5). They are listed here to highlight the selected genes from the array data analysis and other interesting virulence related genes.

4.6.1 Pneumolysin

Here, the expression of *ply*, its production and lytic activity are considered examples of the key virulence gene in pneumococci. To choose the best cDNA concentration for the qRT-PCR reactions, *ply* was tested after preparing three serial dilutions; neat, 1:10 and 1:50. Their associated controls, which had no reverse transcriptase, were similarly diluted.

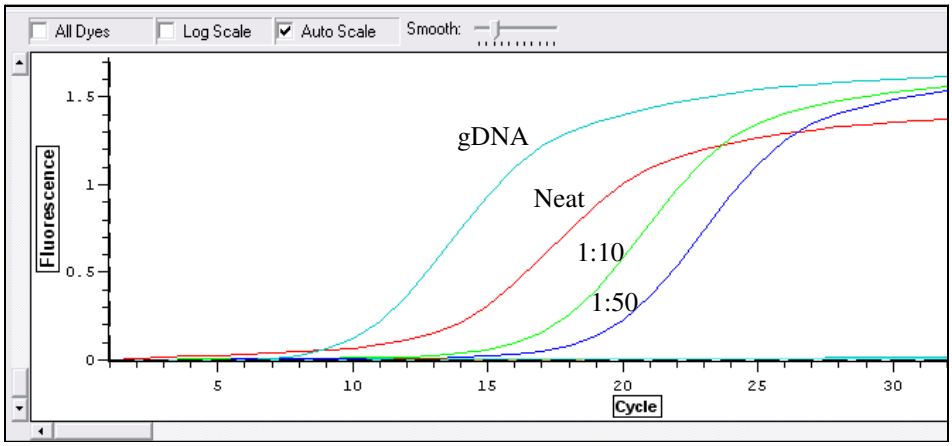


Figure 4-4 qRT-PCR for different concentrations of cDNA

Several dilutions performed after cDNA preparation for detecting the best concentration before using qRT-PCR. The tested dilutions of 1:50 (the blue curve) were the choice as it was between 15 and 25, in the middle. The green curve represents the 1:10 dilution and showed higher levels of mRNA, whereas the neat (its original concentration in the reaction was ng/µl from which 1µl was taken when the above two concentrations were prepared, 1:10 and 1:50) had the highest concentration (the red curve). The top curve was the positive control used, TIGR4 gDNA. qRT-PCR data analysis was performed with MJ OpticonMonitor version 3.1. Each curve represents the mean of triplicate readings.

Once the best concentration was detected after performing qRT-PCR for three serially diluted samples (Figure 4-4), the dilution factor 1:50 was used to prepare all of the other qRT-PCR samples, listed in Table 4-2, which have been recovered from different pneumococcal growth conditions, oxygenated and anaerobic.

qRT-PCR was performed to confirm any differences in the pneumolysin mRNA levels when oxygenated compared to absence of oxygen. Its level was 2.081-fold greater when the clinical isolate TIGR4 was grown in oxygenated versus anaerobic conditions (Figure 4-5).

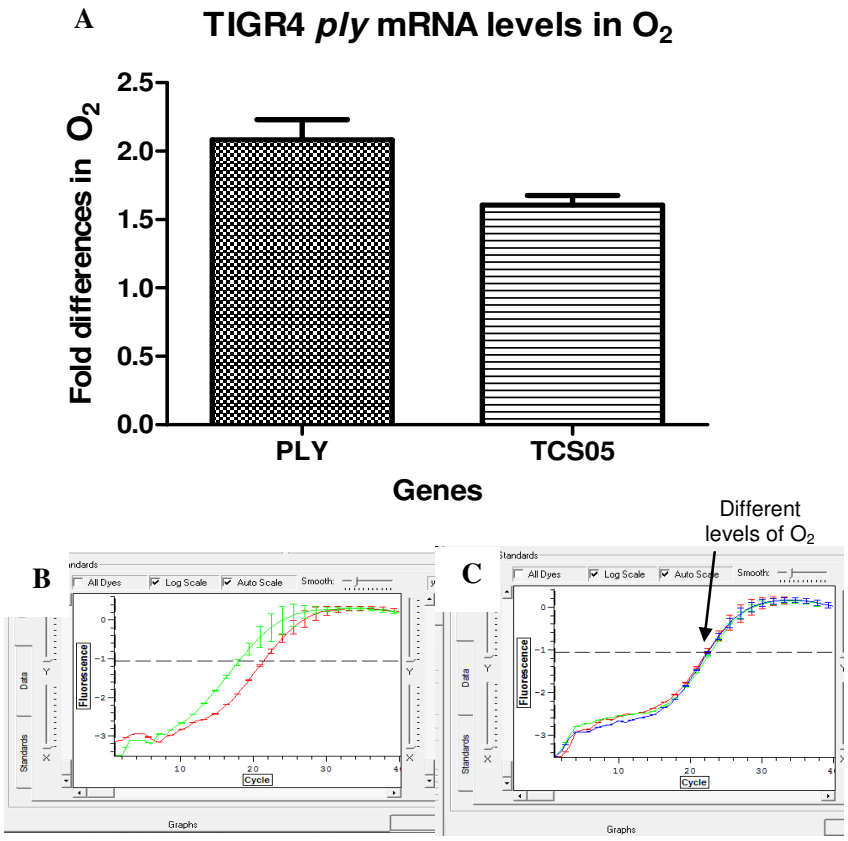


Figure 4-5 qRT-PCR for of *ply* mRNA under oxygenated and anaerobic growth

Pneumolysin fold changes in oxygenated and TCS05, which is known as a stress responder, compared to pneumolysin mRNA levels under the same growth conditions (A), *ply* mRNA averages in aerated and anaerobic environments (B), the green curve represents the mean of triplicates of mRNA levels detected in oxygenated, whereas the red curve shows gene expression during the anaerobic growth condition. The housekeeping gene - *gyrA* was used to be reffered to, and the arrow shows curves with no changes in expression in the three conditions; aerobic, anaerobic and oxygenated (C). The negative control used for all was a sample without the addition of reverse transcriptase, whereas TIGR4 gDNA was used as the positive control. Data analysis was performed with MJ OpticonMonitor version 3.1. and Graph Pad (GraphPad Prism 5, USA 2007) for qRT-PCR analysis.

4.6.1.1 SDS-PAGE and Western Blotting of Ply in TIGR4

To investigate the translation of the pneumolysin gene into protein in *S. pneumoniae* TIGR4, cultures were compared using Western blot analysis performed on cell lysates gathered after growing the pathogen in both environmental conditions, oxygenated and anaerobic. The finding revealed that the pneumolysin intensity was clearly increased in the oxygenated sample compared to the anaerobic one (Figure 4-6).

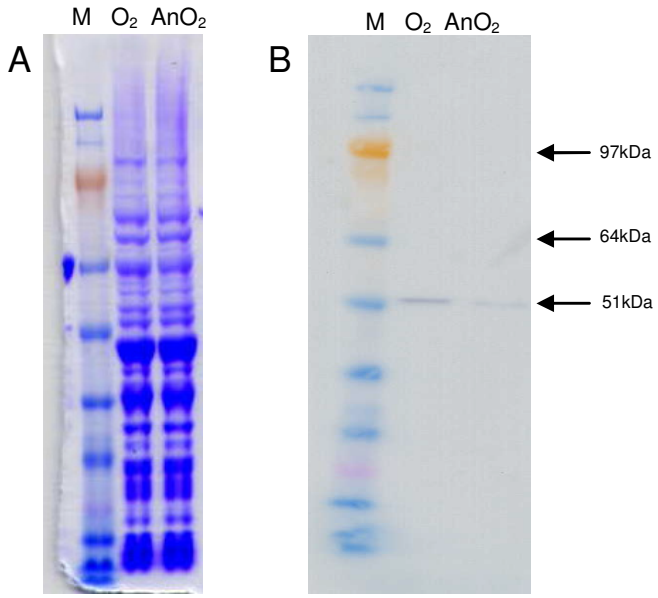


Figure 4-6 Western blot of Ply from oxygenated and anaerobic grown TIGR4

SDS-PAGE for oxygenated and anaerobic bacterial lysates grown until log phases (A) and Western immunoblot (B) of pneumolysin from a corresponding concentration of the bacterium supernatants. A higher intensity of Ply was detected in oxygenated grown cells compared to anaerobically recovered samples (B). The arrow points to Ply with its known molecular weight recognised by the antibodies used. M indicates the marker used (SeeBlue®Plus2, invitrogen, Scotland).

Translational investigation by immunoblot (Figure 4-6) confirms the transcriptional stage seen in qRT-PCR data (Figure 4-5). Both stages proved an enhancement in the protein Ply in oxygenated conditions compared to anaerobic environmental growth.

In light of previous findings, the post-transcriptional step was studied to clarify the protein biology. For example, detecting the haemolytic activity of the toxin pneumolysin after growing *S. pneumoniae* TIGR4 under both levels of oxygen, aerobic and anaerobic.

4.6.1.2 Biological activity

Under the aerobic conditions, there was an increase in levels of pneumolysin as shown with Western Blot (Figure 4-6); therefore, we hypothesised whether or not the lytic activity would increase similarly under the effect of oxygen versus

the anaerobic conditions. Figure 4-7 shows that the biological lytic activity was obviously increased in the aerobic growth conditions.

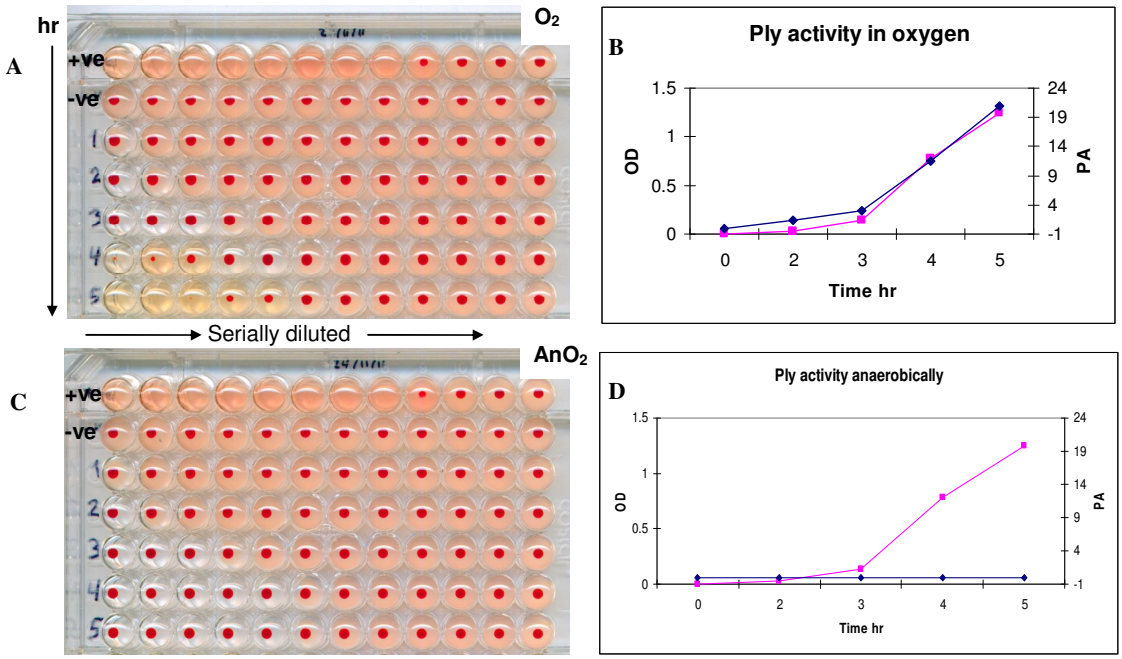


Figure 4-7 Haemolytic assay of TIGR4 Ply from aerobic and anaerobic grown cells

Cells were grown under aerobic and anaerobic conditions until log phases at 37°C, lysed and serially diluted. Samples collected from aerobic growth at different time-points (A), haemolytic activity during bacterial growth in the presence of oxygen, OD represented with a red line shows optical dencity, wheras the blue line PA is the pneumolycin activity (B), haemolytic activity anaerobically at the same time-points, OD represented with a red line shows the bacterial optical dencity, wheras the blue line PA which stands for pneumolycin activity (C) and haemolytic activity during bacterial growth in the absence of oxygen (D). Positive control was a purified pneumolysin. Negative control is D39 Δ*ply*. OD means optical density and Ply stands for pneumolysin. Numbers in the Y axis in the graphs (B and D) are estimated to show the haemolytic activities.

The above figure shows assays of horse red blood cells which were lysed after 4 hr during the growth curve (log phase) once the bacterial population of *S. pneumoniae* reached approximately $1x10^8$ CFU/ml, corresponding to the optical density of around 0.35 of OD_{600} (Figure 4-7, A and B). The anaerobic bacterial growth showed a complete absence of the pneumococcus TIGR4 lytic activity at the same time-points and bacterial population of the oxygenated grown bacteria (Figure 4-7, C and D).

Interestingly, early studies have shown that Ply is oxygen-labile (Smyth *et al.*, 1978). Therefore, one could expect that Ply will lose its activity in the presense

of oxygen. However, haemolytic assays were performed directly with adding DTT which is known as a reducing agent leads to retain all proteins in their reduce state.

In the context of these experiments, it was not clear whether the anaerobic collected pneumolysin is not fully active or it just was not expressed at high levels. To practically do this, purified Ply could be run on a gel after preparing several dilutions to get a similar amount to the anaerobic band as in Figure 4-6. Then, a similar amount can be tested with a haemolytic assay to determine if it is able to lyse RBCs.

However, whatever the reasons are, Figure 4-7 shows that in terms of total haemolytic activity, anaerobic grown pneumococci were not capable of showing any haemolysis, either due to not producing a sufficient amount of the toxin or the toxin was not active. Another possible explanation is that the bacterium itself may express greater *ply* in the URT (where O_2 concentration is close to the natural atmospheric level) to facilitate either host invasion through tissue damage or to obtain more nutrients. Then, pneumococci might accidently spread to the bloodstream and down-regulate *ply* expression as the circulation system contains less oxygen. This hypothesis is supported with a number of experiments including *ply* expression confirmed by qRT-PCR as seen in Figure 4-5, immunoblot of Ply in Figure 4-6 and the Ply haemolytic activity.

We wondered then how Ply in other pneumococcal serotypes would appear on Western blot membrane if the cells have been grown in the same conditions, oxygenated and anaerobic.

4.7 Western blot of Ply in other serotypes

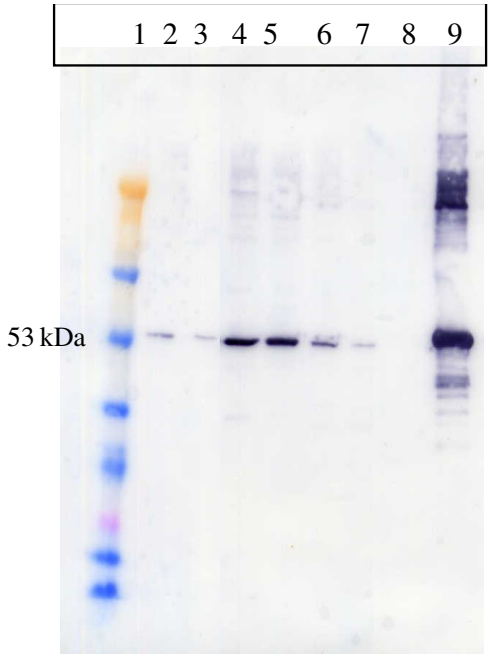


Figure 4-8 Western blot of Ply gathered from oxygenated and anaerobic growth of different bacterial serotypes

Immunoblot membrane of pneumococcal lysates grown in oxygenated and anaerobic growth conditions until the exponential phases. Lane 1: marker (SeeBlue®Plus2, invitrogen, Scotland), 2: Ply detected from oxygenated growth of TIGR4, 3: anaerobic TIGR4, 4: oxygenated P1041, 5: anaerobic P1041, 6: oxygenated OXC141, 7: anaerobic OXC141, 8: PBS with the loading buffer used as a negative control and 9: positive control of purified pneumolysin. There were higher protein intensities of Ply detected in oxygenated grown cells compared with anaerobically collected samples in TIGR4 and OXC141, type four and three, respectively. In contrast, the serotype one isolate P1041 had similar protein produced under both oxygenated and anaerobic growth conditions. The 53kDa illustrated Ply known molecular weight has been successfully recognised with the antibodies used.

The immunoblot for the toxin pneumolysin has shown that the protein may be differently produced in different types of pneumococcal isolates collected after growing under the presence or absence of oxygen. The strains TIGR4 (type 4) and OXC141 (type 3) demonstrated greater toxin intensity after recovering samples from the oxygenated grown cells in comparison to anaerobically grown bacteria (Figure 4-8), whereas the type 1 organisms P1041 showed greater homology of Ply intensity gathered form both cells grown under oxygenated and anaerobic growth environments. One of the possible reasons for these variations when reacting to oxygen is a difference in the gene sequence. To evaluate this, an alignment of the *ply* genes from the different serotypes presented in Figure

4-8 was performed using the CLC Genomics Workbench programme. However, it was found that the majority pneumococci had very similar *ply* coding sequences with similar predicted molecular weights.

4.8 Discussion

TIGR4 growth rates had a significant reduction during exposure to oxygenated compared to anoxygenated environmental conditions (Figure 4-1). As explained in the previous chapter, insufficient biomass in high oxygen levels was reported to be due to pneumococcal production of H_2O_2 which has been shown to be associated with their growth in oxygen availability (Spellerberg *et al.,* 1996). In addition, genetic absence of catalase in pneumococci largely contributes to pneumococcal failure to scavenge H_2O_2 (Pericone et al 2002), as removing H_2O_2 from the bacterial growth cultures escalated the total growth rate in *S. pneumoniae* (Regev-Yochay *et al.,* 2007; Taniai *et al.,* 2008).

Microarray analysis of *S. pneumoniae* TIGR4 gene expression during growth in oxygenated and anaerobic incubation conditions has shown a massive number of phenotypic differences. In total, there were 420 genes of 2,336 (almost 18% of the whole TIGR4 genome) differentially expressed in aerated versus anoxic growth environments. In the oxygenated growth forms, 262 genes were up-regulated (11%) indicating the magnitude of roles of oxygen on the pneumococcal gene profile, whereas 158 genes were down-regulated (representing 7% of TIGR4 genes) during anaerobic growth conditions. Effect of oxygen was also shown to alter the *S. mutans* genome. Its gene profile was differentially expressed by roughly 5% when bacteria were grown in aerated growth conditions (Ahn *et al.,* 2007). Moreover, a recent study described that oxygen availability in the mucosal surface could modulate virulence in *Shigella*, and the investigators showed that oxygen molecules were responsible for activating the bacterial virulence (Marteyn *et al.,* 2010). Furthermore, an early investigation of possible oxygen influences on *S. aureus* pathogenicity determined that staphylococcal growth in aeration conditions was associated with an increase in the bacterial ability to develop abscess (Schmidt *et al.,* 1967). Additionally, virulence of GBS was modulated with oxygen molecules as the streptococcal invasion in animal models was optimal when bacteria were grown in oxygen, but they showed a significant reduction in virulence without growth in oxygen (Johri *et al.,* 2003). They also concluded that bacterial exposure to oxygen could be a strong signal to induce GBS invasiveness.

Some key virulence factors and related genes including *ply*, *pspC*, *blp*, *spxB* and *htrA* were up-regulated in oxygenic conditions relative to hypoxic. *In vivo* studies have documented variation in pneumococcal gene expression from diverse host ecological niches in which oxygen levels are known to be different. Therefore, comparison between *in vivo* gene expressions using *S. pneumoniae* is outlined in Table 4-3. Similar to our data, expression in nasopharynx or during *in vitro* adhesion assays is generally considered to be associated with oxygenated environments, while *in vivo* sites such as blood circulation system and CSF are supposed to have limited levels of oxygen.

Table 4-3 Comparison between pneunococcal expression in different environments

References of studies undertook *in vivo* gene expression are Ogunniyi *et al*., (2002); Orihuela *et al*., (2004) and (Oggioni *et al*., 2006) (their reference was *in vitro* gene expression), LeMessurier *et al*.,(2008), Mahdi *et al*.,(2006), and pneumococcal gene expression in aerobiosis/anaerobiosis conditions (Bortoni *et al*., 2009). *cps*: capsular polysaccharide, *com*: competence, *pspC*: pococcal surface protein C, *htrA*: heat temperature requirement A, *tpx*: thiolperoxide, *spxB*: streptococcus pyruvate oxide B, *sodA*: soper oxide dismutase, NS* indicates not shown, G.R is Glutathione reductase, ECC stands for epithelial cell contact, NP means nasopharynx, CSF is cerebro-spinal fluid, AnO_2: anaerobic and O_2 indicates oxygenated conditions. *** P<0.05 with Benjamini and Hochberg correction applied for multiple testing using 1 way ANOVA test.**

Genes and studies		Expression in nasopharynx (NP) or epithelial cell contact (ECC)	Expression in blood or CSF	Expression from our findings (p <0.05) ***
Gene	Reference			
ply	Ogunniyi	NS*	+3 in blood	
	Orihuela	-9.8 in ECC	-5.6 in CSF	
	LeMessurier	Greater in NP vs blood	Less in blood vs NP	+1.22 in O_2
	Mahdi	Greater vs blood	Less in blood vs NP	
	Oggioni	+0.2 in lung	+1.3 in blood	
pspC	Ogunniyi	NS	In blood as *in vitro*	
	Orihuela	+2.3 in ECC	NS	
	LeMessurier	Greater in NP vs in blood	Less in blood vs NP	+1.50 in O_2
	Mahdi	Up-regulated vs blood	Down-regulated	
	Oggioni	+1.2 in lung	+1.8 in blood	

cps			
cps2A Ogunniyi	NS	+4 in blood	
cps4A Oggioni	+1.6 in lung	+1.2 in blood	
cps4A Orihuela	+2.2 in ECC	NS	-1.21 *cps4F* in O_2
cps4C Orihuela	NS	3.9 in CSF	-1.25 *cps4J*
cpsA LeMessurier	NS	NS	-1.21 *cps4K*
cpsA Mahdi	Greater in NP vs blood	Less vs NP	
comE Orihuela	NS	-15.0 in CSF	
Oggioni	+4.1 in lung	+1.3 in blood	+1.45 in O_2
comF Bortoni	+12 O_2 vs AnO_2	NS	
G.R** Orihuela	NS	-2.2 in CSF	
Bortoni	+8 in O_2 vs AnO_2	NS	+3.27 in O_2
htrA Orihuela	8.2 in ECC	NS	+2.50 in O_2
tpx Bortoni	+12 O_2 vs AnO_2	NS	+6.69 in O_2

spxB	Orihuela	NS	-2.6 in CSF	
	LeMessurier	Up-regulated in NP	Down-regulated in blood	+3.36 in O_2
	Mahdi	Up-regulated in NP	Down-regulated in blood	
sodA	Bortoni	+8 O_2 vs AnO_2	NS	
	Orihuela	NS	-3.5 in CSF	+4.17 in O_2
	Oggioni	+13.9 in lung	+1.5 in blood	

There were a number of DNA related genes found up-regulated in oxygenated conditions when compared to anoxic growth conditions. Examples for these are DNA polymerase (SP_0032), DNA repair (SP_0036), flavoprotein (SP_0165) and DNA helicase (SP_2203). These are known for their responsibility in repairing damaged DNA. Therefore, it could be that their oxygenated induction was to repair and replace damage in pneumococcal nucleic acids resulting from potential H_2O_2 (Mostertz *et al.,* 2003).

Gene expression of several genes involved in metabolism such as carbohydrate transporters (ABC and PTS) that are responsible for nutrient uptake, and radicals removal such as thioredoxin and efflux protein have been found elevated in oxygenated conditions, indicating the greater metabolic energy is required in this growth environment versus anaerobic. This induction supports importance of carbohydrate transporters´ role in oxidative stress response. Consistent with this, expression of certain transporters was greatly increased in *S. mutans* during aerobic growth relative to absence of oxygen (Ahn *et al.,* 2007). We also showed an oxygenated over-expression (up to five-fold) in several PTSs, which is in agreement with another recent related study (Bortoni *et al.,* 2009). It is worthy to mention here that Bortoni et al have shown less gene cohort comparing to our study. This may be attributed to methodology and statistical analysis used in both studies. They provided the pneumococcal cutures with filtered pubbules to generate oxygen, whereas pneumococci were exposed to oxygen in our study by shaken methods. Furthermore, they selected genes which were over-expressed above 2-fold, but we considered expression changes below 2-fold as biologically significant according to what Chaussee et al reported (Chaussee *et al.,* 2002).

Other important genes (SP1869, SP1871 and SP1872) encoding several iron transporters were similarly induced in oxygenated conditions. However, this expression was in contrast to Bortoni and co-workers (2009), as they demonstrated a reduction in iron transporters when oxygenated relative to anaerobic growth conditions, which could be attributed to strain differences used in the two studies. Consistent with the results presented in our work, Mahdi *et al* and other studies have measured mRNA levels in pneumococci recovered from infected animal models. They found that *piaA* gene, an iron transporter, showed variations in the gene transcription between different strains, but it was generally up-regulated in the nasopharynx where oxygen is high as compared to

the blood collected samples (Mahdi *et al.*, 2008; LeMessurier *et al.*, 2006). The authors have also reported another example of genes variation from using different strains. They stated that although *spxB* was largely elevated in the brain for WCH16, its transcription was opposite in the same environmental niche for the strain WCH43.

In fact, iron is well known as a fundamental growth factor for pneumococci as well as for other bacteria and it could be that pneumococci require iron irrespective of the environmental conditions they grow in. This might be explained by other studies which illustrate that *piaA* in pneumococci was up-regulated in the nasopharyngeal niche (LeMessurier *et al.,* 2006; Mahdi *et al.,* 2008), and that may translate the magnitude of the pneumococcal needed for iron consumption as a survival factor even if this metal increases oxidative stress.

Most importantly, the hypothetical proteins SP2187 and SP0095 showed tremendous increase in expression, 10.33-fold and 36-fold respectively, suggesting their fundamental roles in oxidative stress defence in *S. pneumoniae* TIGR4. Genomic analysis using CLC Genomics Bench Work programme revealed that both flanking genes are also hypothetical proteins.

Clp protease was over-expressed in our data analysis with 26-fold in oxygenated conditions, and its key role in degrading damaged proteins has been well documented in *E. coli* (Gottesman 1996). In bacteria and higher plants, Clp proteases are mainly for degrading misfolded and damaged proteins as a result of stress-fluctuation due to adverse environments (Clarke 1999). Further investigation in pneumococci has illustrated that both ClpP and HtrA are involved in responding to oxidative stress and virulence (Ibrahim *et al.,* 2005). Moreover, Clp proteases were reported in pneumococci to play critical roles in oxidative stress response (Charpentier *et al.,* 2000; Park *et al.,* 2010). Similar to our findings, ClpB protease and virulence attributes such as bacteriocins in *S. mutans* were profoundly induced in oxygenated versus anaerobic growth conditions (Ahn *et al.,* 2007). In addition, Charpentier *et al* stated that pneumococcal deficiency in ClpC has resulted in an inability to express several virulence factors including the two important determinants Ply and PspC (Charpentier *et al.,* 2000). Furthermore, *clpP* mutants were unable to colonise

mice nasopharynx, did not survive in the lungs after intranasal challenge, were cleared faster by macrophages cell line and the bacterial virulence was attenuated (Kwon *et al.,* 2004).

The cytolytic Ply was produced differentially between serotypes grown in different levels of oxygen (Figure 4-8). Unlike other tested types, serotype 1 pneumococci (ST217) expressed similar amounts of toxin under both growth conditions. It seemed that oxygen does not regulate their Ply production. However, it is not known whether the haemolytic activity is active or inactive after pneumococcal growth in the presence/absence of oxygen. These type 1 representatives are hyper-virulent (Harvey *et al.,* 2011b; Williams *et al.,* 2012), and it might be able to develop severe manifestations in all environments regardless of oxygen availability. Pneumococcus type 1 has been described as associating with bacteraemia, pneumonia in North America and Europe, and meningitis in Ghana (Kirkham *et al.,* 2006).

Chapter 5 - Effect of Oxygen on Pneumococcal Adhesion Factors and Pathogenicity

5 Effect of Oxygen on Pneumococcal Pathogenicity

As reported in the previous chapter, an oxygenated condition has significantly affected *ply* gene expression, production and its haemolytic activity. In this chapter, potential effects of oxygenated growth environments on other key virulence determinants are investigated, principally some of the pneumococcal adhesion factors. Microarray findings (as detailed in Chapter 4) showed a significant increase in several CBPs including the adhesin PspC in oxygenated growth conditions compared to anaerobic. This induction was ensured with Western blot and adhesion assays herein.

We also investigate in this chapter the potential effect of oxygenated growth patterns on pneumococcal adhesion to human cell line using different strains representing diverse serotypes. Furthermore, measuring mRNA for 10 selected TCSs to confirm the array data, the expression of seven genes responsible for pneumococcal pili, detecting pilins production with immunoblot, biofilm formation and finally capsular formation were investigated in this chapter.

5.1 Pneumococcal Surface Protein C (PspC)

A number of CBPs such as PspC, CbpD, CbpF and CbpJ and two choline transporters were up-regulated under the effect of oxygenated compared to hypoxic growth conditions. These are listed in Table 5-1; their gene numbers, fold change in oxygenated relative to anaerobic growth, as well as their biological roles that have been studied.

Table 5-1 Effect of oxygen on gene expression of choline-binding proteins (CBPs)

Data analysis for gene expression of a number of pneumococcal choline-binding proteins showed that *pspC*, *cbpD*, *cbpF* and *cbpJ* have been up-regulated in oxygenated versus anaerobic growth. Furthermore, choline transporters were affected similarly during oxygenated incubation. These transporters have been analysed using CLC Genomics Workbench programme. P<0.05 with Benjamini and Hochberg correction applied for multiple testing using 1 way ANOVA test.

CBPs	Gene number	Fold change in O_2	Biological function	Reference
PspC	SP_2190	1.50+	binds to epithelial cells	(Ma *et al.*, 2007)
			binds immunoglobulin receptor that transfers IgA	(Hammerschmidt *et al.*, 1997)
			. binds factor H	(Dave *et al.*, 2004a)
			binds complement 3	(Cheng *et al.*, 2000)
			induces IL-8	(Madsen *et al.*, 2000)
			regulated by TCS06	(Standish *et al.*, 2005)
CbpD	SP_2201	1.38+	competence-induced cell lysis	(Hakenbeck *et al.*, 2009; Kausmally *et al.*, 2005)
			plays a role in colonization	(Bergmann *et al.*, 2006)
CbpF	SP_0391	1.43+	inhibits LytC activity but not LytA	(Molina *et al.*, 2009)
CbpJ	SP_0378	1.32+	binds to C-Reactive Prptein (CRP)	(Frolet *et al.*, 2010)
Chol-transp.	SP_1861	2.82+	transporting CBPs	CLC
Chol-transp.	SP_1860	2.6+	transporting CBPs	CLC

In addition to the up-regulated *pspC*, *cbpD*, *cbpF and cbpJ*, genes encoding choline transporters including SP1861 and SP1860 were also up-regulated in oxygenated growth suggesting that these aerated conditions may contribute to binding more CBPs onto the pneumococcal cell wall. We further confirmed *pspC* transcription with measuring its mRNA levels. This showed that the protein was slightly induced in the oxygenated pneumococcal growth 1.23-fold relative to anoxygenated conditions. It was decided then to test the protein translational stage to confirm whether more PspC is produced in oxygenated conditions when compared to anaerobic growth.

5.1.1 SDS-PAGE and Western blot of PspC

In order to investigate whether the choline-binding protein PspC is found on the bacterial cell surfaces or intracellularly in the bacterium, immunoblotting of

bacterial cell wall washes and the bacterial cell content were performed. SDS-PAGE and Western blots were performed using specific antibodies to the PspC as shown in Figure 5-1.

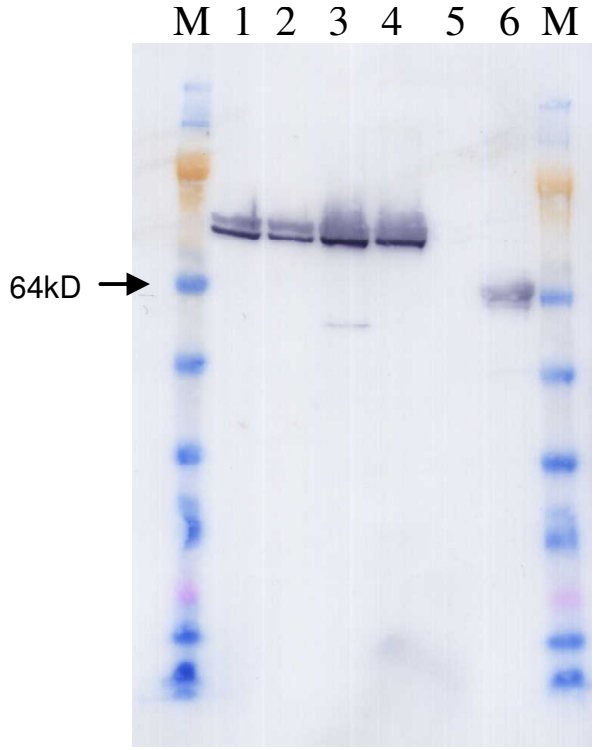


Figure 5-1 Western blot of PspC extracted from TIGR4 grown both oxygenated and anaerobically

Lysates of *S. pneumoniae* TIGR4 gathered from cultures grown under different oxygen levels (oxygenated and anaerobic) until the log phases were at about optical density OD_{600} = 0.35. Western blot of PspC found in the cytoplasm of the oxygenated grown bacteria with total protein content measured as 24.10 mg/ml (1), cytoplasmic PspC collected from anaerobically grown TIGR4 measured as 24.38 mg/ml (2), oxygenated cell surface PspC measured as 2.52 mg/ml (3), anaerobic cell surface PspC measured as 2.85 mg/ml (4), PBS as a negative control was 0.00 mg/ml (5) and part of PspC as a positive control, which was purefied previously by a PhD student in TJM group (6). All samples were collected at the same oxygenated and anaerobic ODs. M: indicates the marker used (SeeBlue®Plus2, Invitrogen, Scotland).

Western blot of PspC protein on the bacterial cell surface and in the cytoplasmic proteins resulted in greater protein intensity on the cell wall (extracellular) when TIGR4 cells were grown in oxygen compared to the anaerobic grown bacteria.

It is worth mentioning here that the two component system 6 (TCS06) has been reported to regulate the adhesive agent PspC, and the expression of this CBP (SP_2190) was found in our microarray findings as well as TCS06 (SP_2192 &

SP_2193) to be significantly up-regulated in oxygenated relative to anoxic grown cells (Chapter 4, Table 4-1). From this, it was predicted that the pathogen *S. pneumoniae* TIGR4 is able to bind to human epithelial cells more efficiently if it is grown in oxygen when compared to anaerobically grown organisms. To assess this hypothesis, the clinical strain was grown until the log phase (optical density OD_{600}=0.35) in both oxygenated and anoxygenated growth conditions before being seeding on confluent lung cells (pneumocytes II, A549 cell line). One hour was allowed for bacterial adhesion.

5.1.2 Effect of aerobiosis on PspC in TIGR4 adhesion

Adhesion assays for TIGR4 grown in both oxygenated and anaerobic environments were performed to test the bacterial adhesion in the presence or absence of oxygen.

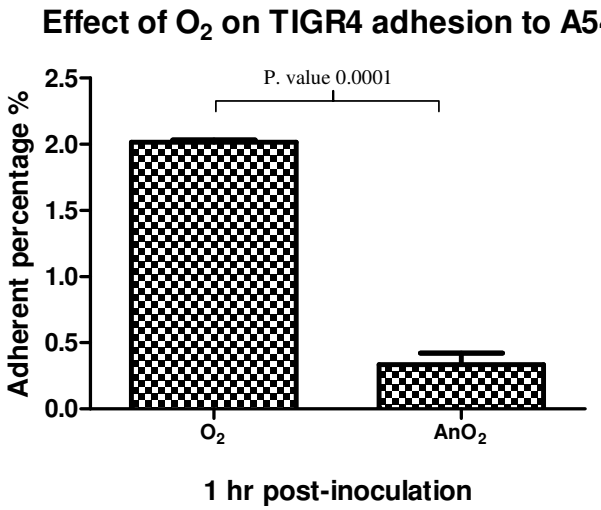


Figure 5-2 Effect of oxygen on TIGR4 adhesion to the human pneumocytes A549

WT TIGR4 grown in oxygenated environment adhered significantly better (p-value=0.0001) to the host cells A549 compared to what the anaerobic grown pneumococci did. Percentage of bacterial adherence was calculated after one hour following seeding oxygenated or anaerobic grown TIGR4 on a monolayer grown cell line. O_2 indicates pneumococci which have been grown in the oxygenated growth conditions, whereas AnO_2 means the anaerobically grown TIGR4 cells. Statistical analysis was performed using *t.test* in Graph Pad (GraphPad Prism 5, USA 2007). Each bar is an average of three replicates, and the error bars represent the standard error of the mean.

Figure 5-2 illustrates that around 2% of the inoculated *S. pneumoniae* TIGR4 were able to significantly (p=0.0001) adhere after bacterial growth in oxygenated versus anaerobic. Anaerobiosis conditions led to significantly weak adhesion, indicating less pneumococcal adhesion ability when oxygen is absent.

This low adherence percentage could be due to the presence of the capsular polysaccharide (CPS) as pneumococci possess this key virulence factor which coats most the bacterial cell wall, preventing proper adhesion. As a consequence, a reduction in CPS thickness may facilitate bacterial-host interaction that could result in better microbial attachment.

The significant oxygenated adhesion ability to the human cell line A549 reported here (Figure 5-2) could be attributed to two reasons; i. Oxygenated reduction occurred in the CPS gene expression, ii. Oxygenated increase in both the adhesive factor PspC and its transporters, which also have been proved in this chapter as can be seen in Table 5-1. Therefore, it was necessary to investigate the role of the capsule in these assays and these two reasons have led us to repeat the adherence assay experiments using the same strain but lacking the capsule. It was expected then that the adhesion ability would be increased when pneumococci are grown in oxygenated growth environments.

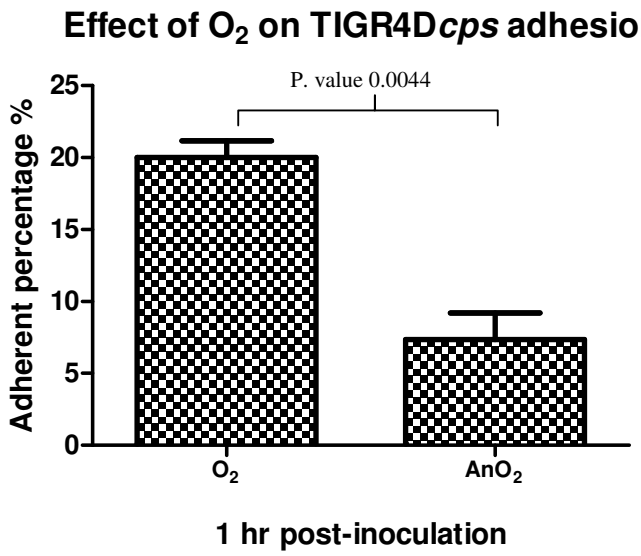


Figure 5-3 Effect of oxygenated growth on TIGR4Δ*cps* adhesion to the human pneumocytes A549

The unencapsulated TIGR4 had the ability to bind the epithelial monolayer significantly after growing in the oxygenated incubation conditions as compared to the anaerobically grown cells with 0.0044 p-value. TIGR4Dcps is the mutant that lacks capsular polysaccharide used in this adherence assay. Statistical analysis was performed using *t.test* in Graph Pad (GraphPad Prism 5, USA 2007). Each bar is an average of three replicates, and the error bars represent the standard error of the mean.

TIGR4Δ*cps* was able to bind the host cell line after growing in oxygenated environment significantly greater than the anaerobic grown bacteria (p-value=0.0044) (Figure 5-3), and the percentage of the adherent pneumococci rose from 2 % when using the WT TIGR4 (Figure 5-2) to about 20 % under the oxygenated conditions. This high ability of adhesion was expected due to the vast effect of the capsule phase variation as the pathogen is known to reduce CPS expression during colonising the host nasopharynx as compared to its expression in the blood circulation system, and the reduction was comparable with the capsular amounts observed in R6, the non-encapsulated strain (Hammerschmidt *et al.*, 2005). Figure 5-4 illustrates how the capsule thickness could coat the whole bacterium.

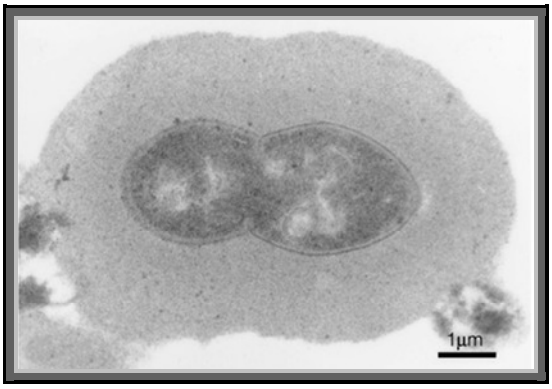


Figure 5-4 Capsular polysaccharide (CPS) of *S. pneumoniae*

The bacterium is coated with a thick CPS covering cell wall agents including CBPs. Quoted from Nature Reviews, Genetics, (Joyce *et al.*, 2002).

This significant increase of pneumococcal binding to the human cell line under oxygenated compared to anaerobic growth conditions suggested a possible role for PspC in this adhesion process. To investigate this, TIGR4Δ*pspC* was used and the adherence assay experiments were repeated under both of the incubation growth conditions, oxygenated and anaerobic (Figure 5-5).

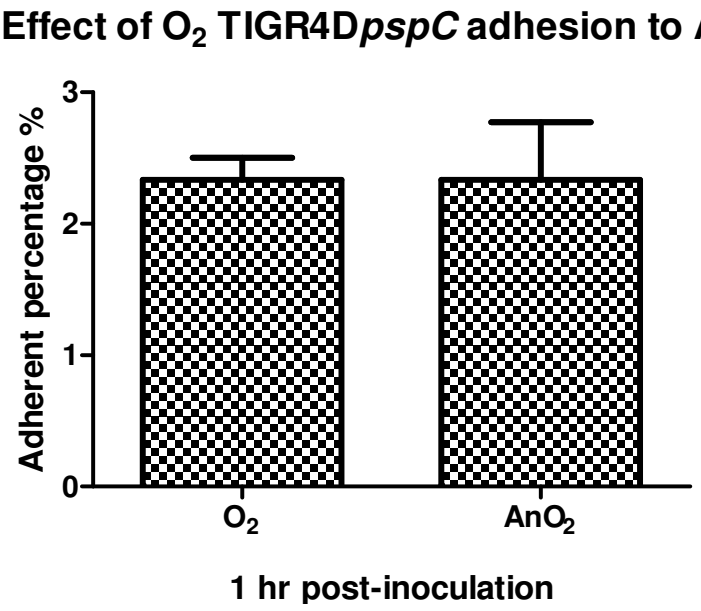


Figure 5-5 Adherence assay of TIGR4Δ*pspC* in oxygenated and anaerobic environments

Ablation of pneumococcal surface protein C in TIGR4 (TIGR4D*pspC*) revealed approximately analogue percentages of adherent microbes to the host cells used after growing the bacteria under oxygenated and anaerobic incubation growth forms. Each bar is an average of three replicates, and the error bars represent the standard error of the mean and no significance variation was found using Graph Pad (GraphPad Prism 5, USA 2007).

It is interesting that the previous significant differences in the bacterial-host adhesion, as shown in Figure 5-2 and Figure 5-3, using the WT TIGR4 under both oxygenated and anaerobic conditions disappeared in Figure 5-5, suggesting that PspC plays a key role in pneumococcal ability for binding the human lung cells once they are exposed to oxygenated growth conditions. Moreover, our previous findings for the transcriptional and post-transcriptional stages after scanning the whole bacterial genome and after blotting the protein showed increased levels of PspC on the bacterial cell wall that supported these adhesion assays. This confers a greater bacterial attachment under oxygenated conditions to the epithelial tissues used. Hence, it was important to determine this ability if repeating the adhesion assays but using different pneumococcal serotypes grown in the same growth forms.

5.1.3 Adhesion by other pneumococcal serotypes

Due to the significant adhesion ability observed in serotype 4 pneumococci grown in oxygenated environments, other serotypes (type 3, OXC141 and type 1,

P1041) have been studied here for their adhesion to the same human cell line A549. *S. pneumoniae* OXC141 is mostly found in human colonisation rather than causing an invasive pneumococcal disease.

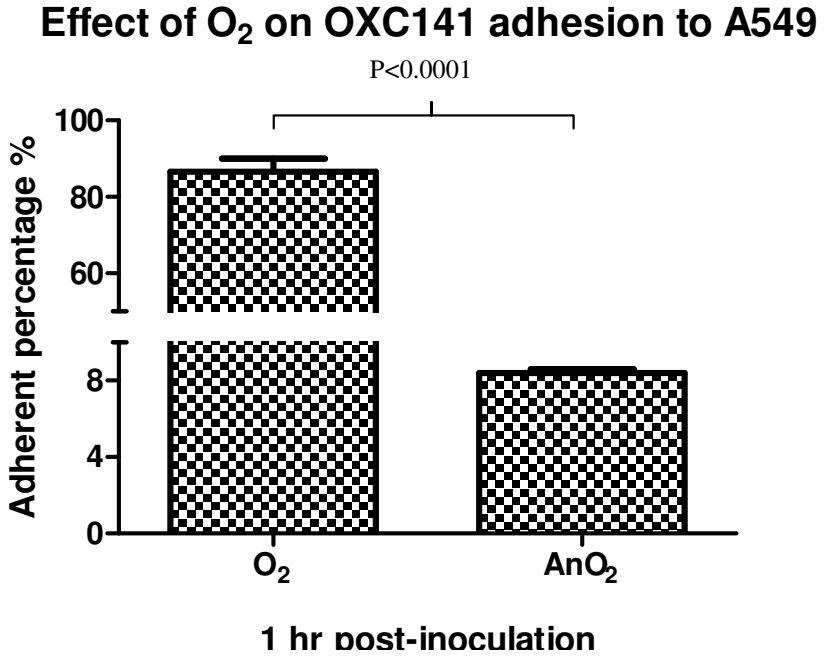


Figure 5-6 Adhesion assay for OXC141 grown in oxygenated and anaerobic growth conditions

Significant (p-value<0.0001) ability of pneumococcal strain OXC141 associated with colonisation once it was grown in oxygenated versus the anaerobically grown bacteria. Statistical analysis performed used *t.test* in Graph Pad (GraphPad Prism 5, USA 2007). Each bar is an average of three replicates, and the error bars represent the standard error of the mean.

The strain OXC141 associated with colonisation had a significant ability to adhere after growing in the oxygenated incubation conditions in comparison to the anaerobic grown cells (Figure 5-6). This statistical significant reduction ($p<0.0001$) in anaerobic capability of adhesion indicates that the bacterium is highly equipped with adhesion factors when it is exposed to oxygenated growth conditions.

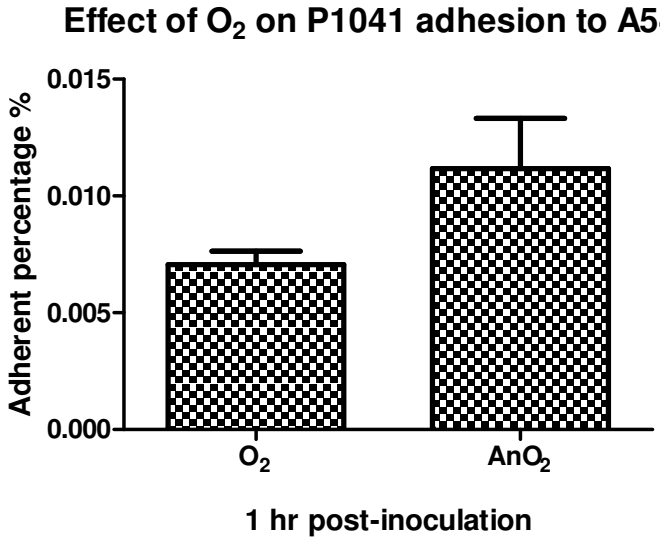


Figure 5-7 Adhesion assay for P1041 grown in oxygen and anaerobically

The hyper-virulent microorganism P1041 revealed no considerable differences in adhesion to the host epithelial cells after growing under the oxygenated or anaerobic growth environments. Statistical analysis was performed using *t.test* in Graph Pad (GraphPad Prism 5, USA 2007) and showed no significant differences between the pneumococcal adhesion in both condotions, aerobic and anaeroboc. Each bar is an average of three replicates, and the error bars represent the standard error of the mean.

In contrast to serotype four (TIGR4) and three (OXC141), the performed adhesion assay (Figure 5-7) of the P1041 clinical isolate, which represents serotype 1, clearly showed no significant differences in the organism adherent percentages under oxygenated and anaerobic growth conditions.

5.2 Pilus

5.2.1 SDS-PAGE AND Western blot for TIGR4 pilus

Pneumococcal pili are well known to participate in colonisation similar to PspC, and it was predicted that the main protein involved in the pili structure, RrgB, could be produced at a higher intensity if it is collected from the oxygenated grown bacteria and compared to the anaerobic growth conditions. Consequently, bacterial cell lysates at optical density OD 0.35, a growth OD which was chosen for the microarray and haemolytic assay experiments (Chapter 4), was separated on SDS-PAGE and electroblotted using Western blot.

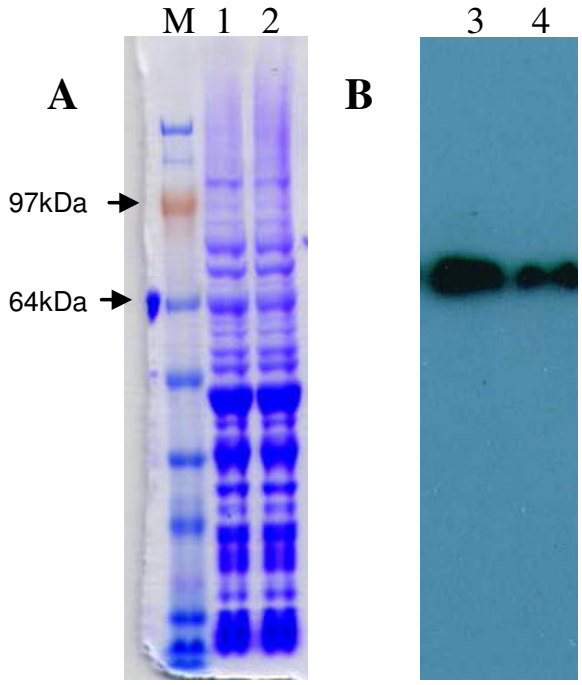


Figure 5-8 Western blot for the pilin RrgB under oxygenated and anaerobic conditions

Bacterial cell lysates (SDS-PAGE) (A), lane 1 aerated (total protein concentration is 24.10 mg/ml) and 2 anaerobic (24.38 mg/ml). Immunoblot for both prepared samples gathered from the two oxygenated and anaerobic conditions (B), lane 3 shows greater RrgB intensity in oxygen compared to lane 4 that represents the anaerobic bacterial sample.

The immunoblot performed on samples gathered from oxygenated and anaerobic grown pneumococci showed an increase in the protein RrgB intensity in aerated grown cells (Figure 5-8, A and B). RrgB forms the backbone of the bacterial pili. This suggests a possible oxygenated enhancement in those genes, which are involved in structuring and assembling the pneumococcal pilus.

These results were supported by another similar investigation performed by Jenny Herbert in a thesis submitted to the University of Glasgow in 2012. In addition, she also proved this with FACS and stated:

> "A clear shift to the left in the FITC pilus positive population grown under anaerobic conditions, suggesting a decrease in the amount of pili on a single cell."

To assess how TIGR4 pilus expression is controlled under both conditions used, mRNA levels were measured by doing qRT-PCR for those genes which are

responsible for both structuring (including *rrgA, rrgB, rrgC, rlrA)*, and assembling enzymes (*srtB, srtC, srtD)*.

5.2.2 qRT-PCR for TIGR4 pilus RNA genes

Quantitative RT-PCR showed an oxygenated up-regulation in all of those genes responsible for controlling the pili structuring and assembling enzymes, which localise pneumococcal pilins on the bacterial cell wall.

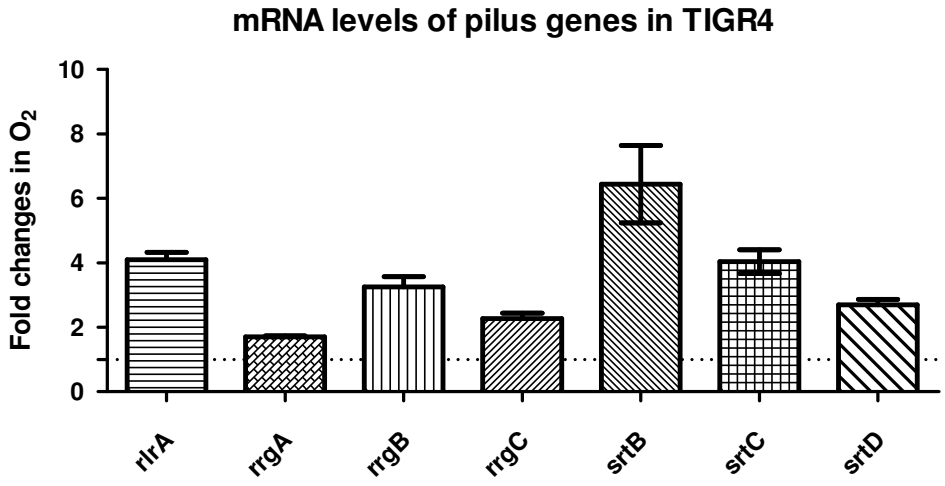


Figure 5-9 qRT-PCR for structural and assembling pili genes on oxygenated versus anaerobic

All pilins mRNA levels are up-regulated in pneumococci grown in oxygen in comparison to the anaerobic microbial growth. RrgA and RrgC are ancillary to each other. RrgA is located on the bacterial pilus shaft at the distal tip, whereas RrgC is placed in the pilus proximal. RrgB forms the pilus itself. Srt proteins are involved in pili assembling and all of their gene expressions were enhanced in oxygenated growth conditions compared to anaerobic. Each bar is an average of three replicates, and the error bars represent the standard error of the mean using Graph Pad (GraphPad Prism 5, USA 2007).

Figure 5-9 shows an oxygenated increase in mRNA levels in all of the tested pili involved genes. Except the *rrgA* gene, genes involved in PI-1 expression were detected to be above two-fold in oxygenated growth. RrgA and RrgC are known to play a complementary role. RrgA is located on the bacterial shaft of the pilus at the distal tip, whereas RrgC is placed in the pilus proximal. RrgB has a key motif and functions as a structural protein involved in the pili formation. Its mRNA during pneumococcal oxygenated growth was induced 3.5-fold compared to anaerobic.

Together, the pilus is definitely up-regulated in oxygenated growth which was expected in terms of colonisation. One possible mechanism for that induction is changes in expression of TCSs as they may involve in regulating pneumococcal pili, which is still exclusive. However, up-regulation in a TCS does not always mean that there is an induction in a gene due to phosphorylation.

5.2.3 Genes coding Two-Component Systems (TCSs)

Investigation of gene expression of the PspC showed a high potential relationship between the availability of oxygen and the adhesion to human cells. Moreover, immunoblotting of the pneumococcal pilus PI-1 and its structurally involved subunits both revealed greater expression in oxygenated rather than anaerobic environments. It was decided to confirm all of the TCS genes that have been found by the microarray data to be elevated in oxygenated conditions and even other important TCSs, listed in Table 5-2. The table shows TCSs, gene number of both HK and RR, p-value, fold change in oxygenated compared to anaerobic growth conditions and the potential product.

Table 5-2 List of up-regulated TCS genes in oxygenated against anaerobic conditions

P<0.05 with Benjamini and Hochberg correction applied for multiple testing using 1 way ANOVA test.

TCS	Gene	p<0.05	Product
TCS02 RR	SP_1226	0.00581	sensory box sensor histidine kinase
TCS02 HK	SP_1227	0.0047	DNA-binding response regulator
TCS03 HK	SP_0386	0.00411	sensor histidine kinase
TCS03 RR	SP_0387	0.0479	DNA-binding response regulator
TCS04 RR	SP_2082	0.009	response regulator
TCS05 RR	SP_0798	0.0302	DNA-binding response regulator CiaR
TCS05 HK	SP_0799	0.0151	sensor hitidine kinase CiaH
TCS06 RR	SP_2193	0.0119	DNA-binding response regulator
TCS06 HK	SP_2192	0.0119	sensor hesitidine kinase
TCS08 RR	SP_0083	0.0146	DNA-binding response regulator
TCS08 HK	SP_0084	0.021	sensor histidine kinase
TCS11 RR	SP_2000	0.00976	DNA-binding response regulator

TCS12 RR	SP_2235	0.0273	response regulator ComE
TCS13 RR	SP_0526	0.04	response regulater BlpR
Orf RR	SP_0376	0.0237	response regulator

The above table demonstrates that the majority of the 13 known Two-component system (TCS) genes are up-regulated significantly in the oxygenated growth states. The table shows that analysis of the microarray data (Chapter 4, Table 4-1) revealed that there were 10 significant over-expressed TCSs in oxygenated growth conditions out of the 13. Therefore, in order to confirm gene expression for these particular TCSs, mRNA levels for each gene have been measured in the following step, focusing on some of the TCSs which have been proved by other published studies to be involved in regulating pneumococcal PI-1.

5.2.4 qRT-PCR for confirming gene expression of TCSs

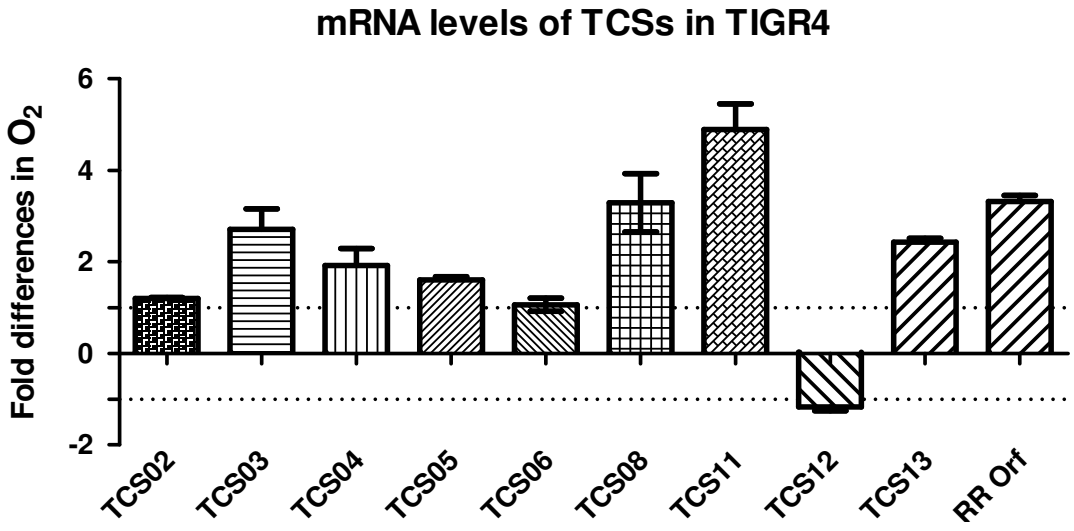


Figure 5-10 qRT-PCR for Two-component system genes and the response regulator *orf*

Most of the studied TCSs and the orf rr have been up-regulated in oxygen to different levels including those which are involved in controlling the PI-1 such as TCS03 and TCS08. TCS11 had almost 6-fold up-regulation in oxygenated growth. Each bar is an average of three replicates, and the error bars represent the standard error of the mean using Graph Pad (GraphPad Prism 5, USA 2007).

mRNA levels of the tested TCSs are up-regulated in bacterial cells grown in oxygenated growth, except TCS12 (Figure 5-10). Approximately, TCS02 and 06

modestly exceeded 1-fold of expression in the aerated environments. Indeed, *rr06* has been shown to positively involve in regulating *pspC* (Standish *et al.,* 2005), but mRNA levels of *rr06* in Figure 5-10 did not show an over-expression.

Moreover, TCS12 was slightly down-regulated under the same oxygenated growth forms. In fact, this supports the magnitude of qRT-PCR validation after microarray experiments. However, quantitative RT-PCR showed in the above figure (Figure 5-10) an induction in TCS03, 04, 05, 08, 11, 13 and the orf response regulator. Expression of an important oxidative stress response gene, *tpx,* was previously detected in this study (Chapter 4) with an induction of 7-fold in oxygenated growth was induced 180-fold using qRT-PCR.

Evidence has shown that some of these signalling systems including TCS03 and TCS08 are implicated in regulating pneumococcal pilus expression (Song *et al.,* 2009). They and other laboratories also reported that TCS05 is involved in regulating *htrA* and contributes to the bacterial colonisation (Sebert *et al.,* 2002). Furthermore, TCS11 has been documented to be involved in the pneumococcal stress response when exposed to the antibiotic vancomycin (Haas *et al.,* 2005). These TCSs had been previously found in this study with an induction in their gene expressions under the oxygenated growth conditions (Chapter 4, Table 4-1).

As the expression of PI-1 was shown in this study by microarray, qRT-PCR and Western blot to be increased in oxygenated growth, there is a possible elevation in biofilm formation for pneumococci grown in oxygenated growth because pili are thought to highly contribute to forming bacterial biofilm.

5.2.5 Biofilm formation

Recent studies have shown that biofilm formation and bacterial adhesion are both associated with the pilus that exists on bacterial cell wall. Therefore, in order to investigate this pili-biofilm relationship, a biofilm formation assay for TIGR4 was performed (Figure 5-11). The pathogen was grown until its decline phase in oxygenated and anaerobic conditions.

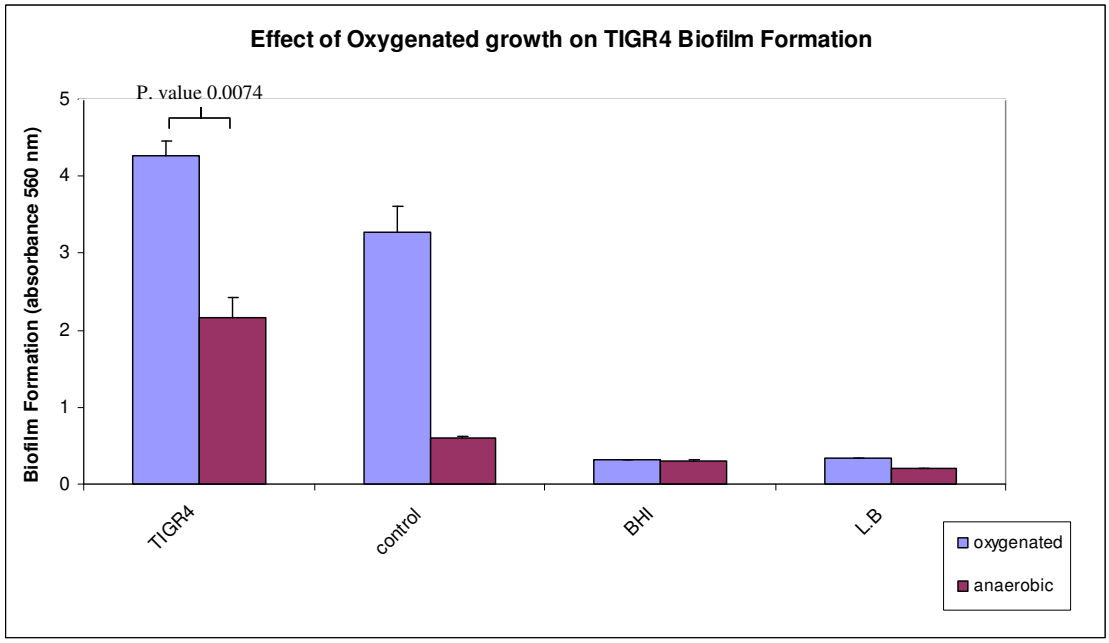


Figure 5-11 TIGR4 biofilm formation under oxygenated and anaerobic conditions

TIGR4 formed significantly greater biofilm (p=0.0074) in oxygenated compared to the anaerobic growth incubation conditions. The control is *P. aeruginosa* (as a positive control), BHI is the medium alone which used for growing TIGR4 and LB to grow *P. aeruginosa*. Error bars represent standarad error of the mean using Microsoft Office Excel 2003, whereas statistical analysis performed using *t.test* in Graph Pad (GraphPad Prism 5, USA 2007).

The absorbance of biofilm formation for *S. pneumoniae* TIGR4 was measured as 4.2% in pneumococci grown in oxygen representing a significant increase ($p<0.05$) in this condition (See Figure 5-12), whereas the anaerobic biofilm absorbance was detected at 2% (Figure 5-11). Moreover, Rgg regulators have been found in streptococci to be involved in oxidative stress response and biofilm formation, as it was described recently that *rgg* deficiency in unencapsulated type 2 *S. pneumoniae* showed a significant reduction in forming pneumococcal biofilms; and most importantly, the organisms were more sensitive to oxygen relative to parental strains (Bortoni *et al.*, 2009).

Due to this increase in the aerated biofilm formation in TIGR4, it was decided then to repeat the biofilm formation experiment to study the rest of the pneumococci used in this project to explore their biofilms during their growth in oxygenated and anaerobic growth environments (Figure 5-12).

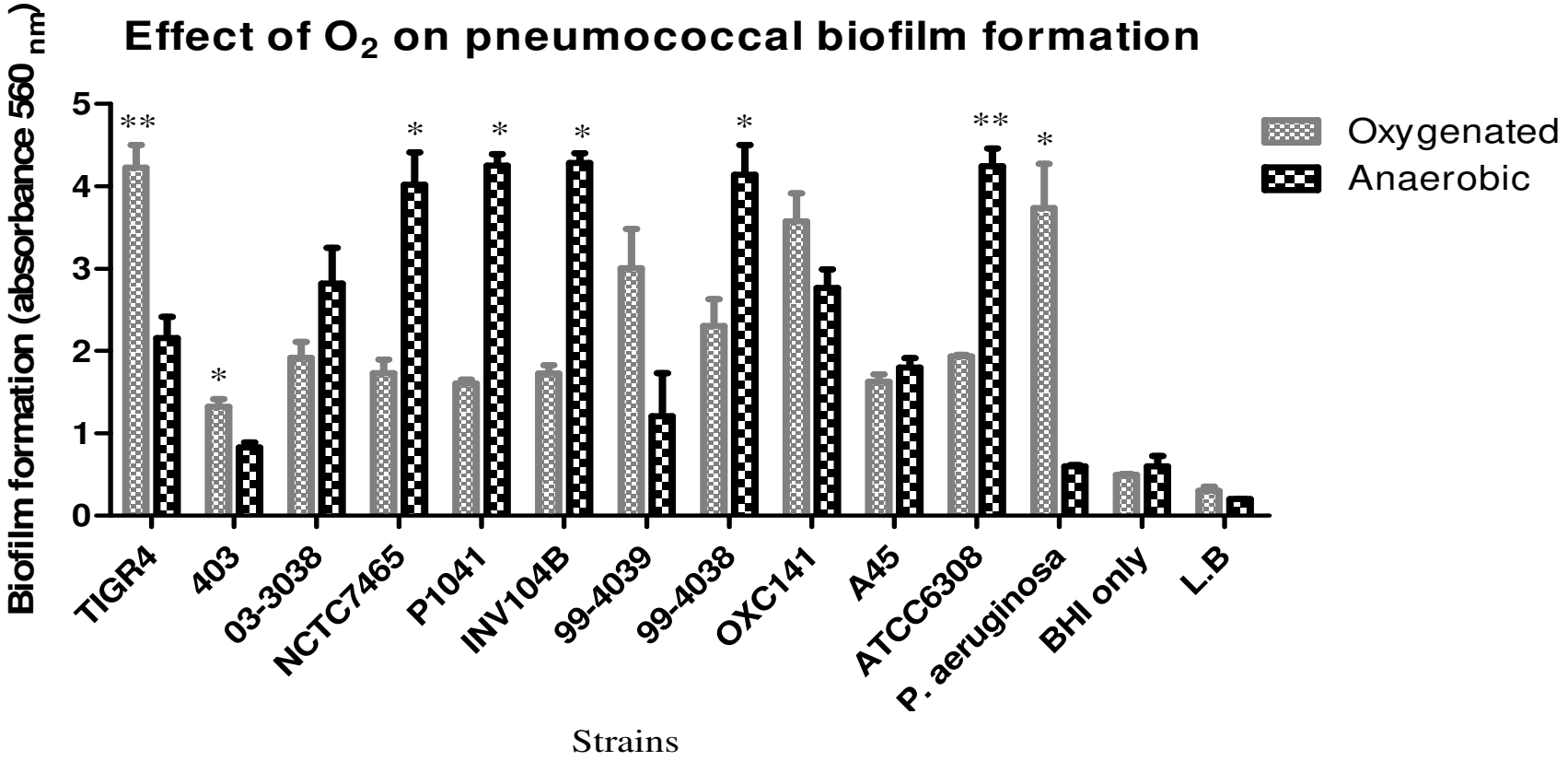


Figure 5-12 Biofilm formation for 11 pneumococcal strains in oxygenated and anaerobic conditions

Different pneumococcal serotypes showed variant abilities to form biofilm. Serotype 1 (03-3038, NCTC7465, P1041 and INV104B) and serotype 8 (ATCC6308) revealed significant increased biofilm formation in the absence of oxygen compared to the aerated condition, except the strain 03-3038 which shows similar biofilm formation under both conditions used. In contrast, serotype 4 (TIGR4 and 403) illustrated greater biofilm formation in oxygen than anaerobically. Members of type 3 show changes in biofilm formation. The experiments performed in triplicates and error bars represent SEM. BHI is the medium alone which used for growing pneumococcal strains and LB to grow *P. aeruginosa*. Statistical analysis was performed using *t.test* in Graph Pad (GraphPad Prism 5, USA 2007). * p<0.05, ** P<0.001 and the stars above the bar represent statistical significance relative to the other biofilm formation growth conditions.

The pneumococcal strains studied for changes in their behaviour in forming biofilm under both conditions belong to different serotypes including serotype 1 (03-3038, NCTC7465, P1041 and INV104B), serotype 3 (99-4039, 99-4038, OXC141 and A45), serotype 4 (TIGR4 and 403) and serotype 8 (ATCC6308). Multi Locus Sequence Type (MLST) and other pneumococcal growth characterisations for all these strains were listed in detail in Chapter 3, Table 3-2.

Biofilm formation assays for these pneumococci (Figure 5-12) showed variations between the strains used. All of the clinical isolates representing serotype 1 (NCTC7465, P1041 and INV104B) and 8 (ATCC6308) formed significantly greater biofilm in the absence of oxygen. Nevertheless, statistical analysis of the pathogen 03-3038 showed no significant differences and was the only bacterium that seemed to be slightly similar in forming biofilms under both conditions, oxygenic and anaerobic.

On the other hand, TIGR4, which represents serotype 4, along with its avirulent derivative strain 403 showed a significant increase. TIGR4 cells significantly formed biofilm in the oxygenated conditions versus the anaerobic incubation forms ($p=0.0074$). This supported our previous data including the oxygenated observed up-regulation in TCS genes which are responsible for regulating pneumococcal pili, up-regulation in mRNA levels of TIGR4 structural and assembling pili proteins and the enhancement of pilus protein RrgB shown by Western blot.

In addition, serotype 3 members behaved differently; 99-4039, which was isolated from a patient´s CSF in 1999, formed greater biofilm in oxygen, but it is not the case with the highly genetically related isolate 99-4038 which behaved oppositely. 99-4038 pneumococci were collected from blood of the same patient, which is considered as an anoxia host environment, and preferred the anaerobic conditions to form biofilms that showed a statistically significant increase. Both (99-4039 and 99-4038) were shown to grow better in anaerobic growth environments (Chapter 3, Section 3.2.).

Together, these closely related strains (99-4039 and 99-4038) reacted to oxygen for forming biofilms completely differently. Therefore and due to that biofilm formation is believed to be a serious clinical barrier against antibiotics and the

immune system, these biofilms variation could affect the medical drugs used, considered as a proper procedure for treating the infection. Studies have shown that changes in forming pneumococcal biofilms under static growth conditions at interval time-points were reported recently in a large number of different clinical isolates representing several serotypes (del Prado *et al.,* 2010; Tapiainen *et al.,* 2010).

A very recent study undertook a comparison between the isolates 99-4039 and 99-4038 and revealed that the pneumococcus 99-4039 is more resistant to antibiotics (Croucher *et al*., 2013, in print). They stated that this may refer to an induction in the PatA/B efflux system.

In some pneumococci, biofilm formation might be linked to a stressful lifestyle. As mentioned earlier in this section, Rgg regulators have been shown to play a role with both pneumococcal biofilm formation and oxidative stress response (Bortoni *et al.,* 2009). Another possible relationship between biofilm formation and oxygen is that TIGR4 and OXC141 growth curves, representing serotype 4 and 3 respectively (as shown in Chapter 3), illustrated no strong variations with or without oxygen availability, whereas serotype 1 growth curves demonstrate an apparent greater shift once grown anaerobically versus the oxygenated growth conditions. Interestingly, del Para *et al* revealed that serotype 3 pneumococci showed the highest biofilm formation during growth under aerobic conditions (del Prado *et al.,* 2010); on the other hand, Tapiain and colleagues demonstrated the opposite using the same strain (Tapiainen *et al.,* 2010). Possibly, differences in experimental procedures/protocols could cause these discrepancies.

5.2.6 Pneumococcal aggregation

It is believed that bacterial ability to aggregate leads to an increase in the microbial clumps and therefore a greater capability to form a biofilm. Furthermore, early work has stated that there is a relationship between bacterial chain length and pathogenicity. Therefore, chains of mid-logarithmic growth for the isolate *S. pneumoniae* TIGR4 were examined microscopically under three different levels of oxygen to study how oxygen variations can affect bacterial aggregation and in turn its biofilm formation (Figure 5-13).

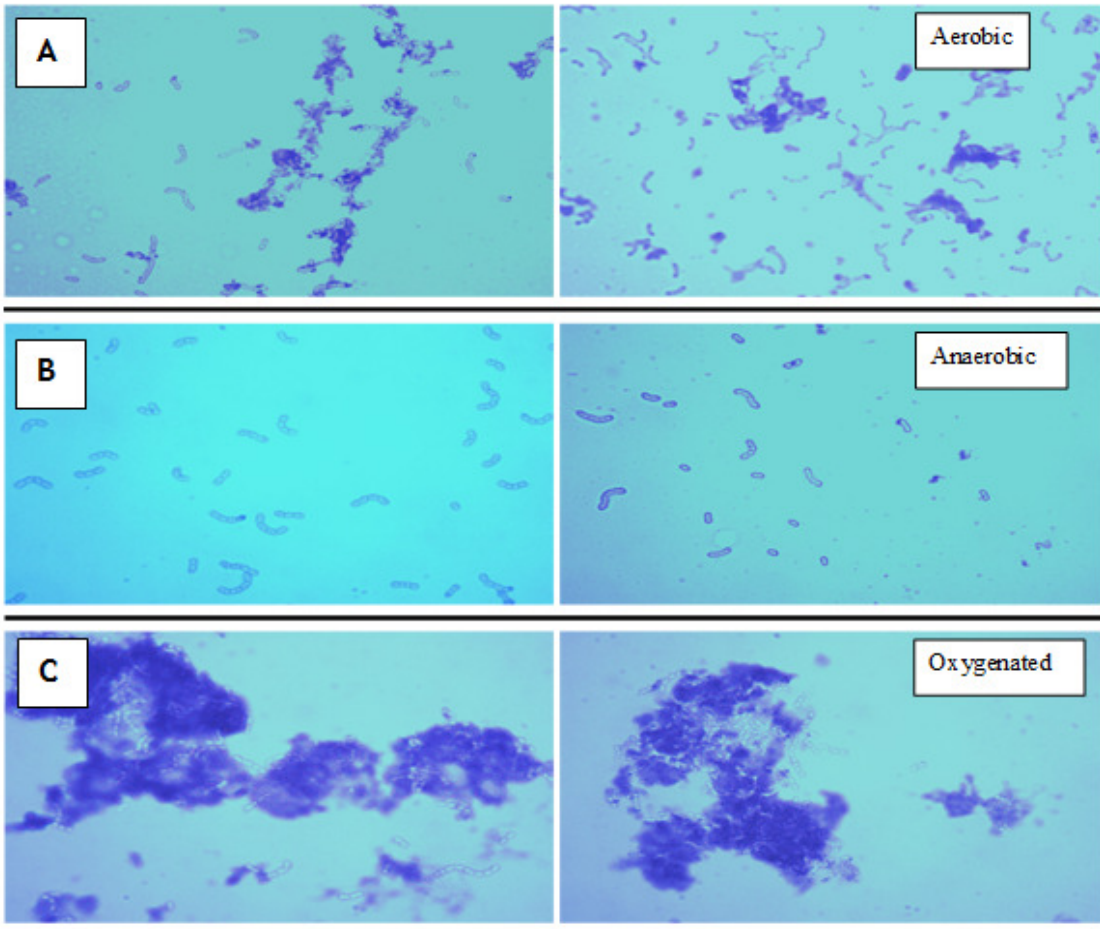


Figure 5-13 TIGR4 aggregation under three different levels of oxygen

Gram stain of un-vortexed mid-log phase TIGR4 grown aerobically showed short pneumococcal chains and a few small bacterial clumps formed (A), anaerobic growth in the same batch of the medium BHI represented no clumps and shorter chain lengths, almost diplococci (B) and the last row illustrates TIGR4 oxygenated growth pattern with long pneumococcal chains and several microcolonies showing the link between aggregation, biofilm formation and oxygen (C). Two films were captured from different aereas on each slide prepared for each condition. The magnification factor used was 40x.

Gram stained examination of the bacterial mid-log phase under three different levels of oxygen (aerobic, anaerobic and oxygenated) showed differences in the pneumococcal aggregation ability (Figure 5-13, A, B and C). This figure demonstrates that the more oxygen provided to the growing bacteria the longer the chains and the greater the biofilms formed following bacterial aggregation. The microscopy inspection of the TIGR4 growth in the anaerobic condition showed very short chains or even diplococci and a complete absence of aggregation (Figure 5-13, B). In contrast, the aerobic growth incubation conditions resulted in bacterial tendency for forming slightly longer chains of a few microbes and a number of aggregated microcolonies, probably an early biofilm formation (Figure 5-13, A). However, when oxygen levels were increased

by shaking bacterial cultures in the oxygenated growth conditions, the bacterial chains made more junctions and initiated biofilm formation as can be seen above in Figure 5-13, C.

As stated earlier in the introduction chapter, it is well known that CPS production can negatively affect microbial biofilm formation. In order to test this in pneumococci, the strain OXC141 as a human coloniser, which prefers oxygenated conditions to form greater biofilms as seen in Figure 5-12, was chosen for studying its CPS production after growing in different levels of oxygen.

5.3 Capsular polysaccharide formation

5.3.1 Effect of anaerobiosis environment on capsule formation

Deu to the well known reduction in *cps* and its phase variation when pneumococci grown in oxygen compared to anaerobic growth (Weiser *et al.*, 1994a; Weiser *et al.*, 1994b), it was decided to study how the bacterial colony formations would be affected after exposing another pneumococcal serotype to oxygen in comparison to the absence of oxygen. Therefore, growth curves of the isolate OXC141 were measured under the two different atmospheric conditions, aerobic and anaerobic. The growth curves are shown in Figure 5-14.

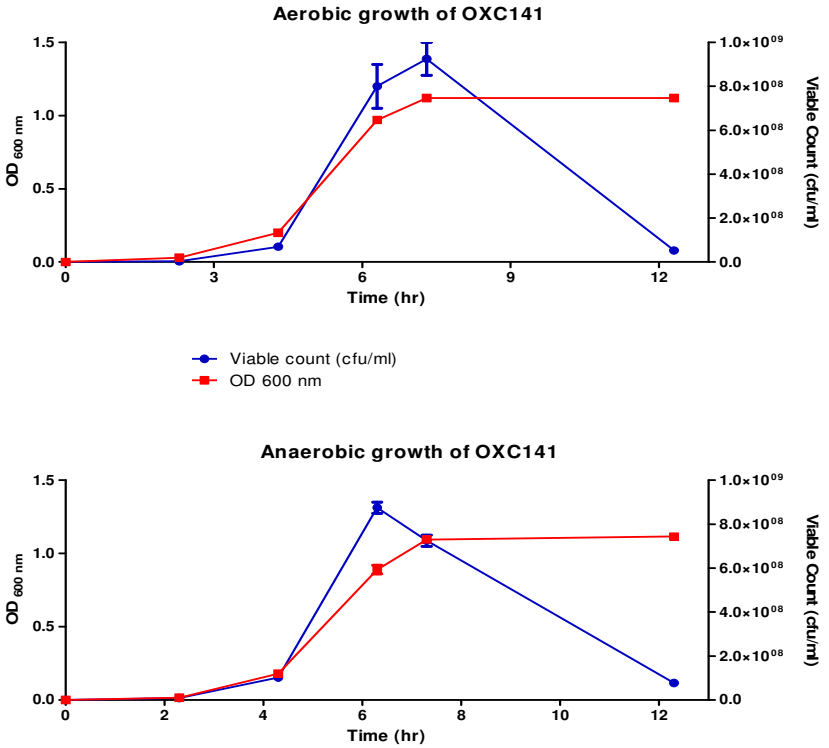
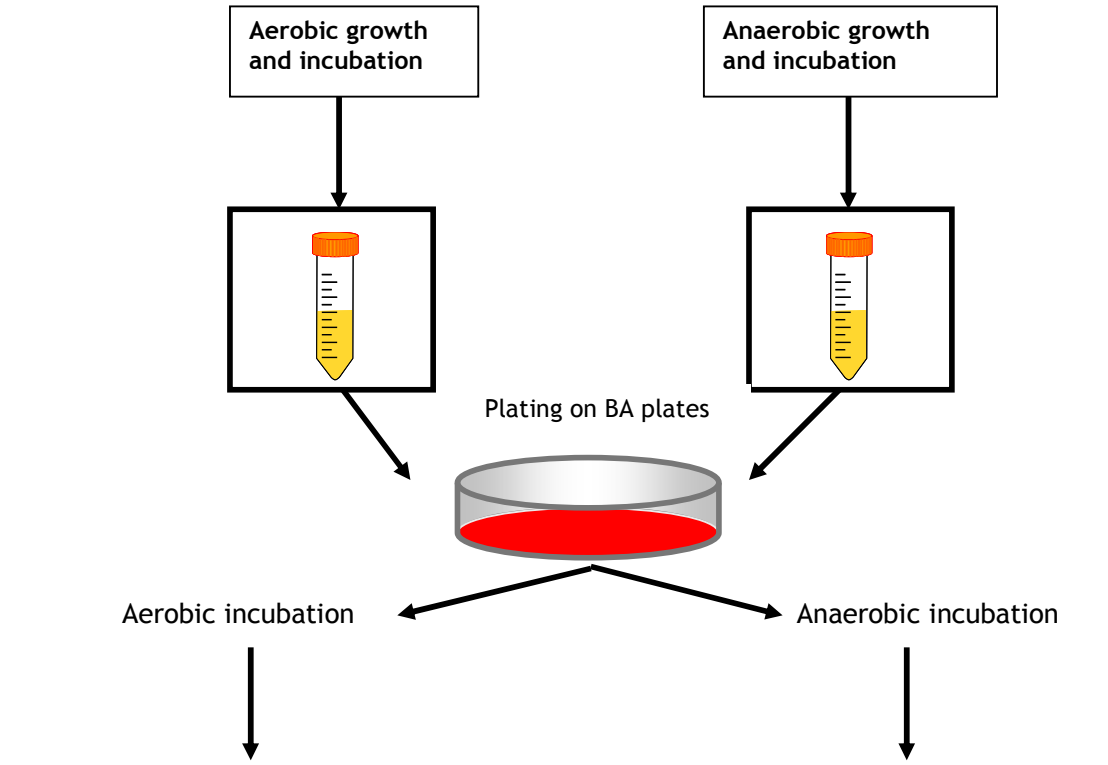


Figure 5-14: OXC141 growth curves aerobically and anaerobically

Under different levels of oxygen, similar growth curves were observed. All lasted 3 hr in their log-phase followed by approximately another 3 hr before entering the death phase, which took a further 5 hr. Error bars show SEM using Microsoft Office Excel 2003.

As Figure 5-14 shows, the growth rates under different conditions used were not considerably different. These *in vitro* growth rates were similar as they took about 3 hr to start the log-phase for approximately another 3 hr before dying after almost 7 hr. While pneumococci were growing at the mid-log phase, samples were gathered and plated directly on BA for overnight incubation as explained in the following diagram.



Capsular formation in OXC141 when grown in different levels of O_2	
Aerobic	Anaerobic
Rough colonies	Mucoid colonies

Figure 5-15 Effect of oxygen availability on OXC141 colony formation

The blood agar (BA) plate on the right side shows mucoid colonies after being incubated anaerobically. In contrast, the left BA plate reflects formation of rough colonies after incubating in the presence of oxygen, aerobically. The table demonstrates a short summary resulting from pneumococci grown in different atmospheric levels. The organisms were grown under each condition until the mid-log phase before plating on blood agar plates.

Phase variation of rough (spotted) and smooth (mucoid) colonies were noticed when culturing OXC141 on BA collected from different growth conditions. The environment in which mucoid colonies formed was the anaerobic condition (Figure 5-15). These phenotypic differences indicate that oxygen availability in the airway tract might suppress the capsule formation in pneumococci, whereas less-oxygenated conditions like the bloodstream triggered its production which is in agreement with several studies where capsule phase variations, transparent and opaque, were observed (Weiser *et al.*, 1994a; Weiser *et al.*, 1994b).

This CPS variation is consistent and supports the previous results in Figure 5-6 as the oxygenated growth environments in Figure 5-6 led to greater than a 10-fold significant increase ($p<0.0001$) in the OXC141 attachment to A549 cell line relative to the adhesion assays using anaerobically grown bacteria. This indicates less capsule formation resulted in an increase in the adhesion ability when cells are grown in oxygen compared to anaerobic growth conditions that Figure 5-15 clearly shows.

5.4 Discussion

There are a series of steps for *S. pneumoniae* to develop IPDs. Colonisation of host nasopharynx is believed to be the first. The pathogen is highly capable of adapting to this anatomical niche, as well as other environmental sites such as the bloodstream and the brain. Each has been reported to require different gene expression (Orihuela *et al.,* 2004; Ogunniyi *et al.,* 2002), and shown to contain variant oxygen concentrations (Burghout *et al.,* 2010; Hathaway *et al.,* 2007; Yesilkaya *et al.,* 2009). In this chapter, our array data analysis of *pspC*, its mRNA levels, protein production and adhesion activity under different levels of oxygen have been studied. All these have demonstrated greater amounts of PspC protein in oxygenated than anaerobically grown bacteria. The immunoblot shows PspC increase in both the pneumococcal cytoplasm and on the bacterial cell surface. Interestingly, the choline transporters, including SP1860 and SP1861, were also over-expressed 2.6 and 2.8-fold in oxygenated growth conditions, respectively, indicating more CBPs are anchored onto the pneumococcal cell wall. Consistent with this, gene expression of the adhesin factor *pspC*, which enhances the bacterial colonisation, was detected with an increase during pneumococcal attachment to human epithelial cells *in vitro* and *in vivo* (LeMessurier *et al.,* 2006; Oggioni *et al.,* 2006; Orihuela *et al.,* 2004a). In addition, Mahdi and coresearchers reported that pneumococci in the nasopharynx express greater *pspC* when compared to cells grown in the bloodstream (Mahdi *et al.*, 2006).

Moreover, transparent phenotype was shown to correlate with colonisation of the nasopharynx, and expressed more PspC proteins on the cell surface versus opaque pneumococci, which have been linked to invasiveness rather than colonisation (Overweg *et al.,* 2000; Rosenow *et al.,* 1997). Further animal studies using pneumococci without the *pspC* gene to assess nasopharyngeal colonisation have shown a 100-fold reduction in the attachment ability of mutant compared to wild-type strains; such differences were not shown when mice were challenged intraperitoneally (Rosenow *et al.,* 1997). Similarly, *in vitro* experiments also revealed that D39 *pspC* deficient bacteria have significantly less adhesion 85% ($p=0.001$) to the cell line A549 (Standish *et al.,* 2005).

Nevertheless, the contribution of PspC, to virulence was described to be strain-dependent (Kerr *et al.*, 2006). According to Kerr and colleagues, not all strains possess PspC; it is not clear to us if oxygen regulates all pneumococci in the same way as shown in our findings, but it does suggest that if adherence is mediated by oxygen in all strains, there must be different mechanism(s) because not all of them have PspC or they may have different adhesion proteins. Therefore, our data (microarray, qRT-PCR and Western blot) led us to examine its role in adhesion to human cells after espousing to oxygen against anaerobic growth conditions.

S. pneumoniae OXC141 showed the highest adhesion rate amongst the other tested isolates (Figure 5-6). This could be due to presense of prophages in most of the pneumococcal colonisers. Unlike TIGR4, OXC141 strains are occasionally isolated from human nasopharynx with prophages carried in their genomes. These prophage carriers have been reported with a greater ability to adhere to nasopharyngeal cells (Loeffler *et al.*, 2006).

In addition to PspC, our findings illustrated the role of other pneumococcal adhesion factors; pili proteins in TIGR4 (six genes encoded structural proteins RrgA, RrgB and RrgC) and their assembling enzymes (SrtB, SrtC and SrtD). Expression of all these genes was elevated when TIGR4 is exposed to oxygenated growth environments (Figure 5-9). Furthermore, 10 of 14 key pneumococcal TCS were up-regulated significantly in oxygenic conditions. For instance, TCS03 was induced in oxygenated growth and its contribution in stress response has been reported (Haas *et al.*, 2005). Similar to our array results, expression of TCS03 was reduced in CSF of infected animals (Orihuela *et al.*, 2004). CSF is considered to be less oxygenated relative to host respiratory airways (Auzat *et al.*, 1999).

Interestingly, unlike serotype 3 (OXC141), serotype 1 (P1041) displayed an extremely low adherece when compared to both serotypes, 4 and 1. A possible explanation behind this could be that type 1 pneumococci are hyper-virulent organisms and rarely isolated from healthy individuals (Harvey *et al.*, 2011b; Williams *et al.*, 2012); therefore, it could be that they have evolved to be more successful as invaders than colonisers (opportunistic pathogens). Nevertheless, more recent studies undertook swabs from hundreds of children at intervals of several days. The researchers were able to detect serotype 1 pneumococci for a

few days in host nasopharynx after which the bacteria disappeared (Abdullahi *et al.,* 2012).

Analysing our biofilm assays for a range of pneumococci (11 strains) representing different serotypes shows biofilm phenotype variations under oxygen availability as shown in Figure 5-12. In fact, several factors have been shown to facilitate pneumococcal biofilms; CBPs, pili and free DNA. Our results illustrated that not only CBPs but also choline transporters were up-regulated in oxygen when compared to anaerobic growth conditions. Investigation of genes involved in pneumococcus biofilms revealed a reduction in biofilm formation with CBP deficient mutants (Moscoso *et al.,* 2006). This was recently supported after constructing a deletion in *lic* operon, which is involved in choline uptake in other bacteria that share the same habitat with pneumococci (Kwak *et al.,* 2002; Rock *et al.,* 2001). They showed that *lic* mutants were unable to form biofilms.

As discussed earlier in this section, we measured mRNA levels for type 1 pili (*rlrA* islet), encoding three pilins and three sortase enzymes, which revealed elevated expression levels in oxygenated growth. Type 1 pili in *E. coli* (Hilleringmann *et al.,* 2009; Wright *et al.,* 2007) and in *S. pyogenes* (Manetti *et al.,* 2007) have been demonstrated to contribute to biofilm formation. Interestingly, we showed in this study that all strains representing serotype 1 pneumococci formed greater biofilms anaerobically, in contrast to serotype 4 pneumococci, TIGR4. Serotype 1 is genetically known to lack type 1 pili. This inability might then explain why oxygen was ineffective to escalate biofilms in serotype 1 pneumococci.

Pneumococci are well characterised as highly transformable pathogens that spontaneously release large amounts of DNA. Free DNA was reported to be required for biofilm formation as treating the pneumococcal biofilm with DNase has significantly reduced the bacterial biomass (Hall-Stoodley *et al.,* 2008).

However, oxygen molecules were found to down-regulate biofilms in *S. mutans* (Ahn *et al.,* 2007). This presumably may reflect variation in nutrients consumption in these two organisms (pneumococcus and mutans) which might result in different metabolic pathways from utilising dissimilar carbohydrates.

Bacterial CPS is another factor counted to play a role in biofilms. It was shown to be down-regulated in pneumococcal biofilms (Hall-Stoodley *et al.,* 2008). A study has documented that oxygen levels are limited inside biofilms, and showed that the deeper they are in biofilms, the more extreme the anaerobic environment detected (Ahn *et al.,* 2007). The capsule has been also reported to negatively affect biofilm formation in *S. pneumoniae* (Moscoso *et al.,* 2006). Bortoni and colleagues explained that pneumococci involved in biofilms were 100-fold greater when using unencapsulated pneumococcal versus encapsulated pneumococci (Bortoni *et al.,* 2009). They also showed that biofilms ability by *rrg* mutants of unencapsulated pneumococci was strongly reduced compared with parent strains, suggesting a relationship between atmospheric environments and biofilm formation in *S. pneumoniae*. Furthermore, accompanied by our data, CPS had a lower expression in the nasopharynx (where oxygen concentration is similar to the atmospheric levels) compared to the bloodstream (Mahdi *et al.,* 2008; Oggioni *et al.,* 2006), indicating that the presence of oxygen reduces expression of the pneumococcal capsule, probably for more adhesion ability and proper colonisation in the URT. In addition to this, obvious evidence supporting this CPS-O_2 hypothesis is that environmental changes during growth at the pneumococcal stationary phase in a bioreactor from anaerobic to aerobic conditions resulted in CPS release in the bacterial growth cultures (Goncalves *et al.,* 2006).

The overall view from the previous chapters suggests that TIGR4 might show greater invasiveness when exposed to oxygen compared to absence of the molecule. We performed general animal model trials to clarify this theory and the results showed a pneumococcal tendency to cause invasive diseases when TIGR4 pneumococci are grown in oxygenated incubation conditions. Similar to these findings, pathogenicity of GBS was shown to be affected by oxygen. The authors demonstrated that growth of GBS in oxygenated conditions has significantly elevated the bacterial invasiveness level (Johri *et al.,* 2003). Therefore, it could be that oxygen molecules somehow increase pneumococcal virulence, but this largely needs to be elucidated.

Furthermore, bacterial virulence in animal models was increased when *Shigella* were grown under oxygenated conditions (Marteyn *et al.,* 2010). Additionally, abscess caused by *S. aureus* was increased once the bacteria were grown in

oxygenated environments. Moreover, animal experiments using Δ*rrg* mutants showed less pneumococcal virulence, indicating poor bacterial resistance to oxidative stress, which possibly has led to adhesion reduction (Bortoni *et al.*, 2009).

Chapter 6 - General Discussion and Future Work

6 General discussion and future work

The pneumococcal infection cycle is challenged by several anatomical niches including nasopharynx colonisation, lungs, blood and CSF. The bacterial survival in these environments definitely requires high genomic flexibility. From the base line growth curves of 11 pneumococci representing several serotypes, growth characteristics showed generally different phenotype traits in response to changes in concentration of oxygen molecules. Differences in oxygen levels mimicked the nasopharynx and blood circulation system in hosts. OXC141, for example, illustrated similar growth patterns and generation times regardless of oxygen availability, whereas P1041 made greater biomass when grown in strictly anaerobic conditions. It could be that oxygen has differentially regulated the bacterial metabolism and/or pathogenicity which might explain why some pneumococcal strains such as OXC141 are carried while some other strains such as P1041 are hyper-virulent and highly considered as invasive strains (Harvey *et al.*, 2011b; Williams *et al.*, 2012).

Growth rates in *S. pneumoniae* TIGR4 and OXC141 were similar under aerobic and anaerobic incubation conditions. However, other pneumococci were largely affected when they were grown aerobically rather than anaerobically. This can be seen for example in serotype 1 pneumococci (P1041) and another two strains representing serotype 3 (99-4039, the CSF isolate and 99-4038, the blood isolate). The peroxide assays results showed that TIGR4 has generated significant amounts of H_2O_2 during growth in oxygenated relative to anaerobic growth conditions, but we do not know what the production levels of H_2O_2 are in these other micro-organisms during growth in oxygen and anaerobic growth environments. It has been reported that determination of peroxides produced by serotype 2 pneumococci was below the detection level when they were grown anaerobically (Pericone *et al.*, 2002). Therefore, it would be interesting to perform H_2O_2 assays for these aerobically affected bacteria to detect the levels of peroxide toxicity they generate in the conditions used. Another possible experiment can be achieved here is to measure inhibition zones on blood agar using H_2O_2 discs. This may reflect different unknown abilities to resist several levels of H_2O_2 that might translate changes observed during growth curves in various O_2 concentrations (Chapter 3). This highlights why some strains such as

OXC141 and TIGR4 grew better in oxygen than others. If this is related to susceptibility to H_2O_2, it might explain why there are differences in virulence in terms of pneumonia. It could be highly beneficial if oxygen levels are tracked indirectly in different anatomical niches during the pneumococcal infection such as nasopharynx, lung, blood, spleen, liver and meninges. This might be feasible when fluorescently tagged genes are well known to be over-expressed with oxygen such as tpx or SP0095, which has been shown in the present study to be induced in oxygenated conditions 36-fold when compared to anaerobic expression. This potentially will reveal where pneumococci are exposed to high levels of oxygen *in vivo*.

It is also possible to genetically compare the *spxB* gene in those pneumococci in order to dissect the gene sequence in all of them. It is worth noting that pneumococci have been shown to differentially generate H_2O_2. For instance, GTC13809 strains representing serotype 19 approximately generates 5mM in the mid-log phase (Taniai *et al.,* 2008), whereas during growth in the stationary phase the serotypes 9V (P10 clinical isolates), 6V (P303 clinical isolates) and 2 (D39) produced 2.7 mM, 2.2 mM and 2.3 mM, respectively (Pericone *et al.,* 2002). In addition, another study has detected 0.45 mM in pneumococci representing serotype 2 in an hour (Overweg *et al.,* 2000). Interestingly, this amount was similar to what host activated neutrophils produced (Pesakhov *et al.,* 2007). Hence, if these organisms can grow in such peroxide differences, they are able then to resist the killing mechanisms used by immune cells.

Pneumococcal gene expression revealed that 420 genes were differentially expressed under oxygenated as compared to anoxygenated growth states. This showed that approximately 20% of the entire TIGR4 genome was affected by oxygen availability. However, the analysis of expression was performed during the bacterial exponential phase where no starvation or waste accumulation had begun. These factors are well known to affect the expression of a large number of genes. Accordingly, scanning the TIGR4 genome during its stationary phase will be different. Conway and Schoolnik stated that using samples collected from the bacterial log phase will not show non-mid-exponential phase-specific genes (Conway *et al.,* 2003). We have not investigated more than one time point during the TIGR4 growth curve. Therefore, it would be more comprehensive and may provide a wider genetic view if gene expression is scanned at a set of time-

points during growth. In addition, we only studied the expression of one strain, TIGR4, but how other serotypes would behave under oxygenated/anaerobic growth conditions is unknown. The only study to undertake *S. pneumoniae* rather than TIGR4 in these environments is Bortoni *et al* (2009) which performed similar work but used serotype 2 instead.

Our data analysis of *ply* mRNA, coded by SP1923 gene, showed that its expression was induced in TIGR4 cells during their oxygenated growth conditions. Its expression was further studied here under the same incubation environments with immunoblots to detect the protein intensity and haemolytic assays testing the biological activity of the toxin biology. All these techniques have demonstrated greater Ply (transcriptional and post-transcriptional) activities when oxygen was available. Western blot showed that Ply intensity in oxygenated versus anaerobic environments in both type 3 (OXC141) and type 4 (TIGR4) was similar as they produce greater amounts in oxygenated conditions. In accordance, *in vivo* investigations of *ply* gene expression using type 2 (D39) have detected an increase in the nasopharynx relative to bacteria recovered from the bloodstream of mice (LeMessurier *et al.,* 2006; Mahdi *et al.,* 2008). Interestingly, the specific antibodies used in the immuno blot have successfully detected same amounts of Ply during anaerobic growth of TIGR4, but these showed no lytic activity. Our thought is that Ply could be either at a very low level and then was not enough to lyse RBCs, or may be the oxygen absence has somehow inactivated the protein after affecting TCS(s). However, blotting of Ply after growing type 1 bacteria (P1041) using the same growth conditions showed bands homology. Although this suggests that oxygen has no impact on *ply* gene expression in the strain P1041, it is not known whether the toxin activity was similar under both conditions. Time permitting, we would have performed haemolytic activity assays for P1041 Ply after growth to the same bacterial population in the presence/absence of oxygen.

Another example showing gene variation under the same niche was reported in *spxB*. Its expression was induced in the brain with WCH16 pneumococci but reduced under the same conditions with the strains WCH43 (Mahdi *et al.,* 2008). This discrepancy in gene expression has been also found in several animal studies. For instance, Oggioni and coworkers have documented similar *ply* expression in TIGR4 under three discrete niches; lung, brain and biofilm (Oggioni

et al., 2006). In contrast, in 2004, expression of *ply* was detected to be greater during TIGR4 attachment to human nasopharyngeal cells *in vitro* than those which bound to mice-CSF *in vivo* (Orihuela *et al.,* 2004). Experimental differences, data analysis and strain-specificly factors were taken into account as being perhaps responsible for these variations.

The results also revealed that high oxygen levels were significantly implicated in positive regulation and served several virulence related factors including different CPSs, a number of CBPs along with two choline transporters, ComE, SpxB, HtrA, Clp pretease, Tpx, SodA, the vast majority of TCSs and several Blp proteins. In other words, the majority of the pneumococcal virulence genes were significantly over-expressed in oxygen relative to strict anaerobic conditions, indicating strong evidence of oxygen being a critical potential external stimulus powering pneumococcal pathogenicity.

Pneumococcal PspC has largely contributed in host adhesion (Ma *et al.,* 2007; Standish *et al.,* 2005). Moreover, its participation in pneumococcal biofilm formation has been shown, as well as Lic, choline uptake protein (Kwak *et al.,* 2002; Moscoso *et al.,* 2006; Rock *et al.,* 2001). Pneumococcal agglutination was apparently increased during oxygenated growth, which might be attributed to PspC induction. The findings presented in this study showed that *pspC* in TIGR4 was significantly expressed in oxygenated relative to anaerobic gathered samples. In addition, adhesion ability to the human cell line used was also significant and the protein intensities detected were greater in both the bacterial cytoplasm and on cell surfaces in oxygenated than anoxygenated conditions. However, it is not known if the PspC-relationship to oxygen was responsible for the bacterial agglutination or not, and it would be useful to perform biofilm assays using a knockout *pspC* mutant in order to test this theory and PspC's possible role(s) in forming pneumococcal biofilms in oxygen.

Overall, the results of pneumococcal virulence expression under the conditions used are relatively in line with their known roles in pathogenicity such as the reported greater adhesion when pneumococci exposed to air, which is believed to mimic human nasopharyngeal conditions *in vivo*. The findings also highlight the proper use of vaccine/s, e.g., PspC against the bacterial infection.

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Nicholas J. Croucher, Andrea M. Mitchell, Katherine A Gould, Donald Inverarity, Lars Barquist, Theresa Feltwell, Maria C. Fookes, Simon R. Harris, Janina Dordel, Susannah J. Salter, Sarah Browall, Helena Z**e**mlickova, Julian Parkhill, Staffan Normark, Birgitta Henriques-Normark, Jason Hinds, Tim J. Mitchell* & Stephen D. Bentley. Dominant Role of Nucleotide Substitution in the Diversification of Serotype 3 Pneumococci over Decades and during a Single Infection. Plos Genetics, Manuscript Accepted/In Press.

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Poster presentations:

Saudi International Conference (SIC04) Saudi minds in action, Characterisation of Pneumococcal Growth and Gene Expression. Hold at University of Manchester, United Kingdom, July 2010

Symposium - pneumonia day, University of Glasgow, Characterisation of Pneumococcal Growth and Gene Expression, United Kingdom, October 2010

Society for General Microbiology (SGM) autumn conference 2012, Characterisation of Pneumococcal Growth and Gene Expression, Hold at University of Warwick, United Kingdom, September 2012

Inaugural Symposium of the Institute of Microbiology and Infection (ISIMI). Hold at University of Birmingham, United Kingdom, December 2012 (attending only).

Appendices

Appendices

Table A - 1 List of genes expressed significantly (p<0.05) during pneumococcal TIGR4 growth in oxygenated as compared to anoxygenated environmental conditions. P<0.05 with Benjamini and Hochberg correction applied for multiple testing using 1 way ANOVA test. Gene expression above 1.5-fold cut-off is used in the table.

Gene number	P<0.05	Fold change in O_2	Biological function
SpTIGR4-0198 (1F13)	0.01	2.62+	hypothetical protein
SpTIGR4-0338 (1J19)	0.05	26+	ATP-dependent Clp protease ATP-binding subunit
SpTIGR4-0372 (1L23)	0	1.54+	hypothetical protein
SpTIGR4-0386 (2B1)	0.05	1.66+	sensor histidine kinase
SpTIGR4-0784 (3F2)	0.03	3.27+	glutathione reductase
SpTIGR4-0869 (3K1)	0.01	7.16+	aminotransferase, class-V
SpTIGR4-1160 (4F1)	0.04	2.6+	lipoate-protein ligase
SpTIGR4-1163 (4A2)	0.01	2.29+	acetoin dehydrogenase, E1 component, beta subunit
SpTIGR4-2016 (6E12)	0.05	2.90+	nicotinate-nucleotide pyrophospharylase
SpTIGR4-2017 (6F12)	0.05	4.34+	hypothetical protein
SpTIGR4-2187 (6G21)	0.04	10.33+	hypothetical protein
SpTIGR4-0024 (1H3)	0.03	1.69+	hypothetical protein
SpTIGR4-0044 (1D6)	0.05	15.43+	phosphoribosylaminomidazole-succinocarboxamide synthase
SpTIGR4-0046 (1F6)	0.04	13.82+	amidophosphorobosyltransferase
SpTIGR4-0060 (1D8)	0.04	2.24+	beta-glactosidase 3
SpTIGR4-0075 (1C10)	0.02	1.65+	phosphorylase Pnp/Udp family protein
SpTIGR4-0083 (1C11)	0.01	1.9+	DNA-binding response regulator
SpTIGR4-0084 (1D11)	0.02	1.82+	sensor histidine kinase
SpTIGR4-0085 (1E11)	0.04	1.75-	30 S ribosomal protein S4
SpTIGR4-0088 (1H11)	0.03	2.06+	hypothetical protein
SpTIGR4-0095 (1G12)	0.01	36+	hypothetical protein
SpTIGR4-0096 (1H12)	0.05	2.25+	hypothetical protein
SpTIGR4-0107 (1K2)	0.02	3.37+	LysM domain-containing protein
SpTIGR4-0120 (1P3)	0.01	1.97+	tRNA udidine 5carboxymethylaminomethyl modification enzyme GidA
SpTIGR4-0145 (1I7)	0.03	1.65+	hypothetical protein
SpTIGR4-0165 (1M9)	0.04	1.88+	flavoprptein
SpTIGR4-0191 (1O12)	0.05	1.53+	hypothetical protein
SpTIGR4-0198 (1F13)	0	2.63+	hypothetical protein
SpTIGR4-0208 (1H14)	0.03	1.63-	30S ribosomal protein S10
SpTIGR4-0220 (1D16)	0.04	1.51-	50S ribosomal protein L24
SpTIGR4-0222 (1F16)	0.05	1.69-	30S ribosomal protein S14
SpTIGR4-0237 (1E18)	0.01	1.55-	50S ribosomal protein L17
SpTIGR4-0267 (1C22)	0.02	2.12+	hypothetical protein
SpTIGR4-0289 (1I13)	0.03	1.54+	dihydropteroate synthase
SpTIGR4-0324 (1L17)	0.02	4+	PTS system, IIC component
SpTIGR4-0325 (1M17)	0.02	4.81+	PTS system, IID component
SpTIGR4-0330 (1J18)	0.01	1.74+	suger binding transcriptional regulator RegR
SpTIGR4-0338 (1J19)	0	26+	ATP-dependent Clp protease ATP-binding subunit
SpTIGR4-0345 (1I20)	0.04	1.52+	IS630-Spn1, transposase
SpTIGR4-0368 (1P22)	0.03	1.52-	cell wall surface anchor family

			protein
SpTIGR4-0369 (1I23)	0.02	1.61+	penicillin-binding protein 1A
SpTIGR4-0386 (2B1)	0	1.66+	sensor histidine kinase
SpTIGR4-0487 (2N1)	0.05	2.31+	hypothetical protein
SpTIGR4-0488 (2O1)	0.01	2.66+	hypothetical protein
SpTIGR4-0489 (2P1)	0.02	1.95+	PAP2 family protein
SpTIGR4-0490 (2I2)	0.01	1.88+	hypothetical protein
SpTIGR4-0507 (2J4)	0.03	1.67-	type I restericton-modification system, S subunit
SpTIGR4-0515 (2J5)	0.05	1.65+	heat-inducibal transcription repressor
SpTIGR4-0529 (2P6)	0.03	1.66+	BlpC ABC transporter
SpTIGR4-0530 (2I7)	0.02	2.14+	BlpC ABC transporter ATP-binding protein
SpTIGR4-0532 (2K7)	0.04	2.88+	bacteriocin BlpJ
SpTIGR4-0545 (2P8)	0.02	1.93+	immunity protein BlpY
SpTIGR4-0547 (2J9)	0.04	1.67+	hypothetical protein
SpTIGR4-0564 (2J11)	0.04	1.66-	hypothetical protein
SpTIGR4-0648 (2F21)	0.04	1.61+	beta-glactosidase
SpTIGR4-0663 (2E23)	0.01	1.85+	hypothetical protein
SpTIGR4-0664 (2F23)	0.03	1.60+	zinc metalloprotease ZmpB
SpTIGR4-0701 (2K16)	0.02	1.67-	orotidine 5-phosphate decarboxylase
SpTIGR4-0702 (2L16)	0.01	1.97-	orotate phosphoribosyltransferase
SpTIGR4-0709 (2K17)	0.04	1.51+	amino acid ABC transporter ATP-binding proten
SpTIGR4-0710 (2L17)	0.04	2.73+	amino acid ABC transporter permease
SpTIGR4-0711 (2M17)	0.03	2.89+	amino acid ABC transporter permease
SpTIGR4-0715 (2I18)	0.02	1.71+	lactate oxidase
SpTIGR4-0716 (2J18)	0.04	2.10+	transcriptional regulator
SpTIGR4-0726 (2L19)	0.02	5.39+	phosphomethylpyrimidine kinase
SpTIGR4-0730 (2P19)	0.01	3.36+	pyruvate oxidase
SpTIGR4-0731 (2I20)	0.01	2.87+	hypothetical protein
SpTIGR4-0766 (2L24)	0.02	4.17+	superoxide dismutase, manganasedependent
SpTIGR4-0783 (3E2)	0.04	2.23-	hypothetical protein
SpTIGR4-0784 (3F2)	0	3.27+	glutathione reductase
SpTIGR4-0789 (3C3)	0.03	1.96+	hypothetical protein
SpTIGR4-0790 (3D3)	0	2.07+	hypothetical protein
SpTIGR4-0798 (3D4)	0.03	1.56+	DNA-binding response regulator CiaR
SpTIGR4-0799 (3E4)	0.02	1.60+	sensor hitidine kinase CiaH
SpTIGR4-0818 (3H6)	0.04	1.53+	IS630-Spn1 transposase
SpTIGR4-0820 (3B7)	0.03	2.08+	ATP-dependent Clp protease ATP-binding subunit
SpTIGR4-0823 (3E7)	0.05	1.52-	amino acid ABC transporter permease
SpTIGR4-0867 (3I1)	0.01	3.87+	ABC transporter ATP-binding protein
SpTIGR4-0868 (3J1)	0.02	5.92+	hypothetical protein
SpTIGR4-0869 (3K1)	0	7.16+	aminotransferase, class-v
SpTIGR4-0870 (3L1)	0.03	4.38+	NifU family protein
SpTIGR4-0875 (3I2)	0.03	1.77-	lactose phosphotransferase system repressor
SpTIGR4-0916 (3J7)	0	2.40-	lysine decarboxylase
SpTIGR4-0918 (3L7)	0.01	2.08-	spermidine synthase
SpTIGR4-0919 (3M7)	0.02	2.05-	hypothetical protein
SpTIGR4-0920 (3N7)	0	1.97-	carboxynorspermidine

			decarboxylase
SpTIGR4-0921 (3O7)	0.01	2.24-	agmatine deiminase
SpTIGR4-0922 (3P7)	0.02	2.18-	carbon-nitrogen hydrolase family protein
SpTIGR4-0959 (3M12)	0.04	1.60-	translation initiation factor factor IF-3
SpTIGR4-0960 (3N12)	0.05	1.78-	50S ribosomal protein L35
SpTIGR4-0961 (3O12)	0.01	1.65-	50S ribosomal protein L20
SpTIGR4-0962 (3P12)	0.02	1.60-	lactoylglutathione lyase
SpTIGR4-0963 (3A13)	0.03	1.95-	dihydroorotate dihydrogenase electron tranfer subunit
SpTIGR4-0964 (3B13)	0.02	1.97-	dihydroorotate dihydrogenase 1B
SpTIGR4-0999 (3E17)	0.01	2.26+	cytochrome c-type biogenesis protein CcdA
SpTIGR4-1000 (3F17)	0.01	2.37+	thioredoxin family protein
SpTIGR4-1041 (3G22)	0.04	1.52-	hypothetical protein
SpTIGR4-1064 (3N13)	0.01	1.87-	IS200 family transposase
SpTIGR4-1117 (3K20)	0.02	1.53-	NAD-dependent DNA ligase ligA
SpTIGR4-1118 (3L20)	0.04	1.55-	pullulanase
SpTIGR4-1125 (3K21)	0.04	1.62-	phosphoserine phosphatase SerB
SpTIGR4-1149 (3K24)	0.01	1.6+	IS630-Spn1, transposase Orf1
SpTIGR4-1160 (4F1)	0	2.60+	lipoate-protein ligase
SpTIGR4-1161 (4G1)	0.04	2.66+	acetoin dehydrogenase complex, E3 component
SpTIGR4-1162 (4H1)	0.01	2.64+	dihydrolipoamide acetyltransferase
SpTIGR4-1163 (4A2)	0	2.29+	acetoin dehyrogenase, E1 component beta subunit
SpTIGR4-1164 (4B2)	0	2.23+	acetoin dehyrogenase, E1 component alpha subunit
SpTIGR4-1180 (4B4)	0.01	1.63-	ribonucleotide-diphsphate reductase subunit deta
SpTIGR4-1208 (4F7)	0.01	1.52-	uridine kinase
SpTIGR4-1225 (4G9)	0.02	1.97+	vicX protein
SpTIGR4-1226 (4H9)	0.01	2.12+	sensory box sensor histidine kinase
SpTIGR4-1227 (4A10)	0	2.21+	DNA-binding response regulator
SpTIGR4-1228 (4B10)	0.02	3.60+	A/G-specific adenine glycosylase
SpTIGR4-1248 (4E12)	0.04	1.71-	ribonuclease
SpTIGR4-1276 (4I4)	0.03	1.98-	carbamoyl phosphate synthase small subunit
SpTIGR4-1277 (4J4)	0.01	2.03-	aspartate carbamoyltransferase catalytic subunit pyrB
SpTIGR4-1278 (4K4)	0.02	2.09-	bifunctional pyrimidine regulatory protein PyrR uracil phosphoribosyltransferase
SpTIGR4-1286 (4K5)	0.03	2.13-	uracil permease
SpTIGR4-1353 (4F13)	0.02	1.94-	hypothetical protein
SpTIGR4-1354 (4G13)	0.05	1.62-	50S ribosomal protein L7/L12
SpTIGR4-1357 (4B14)	0.01	1.72+	ABC transpoter ATP-binding protein/permease
SpTIGR4-1358 (4C14)	0.03	1.63+	ABC transpoter ATP-binding protein/permease
SpTIGR4-1361 (4F14)	0.04	1.53+	homoserine dehydrogenase
SpTIGR4-1415 (4D21)	0.03	2.41+	glucosamine-6-phosphate isomerase
SpTIGR4-1427 (4H22)	0.01	3.37-	U32 family peptidase
SpTIGR4-1428 (4A23)	0.02	3.41-	hypothetical protein
SpTIGR4-1429 (4B23)	0.02	2.73-	U32 family peptidase
SpTIGR4-1438 (4C24)	0.04	2.24+	ABC transpoter ATP-binding protein
SpTIGR4-1458 (4O14)	0.02	1.63+	thioredoxin reductase
SpTIGR4-1462 (4K15)	0.03	1.55+	hypothetical protein

SpTIGR4-1464 (4M15)	0.03	1.75+	acetyltransferase
SpTIGR4-1471 (4L16)	0.03	3.63-	oxidoreductase
SpTIGR4-1472 (4M16)	0.01	4.14-	oxidoreductase
SpTIGR4-1499 (4P19)	0	2.52+	bacteriocin transport accessory protein
SpTIGR4-1532 (4I24)	0.03	1.50-	conserved domain protein
SpTIGR4-1539 (4P24)	0.03	1.52-	30S ribosomal protein S18
SpTIGR4-1546 (5G1)	0.01	4.41+	hypothetical protein
SpTIGR4-1587 (5H6)	0.02	24+	oxalate:formate antiporter
SpTIGR4-1592 (5E7)	0.03	1.61-	hypothetical protein
SpTIGR4-1622 (5C11)	0.02	2.01-	IS200 family transposase
SpTIGR4-1651 (5P2)	0.01	6.96+	thiol peroxidase
SpTIGR4-1690 (5O7)	0.03	2.20+	ABC-transporter supstrate-binding protein
SpTIGR4-1695 (5L8)	0.02	1.53+	acetyl xylan esterase
SpTIGR4-1708 (5I10)	0.05	1.65+	hypothetical protein
SpTIGR4-1716 (5I11)	0.05	1.51+	hypothetical protein
SpTIGR4-1717 (5J11)	0.01	1.71+	ABC transpoter ATP-binding protein
SpTIGR4-1721 (5N11)	0.03	1.86-	fructokinase
SpTIGR4-1724 (5I12)	0.01	1.52-	sucrose-6-phosphate hydrolase
SpTIGR4-1725 (5J12)	0.04	1.64-	sucrose operon repressor
SpTIGR4-1775 (5D18)	0.01	2.26+	hypothetical protein
SpTIGR4-1776 (5E18)	0.04	2.17+	thioredoxin
SpTIGR4-1778 (5G18)	0.01	3.87+	aquaporin
SpTIGR4-1793 (5F20)	0.02	2.03+	hypothetical protein
SpTIGR4-1794 (5G20)	0.02	2.33+	hypothetical protein
SpTIGR4-1807 (5D22)	0	1.57+	acetyltransferase
SpTIGR4-1809 (5F22)	0.02	1.87+	transcriptional regulator
SpTIGR4-1845 (5J15)	0.01	2.03+	exodeoxyribonuclease
SpTIGR4-1846 (5K15)	0.05	1.65+	conserved hypothetical protein
SpTIGR4-1852 (5I16)	0.01	2.47+	galactose-1-phosphate uridylytransferase
SpTIGR4-1860 (5I17)	0.04	2.6+	choline transporter
SpTIGR4-1861 (5J17)	0.02	2.82+	choline transporter
SpTIGR4-1862 (5K17)	0.03	2.89+	hypothetical protein
SpTIGR4-1863 (5L17)	0.03	2.46+	MarR family transcriptional regulator
SpTIGR4-1869 (5J18)	0.01	2.60+	iron-compound ABC transporter permease
SpTIGR4-1871 (5L18)	0.03	2.43+	iron-compound ABC transporter ATP-binding protein
SpTIGR4-1872 (5M18)	0.04	2.29+	iron-compound ABC transporter iron-compound-binding protein
SpTIGR4-1884 (5I20)	0.03	2.25+	trehalose PTS system, IIABC components
SpTIGR4-1894 (5K21)	0.01	2.28+	sucrose phosphorylase
SpTIGR4-1895 (5L21)	0.01	2.21+	sugar ABC tranporter permease
SpTIGR4-1896 (5M21)	0.02	2.54+	sugar ABC tranporter permease
SpTIGR4-1897 (5N21)	0	2.43+	sugar ABC transporter substrate-binding protein
SpTIGR4-1898 (5O21)	0.01	2.51+	alpha-glactosidase
SpTIGR4-1947 (6H3)	0.02	2.23+	hypothetical protein
SpTIGR4-1972 (6A7)	0.02	1.66+	hypothetical protein
SpTIGR4-1988 (6A9)	0.02	1.74+	immunity protein
SpTIGR4-2016 (6E12)	0	2.90+	nicotinate-nucleotide pyrophosphorylase
SpTIGR4-2017 (6F12)	0	4.34+	hypothetical protein
SpTIGR4-2026 (6O1)	0.03	3.30-	bifunctional acetaldehyde-CoA/alcohol dehydrogenase
SpTIGR4-2054 (6K5)	0.02	3.90+	hypothetical protein

SpTIGR4-2056 (6M5)	0.02	2.39+	N-acetylglucosamine-6-phosphate deacetylase
SpTIGR4-2057 (6N5)	0.02	1.61+	hypothetical protein
SpTIGR4-2063 (6L6)	0.02	2.60+	LysM domain-containing protein
SpTIGR4-2073 (6N7)	0.03	1.58+	ABC transpoter ATP-binding protein/permease
SpTIGR4-2075 (6P7)	0.01	1.76+	ABC transpoter ATP-binding protein/permease
SpTIGR4-2084 (6I9)	0.01	1.88+	phosphate ABC transporter phosphate-binding protein
SpTIGR4-2085 (6J9)	0.04	2.08+	phosphate ABC transporter permease
SpTIGR4-2086 (6K9)	0.02	2.09+	phosphate ABC transporter permease
SpTIGR4-2088 (6M9)	0.04	2.10+	phosphate transporter system regulator protein PhoU
SpTIGR4-2096 (6M10)	0.04	1.53+	M20/M25/M40 family peptidase
SpTIGR4-2106 (6O11)	0.01	3.42+	glycogen phosphrylase family protein
SpTIGR4-2107 (6P11)	0.02	3.50+	4-alpha-glucanotransferase
SpTIGR4-2135 (6D15)	0.05	1.51-	3-keto-L-gulonate-phosphate decarboxylase
SpTIGR4-2146 (6G16)	0.03	1.74+	hypothetical protein
SpTIGR4-2181 (6A21)	0.01	2.63+	identified by similarity to EGAD:135254
SpTIGR4-2186 (6F21)	0.01	2.60+	glycerol kinase glpK
SpTIGR4-2188 (6H21)	0.02	2.08+	Hsp 33-like chaperonin
SpTIGR4-2189 (6A22)	0.04	1.96+	NifR3 family TIM-barrel protein
SpTIGR4-2190 (6B22)	0.02	1.50+	choline binding protein A
SpTIGR4-2192 (6D22)	0.01	1.65+	sensor hesitidine kinase
SpTIGR4-2194 (6F22)	0.02	1.91+	ATP-dependent Clp protease ATP-binding subunit
SpTIGR4-2195 (6G22)	0.03	1.82+	transcriptional regulator CtsR
SpTIGR4-2202 (6F23)	0.03	1.61+	hypothetical protein
SpTIGR4-2203 (6G23)	0.04	1.63+	replicative DNA helicase
SpTIGR4-2204 (6H23)	0	1.59+	50S ribosomal protein L9
SpTIGR4-2205 (6A24)	0.02	1.68+	DHH subfamily 1 protein
SpTIGR4-2239 (6K16)	0.01	2.50+	serine protease
SpTIGR4-2240 (6L16)	0.01	2.51+	spspoJ protein

Table A - 2 List of genes expressed significantly (p<0.05) during pneumomococcal TIGR4 growth in oxygenated as compared to aerobic environmental conditions. P<0.05 with Benjamini and Hochberg correction applied for multiple testing using 1 way ANOVA test. Gene expression above 1.5-fold cut-off is used in the table.

Gene number	P<0.05	Fold change in O_2	Biological function
SpTIGR4-0148 (1L7)	0.0954	1.50+	ABC transporter substrate-binding protein
SpTIGR4-0409 (2A4)	0.0877	2.65+	hypothetical protein
SpTIGR4-0459 (2C10)	0.0871	2.21-	formate acetyletransferase
SpTIGR4-0626 (2H18)	0.0954	1.53+	branched-chain amino acid transport system II carrier protein
SpTIGR4-0701 (2K16)	0.0877	2.03-	orotidine 5-phosphate decarboxylase
SpTIGR4-0702 (2L16)	0.0877	2.61-	orotate phosphoribosyltransferase
SpTIGR4-0703 (2M16)	0.0969	1.57-	hypothetical protein
SpTIGR4-0730 (2P19)	0.0994	1.70+	pyruvate oxidase
SpTIGR4-0869 (3K1)	0.0925	1.79+	aminotransferase, class-v
SpTIGR4-0916 (3J7)	0.0871	1.57-	lysine decarboxylase
SpTIGR4-0963 (3A13)	0.0877	2.28-	dihydroorotate dihydrogenase electron tranfer subunit
SpTIGR4-0964 (3B13)	0.0969	2.12-	dihydroorotate dihydrogenase 1B
SpTIGR4-0999 (3E17)	0.0871	2.14+	cytochrome c-type biogenesis protein CcdA
SpTIGR4-1000 (3F17)	0.0925	2.35+	thioredoxin family protein
SpTIGR4-1064 (3N13)	0.0877	1.62-	IS200 family transposase
SpTIGR4-1154 (3P24)	0.0877	1.73+	immunoglobulin A1 protease
SpTIGR4-1175 (4E3)	0.0969	1.55+	hypothetical protein
SpTIGR4-1277 (4J4)	0.0871	1.94-	aspartate carbamoyltransferase catalytic subunit pyrB
SpTIGR4-1278 (4K4)	0.0954	1.85-	bifunctional pyrimidine regulatory protein PyrR uracil phosphoribosyltransferase
SpTIGR4-1286 (4K5)	0.0871	2.35-	uracil permease
SpTIGR4-1499 (4P19)	0.0796	2.52+	bacteriocin transport accessory protein
SpTIGR4-1572 (5A5)	0.0994	2.19-	non-heme iron containing ferritin
SpTIGR4-1622 (5C11)	0.0871	1.88-	IS200 family transposase
SpTIGR4-1651 (5P2)	0.0871	2.45+	thiol peroxidase
SpTIGR4-1721 (5N11)	0.0871	1.71-	fructokinase
SpTIGR4-1775 (5D18)	0.0871	1.64+	hypothetical protein
SpTIGR4-1811 (5H22)	0.0954	1.75+	tryptophan synthase subunit alpha
SpTIGR4-1884 (5I20)	0.0871	2.94-	trehalose PTS system, IIABC components
SpTIGR4-2005 (6B11)	0.0877	1.57+	hypothetical protein
SpTIGR4-2072 (6M7)	0.0994	1.94+	glutamine amidotransferase class I
SpTIGR4-2084 (6I9)	0.0877	2.42-	phosphate ABC transporter phosphate-binding protein
SpTIGR4-2085 (6J9)	0.0877	2.58-	phosphate ABC transporter permease
SpTIGR4-2086 (6K9)	0.0877	2.63-	phosphate ABC transporter permease
SpTIGR4-2088 (6M9)	0.0969	2.88-	phosphate transporter system regulator protein PhoU
SpTIGR4-2187 (6G21)	0.0877	1.56+	hypothetical protein

Table A - 3 List of genes expressed during pneumomococcal TIGR4 growth in anoxygenated as compared to aerobic environmental conditions. P<0.05 with Benjamini and Hochberg correction applied for multiple testing using 1 way ANOVA test. Gene expression above 1.5-fold cut-off is used in the table.

Gene number	P<0.05	Fold change in AnO_2	Biological function
SpTIGR4-0095 (1G12)	0.0397	28.66-	hypothetical protein
SpTIGR4-2054 (6K5)	0.0281	4.61-	hypothetical protein
SpTIGR4-2187 (6G21)	0.0177	6.60-	hypothetical protein
SpTIGR4-0012 (1D2)	0.0477	1.56-	hypoxanthine-guanine phosphoribosyltransferase
SpTIGR4-0013 (1E2)	0.0207	1.52-	cell division protein FtsH
SpTIGR4-0045 (1E6)	0.0119	2.67-	phosphoribosylformylglycinamidine synthase
SpTIGR4-0046 (1F6)	0.0493	3.15-	amidophosphorobosyltransferase
SpTIGR4-0047 (1G6)	0.0494	2.82-	phosphoribosylaminoimidazole synthetase
SpTIGR4-0050 (1B7)	0.0202	2.73-	bifunctional
SpTIGR4-0051 (1C7)	0.0202	1.65-	phosphoribosylamine-glycine ligase
SpTIGR4-0075 (1C10)	0.0427	1.84-	phosphorylase Pnp/Udp family protein
SpTIGR4-0079 (1G10)	0.0494	1.56-	Trk family potassium uptake protein
SpTIGR4-0083 (1C11)	0.0473	1.60-	DNA-binding response regulator
SpTIGR4-0088 (1H11)	0.0269	2.51-	hypothetical protein
SpTIGR4-0089 (1A12)	0.0428	1.56-	hypothetical protein
SpTIGR4-0095 (1G12)	0.00975	28.66-	hypothetical protein
SpTIGR4-0107 (1K2)	0.013	3.62-	LysM domain-containing protein
SpTIGR4-0118 (1N3)	0.0421	1.53-	tRNA-specific MnmA
SpTIGR4-0119 (1O3)	0.0282	1.92-	MutT/nudix family protein
SpTIGR4-0145 (1I7)	0.0322	1.64-	hypothetical protein
SpTIGR4-0191 (1O12)	0.0433	1.86-	hypothetical protein
SpTIGR4-0193 (1A13)	0.0449	1.64-	match to protein family HMM TIGR00250
SpTIGR4-0198 (1F13)	0.00975	2.20-	hypothetical protein
SpTIGR4-0199 (1G13)	0.0213	1.80-	cardiolipin synthetase
SpTIGR4-0220 (1D16)	0.0399	1.543+	50 S ribosomal protein L24
SpTIGR4-0259 (1C21)	0.049	1.52-	holliday junction DNA helicase RuvB
SpTIGR4-0267 (1C22)	0.0277	1.85-	hypothetical protein
SpTIGR4-0299 (1K14)	0.0473	1.52-	IS630-Spn1, transposase Orf1, authentic frameshift
SpTIGR4-0324 (1L17)	0.0391	5.74-	PTS system, IIC component
SpTIGR4-0325 (1M17)	0.0391	8.91-	PTS system, IID component
SpTIGR4-0338 (1J19)	0.00975	28.58-	ATP-dependent Clp protease ATP-binding subunit
SpTIGR4-0385 (2A1)	0.0399	1.59-	hypothetical protein
SpTIGR4-0409 (2A4)	0.0262	2.13+	hypothetical protein
SpTIGR4-0488 (2O1)	0.0202	2.96-	hypothetical protein
SpTIGR4-0489 (2P1)	0.0269	2.22-	PAP2 family protein
SpTIGR4-0490 (2I2)	0.0202	2.09-	hypothetical protein
SpTIGR4-0492 (2K2)	0.0477	2.10-	hypothetical protein
SpTIGR4-0499 (2J3)	0.0477	2.09+	phosphoglycerate kinase
SpTIGR4-0710 (2L17)	0.0289	2.90-	amino acid ABC transporter permease
SpTIGR4-0711 (2M17)	0.0322	2.93-	amino acid ABC transporter permease
SpTIGR4-0716 (2J18)	0.0494	3.07-	transcriptional regulator
SpTIGR4-0717 (2K18)	0.0494	3.45-	hydroxyethylthiazole kinase

SpTIGR4-0726 (2L19)	0.025	3.26-	phosphomethylpyrimidine kinase
SpTIGR4-0730 (2P19)	0.0433	1.97-	pyruvate oxidase
SpTIGR4-0731 (2I20)	0.0213	2.07-	hypothetical protein
SpTIGR4-0789 (3C3)	0.0449	1.85-	hypothetical protein
SpTIGR4-0790 (3D3)	0.00975	2.00-	hypothetical protein
SpTIGR4-0799 (3E4)	0.0473	2.56-	sensor histidine kinase CiaH
SpTIGR4-0820 (3B7)	0.031	2.02-	ATP-dependent Clp protease ATP-binding subunit
SpTIGR4-0868 (3J1)	0.0207	3.28-	hypothetical protein
SpTIGR4-0869 (3K1)	0.0119	4.00-	aminotransferase, class-V
SpTIGR4-0916 (3J7)	0.0289	1.52+	lysine decarboxylase
SpTIGR4-0918 (3L7)	0.0493	1.64+	spermidine synthase
SpTIGR4-0921 (3O7)	0.0466	1.52+	agmatine deiminase
SpTIGR4-1090 (3P16)	0.0405	2.00+	redox-sensing transcriptional repressor Rex
SpTIGR4-1148 (3J24)	0.0268	1.56-	IS630-Spn1, transposase Orf2
SpTIGR4-1149 (3K24)	0.0399	1.63-	IS630-Spn1, transposase Orf1
SpTIGR4-1152 (3N24)	0.0473	1.50+	exonuclease RexA
SpTIGR4-1153 (3O24)	0.0443	1.57+	hypothetical protein
SpTIGR4-1163 (4A2)	0.0174	2.34-	acetoin dehyrogenase, E1 component beta subunit
SpTIGR4-1164 (4B2)	0.0322	2.26-	acetoin dehyrogenase, E1 component alpha subunit
SpTIGR4-1180 (4B4)	0.0174	1.86+	ribonucleotide-diphsphate reductase subunit deta
SpTIGR4-1190 (4D5)	0.0213	2.33+	tagatose 1,6-diphosphate aldolase
SpTIGR4-1192 (4F5)	0.0202	2.04+	glactose-6-phosphate isomerase subunit LacB
SpTIGR4-1227 (4A10)	0.0402	1.50-	DNA-binding response regulator
SpTIGR4-1228 (4B10)	0.00975	2.39-	A/G-specific adenine glycosylase
SpTIGR4-1415 (4D21)	0.0266	2.06-	glucosamine-6-phosphate isomerase
SpTIGR4-1419 (4H21)	0.0174	1.96+	acetyltransferase
SpTIGR4-1420 (4A22)	0.0494	1.73+	NAD synthetase
SpTIGR4-1428 (4A23)	0.0267	1.70+	hypothetical protein
SpTIGR4-1438 (4C24)	0.0399	2.84-	ABC transpoter ATP-binding protein
SpTIGR4-1450 (4O13)	0.0289	1.60+	platelet activation factor
SpTIGR4-1451 (4P13)	0.0414	1.72+	Cof family protein
SpTIGR4-1452 (4I14)	0.0202	1.58+	hypothetical protein
SpTIGR4-1470 (4K16)	0.013	3.03+	thiamine biosynthesis protein ApbE
SpTIGR4-1471 (4L16)	0.0202	4.00+	oxidoreductase
SpTIGR4-1472 (4M16)	0.0372	3.42+	oxidoreductase
SpTIGR4-1546 (5G1)	0.0269	2.69-	hypothetical protein
SpTIGR4-1587 (5H6)	0.0202	9.00-	oxalate: formate antiporter
SpTIGR4-1651 (5P2)	0.0266	2.84-	thiol peroxidase
SpTIGR4-1778 (5G18)	0.0258	3.45-	aquaporin
SpTIGR4-1793 (5F20)	0.0211	4.11-	hypothetical protein
SpTIGR4-1794 (5G20)	0.013	4.07-	hypothetical protein
SpTIGR4-1809 (5F22)	0.0119	1.90-	transcriptional regulator
SpTIGR4-1852 (5I16)	0.0433	2.80-	glactose-1-phosphate uridylyltransferase
SpTIGR4-1860 (5I17)	0.0277	2.00-	choline transporter
SpTIGR4-1861 (5J17)	0.013	2.33-	choline transporter
SpTIGR4-1862 (5K17)	0.00975	2.35-	hypothetical protein
SpTIGR4-1883 (5P19)	0.0174	4.16-	dextran glucosidase DexS
SpTIGR4-1884 (5I20)	0.013	6.62-	trehalose PTS system, IIABC components
SpTIGR4-1894 (5K21)	0.0282	1.95-	sucrose phosphorylase

SpTIGR4-1896 (5M21)	0.0322	2.35-	sugar ABC transporter permease
SpTIGR4-1897 (5N21)	0.0322	2.22-	sugar ABC transporter substrate-binding protein
SpTIGR4-1898 (5O21)	0.00975	2.20-	alpha-glactosidase
SpTIGR4-1988 (6A9)	0.0449	1.71-	immunity protein
SpTIGR4-2010 (6G11)	0.0494	1.58-	penicillen-binding protein 2A
SpTIGR4-2013 (6B12)	0.0289	2.01-	hypothetical protein
SpTIGR4-2016 (6E12)	0.0266	3.40-	nicotinate-nucleotide pyrophosphorylase
SpTIGR4-2054 (6K5)	0.00975	4.52-	hypothetical protein
SpTIGR4-2056 (6M5)	0.0372	2.65-	N-acetylglucosamine-6-phosphate deacetylase
SpTIGR4-2058 (6O5)	0.0146	2.00-	queuine tRNA-ribosyltranferase
SpTIGR4-2063 (6L6)	0.0266	2.78-	LysM domain-containing protein
SpTIGR4-2073 (6N7)	0.0289	1.68-	ABC transpoter ATP-binding protein/permease
SpTIGR4-2076 (6I8)	0.0333	1.58-	DNA mismatch repair protein protein HexA
SpTIGR4-2084 (6I9)	0.0207	4.56-	phosphate ABC transporter phosphate-binding protein
SpTIGR4-2085 (6J9)	0.0289	5.39-	phosphate ABC transporter permease
SpTIGR4-2086 (6K9)	0.0311	5.52-	phosphate ABC transporter permease
SpTIGR4-2088 (6M9)	0.0399	6.05-	phosphate transporter system regulator protein PhoU
SpTIGR4-2106 (6O11)	0.0108	5.19-	glycogen phosphrylase family protein
SpTIGR4-2146 (6G16)	0.0202	2.16-	hypothetical protein
SpTIGR4-2148 (6A17)	0.0399	1.79-	arginine deiminase
SpTIGR4-2150 (6B17)	0.0473	1.72-	ornithine carbamoyltransferase
SpTIGR4-2180 (6H20)	0.0449	1.89-	conserved hypothetical protein
SpTIGR4-2181 (6A21)	0.0262	3.07-	identified by similarity to EGAD:135254
SpTIGR4-2186 (6F21)	0.0267	2.17-	glycerol kinase glpK
SpTIGR4-2187 (6G21)	0.00975	6.60-	hypothetical protein
SpTIGR4-2193 (6E22)	0.0494	1.61-	DNA-binding response regulator
SpTIGR4-2194 (6F22)	0.0289	1.82-	ATP-dependent Clp protease ATP-binding subunit
SpTIGR4-2195 (6G22)	0.0289	2.10-	transcriptional regulator CtsR
SpTIGR4-2210 (6F24)	0.0428	1.56-	hypothetical protein

Table A - 4 List of genes expressed (p<0.1) during pneumomococcal TIGR4 growth in different levels of oxygen. Oxy = oxygenated, Anoxy = anoxygenated, Anaero = anaerobic and Aero = aerobic. P<0.1 with Benjamini and Hochberg correction applied for multiple testing using 1 way ANOVA test.

Oxy. vs Anoxy.		Anaero. vs Aero.	
Gene	P<0.1	Gene	P<0.1
SpTIGR4-0013 (1E2)	0.00852	SpTIGR4-0004 (1D1)	0.0374
SpTIGR4-0014 (1F2)	0.0804	SpTIGR4-0011 (1C2)	0.0669
SpTIGR4-0015 (1G2)	0.071	SpTIGR4-0012 (1D2)	0.0477
SpTIGR4-0016 (1H2)	0.00664	SpTIGR4-0013 (1E2)	0.0207
SpTIGR4-0023 (1G3)	0.0495	SpTIGR4-0016 (1H2)	0.0889
SpTIGR4-0024 (1H3)	0.0265	SpTIGR4-0022 (1F3)	0.0666
SpTIGR4-0025 (1A4)	0.0611	SpTIGR4-0023 (1G3)	0.0768
SpTIGR4-0026 (1B4)	0.0605	SpTIGR4-0026 (1B4)	0.0845
SpTIGR4-0032 (1H4)	0.00451	SpTIGR4-0032 (1H4)	0.0322
SpTIGR4-0036 (1D5)	0.0414	SpTIGR4-0034 (1B5)	0.0907
SpTIGR4-0041 (1A6)	0.0218	SpTIGR4-0044 (1D6)	0.0586
SpTIGR4-0044 (1D6)	0.0486	SpTIGR4-0045 (1E6)	0.0119
SpTIGR4-0045 (1E6)	0.0582	SpTIGR4-0046 (1F6)	0.0493
SpTIGR4-0046 (1F6)	0.0414	SpTIGR4-0047 (1G6)	0.0494
SpTIGR4-0047 (1G6)	0.0618	SpTIGR4-0048 (1H6)	0.0652
SpTIGR4-0048 (1H6)	0.0711	SpTIGR4-0049 (1A7)	0.0551
SpTIGR4-0049 (1A7)	0.0733	SpTIGR4-0050 (1B7)	0.0202
SpTIGR4-0050 (1B7)	0.0556	SpTIGR4-0051 (1C7)	0.0202
SpTIGR4-0051 (1C7)	0.093	SpTIGR4-0053 (1E7)	0.0701
SpTIGR4-0053 (1E7)	0.091	SpTIGR4-0060 (1D8)	0.08
SpTIGR4-0055 (1G7)	0.0702	SpTIGR4-0069 (1E9)	0.0822
SpTIGR4-0056 (1H7)	0.0787	SpTIGR4-0075 (1C10)	0.0427
SpTIGR4-0060 (1D8)	0.0431	SpTIGR4-0079 (1G10)	0.0494
SpTIGR4-0063 (1G8)	0.0733	SpTIGR4-0083 (1C11)	0.0473
SpTIGR4-0075 (1C10)	0.015	SpTIGR4-0084 (1D11)	0.0275
SpTIGR4-0079 (1G10)	0.0179	SpTIGR4-0088 (1H11)	0.0269
SpTIGR4-0083 (1C11)	0.0146	SpTIGR4-0089 (1A12)	0.0428
SpTIGR4-0084 (1D11)	0.021	SpTIGR4-0095 (1G12)	0.00975
SpTIGR4-0085 (1E11)	0.0377	SpTIGR4-0096 (1H12)	0.0525
SpTIGR4-0088 (1H11)	0.032	SpTIGR4-0102 (1N1)	0.0554
SpTIGR4-0089 (1A12)	0.0741	SpTIGR4-0105 (1I2)	0.0473
SpTIGR4-0095 (1G12)	0.00688	SpTIGR4-0107 (1K2)	0.013
SpTIGR4-0096 (1H12)	0.045	SpTIGR4-0118 (1N3)	0.0421
SpTIGR4-0102 (1N1)	0.0377	SpTIGR4-0119 (1O3)	0.0282
SpTIGR4-0104 (1P1)	0.0335	SpTIGR4-0120 (1P3)	0.072
SpTIGR4-0105 (1I2)	0.00722	SpTIGR4-0142 (1N6)	0.0677
SpTIGR4-0106 (1J2)	0.0255	SpTIGR4-0145 (1I7)	0.0322
SpTIGR4-0107 (1K2)	0.0182	SpTIGR4-0168 (1P9)	0.0702
SpTIGR4-0110 (1N2)	0.0162	SpTIGR4-0173 (1M10)	0.0669
SpTIGR4-0118 (1N3)	0.0367	SpTIGR4-0189 (1M12)	0.0666
SpTIGR4-0119 (1O3)	0.0994	SpTIGR4-0191 (1O12)	0.0433
SpTIGR4-0120 (1P3)	0.00976	SpTIGR4-0192 (1P12)	0.0586
SpTIGR4-0142 (1N6)	0.0549	SpTIGR4-0193 (1A13)	0.0449
SpTIGR4-0145 (1I7)	0.033	SpTIGR4-0198 (1F13)	0.00975
SpTIGR4-0148 (1L7)	0.0489	SpTIGR4-0199 (1G13)	0.0213
SpTIGR4-0149 (1M7)	0.0504	SpTIGR4-0202 (1B14)	0.0919
SpTIGR4-0153 (1I8)	0.0531	SpTIGR4-0217 (1A16)	0.0702
SpTIGR4-0163 (1K9)	0.0531	SpTIGR4-0220 (1D16)	0.0399
SpTIGR4-0165 (1M9)	0.0402	SpTIGR4-0222 (1F16)	0.072
SpTIGR4-0168 (1P9)	0.0273	SpTIGR4-0223 (1G16)	0.0554

SpTIGR4-0173 (1M10)	0.0552	SpTIGR4-0228 (1D17)	0.0592
SpTIGR4-0187 (1K12)	0.0733	SpTIGR4-0231 (1G17)	0.0845
SpTIGR4-0189 (1M12)	0.0368	SpTIGR4-0232 (1H17)	0.0954
SpTIGR4-0191 (1O12)	0.0479	SpTIGR4-0234 (1B18)	0.0768
SpTIGR4-0192 (1P12)	0.0237	SpTIGR4-0237 (1E18)	0.0372
SpTIGR4-0193 (1A13)	0.0733	SpTIGR4-0250 (1B20)	0.0673
SpTIGR4-0198 (1F13)	0.00352	SpTIGR4-0255 (1G20)	0.0666
SpTIGR4-0199 (1G13)	0.0295	SpTIGR4-0259 (1C21)	0.049
SpTIGR4-0202 (1B14)	0.0365	SpTIGR4-0261 (1E21)	0.0667
SpTIGR4-0203 (1C14)	0.091	SpTIGR4-0263 (1G21)	0.0561
SpTIGR4-0204 (1D14)	0.0237	SpTIGR4-0267 (1C22)	0.0277
SpTIGR4-0205 (1E14)	0.032	SpTIGR4-0272 (1H22)	0.0202
SpTIGR4-0206 (1F14)	0.045	SpTIGR4-0284 (1D24)	0.0504
SpTIGR4-0208 (1H14)	0.0273	SpTIGR4-0287 (1G24)	0.0846
SpTIGR4-0209 (1A15)	0.0279	SpTIGR4-0290 (1J13)	0.08
SpTIGR4-0210 (1B15)	0.0556	SpTIGR4-0299 (1K14)	0.0473
SpTIGR4-0211 (1C15)	0.0268	SpTIGR4-0311 (1O15)	0.0904
SpTIGR4-0212 (1D15)	0.033	SpTIGR4-0314 (1J16)	0.0859
SpTIGR4-0213 (1E15)	0.0418	SpTIGR4-0324 (1L17)	0.0391
SpTIGR4-0214 (1F15)	0.091	SpTIGR4-0325 (1M17)	0.0391
SpTIGR4-0217 (1A16)	0.0278	SpTIGR4-0330 (1J18)	0.0525
SpTIGR4-0219 (1C16)	0.063	SpTIGR4-0338 (1J19)	0.00975
SpTIGR4-0220 (1D16)	0.0367	SpTIGR4-0342 (1N19)	0.0322
SpTIGR4-0222 (1F16)	0.0497	SpTIGR4-0344 (1P19)	0.0854
SpTIGR4-0223 (1G16)	0.0479	SpTIGR4-0345 (1I20)	0.0787
SpTIGR4-0228 (1D17)	0.0582	SpTIGR4-0354 (1J21)	0.0822
SpTIGR4-0230 (1F17)	0.045	SpTIGR4-0356 (1L21)	0.0666
SpTIGR4-0232 (1H17)	0.0253	SpTIGR4-0358 (1N21)	0.0473
SpTIGR4-0233 (1A18)	0.0173	SpTIGR4-0359 (1O21)	0.0884
SpTIGR4-0234 (1B18)	0.0193	SpTIGR4-0360 (1P21)	0.0845
SpTIGR4-0237 (1E18)	0.0095	SpTIGR4-0368 (1P22)	0.0697
SpTIGR4-0239 (1G18)	0.0701	SpTIGR4-0369 (1I23)	0.00975
SpTIGR4-0240 (1H18)	0.0521	SpTIGR4-0370 (1J23)	0.0473
SpTIGR4-0246 (1F19)	0.0975	SpTIGR4-0371 (1K23)	0.0774
SpTIGR4-0250 (1B20)	0.0769	SpTIGR4-0372 (1L23)	0.055
SpTIGR4-0259 (1C21)	0.095	SpTIGR4-0373 (1M23)	0.0423
SpTIGR4-0261 (1E21)	0.0527	SpTIGR4-0377 (1I24)	0.0847
SpTIGR4-0263 (1G21)	0.0339	SpTIGR4-0378 (1J24)	0.0666
SpTIGR4-0264 (1H21)	0.0554	SpTIGR4-0385 (2A1)	0.0399
SpTIGR4-0266 (1B22)	0.0701	SpTIGR4-0386 (2B1)	0.0702
SpTIGR4-0267 (1C22)	0.0167	SpTIGR4-0387 (2C1)	0.0586
SpTIGR4-0270 (1F22)	0.0618	SpTIGR4-0389 (2E1)	0.0598
SpTIGR4-0271 (1G22)	0.0861	SpTIGR4-0390 (2F1)	0.0964
SpTIGR4-0272 (1H22)	0.00451	SpTIGR4-0402 (2B3)	0.0311
SpTIGR4-0273 (1A23)	0.0108	SpTIGR4-0405 (2E3)	0.0861
SpTIGR4-0275 (1C23)	0.0742	SpTIGR4-0409 (2A4)	0.0262
SpTIGR4-0278 (1F23)	0.0748	SpTIGR4-0412 (2D4)	0.0554
SpTIGR4-0282 (1B24)	0.0974	SpTIGR4-0432 (2H6)	0.0859
SpTIGR4-0283 (1C24)	0.0605	SpTIGR4-0438 (2F7)	0.0741
SpTIGR4-0284 (1D24)	0.0387	SpTIGR4-0453 (2E9)	0.0713
SpTIGR4-0286 (1F24)	0.063	SpTIGR4-0459 (2C10)	0.013
SpTIGR4-0287 (1G24)	0.0899	SpTIGR4-0481 (2I1)	0.0904
SpTIGR4-0288 (1H24)	0.0975	SpTIGR4-0482 (2J1)	0.0854
SpTIGR4-0289 (1I13)	0.0331	SpTIGR4-0483 (2K1)	0.0504
SpTIGR4-0295 (1O13)	0.0504	SpTIGR4-0487 (2N1)	0.0669
SpTIGR4-0299 (1K14)	0.0767	SpTIGR4-0488 (2O1)	0.0202
SpTIGR4-0300 (1L14)	0.0492	SpTIGR4-0489 (2P1)	0.0269
SpTIGR4-0311 (1O15)	0.0753	SpTIGR4-0490 (2I2)	0.0202

SpTIGR4-0324 (1L17)	0.0237	SpTIGR4-0492 (2K2)	0.0477
SpTIGR4-0325 (1M17)	0.021	SpTIGR4-0496 (2O2)	0.0713
SpTIGR4-0330 (1J18)	0.00704	SpTIGR4-0499 (2J3)	0.0477
SpTIGR4-0338 (1J19)	0.00411	SpTIGR4-0500 (2K3)	0.0845
SpTIGR4-0340 (1L19)	0.0546	SpTIGR4-0506 (2I4)	0.0847
SpTIGR4-0344 (1P19)	0.032	SpTIGR4-0507 (2J4)	0.076
SpTIGR4-0345 (1I20)	0.0431	SpTIGR4-0515 (2J5)	0.0693
SpTIGR4-0349 (1M20)	0.063	SpTIGR4-0516 (2K5)	0.0536
SpTIGR4-0351 (1O20)	0.0143	SpTIGR4-0526 (2M6)	0.0494
SpTIGR4-0352 (1P20)	0.0541	SpTIGR4-0527 (2N6)	0.0423
SpTIGR4-0353 (1I21)	0.0691	SpTIGR4-0529 (2P6)	0.0679
SpTIGR4-0354 (1J21)	0.0727	SpTIGR4-0530 (2I7)	0.0254
SpTIGR4-0355 (1K21)	0.0218	SpTIGR4-0532 (2K7)	0.0561
SpTIGR4-0356 (1L21)	0.0444	SpTIGR4-0547 (2J9)	0.0511
SpTIGR4-0357 (1M21)	0.0884	SpTIGR4-0565 (2K11)	0.068
SpTIGR4-0358 (1N21)	0.032	SpTIGR4-0568 (2N11)	0.0473
SpTIGR4-0359 (1O21)	0.04	SpTIGR4-0605 (2C16)	0.0504
SpTIGR4-0360 (1P21)	0.0899	SpTIGR4-0626 (2H18)	0.0954
SpTIGR4-0361 (1I22)	0.0897	SpTIGR4-0645 (2C21)	0.0859
SpTIGR4-0366 (1N22)	0.0728	SpTIGR4-0648 (2F21)	0.0787
SpTIGR4-0368 (1P22)	0.0295	SpTIGR4-0663 (2E23)	0.0494
SpTIGR4-0369 (1I23)	0.0201	SpTIGR4-0666 (2H23)	0.0915
SpTIGR4-0370 (1J23)	0.0237	SpTIGR4-0667 (2A24)	0.0974
SpTIGR4-0372 (1L23)	0.00249	SpTIGR4-0674 (2H24)	0.0689
SpTIGR4-0373 (1M23)	0.00766	SpTIGR4-0675 (2I13)	0.0994
SpTIGR4-0375 (1O23)	0.0674	SpTIGR4-0688 (2N14)	0.0945
SpTIGR4-0376 (1P23)	0.0237	SpTIGR4-0695 (2M15)	0.0702
SpTIGR4-0377 (1I24)	0.066	SpTIGR4-0704 (2N16)	0.0282
SpTIGR4-0378 (1J24)	0.0193	SpTIGR4-0707 (2I17)	0.0554
SpTIGR4-0385 (2A1)	0.0339	SpTIGR4-0709 (2K17)	0.0527
SpTIGR4-0386 (2B1)	0.00411	SpTIGR4-0710 (2L17)	0.0289
SpTIGR4-0387 (2C1)	0.0479	SpTIGR4-0711 (2M17)	0.0322
SpTIGR4-0389 (2E1)	0.0193	SpTIGR4-0715 (2I18)	0.0854
SpTIGR4-0391 (2G1)	0.0367	SpTIGR4-0716 (2J18)	0.0494
SpTIGR4-0393 (2A2)	0.0598	SpTIGR4-0717 (2K18)	0.0494
SpTIGR4-0401 (2A3)	0.075	SpTIGR4-0718 (2L18)	0.0702
SpTIGR4-0412 (2D4)	0.0489	SpTIGR4-0726 (2L19)	0.025
SpTIGR4-0421 (2E5)	0.0632	SpTIGR4-0728 (2N19)	0.0974
SpTIGR4-0430 (2F6)	0.0977	SpTIGR4-0729 (2O19)	0.0787
SpTIGR4-0432 (2H6)	0.0302	SpTIGR4-0730 (2P19)	0.0433
SpTIGR4-0438 (2F7)	0.0414	SpTIGR4-0731 (2I20)	0.0213
SpTIGR4-0440 (2H7)	0.0994	SpTIGR4-0736 (2N20)	0.0701
SpTIGR4-0445 (2E8)	0.071	SpTIGR4-0742 (2L21)	0.0954
SpTIGR4-0453 (2E9)	0.0343	SpTIGR4-0748 (2J22)	0.0635
SpTIGR4-0487 (2N1)	0.0492	SpTIGR4-0783 (3E2)	0.0473
SpTIGR4-0488 (2O1)	0.00704	SpTIGR4-0789 (3C3)	0.0449
SpTIGR4-0489 (2P1)	0.0223	SpTIGR4-0790 (3D3)	0.00975
SpTIGR4-0490 (2I2)	0.0131	SpTIGR4-0791 (3E3)	0.0257
SpTIGR4-0492 (2K2)	0.0508	SpTIGR4-0798 (3D4)	0.0832
SpTIGR4-0494 (2M2)	0.0834	SpTIGR4-0799 (3E4)	0.0473
SpTIGR4-0499 (2J3)	0.0673	SpTIGR4-0806 (3D5)	0.0697
SpTIGR4-0500 (2K3)	0.0302	SpTIGR4-0819 (3A7)	0.0602
SpTIGR4-0501 (2L3)	0.0639	SpTIGR4-0820 (3B7)	0.031
SpTIGR4-0504 (2O3)	0.0565	SpTIGR4-0823 (3E7)	0.0423
SpTIGR4-0506 (2I4)	0.0114	SpTIGR4-0838 (3D9)	0.0726
SpTIGR4-0507 (2J4)	0.0333	SpTIGR4-0842 (3H9)	0.064
SpTIGR4-0508 (2K4)	0.0801	SpTIGR4-0844 (3B10)	0.088
SpTIGR4-0515 (2J5)	0.0477	SpTIGR4-0846 (3D10)	0.0974

SpTIGR4-0516 (2K5)	0.0548	SpTIGR4-0851 (3A11)	0.0262
SpTIGR4-0525 (2L6)	0.0532	SpTIGR4-0857 (3G11)	0.0525
SpTIGR4-0526 (2M6)	0.04	SpTIGR4-0858 (3H11)	0.0266
SpTIGR4-0527 (2N6)	0.075	SpTIGR4-0867 (3I1)	0.0601
SpTIGR4-0529 (2P6)	0.0327	SpTIGR4-0868 (3J1)	0.0207
SpTIGR4-0530 (2I7)	0.0224	SpTIGR4-0869 (3K1)	0.0119
SpTIGR4-0532 (2K7)	0.0425	SpTIGR4-0870 (3L1)	0.0701
SpTIGR4-0533 (2L7)	0.0727	SpTIGR4-0872 (3N1)	0.0854
SpTIGR4-0545 (2P8)	0.0203	SpTIGR4-0876 (3J2)	0.0768
SpTIGR4-0547 (2J9)	0.0429	SpTIGR4-0877 (3K2)	0.0904
SpTIGR4-0548 (2K9)	0.0508	SpTIGR4-0887 (3M3)	0.0736
SpTIGR4-0549 (2L9)	0.0787	SpTIGR4-0893 (3K4)	0.0652
SpTIGR4-0550 (2M9)	0.0632	SpTIGR4-0898 (3P4)	0.0405
SpTIGR4-0564 (2J11)	0.0425	SpTIGR4-0901 (3K5)	0.0449
SpTIGR4-0567 (2M11)	0.0843	SpTIGR4-0902 (3L5)	0.0698
SpTIGR4-0568 (2N11)	0.0733	SpTIGR4-0916 (3J7)	0.0289
SpTIGR4-0579 (2A13)	0.0244	SpTIGR4-0918 (3L7)	0.0493
SpTIGR4-0580 (2B13)	0.0504	SpTIGR4-0919 (3M7)	0.0798
SpTIGR4-0581 (2C13)	0.0279	SpTIGR4-0920 (3N7)	0.0399
SpTIGR4-0601 (2G15)	0.0537	SpTIGR4-0921 (3O7)	0.0466
SpTIGR4-0605 (2C16)	0.0516	SpTIGR4-0922 (3P7)	0.097
SpTIGR4-0613 (2C17)	0.0834	SpTIGR4-0931 (3I9)	0.0949
SpTIGR4-0614 (2D17)	0.0733	SpTIGR4-0938 (3P9)	0.0893
SpTIGR4-0617 (2G17)	0.0404	SpTIGR4-0947 (3I11)	0.0561
SpTIGR4-0618 (2H17)	0.0632	SpTIGR4-0950 (3L11)	0.0289
SpTIGR4-0621 (2C18)	0.0504	SpTIGR4-0961 (3O12)	0.0859
SpTIGR4-0626 (2H18)	0.0729	SpTIGR4-0962 (3P12)	0.0174
SpTIGR4-0629 (2C19)	0.0479	SpTIGR4-0968 (3F13)	0.0907
SpTIGR4-0630 (2D19)	0.0402	SpTIGR4-0995 (3A17)	0.0787
SpTIGR4-0631 (2E19)	0.0801	SpTIGR4-0996 (3B17)	0.0907
SpTIGR4-0633 (2G19)	0.0733	SpTIGR4-1003 (3A18)	0.0587
SpTIGR4-0644 (2B21)	0.0367	SpTIGR4-1004 (3B18)	0.0282
SpTIGR4-0648 (2F21)	0.0431	SpTIGR4-1014 (3D19)	0.0269
SpTIGR4-0649 (2G21)	0.00976	SpTIGR4-1020 (3B20)	0.0473
SpTIGR4-0655 (2E22)	0.0637	SpTIGR4-1027 (3A21)	0.0717
SpTIGR4-0656 (2F22)	0.0497	SpTIGR4-1029 (3C21)	0.0669
SpTIGR4-0663 (2E23)	0.01	SpTIGR4-1034 (3H21)	0.0945
SpTIGR4-0664 (2F23)	0.0295	SpTIGR4-1041 (3G22)	0.0669
SpTIGR4-0666 (2H23)	0.0917	SpTIGR4-1044 (3B23)	0.0494
SpTIGR4-0680 (2N13)	0.0701	SpTIGR4-1068 (3J14)	0.0354
SpTIGR4-0681 (2O13)	0.0504	SpTIGR4-1070 (3L14)	0.0907
SpTIGR4-0682 (2P13)	0.0237	SpTIGR4-1073 (3O14)	0.0838
SpTIGR4-0701 (2K16)	0.0217	SpTIGR4-1074 (3P14)	0.088
SpTIGR4-0702 (2L16)	0.00776	SpTIGR4-1075 (3I15)	0.0752
SpTIGR4-0703 (2M16)	0.0377	SpTIGR4-1076 (3J15)	0.0267
SpTIGR4-0709 (2K17)	0.0371	SpTIGR4-1077 (3K15)	0.0545
SpTIGR4-0710 (2L17)	0.0431	SpTIGR4-1078 (3L15)	0.0859
SpTIGR4-0711 (2M17)	0.0254	SpTIGR4-1088 (3N16)	0.0907
SpTIGR4-0712 (2N17)	0.066	SpTIGR4-1089 (3O16)	0.0601
SpTIGR4-0715 (2I18)	0.0245	SpTIGR4-1090 (3P16)	0.0405
SpTIGR4-0716 (2J18)	0.0444	SpTIGR4-1092 (3J17)	0.0854
SpTIGR4-0717 (2K18)	0.0628	SpTIGR4-1094 (3L17)	0.0613
SpTIGR4-0726 (2L19)	0.0218	SpTIGR4-1095 (3M17)	0.0713
SpTIGR4-0727 (2M19)	0.0841	SpTIGR4-1096 (3N17)	0.0424
SpTIGR4-0729 (2O19)	0.0974	SpTIGR4-1097 (3O17)	0.0473
SpTIGR4-0730 (2P19)	0.00752	SpTIGR4-1100 (3J18)	0.0473
SpTIGR4-0731 (2I20)	0.00714	SpTIGR4-1105 (3O18)	0.0883

SpTIGR4-0743 (2M21)	0.0431	SpTIGR4-1106 (3P18)	0.0473
SpTIGR4-0744 (2N21)	0.054	SpTIGR4-1112 (3N19)	0.0473
SpTIGR4-0745 (2O21)	0.0479	SpTIGR4-1114 (3P19)	0.0254
SpTIGR4-0753 (2O22)	0.0384	SpTIGR4-1118 (3L20)	0.0681
SpTIGR4-0762 (2P23)	0.0702	SpTIGR4-1119 (3M20)	0.0592
SpTIGR4-0766 (2L24)	0.0193	SpTIGR4-1121 (3O20)	0.0602
SpTIGR4-0768 (2N24)	0.0733	SpTIGR4-1122 (3P20)	0.0945
SpTIGR4-0775 (3E1)	0.0497	SpTIGR4-1125 (3K21)	0.0907
SpTIGR4-0783 (3E2)	0.0387	SpTIGR4-1129 (3O21)	0.0973
SpTIGR4-0784 (3F2)	0.00411	SpTIGR4-1130 (3P21)	0.0975
SpTIGR4-0789 (3C3)	0.0255	SpTIGR4-1139 (3I23)	0.0701
SpTIGR4-0790 (3D3)	0.00474	SpTIGR4-1140 (3J23)	0.0854
SpTIGR4-0791 (3E3)	0.0199	SpTIGR4-1142 (3L23)	0.0554
SpTIGR4-0792 (3F3)	0.0504	SpTIGR4-1148 (3J24)	0.0268
SpTIGR4-0793 (3G3)	0.0994	SpTIGR4-1149 (3K24)	0.0399
SpTIGR4-0798 (3D4)	0.0302	SpTIGR4-1151 (3M24)	0.0662
SpTIGR4-0799 (3E4)	0.0151	SpTIGR4-1152 (3N24)	0.0473
SpTIGR4-0801 (3G4)	0.0481	SpTIGR4-1153 (3O24)	0.0443
SpTIGR4-0805 (3C5)	0.0733	SpTIGR4-1154 (3P24)	0.0613
SpTIGR4-0812 (3B6)	0.0826	SpTIGR4-1156 (4B1)	0.0822
SpTIGR4-0818 (3H6)	0.0444	SpTIGR4-1160 (4F1)	0.0602
SpTIGR4-0819 (3A7)	0.0497	SpTIGR4-1161 (4G1)	0.0554
SpTIGR4-0820 (3B7)	0.0268	SpTIGR4-1162 (4H1)	0.0666
SpTIGR4-0823 (3E7)	0.0489	SpTIGR4-1163 (4A2)	0.0174
SpTIGR4-0828 (3B8)	0.0637	SpTIGR4-1164 (4B2)	0.0322
SpTIGR4-0830 (3D8)	0.0709	SpTIGR4-1166 (4D2)	0.0613
SpTIGR4-0838 (3D9)	0.0687	SpTIGR4-1167 (4E2)	0.0666
SpTIGR4-0842 (3H9)	0.0504	SpTIGR4-1168 (4F2)	0.0713
SpTIGR4-0846 (3D10)	0.0504	SpTIGR4-1169 (4G2)	0.0405
SpTIGR4-0847 (3E10)	0.093	SpTIGR4-1175 (4E3)	0.0613
SpTIGR4-0852 (3B11)	0.0504	SpTIGR4-1176 (4F3)	0.0915
SpTIGR4-0853 (3C11)	0.0192	SpTIGR4-1177 (4G3)	0.0399
SpTIGR4-0854 (3D11)	0.0974	SpTIGR4-1178 (4H3)	0.0768
SpTIGR4-0855 (3E11)	0.0218	SpTIGR4-1179 (4A4)	0.0613
SpTIGR4-0857 (3G11)	0.0245	SpTIGR4-1180 (4B4)	0.0174
SpTIGR4-0858 (3H11)	0.0281	SpTIGR4-1182 (4D4)	0.0773
SpTIGR4-0861 (3C12)	0.0867	SpTIGR4-1189 (4C5)	0.0699
SpTIGR4-0864 (3F12)	0.00411	SpTIGR4-1190 (4D5)	0.0213
SpTIGR4-0866 (3H12)	0.0785	SpTIGR4-1191 (4E5)	0.088
SpTIGR4-0867 (3I1)	0.00581	SpTIGR4-1192 (4F5)	0.0202
SpTIGR4-0868 (3J1)	0.0156	SpTIGR4-1193 (4G5)	0.0549
SpTIGR4-0869 (3K1)	0.00352	SpTIGR4-1194 (4H5)	0.0441
SpTIGR4-0870 (3L1)	0.0285	SpTIGR4-1200 (4F6)	0.09
SpTIGR4-0875 (3I2)	0.026	SpTIGR4-1202 (4H6)	0.0439
SpTIGR4-0876 (3J2)	0.0632	SpTIGR4-1203 (4A7)	0.0494
SpTIGR4-0877 (3K2)	0.0508	SpTIGR4-1205 (4C7)	0.0768
SpTIGR4-0880 (3N2)	0.0421	SpTIGR4-1206 (4D7)	0.045
SpTIGR4-0896 (3N4)	0.0273	SpTIGR4-1207 (4E7)	0.0847
SpTIGR4-0904 (3N5)	0.0709	SpTIGR4-1213 (4C8)	0.0504
SpTIGR4-0905 (3O5)	0.0603	SpTIGR4-1225 (4G9)	0.0489
SpTIGR4-0916 (3J7)	0.00451	SpTIGR4-1226 (4H9)	0.0289
SpTIGR4-0918 (3L7)	0.00803	SpTIGR4-1227 (4A10)	0.0402
SpTIGR4-0919 (3M7)	0.0245	SpTIGR4-1228 (4B10)	0.00975
SpTIGR4-0920 (3N7)	0.00494	SpTIGR4-1229 (4C10)	0.0613
SpTIGR4-0921 (3O7)	0.012	SpTIGR4-1233 (4G10)	0.0907
SpTIGR4-0922 (3P7)	0.0194	SpTIGR4-1242 (4G11)	0.0679
SpTIGR4-0936 (3N9)	0.0733	SpTIGR4-1244 (4A12)	0.0656
SpTIGR4-0938 (3P9)	0.0643	SpTIGR4-1245 (4B12)	0.0535

SpTIGR4-0943 (3M10)	0.026	SpTIGR4-1246 (4C12)	0.0314
SpTIGR4-0950 (3L11)	0.0193	SpTIGR4-1247 (4D12)	0.0511
SpTIGR4-0959 (3M12)	0.0436	SpTIGR4-1248 (4E12)	0.0511
SpTIGR4-0960 (3N12)	0.0489	SpTIGR4-1251 (4H12)	0.0887
SpTIGR4-0961 (3O12)	0.00664	SpTIGR4-1255 (4L1)	0.0854
SpTIGR4-0962 (3P12)	0.0224	SpTIGR4-1257 (4N1)	0.0449
SpTIGR4-0963 (3A13)	0.033	SpTIGR4-1267 (4P2)	0.0727
SpTIGR4-0964 (3B13)	0.0151	SpTIGR4-1282 (4O4)	0.0787
SpTIGR4-0967 (3E13)	0.0377	SpTIGR4-1285 (4J5)	0.0586
SpTIGR4-0969 (3G13)	0.0925	SpTIGR4-1287 (4L5)	0.068
SpTIGR4-0970 (3H13)	0.091	SpTIGR4-1291 (4P5)	0.0822
SpTIGR4-0974 (3D14)	0.0431	SpTIGR4-1294 (4K6)	0.0494
SpTIGR4-0980 (3B15)	0.0637	SpTIGR4-1296 (4M6)	0.0536
SpTIGR4-0988 (3B16)	0.0433	SpTIGR4-1306 (4O7)	0.0713
SpTIGR4-0990 (3D16)	0.0596	SpTIGR4-1313 (4N8)	0.0859
SpTIGR4-0992 (3F16)	0.0735	SpTIGR4-1353 (4F13)	0.0945
SpTIGR4-0994 (3H16)	0.0909	SpTIGR4-1356 (4A14)	0.0494
SpTIGR4-0995 (3A17)	0.0873	SpTIGR4-1366 (4C15)	0.0859
SpTIGR4-0999 (3E17)	0.0119	SpTIGR4-1367 (4D15)	0.0666
SpTIGR4-1000 (3F17)	0.00845	SpTIGR4-1382 (4C17)	0.0736
SpTIGR4-1001 (3G17)	0.0556	SpTIGR4-1383 (4D17)	0.0509
SpTIGR4-1003 (3A18)	0.0138	SpTIGR4-1387 (4H17)	0.0891
SpTIGR4-1004 (3B18)	0.0436	SpTIGR4-1388 (4A18)	0.0859
SpTIGR4-1014 (3D19)	0.0481	SpTIGR4-1398 (4C19)	0.0828
SpTIGR4-1016 (3F19)	0.0714	SpTIGR4-1402 (4G19)	0.0876
SpTIGR4-1018 (3H19)	0.0453	SpTIGR4-1403 (4H19)	0.0602
SpTIGR4-1020 (3B20)	0.0697	SpTIGR4-1408 (4E20)	0.0527
SpTIGR4-1023 (3E20)	0.0295	SpTIGR4-1410 (4G20)	0.0859
SpTIGR4-1024 (3F20)	0.032	SpTIGR4-1411 (4H20)	0.0811
SpTIGR4-1027 (3A21)	0.0767	SpTIGR4-1412 (4A21)	0.0289
SpTIGR4-1033 (3G21)	0.0618	SpTIGR4-1415 (4D21)	0.0266
SpTIGR4-1034 (3H21)	0.0556	SpTIGR4-1416 (4E21)	0.0973
SpTIGR4-1035 (3A22)	0.0673	SpTIGR4-1418 (4G21)	0.072
SpTIGR4-1038 (3D22)	0.0561	SpTIGR4-1419 (4H21)	0.0174
SpTIGR4-1041 (3G22)	0.0366	SpTIGR4-1420 (4A22)	0.0494
SpTIGR4-1044 (3B23)	0.0816	SpTIGR4-1421 (4B22)	0.0526
SpTIGR4-1062 (3L13)	0.0656	SpTIGR4-1427 (4H22)	0.0732
SpTIGR4-1064 (3N13)	0.00708	SpTIGR4-1428 (4A23)	0.0267
SpTIGR4-1068 (3J14)	0.0619	SpTIGR4-1429 (4B23)	0.0916
SpTIGR4-1072 (3N14)	0.00664	SpTIGR4-1431 (4D23)	0.0854
SpTIGR4-1073 (3O14)	0.021	SpTIGR4-1435 (4H23)	0.0473
SpTIGR4-1074 (3P14)	0.0556	SpTIGR4-1438 (4C24)	0.0399
SpTIGR4-1076 (3J15)	0.0237	SpTIGR4-1450 (4O13)	0.0289
SpTIGR4-1077 (3K15)	0.0651	SpTIGR4-1451 (4P13)	0.0414
SpTIGR4-1078 (3L15)	0.0556	SpTIGR4-1452 (4I14)	0.0202
SpTIGR4-1087 (3M16)	0.0787	SpTIGR4-1453 (4J14)	0.0525
SpTIGR4-1089 (3O16)	0.054	SpTIGR4-1454 (4K14)	0.054
SpTIGR4-1090 (3P16)	0.0479	SpTIGR4-1470 (4K16)	0.013
SpTIGR4-1092 (3J17)	0.0975	SpTIGR4-1471 (4L16)	0.0202
SpTIGR4-1094 (3L17)	0.0974	SpTIGR4-1472 (4M16)	0.0372
SpTIGR4-1096 (3N17)	0.0643	SpTIGR4-1473 (4N16)	0.0545
SpTIGR4-1099 (3I18)	0.0162	SpTIGR4-1474 (4O16)	0.0701
SpTIGR4-1100 (3J18)	0.0566	SpTIGR4-1475 (4P16)	0.0638
SpTIGR4-1106 (3P18)	0.0343	SpTIGR4-1476 (4I17)	0.0746
SpTIGR4-1107 (3I19)	0.0591	SpTIGR4-1489 (4N18)	0.0822
SpTIGR4-1117 (3K20)	0.0199	SpTIGR4-1498 (4O19)	0.0893
SpTIGR4-1118 (3L20)	0.0431	SpTIGR4-1506 (4O20)	0.077
SpTIGR4-1122 (3P20)	0.0602	SpTIGR4-1507 (4P20)	0.0726

SpTIGR4-1125 (3K21)	0.0433	SpTIGR4-1514 (4O21)	0.0974
SpTIGR4-1142 (3L23)	0.0932	SpTIGR4-1523 (4P22)	0.0666
SpTIGR4-1143 (3M23)	0.0644	SpTIGR4-1529 (4N23)	0.0427
SpTIGR4-1144 (3N23)	0.0974	SpTIGR4-1531 (4P23)	0.0937
SpTIGR4-1148 (3J24)	0.0281	SpTIGR4-1532 (4I24)	0.0862
SpTIGR4-1149 (3K24)	0.0149	SpTIGR4-1533 (4J24)	0.0494
SpTIGR4-1151 (3M24)	0.0444	SpTIGR4-1534 (4K24)	0.077
SpTIGR4-1152 (3N24)	0.0259	SpTIGR4-1539 (4P24)	0.0787
SpTIGR4-1153 (3O24)	0.0497	SpTIGR4-1543 (5D1)	0.0202
SpTIGR4-1154 (3P24)	0.0857	SpTIGR4-1545 (5F1)	0.0289
SpTIGR4-1156 (4B1)	0.0727	SpTIGR4-1546 (5G1)	0.0269
SpTIGR4-1157 (4C1)	0.0733	SpTIGR4-1560 (5E3)	0.0174
SpTIGR4-1159 (4E1)	0.0849	SpTIGR4-1562 (5G3)	0.0954
SpTIGR4-1160 (4F1)	0.00411	SpTIGR4-1563 (5H3)	0.0935
SpTIGR4-1161 (4G1)	0.0434	SpTIGR4-1572 (5A5)	0.0649
SpTIGR4-1162 (4H1)	0.00663	SpTIGR4-1573 (5B5)	0.0854
SpTIGR4-1163 (4A2)	0.00263	SpTIGR4-1575 (5D5)	0.0621
SpTIGR4-1164 (4B2)	0.00418	SpTIGR4-1576 (5E5)	0.0994
SpTIGR4-1166 (4D2)	0.0779	SpTIGR4-1583 (5D6)	0.0289
SpTIGR4-1169 (4G2)	0.032	SpTIGR4-1587 (5H6)	0.0202
SpTIGR4-1176 (4F3)	0.075	SpTIGR4-1588 (5A7)	0.0313
SpTIGR4-1177 (4G3)	0.0656	SpTIGR4-1589 (5B7)	0.0449
SpTIGR4-1178 (4H3)	0.087	SpTIGR4-1590 (5C7)	0.0789
SpTIGR4-1179 (4A4)	0.0793	SpTIGR4-1591 (5D7)	0.0787
SpTIGR4-1180 (4B4)	0.0102	SpTIGR4-1631 (5D12)	0.0662
SpTIGR4-1183 (4E4)	0.0852	SpTIGR4-1651 (5P2)	0.0266
SpTIGR4-1189 (4C5)	0.0592	SpTIGR4-1661 (5J4)	0.0422
SpTIGR4-1190 (4D5)	0.0673	SpTIGR4-1663 (5L4)	0.0638
SpTIGR4-1192 (4F5)	0.0508	SpTIGR4-1674 (5O5)	0.0745
SpTIGR4-1203 (4A7)	0.0596	SpTIGR4-1688 (5M7)	0.055
SpTIGR4-1208 (4F7)	0.0108	SpTIGR4-1690 (5O7)	0.0586
SpTIGR4-1212 (4B8)	0.0917	SpTIGR4-1691 (5P7)	0.0831
SpTIGR4-1213 (4C8)	0.0199	SpTIGR4-1704 (5M9)	0.0956
SpTIGR4-1215 (4E8)	0.0793	SpTIGR4-1717 (5J11)	0.0207
SpTIGR4-1225 (4G9)	0.0179	SpTIGR4-1724 (5I12)	0.0794
SpTIGR4-1226 (4H9)	0.00581	SpTIGR4-1725 (5J12)	0.055
SpTIGR4-1227 (4A10)	0.0047	SpTIGR4-1734 (5C13)	0.0337
SpTIGR4-1228 (4B10)	0.0237	SpTIGR4-1774 (5C18)	0.08
SpTIGR4-1229 (4C10)	0.0984	SpTIGR4-1775 (5D18)	0.0494
SpTIGR4-1241 (4F11)	0.0537	SpTIGR4-1776 (5E18)	0.0854
SpTIGR4-1242 (4G11)	0.0882	SpTIGR4-1778 (5G18)	0.0258
SpTIGR4-1245 (4B12)	0.0047	SpTIGR4-1786 (5G19)	0.0551
SpTIGR4-1246 (4C12)	0.0199	SpTIGR4-1793 (5F20)	0.0211
SpTIGR4-1247 (4D12)	0.0254	SpTIGR4-1794 (5G20)	0.013
SpTIGR4-1248 (4E12)	0.04	SpTIGR4-1802 (5G21)	0.0207
SpTIGR4-1255 (4L1)	0.091	SpTIGR4-1804 (5A22)	0.0854
SpTIGR4-1262 (4K2)	0.0608	SpTIGR4-1809 (5F22)	0.0119
SpTIGR4-1267 (4P2)	0.0152	SpTIGR4-1845 (5J15)	0.0693
SpTIGR4-1275 (4P3)	0.0637	SpTIGR4-1846 (5K15)	0.0502
SpTIGR4-1276 (4I4)	0.0299	SpTIGR4-1851 (5P15)	0.0701
SpTIGR4-1277 (4J4)	0.01	SpTIGR4-1852 (5I16)	0.0433
SpTIGR4-1278 (4K4)	0.0151	SpTIGR4-1853 (5J16)	0.089
SpTIGR4-1279 (4L4)	0.0596	SpTIGR4-1860 (5I17)	0.0277
SpTIGR4-1280 (4M4)	0.0481	SpTIGR4-1861 (5J17)	0.013
SpTIGR4-1285 (4J5)	0.0415	SpTIGR4-1862 (5K17)	0.00975
SpTIGR4-1286 (4K5)	0.032	SpTIGR4-1863 (5L17)	0.0768
SpTIGR4-1296 (4M6)	0.063	SpTIGR4-1874 (5O18)	0.0202
SpTIGR4-1299 (4P6)	0.0629	SpTIGR4-1875 (5P18)	0.0595

SpTIGR4-1306 (4O7)	0.0199	SpTIGR4-1877 (5J19)	0.0473
SpTIGR4-1311 (4L8)	0.0937	SpTIGR4-1878 (5K19)	0.0174
SpTIGR4-1313 (4N8)	0.0387	SpTIGR4-1879 (5L19)	0.0504
SpTIGR4-1353 (4F13)	0.021	SpTIGR4-1880 (5M19)	0.0473
SpTIGR4-1354 (4G13)	0.0479	SpTIGR4-1881 (5N19)	0.0854
SpTIGR4-1355 (4H13)	0.0477	SpTIGR4-1883 (5P19)	0.0174
SpTIGR4-1356 (4A14)	0.0739	SpTIGR4-1884 (5I20)	0.013
SpTIGR4-1357 (4B14)	0.00562	SpTIGR4-1886 (5K20)	0.0961
SpTIGR4-1358 (4C14)	0.0344	SpTIGR4-1891 (5P20)	0.0954
SpTIGR4-1359 (4D14)	0.0378	SpTIGR4-1894 (5K21)	0.0282
SpTIGR4-1360 (4E14)	0.0409	SpTIGR4-1895 (5L21)	0.0527
SpTIGR4-1361 (4F14)	0.0425	SpTIGR4-1896 (5M21)	0.0322
SpTIGR4-1363 (4H14)	0.071	SpTIGR4-1897 (5N21)	0.0322
SpTIGR4-1364 (4A15)	0.0733	SpTIGR4-1898 (5O21)	0.00975
SpTIGR4-1366 (4C15)	0.0632	SpTIGR4-1940 (6A3)	0.0586
SpTIGR4-1367 (4D15)	0.04	SpTIGR4-1941 (6B3)	0.0598
SpTIGR4-1369 (4F15)	0.0182	SpTIGR4-1942 (6C3)	0.0961
SpTIGR4-1383 (4D17)	0.0254	SpTIGR4-1944 (6E3)	0.054
SpTIGR4-1385 (4F17)	0.0505	SpTIGR4-1950 (6C4)	0.0892
SpTIGR4-1386 (4G17)	0.0781	SpTIGR4-1964 (6A6)	0.0391
SpTIGR4-1387 (4H17)	0.0833	SpTIGR4-1966 (6C6)	0.077
SpTIGR4-1388 (4A18)	0.0376	SpTIGR4-1968 (6E6)	0.0446
SpTIGR4-1389 (4B18)	0.0429	SpTIGR4-1969 (6F6)	0.0974
SpTIGR4-1394 (4G18)	0.0917	SpTIGR4-1972 (6A7)	0.0822
SpTIGR4-1402 (4G19)	0.0504	SpTIGR4-1973 (6B7)	0.09
SpTIGR4-1403 (4H19)	0.0255	SpTIGR4-1976 (6E7)	0.0473
SpTIGR4-1408 (4E20)	0.091	SpTIGR4-1986 (6G8)	0.0433
SpTIGR4-1414 (4C21)	0.0576	SpTIGR4-1987 (6H8)	0.0702
SpTIGR4-1415 (4D21)	0.033	SpTIGR4-1988 (6A9)	0.0449
SpTIGR4-1417 (4F21)	0.0193	SpTIGR4-1989 (6B9)	0.0262
SpTIGR4-1418 (4G21)	0.0434	SpTIGR4-1990 (6C9)	0.0473
SpTIGR4-1419 (4H21)	0.0731	SpTIGR4-1991 (6D9)	0.0647
SpTIGR4-1420 (4A22)	0.091	SpTIGR4-1994 (6G9)	0.0494
SpTIGR4-1421 (4B22)	0.0674	SpTIGR4-2008 (6E11)	0.096
SpTIGR4-1426 (4G22)	0.0909	SpTIGR4-2010 (6G11)	0.0494
SpTIGR4-1427 (4H22)	0.00803	SpTIGR4-2013 (6B12)	0.0289
SpTIGR4-1428 (4A23)	0.0201	SpTIGR4-2014 (6C12)	0.0266
SpTIGR4-1429 (4B23)	0.0216	SpTIGR4-2016 (6E12)	0.0266
SpTIGR4-1431 (4D23)	0.032	SpTIGR4-2017 (6F12)	0.0527
SpTIGR4-1433 (4F23)	0.0727	SpTIGR4-2020 (6I1)	0.0702
SpTIGR4-1438 (4C24)	0.0431	SpTIGR4-2021 (6J1)	0.0494
SpTIGR4-1450 (4O13)	0.0295	SpTIGR4-2032 (6M2)	0.0637
SpTIGR4-1451 (4P13)	0.0193	SpTIGR4-2041 (6N3)	0.0787
SpTIGR4-1452 (4I14)	0.0497	SpTIGR4-2045 (6J4)	0.0369
SpTIGR4-1453 (4J14)	0.0444	SpTIGR4-2054 (6K5)	0.00975
SpTIGR4-1454 (4K14)	0.0828	SpTIGR4-2055 (6L5)	0.0511
SpTIGR4-1456 (4M14)	0.0314	SpTIGR4-2056 (6M5)	0.0372
SpTIGR4-1458 (4O14)	0.0237	SpTIGR4-2057 (6N5)	0.0174
SpTIGR4-1462 (4K15)	0.032	SpTIGR4-2058 (6O5)	0.0146
SpTIGR4-1463 (4L15)	0.0828	SpTIGR4-2060 (6I6)	0.0635
SpTIGR4-1464 (4M15)	0.0275	SpTIGR4-2063 (6L6)	0.0266
SpTIGR4-1470 (4K16)	0.0738	SpTIGR4-2072 (6M7)	0.0743
SpTIGR4-1471 (4L16)	0.0343	SpTIGR4-2073 (6N7)	0.0289
SpTIGR4-1472 (4M16)	0.00581	SpTIGR4-2075 (6P7)	0.088
SpTIGR4-1473 (4N16)	0.0632	SpTIGR4-2076 (6I8)	0.0333
SpTIGR4-1474 (4O16)	0.00451	SpTIGR4-2084 (6I9)	0.0207
SpTIGR4-1475 (4P16)	0.0404	SpTIGR4-2085 (6J9)	0.0289

SpTIGR4-1476 (4I17)	0.0548
SpTIGR4-1477 (4J17)	0.0505
SpTIGR4-1488 (4M18)	0.0427
SpTIGR4-1491 (4P18)	0.071
SpTIGR4-1498 (4O19)	0.0661
SpTIGR4-1499 (4P19)	0.00451
SpTIGR4-1504 (4M20)	0.0282
SpTIGR4-1507 (4P20)	0.03
SpTIGR4-1519 (4L22)	0.0801
SpTIGR4-1521 (4N22)	0.0666
SpTIGR4-1522 (4O22)	0.0617
SpTIGR4-1531 (4P23)	0.0415
SpTIGR4-1532 (4I24)	0.0265
SpTIGR4-1533 (4J24)	0.00597
SpTIGR4-1534 (4K24)	0.0828
SpTIGR4-1535 (4L24)	0.0733
SpTIGR4-1539 (4P24)	0.0339
SpTIGR4-1543 (5D1)	0.00704
SpTIGR4-1544 (5E1)	0.0994
SpTIGR4-1546 (5G1)	0.0119
SpTIGR4-1550 (5C2)	0.0793
SpTIGR4-1552 (5E2)	0.0244
SpTIGR4-1560 (5E3)	0.0548
SpTIGR4-1563 (5H3)	0.0429
SpTIGR4-1564 (5A4)	0.0404
SpTIGR4-1565 (5B4)	0.0568
SpTIGR4-1568 (5E4)	0.0898
SpTIGR4-1569 (5F4)	0.091
SpTIGR4-1573 (5B5)	0.0397
SpTIGR4-1575 (5D5)	0.0205
SpTIGR4-1578 (5G5)	0.0214
SpTIGR4-1582 (5C6)	0.0371
SpTIGR4-1583 (5D6)	0.0201
SpTIGR4-1584 (5E6)	0.0436
SpTIGR4-1587 (5H6)	0.0192
SpTIGR4-1588 (5A7)	0.0199
SpTIGR4-1589 (5B7)	0.0173
SpTIGR4-1590 (5C7)	0.0371
SpTIGR4-1591 (5D7)	0.0787
SpTIGR4-1592 (5E7)	0.028
SpTIGR4-1593 (5F7)	0.0384
SpTIGR4-1602 (5G8)	0.0216
SpTIGR4-1612 (5A10)	0.091
SpTIGR4-1622 (5C11)	0.0193
SpTIGR4-1624 (5E11)	0.0332
SpTIGR4-1625 (5F11)	0.0909
SpTIGR4-1630 (5C12)	0.0199
SpTIGR4-1631 (5D12)	0.04
SpTIGR4-1633 (5F12)	0.0863
SpTIGR4-1646 (5K2)	0.0333
SpTIGR4-1651 (5P2)	0.00704
SpTIGR4-1663 (5L4)	0.0689
SpTIGR4-1670 (5K5)	0.0975
SpTIGR4-1671 (5L5)	0.0733
SpTIGR4-1674 (5O5)	0.091
SpTIGR4-1679 (5L6)	0.0605
SpTIGR4-1684 (5I7)	0.0582
SpTIGR4-1686 (5K7)	0.0735
SpTIGR4-2086 (6K9)	0.0311
SpTIGR4-2087 (6L9)	0.0536
SpTIGR4-2088 (6M9)	0.0399
SpTIGR4-2096 (6M10)	0.0446
SpTIGR4-2097 (6N10)	0.0504
SpTIGR4-2106 (6O11)	0.0108
SpTIGR4-2107 (6P11)	0.0554
SpTIGR4-2113 (6N12)	0.0269
SpTIGR4-2114 (6O12)	0.0907
SpTIGR4-2134 (6C15)	0.0741
SpTIGR4-2136 (6E15)	0.0592
SpTIGR4-2143 (6D16)	0.077
SpTIGR4-2146 (6G16)	0.0202
SpTIGR4-2148 (6A17)	0.0399
SpTIGR4-2150 (6B17)	0.0473
SpTIGR4-2151 (6C17)	0.0602
SpTIGR4-2153 (6E17)	0.0854
SpTIGR4-2160 (6D18)	0.0822
SpTIGR4-2171 (6G19)	0.0266
SpTIGR4-2172 (6H19)	0.0694
SpTIGR4-2173 (6A20)	0.0768
SpTIGR4-2174 (6B20)	0.0588
SpTIGR4-2175 (6C20)	0.0822
SpTIGR4-2176 (6D20)	0.0787
SpTIGR4-2180 (6H20)	0.0449
SpTIGR4-2181 (6A21)	0.0262
SpTIGR4-2186 (6F21)	0.0267
SpTIGR4-2187 (6G21)	0.00975
SpTIGR4-2189 (6A22)	0.0586
SpTIGR4-2190 (6B22)	0.0961
SpTIGR4-2191 (6C22)	0.0859
SpTIGR4-2192 (6D22)	0.0961
SpTIGR4-2193 (6E22)	0.0494
SpTIGR4-2194 (6F22)	0.0289
SpTIGR4-2195 (6G22)	0.0289
SpTIGR4-2201 (6E23)	0.0213
SpTIGR4-2203 (6G23)	0.0697
SpTIGR4-2204 (6H23)	0.0752
SpTIGR4-2205 (6A24)	0.055
SpTIGR4-2206 (6B24)	0.0893
SpTIGR4-2209 (6E24)	0.0322
SpTIGR4-2210 (6F24)	0.0428
SpTIGR4-2222 (6J14)	0.0213
SpTIGR4-2225 (6M14)	0.0561
SpTIGR4-2226 (6N14)	0.0907
SpTIGR4-2239 (6K16)	0.0511
SpTIGR4-2240 (6L16)	0.0702

SpTIGR4-1689 (5N7)	0.0727
SpTIGR4-1690 (5O7)	0.0343
SpTIGR4-1691 (5P7)	0.0787
SpTIGR4-1695 (5L8)	0.0218
SpTIGR4-1698 (5O8)	0.0431
SpTIGR4-1699 (5P8)	0.0804
SpTIGR4-1704 (5M9)	0.0317
SpTIGR4-1708 (5I10)	0.0481
SpTIGR4-1710 (5K10)	0.048
SpTIGR4-1711 (5L10)	0.0564
SpTIGR4-1712 (5M10)	0.0804
SpTIGR4-1716 (5I11)	0.0497
SpTIGR4-1717 (5J11)	0.0095
SpTIGR4-1720 (5M11)	0.0527
SpTIGR4-1721 (5N11)	0.0285
SpTIGR4-1722 (5O11)	0.0394
SpTIGR4-1724 (5I12)	0.0121
SpTIGR4-1725 (5J12)	0.0366
SpTIGR4-1734 (5C13)	0.0217
SpTIGR4-1762 (5G16)	0.0698
SpTIGR4-1774 (5C18)	0.0619
SpTIGR4-1775 (5D18)	0.00664
SpTIGR4-1776 (5E18)	0.0365
SpTIGR4-1777 (5F18)	0.052
SpTIGR4-1778 (5G18)	0.00664
SpTIGR4-1786 (5G19)	0.0574
SpTIGR4-1787 (5H19)	0.0994
SpTIGR4-1793 (5F20)	0.0237
SpTIGR4-1794 (5G20)	0.0237
SpTIGR4-1801 (5F21)	0.0201
SpTIGR4-1802 (5G21)	0.0605
SpTIGR4-1803 (5H21)	0.091
SpTIGR4-1804 (5A22)	0.0237
SpTIGR4-1805 (5B22)	0.0564
SpTIGR4-1807 (5D22)	0.00451
SpTIGR4-1809 (5F22)	0.0223
SpTIGR4-1811 (5H22)	0.091
SpTIGR4-1814 (5C23)	0.0207
SpTIGR4-1837 (5J14)	0.0431
SpTIGR4-1845 (5J15)	0.0102
SpTIGR4-1846 (5K15)	0.0474
SpTIGR4-1851 (5P15)	0.0881
SpTIGR4-1852 (5I16)	0.00581
SpTIGR4-1853 (5J16)	0.06
SpTIGR4-1860 (5I17)	0.0368
SpTIGR4-1861 (5J17)	0.0245
SpTIGR4-1862 (5K17)	0.032
SpTIGR4-1863 (5L17)	0.0301
SpTIGR4-1869 (5J18)	0.00664
SpTIGR4-1870 (5K18)	0.0568
SpTIGR4-1871 (5L18)	0.0254
SpTIGR4-1872 (5M18)	0.0429
SpTIGR4-1874 (5O18)	0.0481
SpTIGR4-1875 (5P18)	0.0221
SpTIGR4-1877 (5J19)	0.0232
SpTIGR4-1878 (5K19)	0.00752
SpTIGR4-1879 (5L19)	0.0716
SpTIGR4-1880 (5M19)	0.00664

SpTIGR4-1883 (5P19)	0.0502
SpTIGR4-1884 (5I20)	0.0265
SpTIGR4-1886 (5K20)	0.0492
SpTIGR4-1894 (5K21)	0.0139
SpTIGR4-1895 (5L21)	0.0094
SpTIGR4-1896 (5M21)	0.0201
SpTIGR4-1897 (5N21)	0.0047
SpTIGR4-1898 (5O21)	0.00722
SpTIGR4-1901 (5J22)	0.0791
SpTIGR4-1903 (5L22)	0.0906
SpTIGR4-1905 (5N22)	0.0949
SpTIGR4-1906 (5O22)	0.0102
SpTIGR4-1911 (5L23)	0.093
SpTIGR4-1912 (5M23)	0.0733
SpTIGR4-1913 (5N23)	0.0975
SpTIGR4-1914 (5O23)	0.0851
SpTIGR4-1915 (5P23)	0.0295
SpTIGR4-1923 (5P24)	0.0237
SpTIGR4-1924 (6A1)	0.0158
SpTIGR4-1925 (6B1)	0.0898
SpTIGR4-1939 (6H2)	0.0577
SpTIGR4-1940 (6A3)	0.0201
SpTIGR4-1947 (6H3)	0.0164
SpTIGR4-1953 (6F4)	0.0799
SpTIGR4-1959 (6D5)	0.0474
SpTIGR4-1964 (6A6)	0.0444
SpTIGR4-1966 (6C6)	0.0855
SpTIGR4-1972 (6A7)	0.0213
SpTIGR4-1973 (6B7)	0.058
SpTIGR4-1976 (6E7)	0.00451
SpTIGR4-1986 (6G8)	0.0751
SpTIGR4-1987 (6H8)	0.0339
SpTIGR4-1988 (6A9)	0.0154
SpTIGR4-1989 (6B9)	0.0531
SpTIGR4-1990 (6C9)	0.0366
SpTIGR4-1991 (6D9)	0.00704
SpTIGR4-1992 (6E9)	0.0218
SpTIGR4-1993 (6F9)	0.0619
SpTIGR4-1997 (6B10)	0.033
SpTIGR4-1998 (6C10)	0.0924
SpTIGR4-2000 (6E10)	0.00976
SpTIGR4-2001 (6F10)	0.0576
SpTIGR4-2002 (6G10)	0.0444
SpTIGR4-2003 (6H10)	0.031
SpTIGR4-2005 (6B11)	0.0643
SpTIGR4-2013 (6B12)	0.00581
SpTIGR4-2014 (6C12)	0.0367
SpTIGR4-2015 (6D12)	0.0787
SpTIGR4-2016 (6E12)	0.00411
SpTIGR4-2017 (6F12)	0.00411
SpTIGR4-2021 (6J1)	0.0841
SpTIGR4-2022 (6K1)	0.0224
SpTIGR4-2026 (6O1)	0.0265
SpTIGR4-2032 (6M2)	0.075
SpTIGR4-2040 (6M3)	0.0889
SpTIGR4-2047 (6L4)	0.0849
SpTIGR4-2052 (6I5)	0.0841
SpTIGR4-2053 (6J5)	0.0681

SpTIGR4-2054 (6K5)	0.0214
SpTIGR4-2056 (6M5)	0.0237
SpTIGR4-2057 (6N5)	0.0201
SpTIGR4-2058 (6O5)	0.0656
SpTIGR4-2060 (6I6)	0.0729
SpTIGR4-2063 (6L6)	0.0182
SpTIGR4-2072 (6M7)	0.0899
SpTIGR4-2073 (6N7)	0.0293
SpTIGR4-2074 (6O7)	0.0484
SpTIGR4-2075 (6P7)	0.00562
SpTIGR4-2076 (6I8)	0.0566
SpTIGR4-2082 (6O8)	0.009
SpTIGR4-2083 (6P8)	0.0728
SpTIGR4-2084 (6I9)	0.014
SpTIGR4-2085 (6J9)	0.0398
SpTIGR4-2086 (6K9)	0.0236
SpTIGR4-2087 (6L9)	0.0504
SpTIGR4-2088 (6M9)	0.0357
SpTIGR4-2094 (6K10)	0.0543
SpTIGR4-2095 (6L10)	0.0582
SpTIGR4-2096 (6M10)	0.0414
SpTIGR4-2097 (6N10)	0.0735
SpTIGR4-2099 (6P10)	0.0591
SpTIGR4-2106 (6O11)	0.00562
SpTIGR4-2107 (6P11)	0.0154
SpTIGR4-2113 (6N12)	0.0512
SpTIGR4-2114 (6O12)	0.0596
SpTIGR4-2117 (6B13)	0.0889
SpTIGR4-2120 (6E13)	0.0582
SpTIGR4-2124 (6A14)	0.0783
SpTIGR4-2126 (6C14)	0.0701
SpTIGR4-2134 (6C15)	0.0757
SpTIGR4-2135 (6D15)	0.0471
SpTIGR4-2136 (6E15)	0.0429
SpTIGR4-2141 (6B16)	0.0725
SpTIGR4-2142 (6C16)	0.0914
SpTIGR4-2143 (6D16)	0.0733
SpTIGR4-2146 (6G16)	0.033
SpTIGR4-2150 (6B17)	0.0574
SpTIGR4-2151 (6C17)	0.0589
SpTIGR4-2153 (6E17)	0.0834
SpTIGR4-2168 (6D19)	0.0281
SpTIGR4-2170 (6F19)	0.0787
SpTIGR4-2171 (6G19)	0.0497
SpTIGR4-2172 (6H19)	0.0546
SpTIGR4-2173 (6A20)	0.0257
SpTIGR4-2174 (6B20)	0.0367
SpTIGR4-2175 (6C20)	0.0874
SpTIGR4-2176 (6D20)	0.0897
SpTIGR4-2179 (6G20)	0.0224
SpTIGR4-2181 (6A21)	0.0117
SpTIGR4-2184 (6D21)	0.0224
SpTIGR4-2185 (6E21)	0.0661
SpTIGR4-2186 (6F21)	0.00913
SpTIGR4-2187 (6G21)	0.00411
SpTIGR4-2188 (6H21)	0.0158
SpTIGR4-2189 (6A22)	0.0415
SpTIGR4-2190 (6B22)	0.022

SpTIGR4-2191 (6C22)	0.0729
SpTIGR4-2192 (6D22)	0.0119
SpTIGR4-2193 (6E22)	0.0119
SpTIGR4-2194 (6F22)	0.0193
SpTIGR4-2195 (6G22)	0.0293
SpTIGR4-2201 (6E23)	0.0339
SpTIGR4-2202 (6F23)	0.0268
SpTIGR4-2203 (6G23)	0.0365
SpTIGR4-2204 (6H23)	0.0049
SpTIGR4-2205 (6A24)	0.0199
SpTIGR4-2209 (6E24)	0.0217
SpTIGR4-2210 (6F24)	0.00803
SpTIGR4-2214 (6J13)	0.0367
SpTIGR4-2215 (6K13)	0.031
SpTIGR4-2218 (6N13)	0.0634
SpTIGR4-2219 (6O13)	0.0994
SpTIGR4-2220 (6P13)	0.033
SpTIGR4-2221 (6I14)	0.03
SpTIGR4-2222 (6J14)	0.00742
SpTIGR4-2224 (6L14)	0.0941
SpTIGR4-2228 (6P14)	0.0899
SpTIGR4-2235 (6O15)	0.0273
SpTIGR4-2239 (6K16)	0.00704
SpTIGR4-2240 (6L16)	0.0146