Differential Expression of *Streptococcus Pneumoniae* Genes during Pathogenesis

Kim Suk LeMessurier, B Biotech (Hons)

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School of Molecular and Biomedical Sciences
The University of Adelaide
Adelaide, S.A., Australia

Chapter Two: Materials and Methods

Chapter Three: Optimisation of a mouse model of invasive pneumococcal disease and methods for extraction of RNA from *in vivo*-derived pneumococci

Abstract

Streptococcus pneumoniae is a nasopharyngeal commensal in most healthy individuals. However, it can translocate from this niche to deeper tissues, causing diseases such as otitis media, meningitis, sepsis and pneumonia, which are responsible for significant morbidity and mortality worldwide. At the commencement of this work, inherent difficulties in harvesting sufficient bacterial numbers from experimental animals restricted the examination of pneumococcal gene expression during pathogenesis, and thus virulence gene transcription patterns were largely unknown outside of an *in vitro* environment. This thesis aimed to investigate such transcriptional patterns *in vivo*, and to hence gain a better understanding of pneumococcal behaviour during colonisation and disease.

This work describes refinement of an intranasal *S. pneumoniae* infection model in CD-1 mice that enables pneumococci to be harvested from multiple niches with low contamination by nasopharyngeal microflora or host tissue, and minimal cross-contamination with circulating pneumococci in the vascular system. The challenge route simulates the acquisition of *S. pneumoniae* in the human population, and progression to IPD occurs naturally. RNA extraction, enrichment and linear amplification procedures were optimised so that RNA could be obtained from *in vivo* site in sufficient quantities and with sufficient integrity to be used in semi-quantitative assays. Linear amplification allowed the examination of gene expression in niches where low bacterial numbers had previously prevented such analyses.

Real-time RT-PCR and microarray analyses were used to examine bacterial RNA samples recovered from the nasopharynx, lungs, blood and brains of CD-1 mice, providing the first comparative transcriptional data for pneumococci during carriage and disease, within the same animal model. Two pneumococcal serotypes were examined; a type 2

(D39) and a type 6A (WCH16) strain. CbpA, Ply, and SpxB were shown to be important for carriage in both strains, with pneumococci up-regulating the expression of the genes encoding these virulence proteins in the nasopharynx. This provides *in vivo* evidence supporting the ascribed roles of these proteins in reducing the level of competing microflora and promoting nasopharyngeal adherence. Similarly, D39 *nanA* and *pspA* transcription levels were up-regulated in the nasopharynx. The level of *pspA* mRNA was also higher in the blood than the lungs, suggesting an increased requirement in the bloodstream, where PspA is involved in reducing complement-mediated opsonisation. Despite the anti-phagocytic role of the pneumococcal polysaccharide capsule in the bloodstream, D39 *cpsA* mRNA was present in similar quantities in the nasopharynx, lungs and blood, which may support previous studies indicating post-transcriptional regulation of capsule expression. However, *cpsA* expression was up-regulated in the blood for WCH16. These results may indicate the existence of strain-specific differences in virulence gene regulation.

Microarray analysis of *in vivo*-harvested *S. pneumoniae* D39 found that mRNAs encoding components of phosphotransferase systems, CbpA, a putative neuraminidase, and v-type sodium ATP synthase subunits were significantly higher in bacteria involved in carriage than bacteraemia. Conversely, the expression of genes involved in competence, and *dinF* (present on a competence-induced operon), were up-regulated in the blood compared to the nasopharynx, providing evidence that competence is induced during bacteraemia. Pneumococci also showed increased expression of genes involved in fatty acid metabolism, *pgdA*, *lytB* and *cbpG* in the blood compared to the nasopharynx. This study used a single pneumococcal strain and infection model and, therefore, overcomes inherent issues of serotype/strain- and animal model- specific gene expression that may have complicated interpretation of data in previous studies.

This thesis reports some of the first *in vivo* pneumococcal gene expression data gained using a single animal model and pneumococcal strain. The data reinforce the

putative roles of several virulence factors, and provides novel transcription data for pneumococci during carriage. Results suggest the existence of core genes that are essential for infection in multiple pneumococcal serotypes, whereas other genes appear to have strain-specific roles.

Declaration

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List of abbreviations

Abbreviations acceptable to the American Society for Microbiology are used without definition in this thesis. Additional and frequently used abbreviations are defined when first used in the text, and are listed below.

A_{260}, A_{280}, A_{600}	Absorbance at 260nm, 280 nm, or 600nm respectively
BA	Blood agar
BBB	Blood-brain barrier
BMEC	Brain microvascular endothelial cells
C3b	Complement component 3b
CbpA	Choline binding protein A
CBP	Choline binding protein
CBR	Choline binding region
CFU	Colony forming units
ChoP	Phosphorylcholine
CPS	Capsular polysaccharide
CSF	Cerebrospinal fluid
CSP-1	Competence stimulating peptide 1
CSP-2	Competence stimulating peptide 2
C-terminus	Carboxy terminus
Ct	Cycle threshold
DFI	Differential fluorescence induction
DIG	Digoxigenin
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylene diamine tetraacetic acid
Ery	Erythromycin
g	Gravity units
hr	Hour(s)
Ig	Immunoglobulin
IN	Intranasal
IP	Intraperitoneal
IPD	Invasive pneumococcal disease
Ig	Immunoglobulin
kDa	Kilodalton(s)
LB	Luria Bertani broth

LytA	Autolysin A
M	Molar
min	Minute(s)
mRNA	Messenger ribonucleic acid
NanA	Neuraminidase A
N-terminus	Amino terminus
O/N	Overnight
ORF	Open reading frame
PAF	Platelet activating factor
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PiaA	Pneumococcal iron acquisition A
pIgR	Polymeric immunoglobulin receptor
Ply	Pneumolysin
PS	Polysaccharide
PsaA	Pneumococcal surface antigen A
PspA	Pneumococcal surface protein A
PTS	Phosphotransferase systems
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse-transcription polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulphate
sec	Second(s)
SEM	Standard error of the means
STM	Signature-tagged mutagenesis
THY	Todd-Hewitt broth supplemented with yeast extract
TLR	Toll-like receptor
WCL	Whole cell lysate
WT	Wild-type

CHAPTER 1 - Introduction

1.1 The history of the pneumococcus

In 1880, Louis Pasteur and George Sternberg independently isolated an organism present in human saliva that was capable of causing systemic disease in rabbits. This gram-positive, catalase-negative bacterium has since been referred to as *Microbe septicemique du salive* (Pasteur and Chamberland, 1881), *Micrococcus pasteuri* (Sternberg, 1885), *Pneumococcus* (Fraenkel, 1886), and *Diplococcus pneumoniae* (Winslow *et al.*, 1920). Due to the propensity of the pathogen to form chains in liquid culture media and its association with pneumonia, it was termed *Streptococcus pneumoniae* in 1974, the name by which it is known today. Over twelve decades since its initial identification, *S. pneumoniae* (commonly referred to as the pneumococcus) continues to be responsible for a spectrum of diseases ranging from relatively minor ailments such as otitis media and conjunctivitis, to potentially fatal pneumonia, septicaemia, and meningitis. Young children, the elderly and people with underlying medical conditions such as AIDS or sickle-cell disease are particularly susceptible to

pneumococcal infection, and at least one in every 500 Australian children will develop pneumococcal meningitis or septicaemia by the age of two years (Mackenzie *et al.*, 2005). In developing countries, the number of pneumococcal-related deaths has changed very little since the first isolation of the pathogen, with at least a million deaths per annum amongst children under 5 years of age attributed to pneumococcal pneumonia (World Health Organisation, 2007).

Since 1880, the pneumococcus has carved an illustrious history in the field of biology (Austrian, 1981b). It was the first bacterium observed to retain aniline-gentian violet stain, which lead to the development of the Gram-stain (Gram, 1884), and the discovery that capsular swelling occurs when pneumococci are exposed to specific antisera pioneered the development of the 'Quellung reaction', which is now commonly used to serotype human isolates (Armstrong, 1931; Austrian, 1981b). Klemperer and Klemperer (1891) showed that serum from rabbits injected with heat-killed pneumococci was able to elicit protection against re-infection with the challenge strain, but not necessarily against other clinical isolates. Furthermore, when non-sensitised rabbits received an infusion of serum from immunised rabbits they were protected against infection with the same strain, introducing the concept of humoral immunity. In the 1920s, the capsular polysaccharide (CPS) was shown to be responsible for pneumococcal serological activity, and was also demonstrated to elicit strain-specific protection in mice, making it the first identified non-protein antigen (Heidelberger and Avery, 1923; Heidelberger, 1927).

However, perhaps the most important contribution of *S. pneumoniae* to molecular science was a discovery published by Avery, Macleod and McCarty in 1944. In 1928, Griffith reported that co-injection of mice with heat-killed smooth pneumococci and live

un-encapsulated (avirulent) pneumococci resulted in the death of some animals. Examination of bacteria recovered from the dead mice revealed that the live un-encapsulated strain had acquired CPS serologically identically to the heat-killed cells in the inoculum (Griffith, 1928). The concept of bacterial transformation was confirmed by Neufeld shortly afterwards (Neufeld and Schnitzler, 1928). However, it wasn't until 1944 that the genetic carrier responsible for transformation was identified as DNA (Avery *et al.*, 1944). Further experiments by McCarty and Avery confirmed this finding, demonstrating that the addition of pancreatic DNase resulted in loss of transformation (McCarty and Avery, 1946). The report that DNA is the carrier of genetic information remains one of the most important discoveries in the molecular biosciences.

1.2 Cell structure

The pneumococcus is a gram-positive organism. Its cell envelope is comprised of a plasma membrane, cell wall, and polysaccharide capsule (as shown in Figure 1.1). The plasma membrane consists of a lipid bilayer, and is approximately 75 nm thick (Tomasz, 1981). The outer cell wall contains peptidoglycan, teichoic and lipoteichoic acids. The latter two components are decorated with phosphorylcholine (ChoP), which promotes adherence by recognising the platelet-activating factor (PAF) receptor on host cells. The phosphylcholine moieties are also a site of anchorage on the cell surface for pneumococcal choline-binding proteins via their choline binding domains (Cundell *et al.*, 1995a).

Perhaps the most defining pneumococcal virulence factor is its polysaccharide capsule (CPS), which possesses antiphagocytic properties. (Briles *et al.*, 1998). The capsule, noted by Pasteur in 1890 as being "a sort of aureole which corresponds perhaps to

Figure 1.1 **Diagram of the pneumococcal surface**

Major pneumococcal surface components and important virulence factors are indicated. CbpA and PspA dimers, and autolysin (LytA) are shown bound to phosphorylcholine resides on teichoic and lipoteichoic acids. Other important virulence factors depicted are capsular polysaccharide, neuraminidase and the cytotoxin pneumolysin. Adapted from Briles *et al.* (1998).

a material substance" surrounding pneumococci, was the first pneumococcal virulence factor to be described in the literature (Austrian, 1981a). Subsequently, antibodies to the capsule were found to be protective in animal models, which made it a target for vaccine development (Section 1.5). The pneumococcal CPS varies from approximately 200-400 nm in thickness (Sorensen *et al.*, 1988). Within a particular pneumococcal strain and serotype, a thinner CPS has been correlated with a decrease in virulence, with strains completely lacking a capsule (or 'rough' strains) being avirulent (Austrian, 1981a; MacLeod and Kraus, 1950). The capsule composition is variable. Over 90 different CPS types have been identified, which have negligible immunological cross-reactivity (Garcia *et al.*, 2000). This complicates the development of CPS-based vaccines, as such vaccines are specific to the serotypes included in the formulation. The genes involved in CPS production are located in a locus and typically encode a flippase, polymerase, and various transferases that catalyse the formation of linkages, the addition of sugars and modification of the repeat unit (Paton and Morona, 2000). The first four genes of the locus, referred to as *cpsA-D*, are highly conserved between all serotypes except types 3 and 37 and have been implicated in CPS regulation (Llull *et al.*, 2001; Morona *et al.*, 1999).

The amount of CPS produced by the pneumococcus varies in different environmental conditions, which possibly affects the degree of exposure of surface proteins believed to be important in pneumococcal pathogenesis (Kim and Weiser, 1998). In some niches, particularly the bloodstream, many such factors associated with the cell wall are partially or completely masked by the polysaccharide capsule. This is thought to prevent the recognition by phagocytes of complement components deposited on the cell surface, thereby reducing pneumococcal clearance from the bloodstream. It also may hamper antibody-mediated clearance of bacteraemic pneumococci. Conversely, a lower

level of CPS may increase the exposure of surface proteins such as choline binding protein A (CbpA) and pneumococcal surface protein A (PspA). These proteins are believed to partially extend beyond the polysaccharide capsule, and perform various functions in pathogenesis, such as preventing complement-mediated opsonisation and promoting epithelial adherence and invasion (Rosenow *et al.*, 1997; Tu *et al.*, 1999; Zhang *et al.*, 2000). CbpA and PspA are attached to the pneumococcal surface through interactions with ChoP moieties on teichoic and lipotechoic acids embedded in the peptidoglycan backbone of the cell wall and cell membrane, respectively (see Figure 1.1). These virulence factors are described in Section 1.6.1.

1.3 Pneumococcal pathogenesis

1.3.1 Nasopharyngeal colonisation

Asymptomatic colonisation of the upper respiratory tract by *S. pneumoniae* almost invariably precedes localised and systemic disease, and is also a major factor in horizontal transmission of pneumococcal disease (Malfroot *et al.*, 2004). Prior to the common use of antibiotics, up to 40% of adults were colonised in the nasopharynx by at least one pneumococcal strain. Although these figures are estimated to be lower today, the pneumococcus is still carried asymptomatically by many healthy individuals (Musher, 1992). In most adults, serotype-specific antibodies are produced following acquisition of *S. pneumoniae* and the pneumococcus is cleared after a period of weeks, although in some cases carriage may last for up to one year (Catterall, 1999; Musher, 1992). Carriage is more prevalent in the young, perhaps as a result of a poorly developed immune system and a high degree of contact between individuals, with up to 95% of healthy children colonised

by at least one pneumococcal serotype in some studies (Crook *et al.*, 2004). Nearly all infants are colonised at least once by *S. pneumoniae* by the age of two. Additionally, young infants exhibit a longer duration of colonisation, increasing the opportunity for transmission of the strain to non-colonised individuals (Gray *et al.*, 1980).

Adherence to the mucosal epithelium is crucial for pneumococcal persistence in the nasopharynx (Andersson *et al.*, 1983). Although non-specific factors such as surface charge, pH and hydrophobic interactions are likely to influence bacterial adherence, attachment of *S. pneumoniae* to host cells in the nasopharynx is thought to be largely mediated by specific interactions between the bacterium and the host (Andersson *et al.*, 1983; Cundell *et al.*, 1995a; Rosenow *et al.*, 1997). However, the nature of the host receptors and bacterial adhesin(s) involved is yet to be fully characterised. Pneumococcal adherence to nasopharyngeal cells has been suggested to occur through interactions with the N-acetyl-D-glucosamine β1–3 galactose (GlcNAcβ1–3Gal) disaccharide, part of a glycolipid receptor displayed on pharyngeal epithelial cells (Andersson *et al.*, 1983). ChoP-PAF receptor interactions have been demonstrated to mediate adherence to, and invasion of, lung and tracheal epithelia (Ishizuka *et al.*, 2001). However, the addition of a PAF receptor antagonist has no effect on the binding of pneumococci to Detroit-562 nasopharyngeal cells *in vitro*, suggesting that it does not play a similar role in adherence in the nasopharynx (Zhang *et al.*, 2000). Instead, the pneumococcal surface protein CbpA (Section 1.6.1.1) has been implicated in adherence to the nasopharyngeal epithelium and the subsequent invasion of these cells, for some *S. pneumoniae* strains. A CbpA$^-$ strain is attenuated almost 100-fold in its ability to establish nasopharyngeal carriage in infant rats, and exhibits over a 90% reduction in its ability to invade nasopharyngeal cells (Rosenow *et al.*, 1997; Zhang *et al.*, 2000). Knockout mice defective in pIgR trafficking showed a

delayed onset of bacteraemia after IN challenge (Zhang *et al.*, 2000). Together, these studies suggest roles for CbpA in both carriage and the progression of disease. This is discussed further in Section 1.6.1.1. Pneumococcal adherence and virulence factor A (PavA) may also contribute to adherence in this niche, as it has been shown to bind to immobilized fibronectin (Holmes *et al.*, 2001). In addition, there are several pneumococcal proteins (discussed in Section 1.6) that may contribute to colonisation, either by directly promoting adherence, or indirectly, for example by preventing pneumococcal killing by apolactoferrin or reducing competition by other nasopharyngeal bacteria (Shakhnovich *et al.*, 2002; Shaper *et al.*, 2004; Spellerberg *et al.*, 1996; Tu *et al.*, 1999).

Exactly what provokes the change from a benign commensal to an aggressively invasive pathogen is unclear, although invasive disease often occurs shortly after the acquisition of a new serotype, and is rarely associated with prolonged carriage (Boulnois, 1992; Gray *et al.*, 1980). Developing vaccines capable of interfering with asymptomatic carriage may be desirable, because such carriers are the principal reservoir for infection (Briles *et al.*, 2000). Thus, eliminating carriage in the individual will confer protection against invasive disease in both the vaccinated and non-vaccinated individuals (herd immunity). However, vaccines directed against carriage face strong selective pressure in the nasopharynx, and may also leave binding sites for pneumococci available for other pathogens.

1.3.2 Risk factors for developing pneumococcal disease

Although carriage of *S. pneumoniae* is common within the population, most occurrences of serious pneumococcal disease occur in children < 2 years of age and adults

> 65 years of age (Parsons and Dockrell, 2002). Infants have an immature immune system which is less able to respond to polysaccharide antigens, leaving them vulnerable to encapsulated bacteria. Additionally, pneumococcal carriage and acquisition of new strains is most frequent in young children, which may contribute to the higher incidence of disease (Crook *et al.*, 2004; Hussain *et al.*, 2005). Some underlying medical conditions predispose to pneumococcal infections, such as congenital immunodeficiency, human immunodeficiency virus (HIV) infection, leukaemia and lymphoma. Sufferers of sickle-cell disease are particularly vulnerable, as they exhibit defective reticulo-endothelial clearance of encapsulated bacteria from their bloodstream. Patients taking immunosuppressive medication, such as transplant patients, are also at increased risk of pneumococcal infection. Individuals with respiratory conditions including viral respiratory infections, chronic bronchitis, and chronic obstructive pulmonary disease, or behavioural conditions such as alcoholism and smoking, also face increased chances of developing invasive pneumococcal disease (Centers for Disease Control and Prevention, 1997). Smokers are 4.1 times more likely to develop pneumococcal diseases than individuals who are not exposed to cigarette smoke, and passive smokers have a 2.5-fold increased risk (Nuorti *et al.*, 2000). This may be a result of impaired ciliary and mucociliary defence, epithelial damage, increased bacterial colonisation and adherence, and reduced immunoglobulins. Other high risk factors include low socio-economic conditions, accommodation in a nursing home and attendance at a day-care facility (Kalin *et al.*, 2000; Levine *et al.*, 1999). Certain indigenous populations also have increased risk of pneumococcal disease. Alaskan natives are 8- to 10-fold more likely to develop meningitis and bactaeremic pneumonia then other population groups in the USA (Davidson *et al.*, 1994). In Australia, the incidence of invasive pneumococcal disease in Australian aboriginal children is the highest in the world (Torzillo *et al.*, 1995).

1.3.3 Pneumococcal disease

Pneumococcal disease occurs when colonising organisms in the nasopharynx translocate from this niche to the middle ear, alveolar space or bloodstream causing otitis media, pneumonia and bacteraemia, respectively (McCullers and Tuomanen, 2001). Occasionally, bacteria also traverse the blood-brain barrier and cause meningitis. Otitis media, a common manifestation of pneumococcal infection in young children, results from the progression of colonising pneumococci in the nasopharynx up the Eustachian tube and into the middle ear cavity, where they establish infection. Once in this niche, bacterial replication results in an influx of neutrophils, and cell wall components appear to induce the inflammation characteristic of otitis media (Carlsen *et al.*, 1992). Recurrent bouts of otitis media can result in hearing impairment. Although not fatal, otitis media is a considerable economic and health burden in developed countries. *S. pneumoniae* is the most common bacterial cause of otitis media in the United States of America, where it is responsible for an estimated seven million middle ear infections per annum (Dowell *et al.*, 1999).

The spontaneous aspiration of bacteria from the upper respiratory tract into the alveolar space can result in pneumonia. Pneumococci are thought to attach to pneumocytes via interactions with host glycoconjugates containing GlcNAcβ1-3Gal and GlcNAcβ1-4Gal sequences (Andersson *et al.*, 1981; Cundell and Tuomanen, 1994). A second interaction between ChoP on bacterial teichoic acid and the host cell platelet activating factor receptor has been suggested to mediate both adherence and invasion of the lung and lower tracheal epithelia (Cundell *et al.*, 1995a; Ishizuka *et al.*, 2001). The PAF receptor is known to be rapidly internalised following binding of its natural ligand,

and the pneumococcus hijacks this pathway to transmigrate through the cell (Le Gouill *et al.*, 1997). Moreover, histological studies using pneumococci expressing green fluorescent protein (GFP) have found bacteria inside lung epithelial cells, but not in the tight junctions of these cells, confirming that invasion can occur via trans-cellular migration (Kadioglu *et al.*, 2001). Studies have indicated that CbpA-mediated interactions activate vascular endothelium, resulting in increased capillary permeability and the subsequent leakage of proteinaceous fluid, red blood cells and macrophages into the alveoli. Proteinaceous fluid provides a medium that promotes pneumococcal growth and dissemination. The activation of endothelial cells also increases the amount of surface-expressed PAF receptor, which may promote invasion of these cells (Cundell *et al.*, 1995a). In addition, several pneumococcal components are able to provoke the up-regulation in expression of various cytokines and other pro-inflammatory mediators by resident alveolar macrophages, resulting in the recruitment of polymorphonuclear lymphocytes (Bergeron *et al.*, 1998). It is likely that the activation of vascular endothelium (resulting in increased PAF receptor and capillary permeability) facilitates the entry of pneumococci into the bloodstream, causing potentially fatal bacteraemia. Pneumococci can also enter the bloodstream from the nasopharynx via intracellular translocation facilitated by interactions with the human polymeric immunoglobulin receptor human pIg receptor (pIgR), as discussed in Section 1.6.1.1 (Cundell *et al.*, 1995a; Kadioglu *et al.*, 2001).

Once in the blood, CPS is critical for preventing clearance by host phagocytic cells (Section 1.2). Circulating bacteria can adhere to, and subsequently invade, brain microvascular endothelial cells (BMEC), a process requiring CbpA (Ring *et al.*, 1998). Transmigration across BMEC may also occur via the PAF receptor (Cundell *et al.*, 1995a; Koedel *et al.*, 2002). Thus, invasion of BMEC allows pneumococci to cross the blood-

brain barrier (BBB) and enter the sub-arachnoid space. Prior to bacteria breaching the BBB, the sub-arachnoid space is almost completely devoid of general host defense mechanisms such as polymorphonuclear leucocytes, complement components, and immunoglobulins (Koedel *et al.*, 2002; Simberkoff *et al.*, 1980; Smith and Bannister, 1973). Leukocytes are recruited to the sub-arachnoid space following pneumococcal invasion. However, the cerebrospinal fluid (CSF) lacks sufficient complement concentrations to achieve opsonic activity by leukocytes (Simberkoff *et al.*, 1980), which allows pneumococci to proliferate in this niche without being cleared by the immune system. Pneumococcal components and products such as peptidoglycan, lipoteichoic acid, pneumolysin, hydrogen peroxide and bacterial DNA cause brain cell death and immune activation (Koedel *et al.*, 2002). This results in neuronal damage and massive inflammation, symptomatic of meningitis.

Bacteraemia and meningitis are both referred to as ‘invasive pneumococcal disease’ (IPD). IPD is the leading cause of death in children younger than two years of age in developing countries, and is associated with an overall case fatality rate of approximately 25%, even with antibiotic treatment (Pallares *et al.*, 1995). Although mortality rates are generally lower in developed countries, the financial cost is high, with the economic burden of pneumococcal infection in the USA in excess of 2 billion dollars per annum (Cunha *et al*., 1997). Before the introduction of subsidised Prevnar vaccinations for infants in Australia, approximately one in 500 Australian children developed IPD before two years of age (Mackenzie *et al.*, 2005). Furthermore, *S. pneumoniae* meningitis has a case-fatality rate of approximately 20%, which is the highest for community acquired meningitis in the USA (Schuchat *et al.*, 1997).

1.3.4 Animal models of pathogenesis

S. pneumoniae is considered a strict human pathogen, and humans are the principle reservoir for infection. However, a serotype 3 clone has been associated with lower airway disease in horses (Burrell *et al.*, 1986; Neufeld and Schnitzler, 1928; Timoney, 2004). Like its human counterparts, equine *S. pneumoniae* is able to exist asymptomatically in the upper respiratory tracts of healthy animals (Timoney, 2004). Bacteraemia occasionally occurs in foals, although it is rarely associated with adult horses (Meyer *et al.*, 1992). Interestingly, equine isolates have deletions in *ply* and *lytA* genes (encoding pneumolysin and autolysin, respectively), and exhibit a lack of autolytic and haemolytic activity (Whatmore *et al.*, 1999). Regardless, *S. pneumoniae* is still isolated from horses with respiratory infections and bacteraemia, and is able to induce disease in experimental models (Blunden *et al.*, 1994; Timoney, 2004). Clinically, pneumococci are usually isolated from horses already infected with other pathogens, suggesting that *S. pneumoniae* may be an opportunistic pathogen in horses (Chapman *et al.*, 2000).

Although *S. pneumoniae* is only a natural pathogen for humans and horses, several other animals are capable of replicating the pathogenesis observed in humans to varying degrees, thus allowing it to be examined *in vivo*. Chinchillas are the favoured animal in which to study otitis media, while meningitis is often examined using a rabbit model (Giebink, 1999; O'Donoghue *et al.*, 1974). However, due to the handling, expense and availability considerations involved in using these larger animals, the mouse model of infection is most frequently used for *in vivo* experiments. Depending on the strain of mouse and *S. pneumoniae*, experimental mice can develop a range of infections, including nasopharyngeal colonisation, pneumonia, bacteraemia and meningitis (Orihuela *et al.*, 2003; Wu *et al.*, 1997). Several different routes of infection are commonly used in

pneumococcal disease models. Direct challenge models, such as intraperitoneal (IP), intracisternal (IC) or transbullar (TB), involve the injection of bacteria directly into the peritoneal, intracisternal, or middle ear cavities, respectively (Barry *et al.*, 1996; Giebink, 1999; Krontz and Strausbaugh, 1980). Such challenge models allow a specific facet of disease to be examined, as a controlled number of bacteria are introduced into the niche. Because the pneumococcus is primarily a respiratory pathogen with transmission occurring by the aerosol route, an intranasal (IN) challenge model more accurately represents natural acquisition of the pathogen than direct challenge models. Nasopharyngeal colonisation is established following IN infection. From this niche, diseases such as pneumonia, bacteraemia and meningitis can occur naturally. Anaesthetising the animals prior to IN infection relaxes the tracheal cilia, promoting the aspiration of pneumococci in the challenge inocula into the lungs and resulting in a more uniform level of infection.

1.4 Antibiotic therapy and resistance

Since the late 1940s, antibiotic therapy has been employed extensively to treat bacterial diseases, including those resulting from pneumococcal infection. Exposure of bacteria to antibiotics inevitably selects for acquisition of resistance. This problem is particularly significant considering the ability of the pneumococcus, a naturally transformable organism, to acquire resistance by horizontal gene transfer, and the rapid global dissemination of resistant clones (Witte, 1999). In addition, the propensity of the pneumococcus to colonise the nasopharynx of carriers for many months increases the chance of antibiotic exposure. Strains of *S. pneumoniae* with reduced sensitivity to penicillin have been reported since the mid 1960s. Pneumococcal penicillin resistance results from a decreased affinity of the antibiotic for altered penicillin binding proteins

(PBPs), which are transcarboxypeptidases involved in cell wall synthesis (Zighelboim and Tomasz, 1980). Penicillin interacts with the PBPs, due to the analogous nature its β-lactam ring and the D-Alanyl-D-Alanine terminus of the peptidoglycan precursors. This prevents transpeptidation from occurring, disrupting cell wall synthesis and leading to cell lysis (Tipper and Strominger, 1965). Currently, over a third of pneumococcal strains exhibit resistance to penicillin (Doern *et al.*, 2001). The incidence is much higher in countries where antibiotic use has been poorly regulated, and is most prevalent for the 'paediatric' serotypes such as 6B, 9V, 9A, 14 and 19F (Schrag *et al.*, 2000). Many penicillin-resistant strains also exhibit resistance to other classes of antibiotics including tetracyclines, chloramphenicol, macrolides and co-trimoxazole. In the United States of America, approximately 11% of *S. pneumoniae* clinical isolates are resistant to three or more different classes of antibiotics (Sahm *et al.*, 2001). Such multi-drug resistance complicates the treatment of pneumococcal disease, often requiring the use of more expensive antimicrobial agents, and also contributes to outbreaks in hospitals and other care facilities, where antibiotic use is high and individuals are more prone to disease (Appelbaum, 1995; Butler and Cetron, 1999). Although much effort is being directed towards developing new antimicrobials, it is clear that the life-spans of individual antibiotics are limited in terms of their clinical efficacy, and that alternative methods of treatment and prevention must be sought if pneumococcal disease is to be kept under control.

1.5 Pneumococcal vaccines

Antibiotic resistance does not appear to be a problem that will be alleviated simply by the development of new antibiotics. The regular use of antibiotics for children, the elderly, and in the nosocomial setting, both augments the development of resistance and

increases the prevalence of resistant clones amongst the groups at highest risk of IPD. The limited life-spans and complications of antibiotics have made vaccination against *S. pneumoniae* an increasingly attractive alternative.

1.5.1 Polysaccharide vaccines

A vaccine comprising 14 purified CPS from the most prevalent serotypes was introduced in 1977. In 1983, coverage was increased to 23 CPS serotypes, namely 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F (Mulholland, 1999). The 23-valent vaccine, Pneumovax 23, covers the serotypes responsible for approximately 85% of disease in the USA and Europe (Robbins *et al.*, 1983). In adults, the polysaccharide vaccine is able to elicit a humoral immune response in the majority of vaccinated individuals, thereby resulting in the production of antibodies that enhance opsonisation and phagocytosis of pneumococci (Musher *et al.*, 1990). However, as polysaccharides are T-independent antigens, such CPS -based vaccines are poorly immunogenic in the groups most at risk of developing pneumococcal disease: elderly people, young children and immunocompromised individuals, and also fail to induce immunological memory (Briles *et al.*, 1998).

1.5.2 Polysaccharide-protein conjugate vaccine

When conjugated to a protein carrier, CPS induces a T-dependent immune response and hence has higher success in inducing a lasting response and immunological IgA memory in children under two years of age (Choo *et al.*, 2000; Robbins and Schneerson, 1990). Protein conjugate vaccines also protect against pneumococcal carriage in the nasopharynx, thus resulting in protection to the community as well as the individual (Mbelle *et al.*, 1999; Russell *et al.*, 2000). A vaccine comprising seven pneumococcal

capsular types conjugated to diphtheria toxiod (Prevnar) was licensed in the USA in 2000. These seven serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) accounted for over 80% of IPD in children under five years of age in the USA before the introduction of Prevnar (Centers for Disease Control and Prevention, 2005). In the subsequent three years, IPD decreased by 94% in children under two years in the USA (Centers for Disease Control and Prevention, 2005). The incidence of disease caused by the serotypes included in the conjugate vaccine was also reduced by 55% in adults 50 years or older, a high risk group that was not a vaccination target (Lexau *et al.*, 2005). In addition, the incidence of IPD in infants too young to be vaccinated also decreased significantly, demonstrating significant protection of non-vaccinated individuals by herd immunity (Poehling *et al.*, 2006).

Unfortunately, the high cost of producing the polysaccharide conjugate restricts the number of serotypes that can be included in a single vaccine, which results in limited cover and hence serotype replacement. Currently, a course of immunisations with Prevnar costs about ten times more than immunisation with Pneumovax 23. This cost prohibits the use of Prevnar in developing countries where IPD is a major cause of infant mortality. In addition, conjugate vaccines do not prevent recurrent otitis media (van Heerbeek *et al.*, 2006).

1.5.3 Serotype replacement

Given the negligible immunological cross-reactivity between serotypes of anti-CPS antibodies, polysaccharide vaccines face a high risk of redundancy should strains undergo CPS type switching by recombination at the capsule locus between included and non-included serotypes (Bogaert *et al.*, 2004b). Additionally, the niche previously occupied by vaccine serotypes may be colonised by other serotypes either newly acquired or previously

present in low numbers, resulting in a serotype shift. Epidemiological studies have confirmed that such serotype replacement is a real issue in practice, demonstrating that although vaccination with capsule-based formulations results in a reduction in carriage of the included serotypes, there is an increase in carriage of non-vaccine serotypes in immunised individuals (Mbelle *et al.*, 1999). A recent study in the USA indicated that, since the introduction of Prevnar, total incidence of pneumococcal bacteraemia has decreased by 57%. Bacteraemia caused by vaccine serotypes decreased from 85% to 34%. (Steenhoff *et al.*, 2006). Initially, it was suggested that some vaccine-included serotypes would elicit a degree of cross-reactive protection against pneumococci within a serogroup. For example, the 6A and 6B CPS differ only by a single glycosidic bond in the structural repeat unit; 6A possesses a rhammopyranosyl-(1 → 3)-D-ribitol bond and type 6B has a rhamnopyranosyl-(1 → 4)-D-ribitol bond (Robbins *et al.*, 1979). Similarly, serotypes 19A and 19F differ only by the nature of the linkage between CPS repeat units (Katzenellenbogen and Jennings, 1983). Therefore, immunisation with one capsular type was expected to confer cross-protection against the other due to the antigenic similarity. However, data collected by Steenhoff *et al.* (2006) indicated that the most notable increase in serotype prevalence during the course of their study was for the "vaccine-related" serotypes 19A and 6A.

1.5.4 Protein vaccines

Until recently, the focus of vaccine development has been on the pneumococcal capsule. Pneumococcal strains lacking a capsule were found to be avirulent, and there was a profound gap in knowledge regarding other surface antigens. However, as there are numerous pneumococcal serotypes associated with invasive disease, the development of capsule-based vaccines has been difficult (as discussed above). Recent advances in the

understanding of the structure and function of certain virulence proteins has raised the possibility of using protein antigens in vaccine formulations. A protein-based vaccine has several advantages over current CPS-based vaccines. Firstly, proteins are T-dependent antigens, whereas carbohydrates are not. Therefore, a protein vaccine would have increased immunogenicity in infants and in the elderly. Additionally, immunological memory would be stimulated, thus providing longer-term protection. Secondly, a protein vaccine would be cheaper to produce than conjugate vaccines, making it feasible for use in developing countries. Thirdly, some pneumococcal virulence proteins appear to be conserved between most serotypes, preventing the occurrence of serotype shifting following a vaccination regime with a protein-based formulation, and increasing vaccine coverage.

1.6 Important pneumococcal virulence factors

S. pneumoniae is primarily a colonising organism, existing in the nasopharynx of individuals where it establishes a balance between bacterial aggression and the host's defence system, allowing it to persist in this niche asymptomatically. The development of IPD is incidental and occurs in only a small minority of infected individuals. As the spread of pneumococci occurs most readily via aerosol transfer between a colonised and uncolonised individual, causing systemic disease that might kill the host would be counterproductive to persistence in a population. In this regard, invasive disease is not a favourable outcome and hence the pneumococcus experiences little selective pressure to maintain factors that enhance systemic virulence alone. As a consequence, it has become increasingly apparent that many pneumococcal virulence factors also play an important role in establishing and maintaining stable nasopharyngeal carriage.

1.6.1 Choline Binding Proteins (CBP)

The pneumococcus possesses a number of surface proteins termed 'choline binding proteins' (CBPs) that attach to cell wall teichoic and lipoteichoic acids (Garcia *et al.*, 1998; Rosenow *et al.*, 1997). CBPs share a common Choline Binding Region (CBR) comprising between two and 10 highly conserved 20 amino acid repeats which bind non-covalently to ChoP residues (Giffard and Jacques, 1994). For most CBPs, the CBR is at the C-terminus. The major pneumococcal autolysin, LytA, and PspA (Section 1.6.1.2), were the first two pneumococcal CBPs to be extensively characterised (Garcia *et al.*, 1986; Talkington *et al.*, 1991; Yother and Briles, 1992). Several more CBPs, exhibiting diverse functions, were identified using an immobilised choline affinity matrix, and of these CbpA was the most abundant (Rosenow *et al.*, 1997) (Section 1.6.1.1). Subsequently, other pneumococcal CBPs have been identified, including cell wall hydrolases (LytB and LytC), the putative amidase CbpD and putative serine protease CbpG (Garcia *et al.*, 1999a; Garcia *et al.*, 1999b; Gosink *et al.*, 2000; Kausmally *et al.*, 2005). To date, at least 10 pneumococcal CBPs have been described (Garcia *et al.*, 1986; Garcia *et al.*, 1999a; Garcia *et al.*, 1999b; Gosink *et al.*, 2000; Kausmally *et al.*, 2005; Rosenow *et al.*, 1997; Yother and Briles, 1992).

1.6.1.1 Choline binding protein A (CbpA)

CbpA, also referred to as PspC (pneumococcal surface protein C) and SpsA (*Streptococcus pneumoniae* secretory IgA binding protein), is a surface-exposed CBP. Some pneumococcal strains possess Hic (H-binding inhibitor of complement) instead of CbpA. While Hic exhibits high sequence homology with the N-terminus of CbpA and is thought to share many of its functions, it lacks a choline binding domain. Instead, it

possesses a LPXTG motif and is anchored to the pneumococcal surface in a sortase-dependent manner (Janulczyk *et al.*, 2000). The role of CbpA as an adhesin was suggested by Rosenow *et al.* (1997), who demonstrated a decreased ability of a CbpA-negative *S. pneumoniae* mutant ($CbpA^-$) to bind cytokine-activated human lung epithelial cells and endothelial cells when compared to the parent strain, and an almost 100-fold reduction in nasopharyngeal carriage in an infant rat model (Rosenow *et al.*, 1997). Likewise, *S. pneumoniae* mutants lacking CbpA are deficient in nasopharyngeal carriage in a mouse model, and are unable to infect and multiply in the lungs (Balachandran *et al.*, 2002). CbpA has also been implicated in pneumococcal adherence to the brain microvascular endothelium and subsequent invasion of the subarachnoid space (Ring *et al.*, 1998).

Zhang *et al.* (2000) reported *in vitro* interactions between CbpA and human pIgR. The group reported that a deficiency in CbpA reduced the ability of mutant pneumococci to invade nasopharyngeal cells by over 90% when compared to the parent strain. Furthermore, knockout mice defective in pIgR trafficking showed a delayed onset of bacteraemia after intranasal challenge. These observations led to the proposal that pneumococci gain access to the interior of the cell by subversion of the pIgR. This is consistent with the primary function of pIgR in apical-basolateral transcytosis of its endogenous ligands IgA and IgM (Mostov, 1994), and with previous reports indicating a strong association between CbpA and secretory IgA (Hammerschmidt *et al.*, 1997). However, studies performed by a separate group indicate that the CbpA-pIgR mediated pneumococcal invasion observed by Zhang *et al.* (2000) was restricted to both the strain of *S. pneumoniae* and the cell type used (Brock *et al.*, 2002). Detroit-562 cells were the only cell line in which CbpA-pIgR-mediated invasion was observed, despite several human pIgR expressing lines being examined. Additionally, of the nine clinical isolates and

laboratory strains of *S. pneumoniae* tested, only R6x, the non-encapsulated strain used by Zhang *et al*. (2000), showed significant invasion of Detroit-562 cells.

In addition to its active role as an adhesin and putative invasive determinant, CbpA has also been shown to bind factor H (Dave *et al.*, 2001). Factor H is a serum protein that inhibits the binding of factor B to C3b, thus protecting host cells from being attacked by its own complement pathway (Dave *et al.*, 2001; Jarva *et al.*, 2003). It is possible that, by binding pneumococcal CbpA, factor H is then able to bind C3b deposited on the cell surface, arresting the complement pathway at this critical stage.

Due to its multiple roles in both carriage and pathogenesis, CbpA presents as a suitable vaccine candidate. Additionally, CbpA extends beyond the capsular layer. Therefore, carriage could be abrogated by either opsonic or neutralising antibodies. Indeed, immunization studies have shown CbpA to be highly protective in mice following IP challenge (Brooks-Walter *et al.*, 1999; Ogunniyi *et al.*, 2001).

1.6.1.2 Pneumococcal surface protein A (PspA)

PspA is a highly immunogenic, surface exposed protein with dual functionality. The protein is tethered to the cell wall via interactions between ChoP moieties on teichoic and lipoteichoic acids and its C-terminal CBR, which shares 90-95% sequence homology with the CBR of CbpA (Brooks-Walter *et al.*, 1999). The highly charged N-terminal region extends beyond the capsule (Yother and Briles, 1992). PspA is found in all clinically important *S. pneumoniae* serotypes, although its molecular size varies from 67-99 kDa (Crain *et al.*, 1990; Waltman *et al.*, 1990). Despite inter-strain variation in amino acid composition of the N-terminus, key protective epitopes exhibit high cross-reactivity

(Briles *et al.*, 1988; Nabors *et al.*, 2000). The α-helical N-terminal portion of PspA is particularly immunogenic, and immunisation with a 43 kDa fragment containing this region has been demonstrated to provide protection against intravenous and intratracheal challenge of mice (Talkington *et al.*, 1991). In human clinical studies, pre-existing antibodies (IgG and IgA) to PspA, in particular the N-terminal region, have been associated with protection against the acquisition of carriage. Indeed, the pre-existence of antibodies to PspA appeared to be the only pneumococcal factor that affected susceptibility in the subjects examined (McCool *et al.*, 2002). This presents PspA as a promising vaccine candidate.

The region 2 of the α-helical domain of PspA has been shown to bind lactoferrin (Hakansson *et al.*, 2001). Lactoferrin is the only identified ligand of PspA, and hence the protein was initially believed to be involved in sequestering iron at respiratory mucosal sites, where the availability of free iron is limited (Hammerschmidt *et al.*, 1999). Presence of lactoferrin alone, however, is not sufficient to support pneumococcal growth in iron-deficient media (Tai *et al.*, 1993). In addition, pneumococci lacking a full-length PspA, but producing a truncated form incapable of binding lactoferrin, have been shown to successfully colonise the human nasopharynx (McCool *et al.*, 2002). Shaper *et al.* (2004) observed that incubation of pneumococci with recombinant PspA could protect pneumococci against the bactericidal effects of apolactoferrin (the iron-depleted form of lactoferrin). This was supported by the ability of antibodies to PspA to increase apolactoferrin-mediated death of pneumococci, indicating that PspA may promote persistence in the nasopharyngeal by preventing killing of pneumococci by apolactoferrin (Shaper *et al.*, 2004).

A second role for PspA is suggested by several observations. Firstly, after intravenous infection, PspA$^-$ pneumococci are rapidly cleared from the blood, whereas levels of an otherwise isogenic PspA$^+$ strain increased over the same time (Tu *et al.*, 1999) (McDaniel *et al.*, 1987). Secondly, mice infected with PspA$^-$ pneumococci have been observed to have significantly decreased C3 concentrations in the serum within 30 minutes after intravenous injection, whereas no significant consumption of C3 was measured in the serum of mice infected with a PspA-positive strain (Tu *et al.*, 1999). In addition, both a PspA$^-$ mutant and pneumococci incubated with anti-PspA exhibit increased binding of C3 *in vitro* (Ren *et al.*, 2003; Ren *et al.*, 2004). These observations implicate PspA in preventing complement-mediated opsonisation. In the alternative complement pathway, C3b that has been deposited on the complement-activating surface is bound by factor B. The resulting complex is then cleaved by factor D, forming C3bBb (Volanakis, 1990). Tu *et al*. (1999) showed that, while a PspA$^-$ mutant was rapidly cleared from the blood of WT mice, it was able to cause bacteraemia in both C3$^-$ and factor B$^-$ knock-out mice. Therefore, they proposed that PspA functions by blocking formation of, or accelerating the dissociation of, C3bBb, although the mechanism by which this occurs is yet to be defined (Tu *et al.*, 1999).

1.6.2 Pneumolysin (Ply)

Virtually all clinical isolates of *S. pneumoniae* produce pneumolysin (Ply), a 53 kDa cytotoxin that plays an important role in the pathogenesis of pneumococcal disease (Paton *et al*., 1993). The toxin is able to interact with cholesterol in eukaryotic membranes, triggering its insertion into the lipid bilayer. Oligomerisation of Ply results in formation of transmembrane pores allowing influx of water, ions and some macromolecules, thereby causing cell lysis (Boulnois *et al.*, 1991). Ply is also thought to

induce nitric oxide production by macrophages, resulting in further cell damage (Braun *et al.*, 1999). Unlike related thiol-activated toxins produced by other bacteria, Ply lacks an N-terminal signal sequence required for secretion. In most cases, toxin release coincides with activation of autolysin (LytA), a cell-wall-degrading amidase present in the cell envelope of the pneumococcus (Berry *et al.*, 1989a). Autolysin activation occurs when cell wall biosynthesis ceases during such conditions as stationary growth phase or penicillin treatment. For some strains, Ply has been detected extracellularly during early log phase before significant cell lysis has occurred, suggesting that a LytA-independent release mechanism may also exist in some instances (Balachandran *et al.*, 2001). In most strains, however, Ply release parallels autolysis (Berry *et al.*, 1989a).

It is likely that uncontrolled inflammatory responses contribute to the severity of pneumococcal disease and the high associated mortality rate. This response is due, in part, to cell wall degradation products (Tuomanen *et al.*, 1987). The contribution of Ply to inflammation also appears to be significant, as the toxin is capable of inducing inflammation by several means. Firstly, the purified toxin is able to elicit TNFα, IL-1β, and IL-6 production by blood monocytes (Houldsworth *et al.*, 1994). The toxin has also been shown to stimulate the production of pro-inflammatory cytokines IL-8 and TNFα by neutrophils (Cockeran *et al.*, 2002). High Ply levels are associated with both increases in transcription and release of IL-8, the latter being possibly due to Ply-mediated alterations of the plasma membrane integrity (Cockeran *et al.*, 2002). Secondly, pneumolysin can activate the classical complement pathway (Paton *et al.*, 1984). This has been proposed to occur following the binding of C1, the first component of complement, to a Ply domain exhibiting similarity to the Fc portion of IgG (Mitchell *et al.*, 1991; Rossjohn *et al.*, 1998).

In vitro and *in vivo* models have suggested that Ply has roles in multiple stages of pneumococcal pathogenesis (Paton, 1996). Ply facilitates progression of disease by inhibiting ciliary beating in human respiratory epithelium, thus augmenting the retention of bacteria in the lungs where pneumococci can cause pneumonia (Boulnois *et al.,* 1991), and is capable of disrupting tight junctions between epithelial cells, which may provide an alternate pathway by which the pneumococcus infiltrates the bloodstream (Steinfort *et al.,* 1989). Ply-negative pneumococci have been shown to exhibit reduced proliferation in the lungs, and are compromised in their ability to injure the alveolar-capillary barrier compared to the wild-type (WT) (Rubins *et al.*, 1995). Mice injected IP with a pneumolysin negative mutant survive significantly longer than mice challenged with the WT (Berry *et al.,* 1989b; Berry *et al.,* 1992). Additionally, a significant increase in survival time was observed for mice immunised with purified pneumolysin protein prior to challenge with a virulent strain of *S. pneumoniae* (Paton *et al.,* 1983). Passive immunisation with naturally acquired human antibodies specific for Ply is also protective against IP challenge in mice, implicating the toxin strongly in pneumococcal virulence (Musher *et al.,* 2001). Interestingly, although higher levels of Ply are often associated with invasive disease, the haemolytic level of the Ply appears to be independent of the ability of the strain to cause disease. In some instances, the haemolytic level of Ply has been lower in invasive than in non-invasive isolates (Kirkham *et al.,* 2006; Lock *et al.,* 1996). This may indicate that pneumolysin has an additional function in sepsis.

The role of pneumolysin in pneumococcal carriage is less defined. Ply has been shown to be important for activation of toll-like receptor 4 (TLR-4)-dependent inflammatory responses. This is thought to be an important factor in maintaining a constant level of colonising bacteria in the nasopharynx whilst preventing the development

of invasive disease (Malley *et al.*, 2003). Kadioglu *et al.* (2002) found that a Ply mutation eliminated carriage in the mouse model after only four hours, implicating the toxin strongly in nasopharyngeal colonisation. However, studies by different groups indicate that Ply deficiency does not adversely affect colonisation either *in vitro* or *in vivo* (Ogunniyi *et al.*, 2007; Rayner *et al.*, 1995; Rubins *et al.*, 1998). Recently, the use of real-time bioluminescent imaging has indicated that Ply-negative mutants are attenuated in nasopharyngeal colonisation, translocation to the lungs, and survival in the bloodstream, supporting a role for Ply in carriage and demonstrating the importance of the toxin in multiple stages of pathogenesis (Orihuela *et al.*, 2004a).

1.6.3 Ion acquisition proteins

1.6.3.1 Pneumococcal surface antigen A (PsaA)

PsaA, the third gene in the *psa* operon, encodes a conserved 37 kDa lipoprotein belonging to the lipoprotein receptor-associated antigen family (Johnston *et al.*, 2004). Initially PsaA, and indeed many other members of this family, were considered putative adhesins for their respective pathogens. $PsaA^-$ pneumococci displayed deficient adherence to type II pneumocytes (Berry and Paton, 1996) and to Detroit-562 cells (Romero-Steiner *et al.*, 2003). Additionally, pneumococci pre-incubated with anti-PsaA antisera (absorbed with a PsaA- mutant) showed a decreased ability to bind to Detroit-562 cells (Romero-Steiner *et al.*, 2003). This suggested that PsaA contributes directly or indirectly to pneumococcal adherence. However, the operon was later found to encode components of an ATP-binding cassette Mn^{2+} transporter (Dintilhac *et al.*, 1997). Manganese acts as a cofactor for bacterial proteins involved in glycolysis, gluconeogenesis, sugar and amino acid metabolism, peptide cleavage, nucleic acid degradation, signal transduction, and

oxidative stress defence, and therefore contributes significantly to the survival and growth of many bacterial pathogens during infection (Jakubovics and Jenkinson, 2001). Pneumococci deficient in PsaA are unable to establish colonisation, cause lung disease, bacteraemia, or otitis media in *in vivo* models, which can be attributed to the very low levels of Mn^{2+} present in these niches (Marra *et al.*, 2002b; McAllister *et al.*, 2004). In addition, *psaA* mutants possess a defective oxidative stress response, which contributes to the attenuated virulence exhibited (Tseng *et al.*, 2002). Moreover, intranasal immunisation of mice with PsaA has been shown to protect against pneumococcal carriage in the nasopharynx (Briles *et al.*, 2000; Johnson *et al.*, 2002), which makes it a potential mucosal vaccine candidate.

1.6.3.2 PiaA

PiaA is the 42 kDa lipoprotein component of one of several pneumococcal iron acquisition systems. It has recently been identified as a vaccine candidate due to its immunogencity, ubiquitous presence in all typical pneumococcal strains and conservation between strains, based on RFLP analysis (Brown *et al.*, 2001b; Whalan *et al.*, 2006). Competitive studies have demonstrated that a $PiaA^-$ strain possesses attenuated virulence compared to the WT following co-infection in both a pulmonary and systemic model of infection, indicating that iron uptake via the PiaA ABC transporter is important for full virulence and lung infection (Brown *et al.*, 2001a). In addition, IP immunisation with PiaA protects against systemic challenge and IN immunisation has been shown to be protective against pneumonia (Brown *et al.*, 2001b; Jomaa *et al.*, 2006). The antibodies elicited appear to protect by promoting the clearance of pneumococci by opsonophagocytosis rather than by preventing iron uptake (Jomaa *et al.*, 2005).

1.6.4 Neuraminidase A (NanA)

Many bacterial respiratory pathogens possess at least one neuraminidase, an exoglycosidase that cleaves terminal sialic acid moieties from glycolipids, mucins, glycoproteins and oligosaccharides. *S. pneumoniae* possesses two defined neuraminidases (NanA and NanB) and a third putative neuraminidase (NanC), the most important of which is considered to be NanA. NanA is 108 kDa in size, and is attached to the cell wall by a LPXTG anchor at the C-terminus (Camara *et al.*, 1994). Pneumococci have been shown to form both transparent and opaque colony phenotypes, based on their appearance when viewed on transparent media. The transparent opacity phenotype is commonly associated with nasopharyngeal colonisation and invasion of the BMEC, while bacteria recovered from the blood of mice with sepsis form opaque colonies (discussed in Section 1.7.1). Transparent 6A and 6B pneumococci have been shown to possess a higher level of functional *nanA* mRNA than opaque variants by microarray analysis, suggesting a role for neuraminidase A in the nasopharynx where this phenotype predominates (King *et al.*, 2004) (Weiser *et al.*, 2001). NanA is capable of both desialating the cell surfaces of *Neisseria meningitidis* and *Haemophilus influenzae* and modifying host cell glycoconjugate receptors such as lactoferrin and IgA2 (King *et al.*, 2004; Shakhnovich *et al.*, 2002). This is thought to provide a competitive advantage for the pneumococcus by reducing carriage of other bacterial species that reside in the nasopharynx, whilst promoting adhesion to the epithelia. *In vivo*, a *nanA* mutant has been shown to exhibit attenuated persistence in a chinchilla colonisation model. The mutant was completely cleared from the nasopharynx 14 days post-infection, whereas the parent stain prevailed in this niche for 28 days before being cleared. The *nanA* mutant also showed a decreased ability to persist in the middle ear of chinchillas compared to the WT, although the effect of the NanA deficiency was not apparent until eight days following transbullar inoculation

(Tong *et al.*, 2000). NanA does not appear to play a role in sepsis, as a *nanA* knockout mutant is not attenuated in a murine IP challenge model (Berry and Paton, 2000).

1.6.5 SpxB

SpxB encodes a pyruvate oxidase that catalyses the reduction of pyruvate, free phosphate and oxygen to form carbon dioxide, acetylphosphate and hydrogen peroxide. The latter contributes to inflammation and cell damage during infection, and induces TLR-2 and TLR-4 independent apoptosis in brain endothelial cells (Bermpohl *et al.*, 2005). Hydrogen peroxide is also capable of inhibiting the growth of competing microflora, thereby promoting pneumococcal carriage (Spellerberg *et al.*, 1996). This has been demonstrated *in vitro*, with *Haemophilus influenzae*, *Neisseria meningitidis*, and *Moraxella catarrhalis* showing inhibited growth when co-cultured with pneumococci (Pericone *et al.*, 2000). In addition to catalysing H_2O_2 production, SpxB contributes towards pneumococcal resistance to hydrogen peroxide, with mutants lacking a functional SpxB exhibiting 10^2 to 10^3-fold greater killing in the presence of high levels of hydrogen peroxide than the WT (Pericone *et al.*, 2003). This allows pneumococci to grow in the presence of higher hydrogen peroxide concentrations than many other nasopharyngeal bacteria. Pyruvate oxidase has been shown to be required for pneumococcal growth *in vivo* (Spellerberg *et al.*, 1996), but not *in vitro*. $SpxB^-$ D39 possesses a reduced ability to colonise the nasopharynx of IN infected mice, and is also defective in establishing lung and blood infection (Orihuela *et al.*, 2004a).

1.7 Differential gene expression

With the exception of a few serotype 3 strains that are able to cause disease in horses, *S. pneumoniae* is a strict human pathogen (Timoney, 2004). Nevertheless, the pneumococcus is exposed to the varying environments of the nasopharynx, lung, bloodstream, brain and inner ear during human infection. Numerous gene expression profiles are likely to exist in order to enable the pneumococcus to survive and function while occupying different niches in the human host. It is likely these are regulated by a number of different environment cues, for example extracellular signals such as oxygen tension and pH, or host molecules such as immune factors and receptors.

1.7.1 Phase variation

Pneumococci undergo spontaneous opacity phase variation *in vitro* at a frequency of 10^{-3} to 10^{-6} per generation, giving rise to colonies that appear transparent or opaque when viewed under oblique, transmitted light after growth on transparent agar media (Weiser *et al.*, 1994) (see Figure 1.2). An additional 'intermediate' opacity phase was defined by Weiser *et al*. in 1994. For some strains, pneumococcal opacity phase variation has been shown to be independent of pH, growth media, temperature and supplemented CO_2 *in vitro*, with bacteria grown in these conditions showing similar opacity phase switching to a control (McKesser, 2003). *In vivo*, bacteria recovered from the blood of infected mice are predominantly of opaque variants and possess elevated levels of PspA protein when analysed by Western Immunoblot Analysis (Rosenow *et al.*, 1997; Weiser *et al.*, 1994). Furthermore, immunoelectron microscopy has shown opaque colonies to possess more capsular polysaccharide than transparent colonies, which is important in preventing phagocytosis by monocytes in the bloodstream and masking other

NOTE: This figure is included in the print copy of the thesis held in the University of Adelaide Library.

Figure 1.2 **Pneumococcal opacity phenotype**

Single colonies of *S. pneumoniae* strain 62 (type 18C) following growth on THY/catalase agar when viewed under oblique light. A) Shows the transparent colony morphology, and B) shows an opaque colony. Weiser (1994).

pneumococcal surface structures (Kim *et al.*, 1999). Pneumococci cultured from the blood of patients with IPD are also of the opaque phenotype (Weiser *et al.*, 2001). Conversely, the transparent phenotype is believed to express a higher amount of cell wall teichoic acid (Kim and Weiser, 1998). Elevated protein levels of CbpA and SpxB have also been reported for transparent pneumococci compared to the opaque phenotype, which may promote carriage and facilitate adherence to the nasopharyngeal epithelium (Kim and Weiser, 1998; Overweg *et al.*, 2000). In addition, a reduced content of CPS, when compared to its opaque counterpart, may promote the exposure of pneumococcal surface proteins involved in these processes. This hypothesis is supported by enhanced binding of the transparent phenotype (compared to opaque pneumococci) to nasopharyngeal epithelial, lung epithelial and brain microvascular endothelial cell lines, locations where adherence is essential for the pathogenicity of the pneumococcus (Cundell *et al.*, 1995b; Ring *et al.*, 1998; Weiser *et al.*, 1994). Mice challenged IN with transparent pneumococci develop nasopharyngeal colonisation, whereas opaque pneumococci appear to colonise the nasopharynx poorly (Weiser and Kapoor, 1999). Moreover, pneumococci recovered from human carriers are predominantly the transparent phenotype (Weiser *et al.*, 2001). Although opacity phase has been found to be associated with different niches *in vivo*, the exact mechanism of phase variation is not known.

1.7.2 *In vitro* gene regulation

Environmental conditions also affect pneumococcal gene expression at a transcriptional level. Using differential fluorescence induction (DFI) technology, artificially induced differences in environmental conditions (specifically low iron, high osmolarity, carbon dioxide, blood agar, and temperature shift) have been shown to influence the activity of a number of pneumococcal promoters *in vitro* (Marra *et al.*,

2002a). DFI involves the creation a promoter trap library of DNA fragments cloned upstream of a promoterless *gfp* gene (encoding green fluorescent protein). The level of promoter activity relates directly to the fluorescence under different environment conditions, which is then used to sort the cells by fluorescence-activated-cell sorter (FACS). Interestingly, this method was not reported to identify changes in promoter activity for key pneumococcal virulence factors such as pneumolysin, the choline binding proteins and genes of the capsule locus. This may have been indicative of constitutive or growth-stage-specific expression. Alternatively, DFI may not have been sensitive enough to pick up the changes in promoter induction, or perhaps regulation of these virulence factors does not occur at a transcriptional level. It is also possible that the environment conditions tested are not responsible for changes in transcription for those genes, which is an unavoidable limitation of such *in vitro* experimentation.

Pneumococcal opacity phase has previously been demonstrated to correlate with either epithelial and endothelial cell contact, or disease. King et al. (2004) examined differences in gene expression between 6A and 6B opacity variants using microarray analysis, using opaque and transparent clinical isolates cultured *in vitro*. They observed that the expression of several genes, including *blpN*, *blpY*, and an operon encoding a sugar ABC transporter, appeared up-regulated in transparent 6A and 6B strains compared to their opaque counterparts. The expression of AliA and a fructose-6-phosphate aminotransferase was higher in the opaque phenotype. *nanA* mRNA was found to be 3.61 times higher in the 6A transparent bacteria, and 2.15 times higher in 6B transparent bacteria, than the respective opaque strains. However, like the findings of Marra *et al.* (2002a), no differences in gene expression were observed between opaque and transparent cultures for many of the key virulence genes previously identified in the literature, despite the

association of the distinct phenotypes with invasive disease or carriage *in vivo* (Cundell *et al.*, 1995b; King *et al.*, 2004; Ring *et al.*, 1998; Weiser *et al.*, 1994; Weiser and Kapoor, 1999). This may suggest that the regulation of virulence proteins is not at the transcriptional level, or perhaps that other factors present in a human host, but not in the *in vitro* conditions used in these two studies, are responsible for triggering the regulatory networks.

1.7.3 *In vivo* gene regulation

Although neither Marra *et al.* (2002a) nor King *et al.* (2004) observed differential expression for many key virulence genes in pneumococci that were subjected to different *in vitro* conditions, or between the opacity phenotypes *in vitro*, a study by Ogunniyi *et al.* (2002) suggested that these genes are indeed regulated *in vivo*. This was indicated by changes in mRNA levels in bacteria harvested from the bloodstream of mice following IP infection, when compared to mRNA isolated from bacteria in the original challenge culture. mRNA levels of *pspA*, *ply*, *psaA* and *cps2A* were shown to be higher in the bloodstream of infected mice at both 12 and 24 hr post-challenge. At both time points, the relative levels of *cbpA* transcript were similar to the *in vitro* control, perhaps not surprisingly considering the primary role of CbpA as a nasopharyngeal adhesin. In contrast, when Marra *et al.*, (2002a) performed *in vivo* screenings of a bank of strains expressing GFP under the control of specific pneumococcal promoters, those controlling the expression of major virulence genes such as *lytA*, *ply* and the genes in the *cps* locus were not found to be induced. Strains were examined in three *in vivo* models; a mouse lung infection model, a gerbil otitis media model, and an IP chamber implant model, which involved the surgical insertion of a chamber containing bacteria into the peritoneal cavity of CD-1 mice. Hence, at the commencement of this thesis work, the transcriptional

behaviour of *S. pneumoniae* virulence genes *in vivo* was poorly understood.

In 2004, Orihuela *et al*. published a microarray analysis study comparing pneumococcal gene expression in bacteria harvested from various *in vivo* infection models and cell lines, to a baseline control. The study identified genes that were up- or down-regulated in the bloodstream of BALB/cJ mice following IN challenge with a D39 derivative, or in the brains of New Zealand white rabbits 4 hr after intracisternal infection with a TIGR4 derivative, compared to growth in C+Y media. To simulate nasopharyngeal colonisation an un-encapsulated derivative of TIGR4 was co-cultured with Detroit-562 epithelial cells, and the RNA extracted from the harvested bacteria was compared to that extracted from pneumococci grown in tissue culture medium. In contrast to the *in vitro* studies reviewed in Section 1.7.2, several previously identified virulence factors were found to exhibit niche-dependent expression, including several CBPs; *cbpA* and *cbpJ* expression were up-regulated 2.3- and 3.6-fold, respectively, following incubation with Detroit-562 nasopharyngeal cells, whereas *cbpG* and *cbpF* were induced in the blood of IN infected mice. *lytA* expression was down-regulated in the CSF. In addition, pneumococci repressed *ply* expression in the CSF and when co-cultured with Detroit-562 nasopharyngeal cells. However, the methods used in this study have several limitations, most importantly the need to use different animal models, different infection routes, and different pneumococcal strains to examine disease in each niche. These concerns were also raised by the authors, and are discussed further in Chapter 5.

1.8 Aims of this work

The pneumococcus is capable of causing a number of diseases in the human host which are responsible for significant morbidity, mortality and economic burden worldwide. At the time of commencing this thesis work, the relative expression *in vivo* of specific pneumococcal factors considered to be important in disease pathogenesis was poorly understood. This was largely due to the inability to harvest sufficient RNA from animal hosts to perform quantitative mRNA assays such as real-time RT-PCR. As a consequence, much of the information regarding the role of key pneumococcal virulence factors was gleaned from mutagenesis studies and *in vitro* assays. There was little known about the regulation of the genes encoding these virulence factors *in vivo*.

The aim of this work was to examine differential virulence gene expression in *S. pneumoniae* in an *in vivo* system. Specifically,

1. To develop a suitable *in vivo* mouse model for the harvesting of pneumococci for RNA analysis, and to develop and optimise downstream procedures allowing recovery of RNA of sufficient quality and quantity to perform RNA assays.
2. To examine the expression of key pneumococcal virulence factors in different *in vivo* niches using real-time reverse-transcription PCR analysis.
3. To examine the pneumococcal gene expression in different *in vivo* niches using global cDNA microarray analysis.

CHAPTER 2 - Materials and methods

2.1 General reagents

Most chemicals used were AnalaR grade and were purchased from Ajax Chemicals (NSW, Australia), unless otherwise stated. Antibiotics were purchased from Roche Diagnostics, except gentamicin, which was purchased from Sigma-Aldrich. Sodium dodecyl sulphate was purchased from Sigma-Aldrich (St. Louis, MD., USA). Trypsin was obtained from Trace Biosciences (Sydney, Australia).

2.2 Bacterial strains and growth media

2.2.1 Pneumococcal growth and storage

The pneumococcal strains used in this study are shown in Table 2.1. *S. pneumoniae* were routinely grown in Todd Hewitt broth with yeast (THY) (36.4 g/l Todd Hewitt [Oxoid, England], 10 g/l yeast extract), or on blood agar (BA) plates (39 g/l Columbia base agar [Oxoid]), 5% [v/v] defibrinated horse blood).

For storage, pneumococci were grown in serum broth (10% [v/v] donor horse serum in nutrient broth [10 g/l peptone (Oxoid), 10 g/l Lab Lemco powder (Oxoid) and 5

Strain	Capsular Serotype	Source	Reference
D39	2	Laboratory strain	Avery, 1916
TIGR4	4	T. Mitchell, University of Glasgow	Bricker & Camilli, 1999
WCH43	4	WCH, Adelaide	Clinical isolate
WCH16	6A	WCH, Adelaide	Clinical isolate
D39(LUX)	2	Paton Laboratory, Adelaide	This study
WCH16(LUX)	6A	Paton Laboratory, Adelaide	This study

***Table 2.1*: Pneumococcal strains used in this study**

g/l NaCl]) then stored at –80°C. Alternatively, pneumococci were grown in THY broth, centrifuged upon reaching mid-log phase, and resuspended in THY broth with 30% glycerol prior to storage at –80°C.

2.2.2 Opacity phase determination

For the assessment of opacity phase phenotype, pneumococci were plated on THY with 1.5% agar containing 315 U/ml catalase (Roche Diagnostics, Germany), and incubated at 37°C for approximately 36 hr. Colony morphologies were determined using oblique, transmitted light as described by Weiser *et al.* (1994).

2.2.3 Optochin sensitivity

To confirm bacteria were *S. pneumoniae*, strains were tested for optochin sensitivity by plating on blood agar in the presence of an optochin disc (Oxoid), at 37°C in an atmosphere of 95% air and 5% CO_2. Pneumococci are inherently sensitive to optochin, whereas other Streptococcal spp. are resistant.

2.2.4 Quellung reaction

Production of a specific capsular serotype was confirmed by the Quellung reaction using type-specific pneumococcal antisera (obtained from Statens Seruminstitut, Copenhagen, Denmark). Strains, taken from overnight blood agar cultures, were grown in serum broth (in the presence of the appropriate antibiotic(s)) for 3 hr at 37°C. 5 μl of serum broth culture was then mixed with 4 μl of antiserum and examined microscopically for specific capsular swelling.

2.2.5 Pneumococcal passaging

Pneumococci were grown in serum broth until the culture reached an OD_{600} of approximately 0.8. An aliquot of culture was then centrifuged at 4000 × *g* for 10 min at 4°C. The pellet was resuspended in serum broth and administered IP to CD-1 mice. 24 hr post-challenge, approximately 50 μl of blood was recovered from the infected mice following sacrifice, plated on BA plates and incubated overnight at 37°C in an atmosphere of 95% air and 5% CO_2. THY stocks were prepared from the passaged bacteria, as described in Section 2.2.1.

2.2.6 Growth curve determination

S. pneumoniae strains were cultured in THY following overnight growth on BA. Initial absorbance at 600nm (A_{600}) was measured at the commencement of growth, and strains were subsequently incubated at 37°C for 6-8 hr during which A_{600} readings were taken at predetermined time points. At each of these time points, a 50 μl aliquot of culture was serially diluted in 450 μl sterile phosphate buffered saline (PBS) then plated on BA and incubated overnight at 37°C, to determine the number of CFU (colony forming units).

2.3 Oligonucleotides

The oligonucleotides used in this work were purchased from Sigma-Aldrich and are listed in Table 2.2. LUX fluorogenic primers, labelled with JOE, were obtained from Invitrogen (California, USA) and are listed in Table 2.2.

Name	Sequence 5`→ 3`	Strain (Accession No.)
16S F (JOE)	CAACATCACCGCATAAGAGTGGATG(JOE)TG	R6 (gi: 15459504)
16S R	CAACGCAGGTCCATCTGGTA	R6 (gi: 15459504)
16S R2	AACGCAGGTCCATCTGGTAGTG	R6 (gi: 15459504)
cbpA F (JOE)	GATCGTTTGGTTGGGTAGTTACGACGA(JOE)C	D39 (gi: 6469846)
cbpA R	GCAGAAGCTAAGAAGAAGGTTGAA	D39 (gi: 6469846)
cbpA 6B F	TGCTAAGTTGAAGGAAGCTGTTG	BG9163 (gi: 6469854)
cbpA 6B R (JOE)	CGAGGTAGCTCTCCAGAAACTCCT(JOE)G	BG9163 (gi: 6469854)
pspA F (JOE)	CTAGCGAACTGAGGAGAAAGCCGCGC(JOE)AG	R6 (gi:15902044)
pspA R	GGCTAGATACGCTTGTTGAACTGC	R6 (gi:15902044)
pspA 6B F	TTCCGTGCTCCTCTTCAATCTG	DBL6A (gi: 6752380)
pspA 6B R (JOE)	CGGTGTATTGTCCAGCTTGCTCAC(JOE)G	DBL6A (gi: 6752380)
ply F (JOE)	CAACGAAGGATAGAGGCGACTGTCG(JOE)TG	R6 (gi: 15459416)
ply R	GAAAGAAAGAAGCGGAGCTTG	R6 (gi: 15459416)
psaA F	GAACCACTTCCTGAAGACGTTAAG	R6 (gi:15902044)
psaA R (JOE)	GAAACACAAFCATTGCCACCTGT(JOE)TC	R6 (gi:15902044)
cps2A F (JOE)	CAACTCGCGGGCATTTATGGAG(JOE)TG	R6 (gi: 15457864)
cps2A R	GAAGTGAAGTTCAATCGCACATAG	R6 (gi: 15457864)
piaA F (JOE)	CAGCACAAGCAAATTACGGTGTAAGTGC(JOE)G	R6 (gi: 15458539)
piaA R	TCGTCAAATAGGTTAGCTTTACCA	R6 (gi: 15458539)
nanA F (JOE)	GACGTTGAGCAGTGGTATTTGGAACG(JOE)C	R36A (gi: 587550)
nanA R	CCGAAAGTTGAGTTTCATTTGC	R36A (gi: 587550)
spxB F (JOE)	CACCGGCGACTCACTTGATTAACGG(JOE)G	D39 (gi: 32744844)
spxB R	TTAACTGGACGTGATCCAAGG	D39 (gi: 32744844)

***Table 2.2*: Oligonucleotides used in this study**

The oligonucleotides used in this study are listed in this table. For LUX fluorogenic primers, the JOE label is indicated by (JOE).

2.4 *In vivo* pathogenesis model

2.4.1 Mice

Animal experimentation was conducted in accordance to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th edition). CD-1 outbred female mice, obtained from the Institute of Medical and Veterinary Science (Adelaide, SA, Australia) were used in all experiments. Mice were aged between 5 and 6 weeks at the commencement of experiments, and weighed between 20 g and 24 g with an average weight of 22 g. The animals were known to be free of common mouse pathogens when obtained, and were maintained under contamination barrier conditions during experimentation.

2.4.2 Growth of challenge bacteria

THY broth supplemented with 5% yeast extract was inoculated with *S. pneumoniae* taken from overnight blood agar plates, and grown at 37°C to A_{600} = 0.25 (equivalent to mid logarithmic phase). For challenge of mice, 5 ml of culture was centrifuged at 2200 × *g* for 15 min, and supernatant was carefully decanted. The bacterial pellet was resuspended in 2 ml of sterile PBS, and kept on ice prior to intranasal challenge. The challenge dose administered was determined retrospectively by plating appropriate dilutions of the inoculum on blood agar plates immediately after infection of mice.

2.4.3 IN challenge of mice

Previous studies by Wu *et al.* (1997) indicate that there is no detectable transmission of pneumococci from one mouse to another when animals are co-housed. Therefore, mice were separated into cages of 10-15 animals and maintained in a specific

pathogen-free environment for the duration of the experiment. Prior to challenge, mice were anaesthetised by intraperitoneal injection with Nembutal (pentobarbitone sodium, Rhone-Merieux) at a dose of 66 μg per 1 g of body-weight. 25 μl of the desired pneumococcal strain was pipetted into the nares and involuntarily inhaled. Mice were monitored closely until they had regained consciousness, which took approximately one hour. The number of bacteria in the challenge inoculum was determined retrospectively by growth on BA at 37°C in 95% air, 5% CO_2 O/N.

2.4.4 Euthanasia of mice

The following procedure was adapted from the method originally described by Wu *et al*. (1997). Animals were euthanased by asphyxiation with carbon dioxide at 24, 48 and 72 hr following challenge. The trachea of each mouse was cut below the larynx, and the loosely adherent bacteria in the nasopharynx were collected by washing with 1 ml trypsin buffer (0.5% trypsin, 0.02% ethylenediaminetetracetic acid [EDTA], in PBS, filter sterilised) by insertion of a 26-gauge needle sheathed in tubing into the exposed tracheal end of the upper respiratory tract. Trypsin buffer was allowed to drip into the nasopharynx slowly and was collected from the nose, with each wash taking approximately 40 sec. The left atrium of the heart was cut, and a small sample of blood removed for determination of bacteraemia level. Mice were perfused with a small amount of cold PBS in order to collect 2 ml blood for RNA extraction. Mice were subsequently perfused with sterile PBS until the eluent was clear, to remove blood-borne bacteria from the lungs. Lungs were removed and homogenized on ice in 2 ml sterile PBS using a tissue homogeniser (Cat X120, Germany), which was rinsed in PBS, decontaminated in 70% (v/v) ethanol, and rinsed again in PBS between samples. To separate pneumococci from host cells, lung homogenates and blood samples were centrifuged twice at 855 × *g* for 6 min at 4 °C, an

adaptation of a method described previously (Ogunniyi *et al.*, 2002). For the 6A strain, whole brains were removed from perfused mice through an incision in the skull, and placed in 2 ml sterile PBS. The organ was then homogenised and centrifuged at 850 × *g* for 6 min at 4°C, as for lung and blood samples. Nasopharyngeal washes and lung, blood and brain supernatants were subsequently centrifuged at 15,500 × *g* for 2 min at 4°C, the supernatant decanted, and the bacterial pellet stored at –80°C until further processing.

2.4.5 Enumeration of recovered bacteria

Prior to pelleting harvested bacteria, 40 µl was removed from the nasal washes and the supernatant of the lung, blood and brain samples centrifuged at 850 × *g*. The aliquot was serially diluted in sterile PBS and plated onto blood agar in duplicate in order to enumerate pneumococci present in the sample, and to determine the presence, if any, of contaminating microflora. Blood plates were incubated at 37°C in 95% air, 5% CO_2 O/N. Colonies selected randomly from a subset of samples were plated and tested for Optochin sensitivity, as described in Section 2.2.3.

2.4.6 Assaying mice infected with luminescent pneumococci

Mice were anaethetised by IP injection with Nembutal (pentobarbitone sodium, Rhone-Merieux) at a dose of 66 µg per 1 g of body-weight. After anaesthesia was established, mice were placed in an imaging chamber without restraint and bioluminescence was visualised using an IVIS 100 Imaging System (Xenogen Corporation, Alameda, CA.) with a 500-nm filter to target the luciferase (γ_{max}, 490 nm). Animals were scanned both ventrally and dorsally for between 2 min and 7 min during which time the imaging chamber was maintained at 30°C. The bioluminescent images were superimposed over grayscale reference photographs taken prior to bioluminescence

acquisition. Total photon emission from selected and defined areas within the images of each mouse was quantified using the LivingImage software package (Xenogen Corporation).

2.5 RNA isolation and modifications

2.5.1 Acid-phenol RNA extraction

RNA was isolated from bacterial pellets with acid-phenol:chloroform:isoamyl alcohol (125:24:1; pH 4.5, Ambion, Austin, TX., USA) essentially as described previously (Ogunniyi *et al.*, 2002). Cells were pelleted by centrifugation at 15,500 × *g*. The supernatant was removed, and the cells resuspended in 400 µl acid-phenol:chloroform:isoamyl alcohol, pre-warmed to 65°C. Samples were incubated at 65°C for 5 min, after which 400 µl pre-warmed NAES solution (50 mM sodium acetate, 10 mM EDTA, 1% [w/v] SDS, pH 5.1 treated with diethyl pyrocarbonate [DEPC]) was added to the lysate and mixed by inversion before being incubated at 65°C for a further 5 min. The lysate was then placed on ice for 2 min and centrifuged at 15,500 × *g* for 2 min at 4°C. The aqueous phase was removed and placed in a new RNase-free tube, and the phenol extraction was repeated. Following the phenol extractions, 30 µl of 0.05% (w/v) DEPC-treated 3 M sodium acetate and 750 µl 100% ethanol was added to the aqueous phase, and mixed by inversion. The extract was then precipitated at –80°C overnight in the presence of 40 ng/µl glycogen (Sigma). Following precipitation, nucleic acids were pelleted by centrifugation at 15,500 × *g* for 30 min at 4°C. The pellet was washed with 1 ml 70% ethanol (0.7 vol 100% ethanol and 0.3 vol DEPC-treated water) and centrifuged for 5 min at 15,500 × *g*. The supernatant was aspirated by pipetting, and residual ethanol was

removed by air-drying for 15 min. The nucleic acids were suspended in 18 µl RNase-free, DNase-free water (Roche). Subsequently, the preparation was treated with 10 units of RNase-free DNase (Roche) at 37°C for 30 min in the presence of 1 U/µl recombinant RNasin ribonuclease inhibitor (Promega Life Sciences, WI., USA), after which RQ1 DNase stop buffer (Promega) was added to inactivate the DNase. The purity of the RNA preparation was confirmed by one-step reverse transcription polymerase chain reaction (RT-PCR, Section 2.6.1) with or without reverse transcriptase, using 16S rRNA-specific primers (Table 2.2), and the products were visualised after electrophoresis on a 2% TBE-agarose gel (see Section 2.6.4).

2.5.2 Enrichment for prokaryotic RNA

RNA samples from a specific niche were pooled from 4 to 5 mice, based on the number of CFU recovered and also on the absence or minimal presence of contaminating bacteria and RNA was purified further using a Qiagen RNeasy™ minikit. RNA obtained from lung homogenates was enriched for prokaryotic RNA using the MICROB*Enrich*™ kit (Ambion), in accordance to the manufacturer's instructions. The amount of RNA recovered following purification/enrichment was determined by $A_{260/280}$ measurements.

2.5.3 Linear amplification of RNA

RNA samples were purified by using Qiagen RNeasy™ clean-up columns, according to the manufacturer's instructions. RNA was then amplified using a SenseAmp Plus™ RNA Amplification Kit (Genisphere, Hatfield, PA., USA), in accordance to the manufacturer's instructions for Amplification of Prokaryotic Samples, or Amplification of Degraded Samples (as required). A second round of amplification was performed following primary amplification, in accordance to Genisphere's instructions for Second

Round Amplification. 12.5 µl of tailed RNA was transcribed using either vials 13, 14 and 15 included in the SenseAmp Plus™ kit, or using a MEGAscript High Yield Transcription Kit™ (Ambion). For tailed RNA transcribed using MEGAscript, 2 µl each of ATP, CTP, GTP and UTP solutions, 2 µl reaction buffer, and 2 µl enzyme mix were added to the template RNA, as suggested by Genisphere, and reactions were incubated at 37°C overnight. The remaining 12.5 µl tailed RNA was stored at –70°C for later transcription. The yield of RNA following linear amplification was determined by $A_{260/280}$ measurements. Amplified RNA was stored at –70°C until use.

2.6 RNA analysis

2.6.1 Reverse transcription polymerase chain reaction (RT-PCR)

Oligonucleotide primers used for RT-PCR analysis were obtained from Sigma-Aldrich, and listed in Table 2.2. To test for contamination of RNA samples with DNA, RT-PCR was carried out using the Access RT-PCR System (Promega) in accordance with the manufacturer's instructions. A standard 12 µl RT-PCR reaction mix contained DEPC-treated water, 0.2 mM of each dNTP, 1 µM of each primer, 1 mM $MgSO_4$, AMV/*Tfl* reaction buffer, 0.1 U/µl AMV Reverse Transcriptase, 0.1 U/µl *Tfl* DNA Polymerase, 0.25 µl RQ1 DNase stop solution and 0.3 µl RNA extract. A control reaction mix was performed in tandem, for which RNase-free DNA-free water was substituted for Reverse Transcriptase.

RT-PCR amplification was performed using a Hybaid Touchdown Thermocycler (Hybaid, Middlesex, England). The standard amplification conditions consisted of one

reverse transcription cycle at 48°C for 45 min. The subsequent PCR step consisted of 30 cycles, each consisting of 30 sec of denaturation at 95°C followed by 45 sec of annealing at the optimal temperature and 1 min of elongation at 68°C.

2.6.2 Real-time reverse transcription polymerase chain reaction (Real-time RT-PCR)

2.6.2.1 Real-time RT-PCR using LUX primers

Real-time RT-PCR was performed using LUX fluorogenic primers obtained from Invitrogen, listed in Table 2.2. Real-time RT-PCR was carried out using SuperScript™ III One-Step RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen) in accordance with the manufacturer's instructions. A standard 20 µl reaction contained 0.3 µl Superscript III RT/Platinum *Taq* Mix, 10 µl reaction mix, 1 µM of each primer, and 4 µl RNA extract. Amplification was performed using a Corbett Research Model RG-2000 Real-time Thermal Cycler. The standard amplification conditions consisted of one reverse transcription cycle at 48°C for 45 min. The subsequent PCR step comprised of 40 cycles, each consisting of 15 sec of denaturation at 95°C, followed by 30 sec of annealing at the optimal temperature and 30 sec of elongation at 72°C. The reaction was acquired at both 60°C and 72°C.

2.6.2.2 Real-time RT-PCR analysis

Relative mRNA concentration was calculated using the comparative cycle threshold ($2^{\Delta\Delta Ct}$) method (Livak and Schmittgen, 2001), in which the amount of target mRNA in one niche is compared to the amount of mRNA in another, relative to an internal control (16S rRNA). Results are expressed as relative changes in target gene mRNA levels

in one niche compared another. Standard deviations (SD) were initially determined as the $\sqrt{((\mathrm{SD\,sample})^2 + (\mathrm{SD\,16S})^2)}$, and this was then applied to the formulas: $SD+ = 2^{\Delta\Delta Ct-SD} - 2^{\Delta\Delta Ct}$ and $SD- = 2^{\Delta\Delta Ct} - 2^{\Delta\Delta Ct+SD}$.

2.6.3 Microarray analysis

Total bacterial RNA was labelled and hybridised using the Genisphere Array 900 MPX Kit (Genisphere), according to the manufacturer's instructions. 3 µg of each RNA preparation was used per microarray slide. cDNA synthesis steps specified in the Genisphere Array 900 MPX method were performed using SuperScript III™ (Invitrogen), according to Genisphere's instructions. Microarray slides (Bacterial Microarray Group, St Georges's Hospital, University of London, UK) consisted of PCR products for each of the defined open reading frames of both *S. pneumoniae* TIGR4 and R6, spotted in duplicate. Prior to use, arrays were incubated in filtered pre-hybridisation solution (1% [w/v] BSA, 17.5% [v/v] SCC, 0.1% [v/v] SDS in sterile water) for 30 min at 37°C. Arrays were then washed twice in sterile water to remove all pre-hybridisation solution, washed once in isopropanol, and quickly dried using nitrogen. Subsequent hybridisation of the labelled RNA to the microarray slide was carried out at 65°C. The array was read using an Axon 4000B Dual-wavelength Scanner (Molecular Devices Sunnyvale, CA., USA), and images were acquired using GenePix Pro 4.0 (Axon Instruments, Inc.). Array readings were normalised by adjusting the PMT 532 nm and 635 nm intensities until 16S rRNA ratios were approximately 1: 1. Gene lists were prepared based upon filtration algorithms and signal-to-noise ratios by GenePix Pro 4.0, with a 4- fold cut-off for each spot and with *P* value < 0.01. The *P* value was calculated from Student *t*-tests between the intensities of individual spots. This analysis is described further in Section 5.2.2.

2.6.4 Agarose gel electrophoresis

DNA was electrophoresed through horizontal agarose gels (0.8-2% [w/v] agarose dissolved in TBE buffer [44.5 mM Tris, 44.5 mM boric acid, 1.25 mM EDTA, pH 8.4]) immersed in TBE buffer containing ethidium bromide (100 μg in 2.5 l) at 140-180 V. Prior to loading DNA samples, a one tenth volume of tracker dye was added (15% [w/v] Ficoll, 0.1% [w/v] bromophenol blue, 100 ng/ml RNase A). DNA bands were visualised by short wave UV transillumination and images were captured by a Tracktel video imaging system attached to a Mitsubishi thermal printer. Approximate sizes of visualised fragments were calculated by comparison of their mobility with that of DNA size markers derived from HpaII-digested pUC19 DNA (fragment sizes: 501, 404, 331, 242, 190, 147, 111, 110, 67, 34, 26 base pairs).

2.7 Bacterial transformation

2.7.1 Preparation of competent cells

E. coli cells were grown at 37°C to an absorbance at 600nm (A_{600}) of 0.4, then centrifuged for 10 min at 2880 × *g* at 4°C. The pellet was then resuspended in cold 0.1 M $MgCl_2$, and placed on ice for 10 min before pelleting at 2880 × *g* for 10 min at 4°C. The pellet was then resuspended in a one fifth volume of cold 0.1 M $CaCl_2$, and placed on ice for 90 min. Competent cells were stored at -80°C in 100 μl aliquots containing 20% glycerol.

Pneumococcal cells were grown in c-CAT medium (10 g/l Bacto Casamino acids

[Difco, MD, USA], 5 g/l Bacto Tryptone, 5 g/l NaCl, 10 g/l Bacto yeast extract, 4% [v/v] 0.4 M K_2HPO_4, 0.002% [w/v] glucose, 150 mg/l glutamine) to an A_{600} of 0.25-0.3, then diluted to an A_{600} of 0.01 in 10 ml CTM medium (0.2% [w/v] BSA, 1% [v/v] 0.1 M $CaCl_2$) and grown to an A_{600} of 0.1. The cells were then pelleted at 10,000 × *g* for 10 min, and resuspended in 812 µl CTM-pH 7.8 and 188 µl 80% glycerol, and stored at –80°C in 100 µl aliquots.

2.7.2 Transformation of *E. coli* and *S. pneumoniae*

Transformation of *E. coli* involved adding ~1 µg DNA directly to competent DH5α, and leaving on ice for 15 min. Cells were then heat-shocked at 42°C for 2 min, and placed on ice for a further 15 min. 1 ml LB was then added and after shaking for 45 min at 37°C, the culture was pelleted, resuspended in 200 µl LB, and plated on LB agar plates with the appropriate antibiotic selection.

Transformation of *S. pneumoniae* involved 500 µl of CTM-pH 7.8 and 2.5 µl of 10 µg/ml competence stimulating peptide-1 (CSP-1) (amino acid sequence: MRLSKFFRDFILQRKK [Chirontech (Victoria, Australia)]) or competence stimulating peptide-2 (CSP-2) (amino acid sequence EMRISRIILDFLFLRKK [Mimotopes (Victoria, Australia) (Havarstein *et al.*, 1995)]) were added to an aliquot of competent cells. The cells were then incubated at 37°C for 10 min before addition of approximately 1 ng of DNA, and then incubated at 32°C for 30 mins, followed by 37°C for 2-4 hr. After incubation, cells were plated onto BA plates containing 0.2 µg/ml Ery, and incubated for approximately 16 hr at 37°C in 95% air/ 5% CO_2.

2.8 Protein analysis

2.8.1 Preparation of whole cell lysates (WCLs)

Pneumococci were cultured in THYuntil they reached early to mid-log growth, as determined by A_{600} readings (A_{600} = 0.25). Cells were centrifuged at 2500 × *g* for 15 min. The pellet was resuspended in one tenth of the original culture volume of PBS, thereby concentrating the culture 10-fold. Pneumococcal cells were then lysed by incubating the cultures at 37°C for 10 min in the presence of 0.1% sodium deoxycholate (DOC) solution. The lysate was centrifuged at 15,500 × *g* for 2 min and the supernatant was removed and stored at -20°C until use.

2.8.2 SDS-PAGE

Pellets of 10 ml bacterial cultures were resuspended in 100 µl of LUG (5% [v/v] β-mercaptoethanol, 62.5 mM Tris, 2% [w/v] SDS, 10% [v/v] glycerol, 0.05% [w/v] bromophenol blue). Samples were boiled for 5 min prior to analysis, and stored at -20°C until use.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using the method described by Laemmli, (1970). Proteins were separated by electrophoresis at 170 V through a stacking gel (6% acrylamide) and a separating gel (12 to 20% acrylamide). Proteins were stained by gentle agitation for 1 h at 65°C in 0.06% (w/v) Coomasie Brilliant Blue R250, dissolved in 10% (v/v) glacial acetic acid and 25% (v/v) methanol. Destaining was performed by washing with several changes of 10% (v/v) acetic acid and 10% (v/v) isopropanol.

2.8.3 Western blot analysis

Proteins were separated by SDS-PAGE (as described in Section 2.8.2) and electroblotted onto nitrocellulose (Pall Life Sciences, MI., USA) at 300 mA for 1 hr as described by Towbin *et al*. (1979). After transfer, the membrane was blocked in 5% (w/v) skim milk powder (Diploma) in TTBS (20 mM Tris-HCl, 154 mM NaCl, 0.5% (v/v) Tween-20, pH 7.4) for 20 min, with gentle agitation. The blocking solution was removed and the membrane probed with the appropriate polyclonal antiserum at a dilution of 1/3000 in TTBS overnight at room temperature with gentle agitation. The filters were washed with TTBS 3 times for 10 min, before the addition of blotting grade goat anti-mouse IgG-Alkaline Phosphate conjugate (BioRad Laboratories, Hercules, CA., USA) at a dilution of 1/15,000 in TTBS, and incubated for 1 h at room temperature with gentle agitation. The membrane was washed 4 times for 5 min each with TTBS, and then equilibrated in 15 ml DIG 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 2 min before addition of 45 µl DIG 4 (75 mg/ml nitroblue tetrazolium salt in 70% dimethylformamide), and 35 µl DIG 5 (50 mg/ml 5-bromo-4-chloro-3-indolylphosphate toluidinium salt in dimethylformamide). After the desired colour reaction had taken place, the reaction was stopped by the addition of TE (10 mM Tris, 1 mM EDTA, pH 8.0). The membrane was then rinsed in water and dried.

2.8.4 Haemolysis assay

3% human red blood cell (hRBC) suspension was prepared by centrifuging 2 ml hRBC for 1 min in a microfuge, and washing the pellet twice with sterile PBS. Following washing, the solution was centrifuged at 15,500 × *g* for 2 min, and the RBC pellet was resuspended in 34 ml PBS and 50 µl β-mercaptoethanol.

To determine pneumolysin activity of pneumococcal cultures, haemolysis assays were performed as follows. 100 µl PBS was pipetted into each well of a 96-well round-bottomed microtiter plate (Sarstedt, South Australia), and serial 2-fold dilutions of pneumococcal lysates were performed in duplicate. 100 µl was removed and discarded from the last well of each row. Lysate was not added to four rows, which were used as controls. In one row, an extra 100 µl PBS was added to determine the blank absorbance value. In a further two rows, 100 µl 3% hRBC solution was added to determine the level of lysis in the absence of pneumolysin, and 100 µl 3% hRBC solution with purified Ply added was used as a positive control. 100 µl 3% hRBC solution was pipetted into the wells containing lysate, and the plate was then incubated at 37°C for 30 min. Following incubation, the microtiter plate was centrifuged at 850 × *g* for 10 min. 100 µl of supernatant from each well was transferred to a new flat-bottomed microtiter plate, and the absorbance at 540 nm was read using a Spectramax M2 spectrometer (Molecular Devices). The results were graphed as average absorbance against dilution. The haemolytic activity was determined as the reciprocal of the dilution at which 50% of the RBCs had lysed.

CHAPTER 3 – Optimisation of a mouse model of invasive pneumococcal disease and methods for extraction of RNA from *in vivo*-derived pneumococci

3.1 Introduction

To date, there have been limited *in vivo* studies reported in the literature that examine the expression of pneumococcal virulence genes during pathogenesis. This can be attributed to restrictions of *in vivo* models of infection, including limited bacterial recovery from specific niches such as the nasopharynx, difficulty in obtaining samples without substantial contamination by host cells and resident microflora, and sensitivity of quantitative mRNA assays available. As a result, knowledge of how key pneumococcal virulence factors are expressed and regulated *in vivo* has been mainly extrapolated from mutagenesis studies and *in vitro* assays. The work in this chapter aimed to develop an appropriate and working pneumococcal IN infection model and downstream RNA extraction/amplification protocols that would yield RNA in sufficient amount and purity for quantitative assays to be performed.

3.2 Development of a CD-1 mouse model for RNA recovery

3.2.1 Establishment of an IN infection model

In order to examine gene expression in varying *in vivo* niches, it was essential to use a mouse model of infection from which pneumococci could be harvested from the nasopharynx, lung, blood and brain in numbers sufficient for RNA analysis. An IN infection model was considered the most appropriate, as this simulates the natural route of infection for the pneumococcus. The CD-1 mouse strain (also referred to as the Swiss mouse strain) was chosen for this work for several reasons. Firstly, it is an outbred strain and, therefore, better represents a population of individuals. The use of an inbred population leaves the possibility that results are due to a specific characteristic or phenotype of the mouse strain. Secondly, pneumococcal pathogenesis in CD-1 mice closely resembles the pathogenic progression in its human host. Pneumococci are able to be carried in the nasopharynx and then progress to IPD (Wu *et al.*, 1997), whereas for some other mouse strains, such as BALB/c, the establishment of nasopharyngeal colonisation rarely leads to fulminant infection. The serotype 2 strain D39 was used to develop this CD-1 IN infection model. D39 was first isolated from the blood of a patient in the USA by Avery in 1916 and is a prototype laboratory pneumococcal strain that has been intensively studied. D39 is highly virulent for mice: it is capable of both colonising the nasopharynx and causing invasive disease in CD-1 mice, and continues to be commonly used in models of pneumococcal infection. In addition, the availability of its complete genomic sequence made it particularly attractive for this study (Lanie *et al.*, 2007).

An IN model was adapted from that described by Wu *et al.* (1997). 5- to 6- week old female CD-1 mice were intranasally infected with D39 under Nembutal anaesthesia, as described in Sections 2.4.2 and 2.4.3. Mice were euthanased by CO_2 asphyxiation at 24 and 72 hr post-infection. The nasopharynx of each mouse was washed with 1 ml 0.5% trypsin solution in PBS and then placed on ice until further use. Blood was perfused from each mouse as described in Section 3.2.3. Approximately 2 ml of blood and PBS was recovered from the chest cavity during early perfusion, and held on ice. Following complete perfusion, the lungs were excised and homogenised on ice in 2 ml sterile PBS using a tissue homogeniser. To separate pneumococci from host cells, lung homogenates and blood samples were centrifuged twice at 1000 × *g*, a method adapted from that of Ogunniyi *et al*. (2002). The optimisation of this procedure is described in Section 3.6.2. Nasopharyngeal washes, and lung and blood supernatants were subsequently centrifuged at 15,000 × *g* for 2 min at 4°C, and the bacterial pellet was stored at -80°C until further processing. Prior to pelleting harvested bacteria, an aliquot of the sample was removed, diluted in PBS, and plated on BA in order to enumerate pneumococci and determine the presence of contaminating microflora. This method for euthanasing mice and processing recovered samples is described in more detail in Section 2.4.4. The method was initially tested using 12 female CD-1 mice, aged 5 weeks. Mice were challenged IN with 1.6×10^7 D39 (determined retrospectively by plating on BA for enumeration), and a number of mice were euthanased at 24 and 72 hr post-challenge, as described in this section. Bacterial counts harvested from the nasopharynx, lungs and blood are shown in Figure 3.1.

3.2.2 Isolation of pneumococci from the nasopharynx

A washing procedure to remove loosely adherent bacteria from the nasopharyngeal cavities of mice was described by Wu *et al.* (1997), involving washing the severed

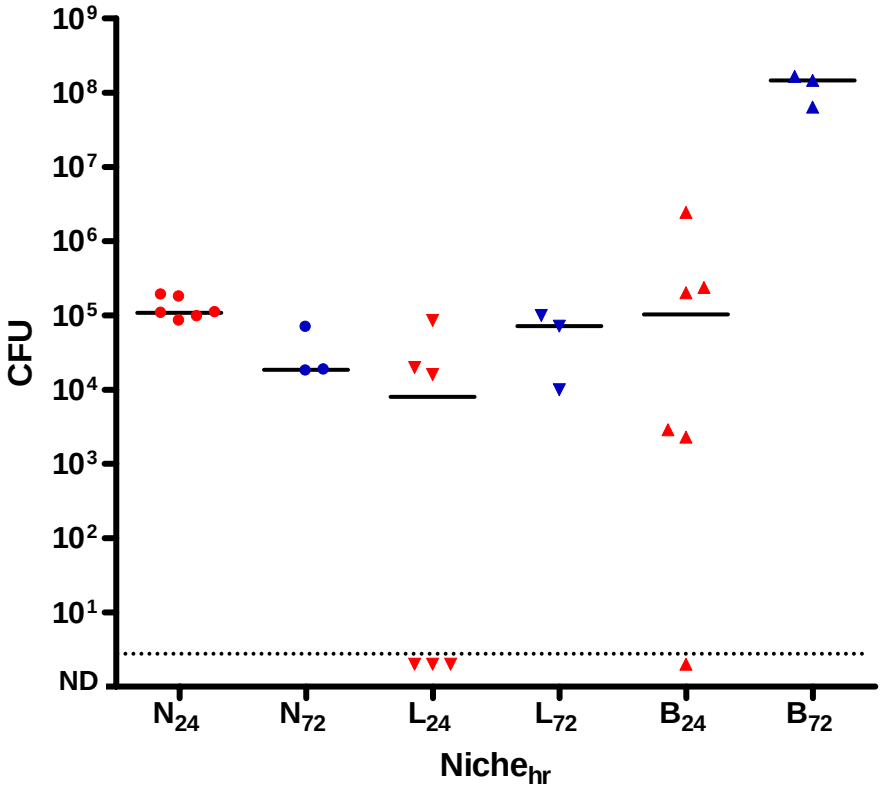


***Figure 3.1*: Disease progression in CD-1 mice following IN infection with D39**

In this pilot experiment, D39 was cultured in THY until mid-exponential phase and 6 CD-1 mice were each challenged IN with 1.6×10^7 *S. pneumoniae* D39. Mice were euthanased at 24 and 72 hr post-challenge, and nasopharyngeal washings (N), lung homogenates (L) and blood samples (B) were diluted and plated on BA for enumeration. Data are CFU per niche for nasal wash and lungs, and CFU/ml for blood. The time point is indicated in subscript. Three mice died between 24 and 72 hr. The median for each time/niche is indicated by a bar, and the limit of detection (40 CFU) is shown by a dotted line. ND denotes no growth, i.e. <40 CFU.

nasopharynx with Ringer's Solution. Bacteria that are associated strongly with, or have invaded, the epithelium can be recovered by excising and homogenising the nasopharyngeal tissue. However, homogenised tissue has been found to contain a significant proportion of other nasopharyngeal bacteria (LeMessurier, 2002). The presence of other bacteria was highly undesirable, as 16S rRNA, which was to be used as an internal control for quantitative RNA assays, is highly conserved amongst bacteria. For this reason, pneumococci residing in the nasopharynx were recovered by washing rather than by removing the nasopharyngeal tissue, as nasopharyngeal washes contain a much lower proportion of contaminating microflora. In an attempt to increase the recovery of more adherent bacteria by washing, colonising pneumococci were loosened and washed out of the nasopharynx of infected mice using a PBS buffer containing trypsin. Trypsin is commonly used at 0.25% to detach epithelial cell lines from the substratum *in vitro* whilst leaving pneumococci intact (Talbot *et al.*, 1996). To test the effect of trypsin on pneumococcal viability, D39 cultures in mid-exponential phase were incubated with PBS (control) or a PBS buffer containing either 0.25% or 0.5% trypsin (0.02% EDTA) for 1 hr and then diluted and plated on BA for enumeration. After overnight incubation at 37°C, similar CFU counts were obtained for the pneumococci incubated with both concentrations of trypsin, and the control culture (Table 3.1). Therefore, pneumococci were considered to retain viability in 0.5% trypsin, which was the concentration used for nasopharyngeal washes in this work.

3.2.3 Blood perfusion

The blood of mice that have developed sepsis commonly contains 10^7-10^9 pneumococci per ml. Circulating pneumococci in the capillaries has often been a factor overlooked in the literature when examining tissue infection and it is possible that small

Culture supplement	CFU/ml
PBS	5.0×10^6
0.25% trypsin	5.1×10^6
0.5% trypsin	4.8×10^6

***Table 3.1*: Survival of D39 in trypsin**

S. pneumoniae D39 were incubated with PBS (control) or a PBS buffer containing either 0.25% or 0.5% trypsin (0.02% EDTA) for 1 hr and then diluted and plated on BA. CFU/ml were calculated after incubation at 37°C ON.

volumes of blood containing pneumococci may artificially elevate numbers of pneumococci recovered from the lungs and the brain. To reduce the contamination of lung and brain samples by bacteria present in the blood, mice were perfused with cold, sterile PBS prior to the removal of the organs. This was achieved by cutting the right atrium of the heart directly following euthanasia, then running PBS from an elevated container and into the heart via tubing and a 26 gauge needle inserted in the left ventricle. Mice were perfused until the PBS flowing from the right atrium was clear of blood, which generally took 2 min. Animals exhibiting lung damage from pneumococcal infection did not perfuse as efficiently as mice with healthy lungs, with the flow of PBS during perfusion often being slower. These mice required approximately 3 min of perfusion until the PBS was clear of blood. Perfusing the animals in this manner was found to effectively remove most, if not all, blood-derived pneumococci from the lung and brain tissues. This was demonstrated by the observation that D39-infected mice possessing a high pneumococcal burden in the blood did not necessarily possess pneumococci in the lungs or brain, as determined by CFU counts for the respective niches. Perfusion with ice-cold PBS also rapidly chilled the tissues.

3.2.4 Detection of bacterial contaminants

A variety of commensal bacteria exist in the respiratory tracts of mice. Additionally, bacteria native to other sites in the body, such as the gastrointestinal tract, can also be found in the murine nasopharynx following experimental infection, due to the poor hygiene exhibited by ill animals. As 16S rRNA was to be used as an internal control for quantitative RNA analysis, the high homology between the 16S rRNA sequences of pneumococci and many other bacterial species, in particular other streptococci (Kawamuru *et al.,* 1995), made the contamination of pneumococcal samples recovered from the mouse

with other bacteria undesirable.

Some nasopharyngeal bacteria can be easily distinguished from *S. pneumoniae* on the basis of morphology on BA. However, in addition to phenotypically distinct bacteria, alpha-haemolytic colonies that appeared almost identical to *S. pneumoniae* were recovered from the lungs and nasopharynx of some CD-1 mice. This contaminant was distinguished from *S. pneumoniae* by growth on optochin plates (to which it was resistant), and was subsequently identified as *Enterococcus faecalis*, a related bacterium commonly present as a commensal in the mammalian intestinal tract and oral cavity (Kayaoglu and Orstavik, 2004). Control mice challenged IN with sterile PBS were found to possess small numbers of *E. faecalis* in their respiratory tracts after 72 hr. When mice were challenged with pneumococci, animals possessed even greater numbers of this contaminant in the lungs at this time point (data not shown). Commonly, pneumococci recovered from the upper respiratory tract are grown on BA in the presence of low levels of gentamicin (to which *S. pneumoniae* is inherently resistant), allowing the growth of pneumococci while suppressing the growth of other respiratory microflora (Converse and Dillon, 1977). However, enterococci recovered from the CD-1 mice were also resistant to low levels of gentamicin, making this unsuitable as a basis for recognition of the contaminant.

Contaminating flora encountered in laboratory animals may often be eliminated by a course of antibiotics prior to challenge. However, as young mice (5-6 wk) were required for this model of infection, and antibiotics take several weeks to be completely eliminated from a treated mouse, this approach did not seem feasible. Therefore, CD-1 mice were sourced from a different supplier for the *in vivo* experiments described in this thesis. Bacterial samples harvested from mice were routinely plated on plain BA to visually

screen for contaminants such as *Staphlycoccus* spp. Any suspicious colonies, and also a random selection of colonies, were then streaked on BA in the presence of an optochin disc, as described in Section 2.2.3. Resistant colonies were deemed to be contaminating bacteria, and the corresponding samples were discarded. Thus, all RNA assays were carried out on samples harvested from mice that did not exhibit contamination. In addition, colonies were randomly selected from samples recovered from all sites examined and grown in serum broth for 3 hr. Cultures were then tested by the Quellung Reaction (Section 2.2.4) to confirm serotype-specific CPS production by all organisms present in the culture.

3.3 Additional studies using serotype 4 challenge strains TIGR4 and WCH43

3.3.1 TIGR4 infection kinetics in CD-1 mice

Using the model established in Section 3.2, several other strains were examined for their pathogenicity in CD-1 mice. Initially, TIGR4 was selected. TIGR4 was isolated from a 30-year-old male patient in Norway in the late 1980s, and was a particularly attractive strain for this work as its complete genomic sequence is available (Tettelin *et al.*, 2001). The strain has been shown to be highly virulent in a MF1 murine pneumonia model, with a median survival time of 28 hr post-challenge (Ibrahim *et al.*, 2004; Ibrahim *et al.*, 2005). In BALB/cJ mice, TIGR4 establishes only low-level lung and blood infection by 72 hr following IN infection (Orihuela *et al.*, 2003). However, results from Oriheula *et al.* (2003) suggested that TIGR4-infected BALB/cJ mice may develop meningitis over a longer time-course. Both MF1 and CD-1 mice are outbred strains. In

addition, we have previously found BALB/c mice to be resistant to disease following IN challenge with the serotype 2 pneumococcal strain D39 compared to CD-1 mice (unpublished data), so it was thought likely that TIGR4 would be able to both colonise and progress to invasive disease in CD-1 mice.

In preliminary IN pathogenicity trials, TIGR4 was unable to cause bacteraemia or lung infection, and could only establish low-level colonisation of the nasopharynx by 72 hr following challenge (data not shown). Subsequently, TIGR4 was grown in serum broth and passaged three times through CD-1 mice as described in Section 2.2.5. To assess the kinetics of TIGR4 infection in CD-1 mice, four female CD-1 mice were challenged IN with 3×10^7 bacteria (Section 2.4.3) in a pilot experiment. Surviving mice were euthanased 72 hr post-challenge and bacteria present in each niche were enumerated, as described in Sections 2.4.4 and 2.4.5. In this trial experiment all mice exhibited colonisation of the nasopharynx at 72 hr, albeit at a level approximately 10-fold lower than that commonly observed for the serotype 2 strain D39. All surviving mice were also found to have lung infection and a high level of bacteraemia (Figure 3.2). Due to these preliminary data indicating the ability of TIGR4 to cause both invasive disease and colonise the nasopharynx, it was initially selected for examination of differences in gene expression between host niches. However, the levels of infection observed in the trial experiment were not reproducible. In subsequent experiments, when TIGR4 was harvested from CD-1 mice at 24 and 72 hr post IN-challenge (challenge dose of 4.2×10^7 bacteria per mouse), bacteria were recovered from nasopharyngeal washes of over half the mice, but were absent from both the lungs and the blood (Figure 3.3A). This experiment was repeated using a challenge dose of 1.4×10^7 bacteria per mouse (Figure 3.3B). Although mice euthanased at 24 hr exhibited pneumococci in the lungs and bloodstream, few

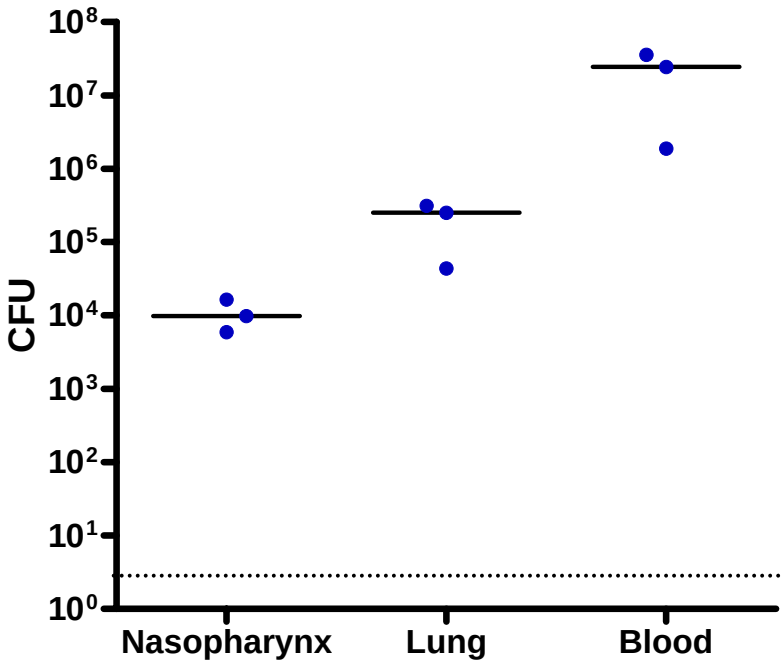
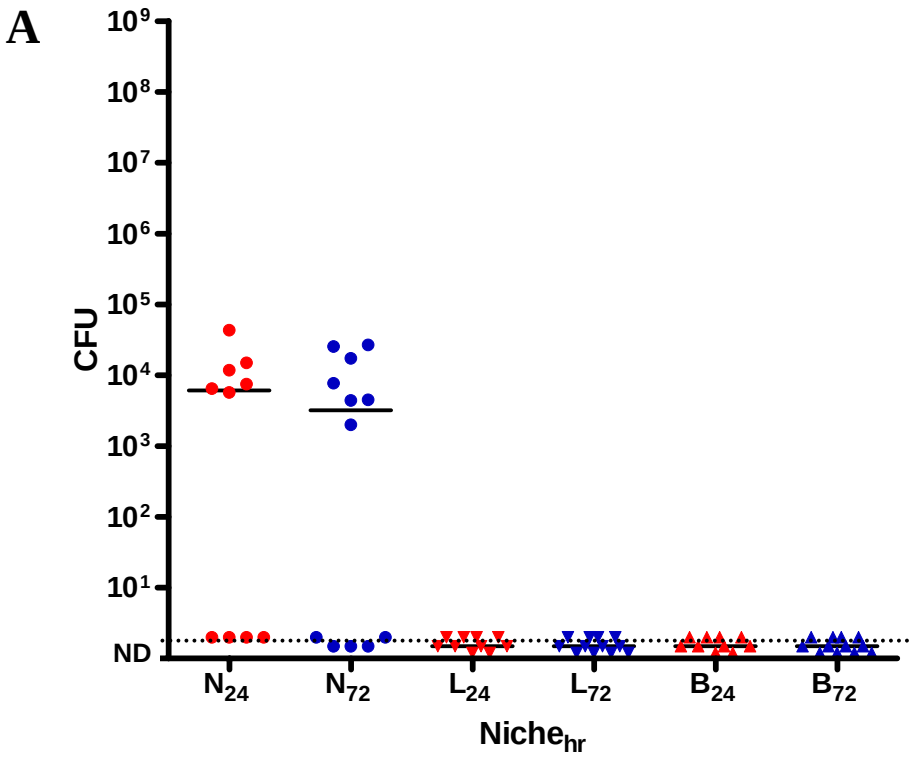


Figure 3.2: **Pilot experiment: Disease progression in CD-1 mice following IN infection with TIGR4**

4 CD-1 mice were challenged IN with 3×10^7 *S. pneumoniae* TIGR4 per mouse, and surviving animals were euthanased 72 hr post-challenge. One mouse died prior to 72 hr from bacteraemia. Nasopharyngeal washings, lung homogenates and blood samples were diluted and plated on BA for enumeration. Data are CFU per niche for nasal wash and lungs, and CFU/ml for blood. The median for each time/niche is indicated by a bar, and the limit of detection (40 CFU) is shown by a dotted line. ND denotes no growth, i.e. <40 CFU.

***Figure 3.3*: Disease progression in CD-1 mice following IN infection with TIGR4**

TIGR4, a serotype 4 clinical isolate, was cultured in THY until mid-exponential phase and CD-1 mice were challenged IN with the culture. Mice were euthanased at 24 and 72 hr post-challenge, and nasopharyngeal washings (N), lung homogenates (L) and blood samples (B) were diluted and plated on BA for enumeration. Data are CFU per niche for nasal wash and lungs, and CFU/ml for blood. The time-point is indicated in subscript. **A)** and **B)** represent data obtained from two independent experiments (challenge dose 4.2×10^7 and 1.4×10^7 CFU respectively) with the median for each time/niche indicated by a bar and the limit of detection (40 CFU) shown by a dotted line. ND denotes no growth, i.e. <40 CFU.


A
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CFU
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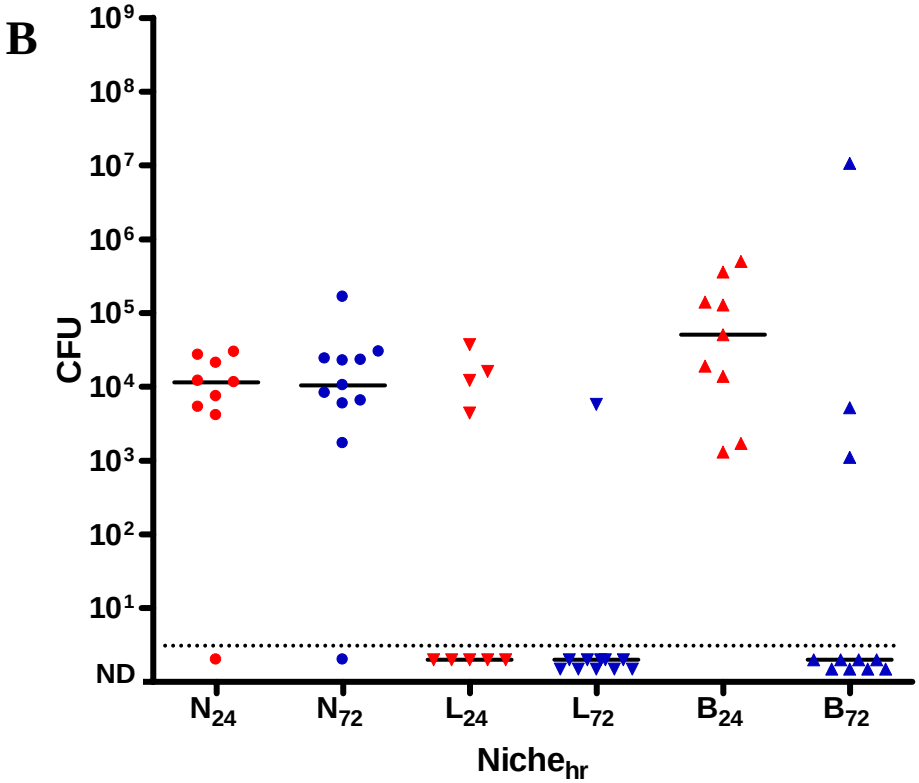
B
10^9
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ND
CFU
N_{24}
N_{72}
L_{24}
L_{72}
B_{24}
B_{72}
$Niche_{hr}$

animals euthanased at 72 hr had bacteria in either of these niches.

3.3.2 TIGR4 growth *in vitro*

Despite the tertiary *in vivo* passaging, TIGR4 still grew poorly in THY broth, with A_{600} readings indicating that growth often ceased after only a few hours of incubation. When growth curves in THY broth were established (Section 2.2.6) and compared to the CFU/ml at each time point measured, a decrease in A_{600} readings coupled with a decrease in CFU/ml was observed after 4.5 hr, suggesting that autolysis may be occurring (Figures 3.4A and 3.4B).

A similar decrease in CFU/ml was observed for cultures grown in THY broth supplemented with 0.5% choline, which is routinely added to pneumococcal cultures during prolonged incubation periods to prevent cell lysis from occurring (Briles *et al.*, 1996). However, the A_{600} reading increased over the time course, indicating that pneumococcal growth and division were occurring with minimal lysis. The decrease observed in CFU/ml may have been due to cell chain formation or clumping. When TIGR4 was grown in THY supplemented with 10% horse serum, both A_{600} and CFU/ml increased during the entire time course (Figures 3.4A and 3.4B). As suggested by the magnitude of the error bars representing SEM between two replicate experiments, the variation in TIGR4 growth was significant between experiments. This experiment was conducted additional times (data not shown) and similar fluctuations in growth curves was observed. As a consequence of poor growth *in vitro* resulting in concomitant uncertainties regarding dosage calculation and unreliable infection of CD-1 mice, it was decided that TIGR4 was not a suitable challenge strain for *in vivo* comparison of gene expression using this model.

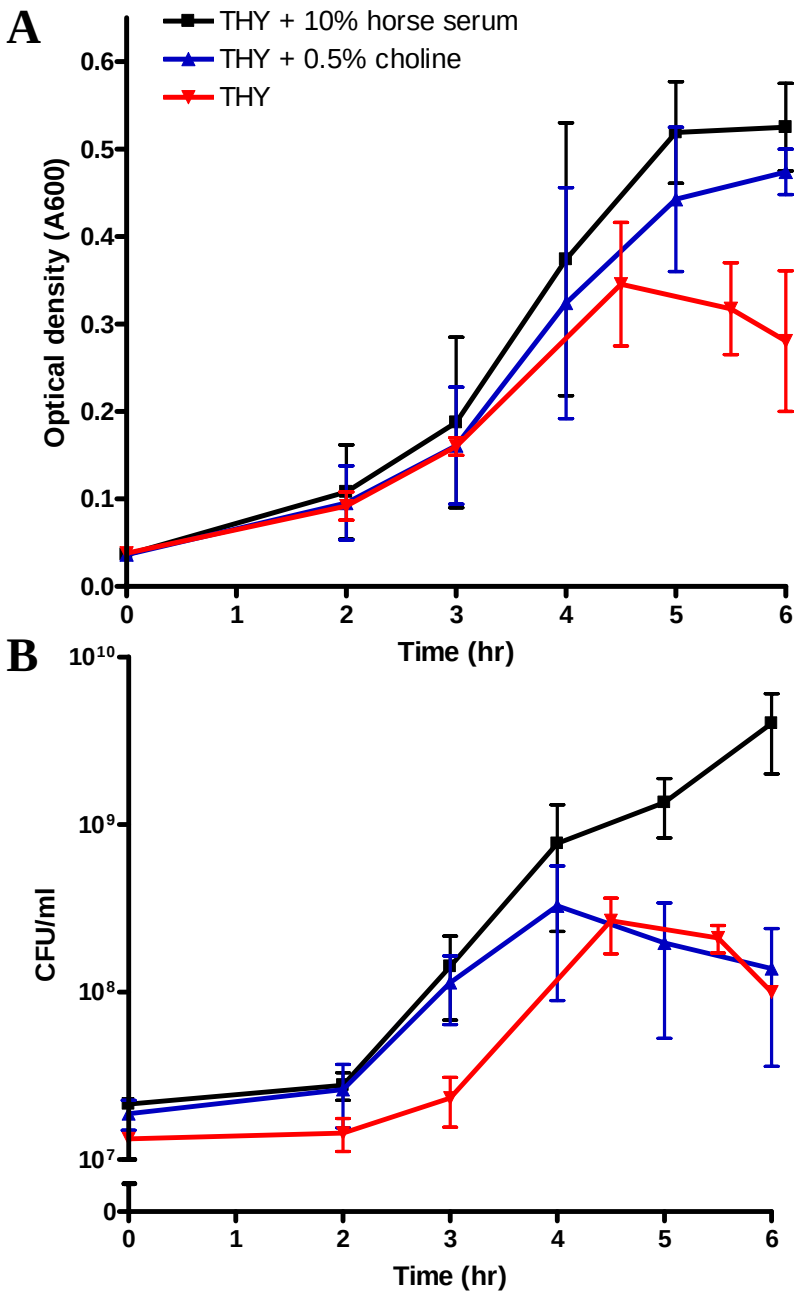


***Figure 3.4*: TIGR4 growth in THY supplemented with serum or choline**

TIGR4, taken from overnight BA plates, was cultured at 37°C in THY media supplemented with either 10% horse serum or 0.5% choline, as indicated. Bacteria were also grown in THY with no supplement. **A)** A_{600} was recorded at predetermined time points over a 6- hr incubation period. **B)** At these time points, culture was diluted and plated for enumeration. The error bars indicate SD between two replicate experiments.

3.3.3 WCH43 growth *in vitro*

As TIGR4 did not seem able to establish stable and reproducible infection in CD-1 mice, a second serotype 4 *S. pneumoniae* strain (WCH43, a clinical isolate obtained from the Women's and Children's Hospital, South Australia) was examined. WCH43 was cultured in THY broth to determine a growth curve (described in Section 2.2.6). Unlike TIGR4, WCH43 exhibited reliable and reproducible growth in THY broth between two separate experiments (Figure 3.5).

3.3.4 WCH43 infection kinetics in CD-1 mice

A pilot experiment was performed to determine the ability of this strain to colonise the nasopharynx and invade other niches. Six female CD-1 mice, aged five weeks, were challenged IN with 4×10^7 bacteria (determined retrospectively by plating on BA) as described in Section 2.4.3. The level of bacteraemia for each challenged mouse was monitored at 24, 48 and 72 hr following challenge by performing tail bleeds to obtain a sample of blood (which involved making a small incision with a sterilised scalpel along the tail vein and removing an aliquot of blood using heparinised tubing), which was then plated on BA (Figure 3.6A). The surviving mice were euthanased 72 hr after challenge, and nasopharyngeal washes, lung and blood samples were obtained. The blood sample was obtained prior to the commencement of perfusion. The numbers of bacteria in each sample were determined by plating on BA (Figure 3.6B). Approximately 10^5 pneumococci were recovered in the nasopharyngeal wash for all infected mice, indicating that WCH43 is able to colonise CD-1 mice for at least three days. However, the strain caused lung disease

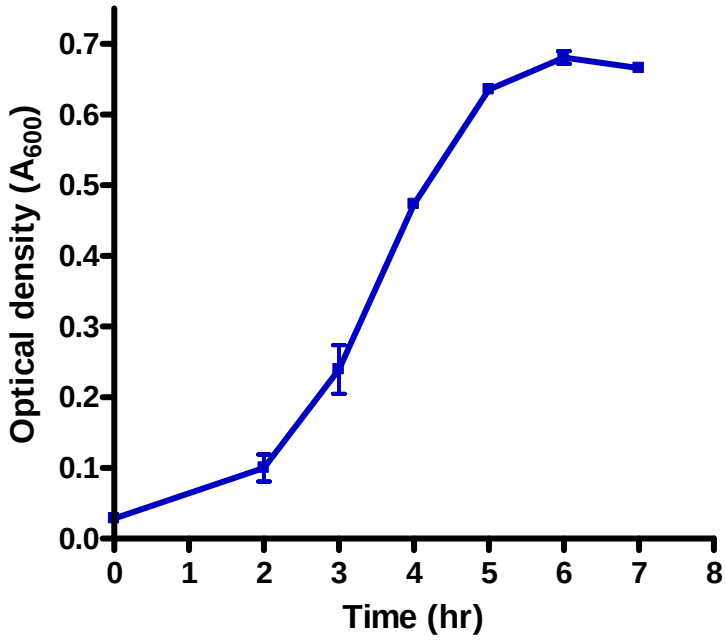
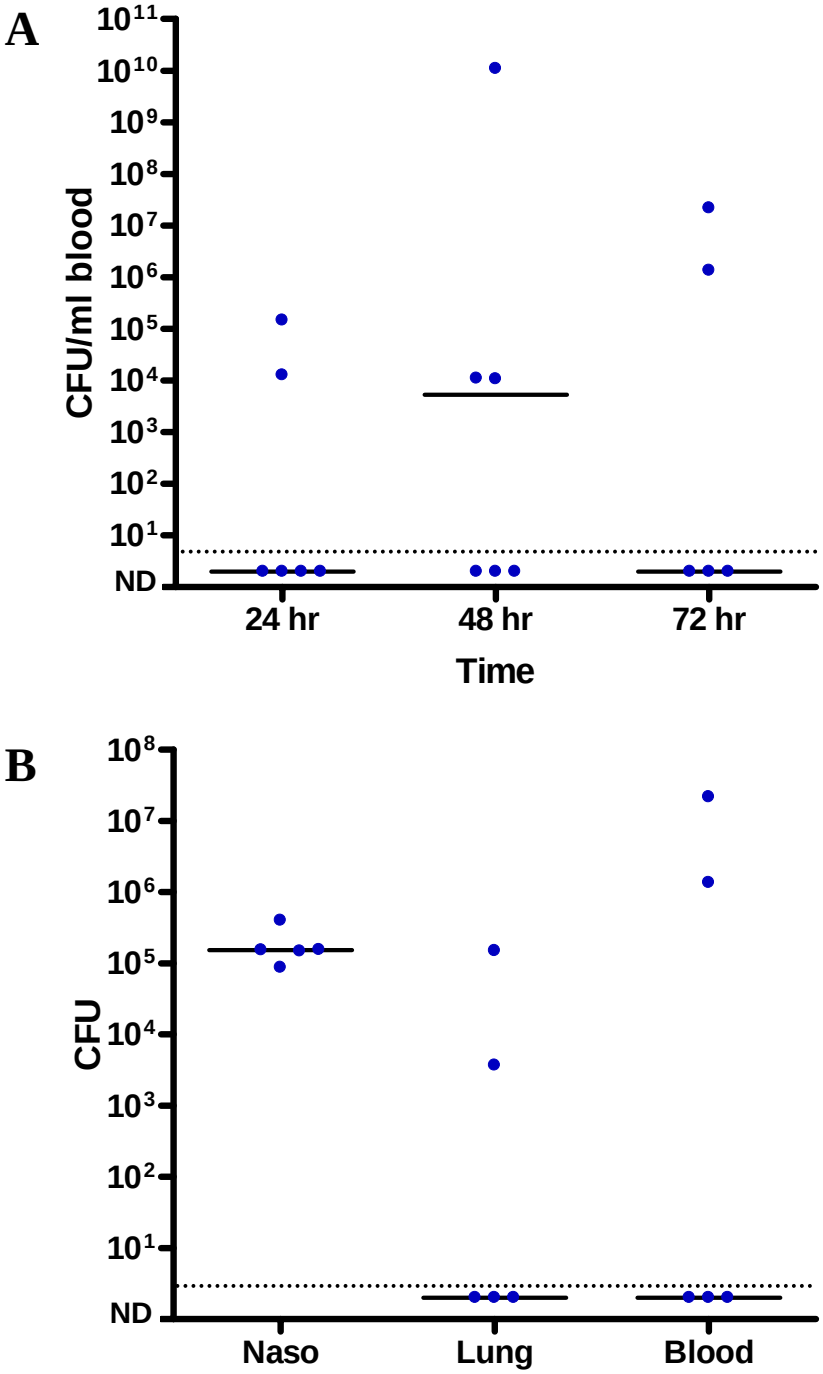


***Figure 3.5*: WCH43 growth in THY**

WCH43, taken from overnight BA plates, was cultured at 37°C in THY media and A_{600} readings were recorded over a 7 hr incubation. The error bars represent SD between two replicate experiments.

***Figure 3.6*: Pilot experiment: Disease progression in CD-1 mice following IN infection with WCH43**

WCH43 (a serotype 4 clinical isolate) was cultured in THY until mid-exponential phase and 6 CD-1 mice were each challenged IN with 4×10^7 bacteria. **A)** depicts the level of bacteraemia observed for each mouse, determined by tail bleeds at 24, 48 and 72 hr post-challenge. One mouse died between 48 and 72 hr time points. **B)** Surviving mice were euthanased at 72 hr and nasopharyngeal washings, lung homogenates and blood samples were diluted and plated on BA for enumeration. Data are CFU per niche for nasal wash and lungs, and CFU/ml for blood. For both **A)** and **B)**, the median is indicated by a bar and the limit of detection (40 CFU) is shown by a dotted line. ND denotes no growth, i.e. <40 CFUs.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ND
CFU/ml blood
24 hr
48 hr
72 hr
Time
B
10^{8}
10^{7}
10^{6}
10^{5}
10^{4}
10^{3}
10^{2}
10^{1}
ND
CFU
Naso
Lung
Blood

and bacteraemia in only two out of the five surviving mice (one mouse died between 48 and 72 hr time points). Interestingly, although the level of bacteraemia reached $10^6 – 10^7$ CFU/ml for mice euthanased at 72 hr, the animals did not display the typical prodromal symptoms of sepsis observed in mice challenged IN with D39 or TIGR4 (hunched appearance, poor hygiene, glazed eyes and lethargy). This strain was not studied further due to the low proportion of mice in which nasopharyngeal colonisation progressed to invasive disease.

3.4 Additional studies using serotype 6A challenge strain WCH16

3.4.1 WCH16 infection kinetics in CD-1 mice

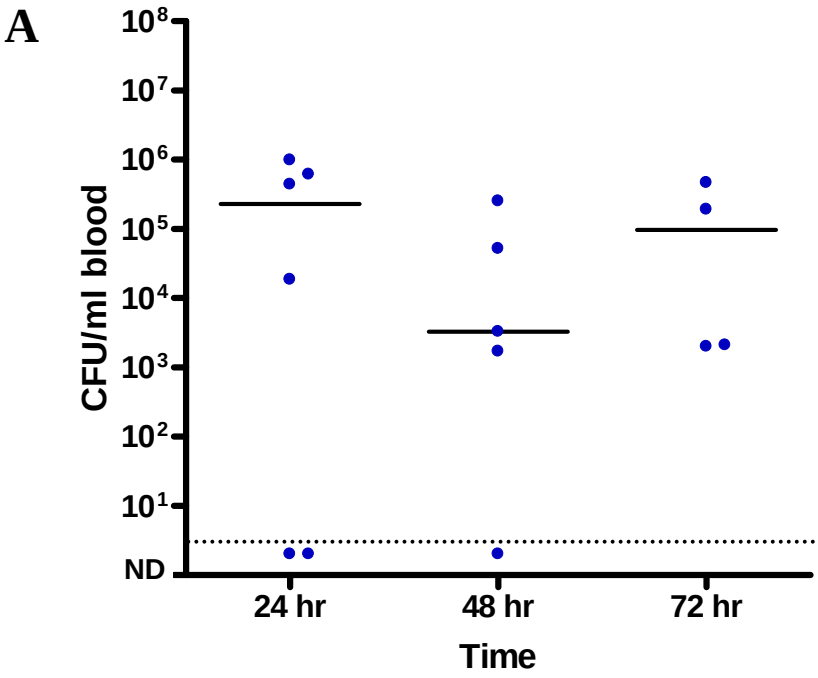
Due to the unreliable progression of the type 4 strains TIGR4 and WCH43 from nasopharyngeal colonisation to invasive disease, a serotype 6A strain was examined. Serogroup 6 comprises of serotypes 6A and 6B, which differ from each other by a the nature of the glycosidic linkage between the rhamnose and ribitol moieties in the polysaccharide repeat unit (Robbins *et al.*, 1979). For these experiments, the type 6A clinical isolate WCH16 (obtained from the Women's and Children's Hospital, South Australia) was used. This strain had been characterised previously in our laboratory. It had been shown to both exhibit reliable growth *in vitro*, and is virulent for mice when challenged IP (A.D. Ogunniyi, personal communication).

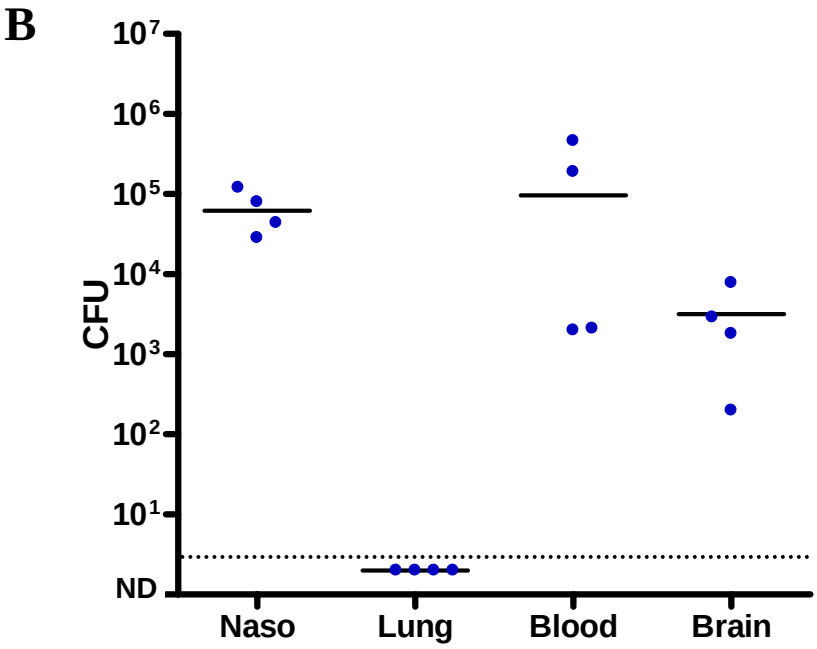
Six female CD-1 mice, aged five weeks, were challenged IN with 2×10^7 bacteria (determined retrospectively by plating on BA) as described in Section 2.4.3. The level of bacteraemia for each challenged mouse was monitored at 24, 48 and 72 hr following

challenge by performing tail bleeds to obtain a small sample of blood, which was then diluted and plated on BA. The numbers of bacteria present in the blood of each mouse at these time points are shown in Figure 3.7A. Surviving mice at 72 hr post-challenge were euthanased by CO_2 asphyxiation, and nasopharyngeal washes, lung, blood, and brain samples were obtained. The blood sample was obtained prior to the commencement of perfusion. The numbers of bacteria in each sample were determined by plating on BA (Sections 2.4.4 and 2.4.5). A moderate level of bacteraemia (approximately 10^5 CFU/ml of blood) was observed for four out of the six mice at 24 hr post challenge, and this level was maintained over the three-day trial. One mouse died presumably due to pneumococcal infection, as indicated by the heavy bacterial load in the bloodstream, between the 48- and 72- hr time points. Additionally, one mouse died during tail bleeding at 24 hr and was found to have 4.4×10^5 CFU/ml of blood in its bloodstream. A similar level of nasopharyngeal colonisation was observed for mice infected with WCH16 as for D39, with all animals euthanased at 72 hr having approximately 10^5 pneumococci per nasopharyngeal wash. No bacteria were recovered from the lungs of the mice euthanased at this time point, although all surviving animals were bacteraemic. The level of infection in these three sites is shown in Figure 3.7B. All mice challenged IN with WCH16 developed bacteraemia, albeit at a lower level than for D39. Although mice in the pilot experiment did not possess pneumococci in the lungs at 72 hr post-challenge, bacteria were present in the brain tissue, suggesting the onset of meningitis. The differences in pathogenicity between WCH16 and D39 made it an attractive strain to use in further experiments examining gene expression in these different niches.

***Figure 3.7*: Pilot experiment: Disease progression in CD-1 mice following IN infection with WCH16**

WCH16 (a serotype 6A clinical isolate) was cultured in THY until mid-exponential phase, and 6 CD-1 mice were each challenged IN with 2×10^7 bacteria. **A)** depicts the level of bacteraemia observed for each mouse, determined by tail bleeds at 24, 48 and 72 hr post-challenge. One mouse died during tail bleeding at 24 hr. Additionally, one bacteraemic mouse died between the 48- and 72- hr time points. **B)** Surviving mice were euthanased at 72 hr and nasopharyngeal washings, lung and brain homogenates and blood samples were diluted and plated on BA for enumeration. Data are CFU per niche for nasal wash and lungs, and CFU/ml for blood. For both **A)** and **B)**, the median is indicated by a bar, and the limit of detection (40 CFU) is shown by a dotted line. ND denotes no growth, i.e. <40 CFU.


A
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
ND
CFU/ml blood
24 hr
48 hr
72 hr
Time


B
10^7
10^6
10^5
10^4
10^3
10^2
10^1
ND
CFU
Naso
Lung
Blood
Brain

3.5 Comparison between D39 and WCH16 pathogenesis in CD-1 mice

While euthanasing mice and enumerating bacteria present in harvested samples at selected time-points is a quantitative way of examining pathogenesis, it is not possible to follow the disease course within a single mouse. This method does not account for the possibility that each euthanased mouse may have cleared existing disease, developed further disease, or died. To account for mouse-to-mouse variability in susceptibility to infection, the number of mice euthanased at any one time point has to be sufficiently large to be considered representative of the experimental population, which may not necessarily be possible due to technical and ethical considerations. An alternative method of monitoring pathogenesis is to use challenge bacteria expressing a reporter such as GFP or bacterial luciferase (Lux) that can emit a detectable signal in live mice, such that an infection can be monitored in individual mice over a time course (Lee and Camilli, 2000). That is, the course of disease can be followed in individual mice. In this experiment, the *lux* cassette was chosen as a reporter for several reasons. Firstly, the Lux system requires live cells for signal generation, because it utilises reduced flavin mononucleotide as an energy source (Hill *et al.*, 1993). Therefore, any detectable signal can be almost completely attributed to viable bacterial cells. Secondly, GFP imaging suffers high-background fluorescence, whereas background luminescence is relatively low. Thirdly, GFP is relatively stable, with a half-life of 26 hr (Corish and Tyler-Smith, 1999). Although a derivate has been engineered that possesses a half-life of 6 hr, it is still significantly more stable than Luc and Lux luciferase proteins (Qazi *et al.*, 2001; Thompson *et al.*, 1991). GFP fluorescence also transmits poorly through tissue, making it less suitable for use in a live animal model than luciferase (Lee and Camilli, 2000). To further investigate the pathogenesis of D39 and WCH16, bioluminescent derivates were

used to monitor the disease progression of each strain in real time.

3.5.1 Construction of luminescent D39 and WCH16 strains

Pneumococcal strains D39LUX and WCH16LUX were constructed by transforming D39 and WCH16 with pAL2 (Beard *et al.*, 2002), a plasmid possessing a LUX cassette from *Photorhabdus* (*Xenorhabdus*) *luminescens* (kindly provided by Dr. Habib Alloush, University of Western England, UK), as described in Section 2.7. The plasmid is shown in Figure 3.8 and contains the entire *luxCDABE* operon under the control of the constitutive *ami* promoter (Alloing *et al.*, 1990), so is able to luminesce without the addition of exogenous substrate. Furthermore, the enzymes encoded by the *luxCDABE* operon of *P. luminescens* are functional up to 45°C, which is higher than other bacterial luciferases used in the past (Meighen, 1991). This makes *P. luminescens* luciferase particularly attractive for *in vivo* work as bioluminescence can occur over the spectrum of temperatures a pathogen may encounter during infection. *E. coli* DH5α cells were transformed with pAL2 and cultured in LB broth in the presence of 50 µg/ml Ery O/N. *S. pneumoniae* D39 or WCH16 were then transformed with pAL2 extracted from the DH5α cells, in the presence of either 2.5 µl of 10 µg/ml CSP-1, or 2.5 µl of 10 µg/ml CSP-1 and 5 µl of 10 µg/ml CSP-2, for the respective strains, as described in Section 2.7.2 (Table 2.1). Transformants were identified following O/N growth on BA supplemented with 0.2 µg/ml Ery, and colonies were then re-streaked on BA containing 50 µg/ml Ery and incubated at 37°C with 5% CO_2 overnight. The plates were examined for bioluminescence using IVIS 100 Imaging System (Xenogen), and luminescent colonies were then cultured in THY in the presence of 50 µg/ml Ery and glycerol stocks were prepared and stored at –80°C. Glycerol stocks were streaked on BA and examined for luminescence again prior to mouse challenge (Figure 3.9).

***Figure 3.8*: pAL2 plasmid map**

Arrows indicate the locations and orientations of open reading frames. Gram-positive ribosomebinding sites, located upstream of *luxA*, *luxC*, and *luxE* in the modified *lux* cassette, are represented by shaded regions at the 5' ends of open reading frames. Em-R indicates erythromycin resistance gene (Beard *et al.*, 2002).

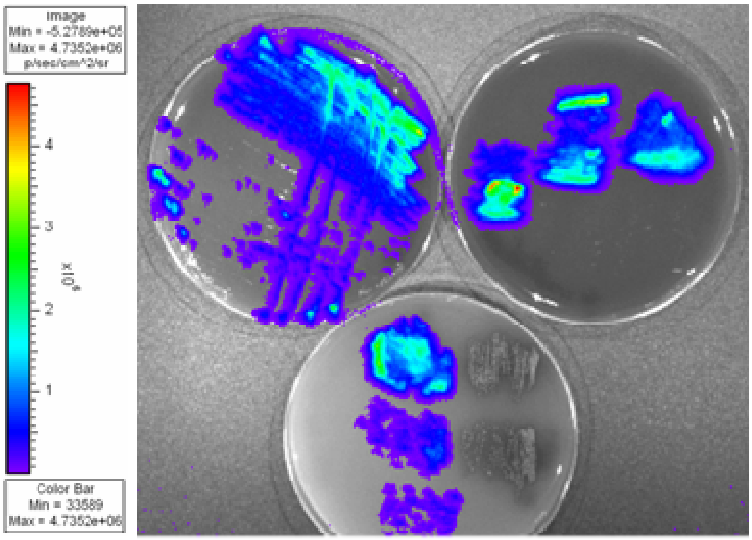


***Figure 3.9*: Bioluminescent pneumococcal strains prior to mouse challenge**

Photon emissions from D39LUX and WCH16LUX grown O/N on BA containing 50 µg/ml Ery were visualised using a Xenogen IVIS 100 Imaging System. Clockwise from the top left: D39LUX recovered from the blood of an IN infected CD-1 mouse with bacteraemia 3 days post-challenge; D39LUX colonies following transformation with pAL2; WCH16LUX colonies following transformation with pAL2.

3.5.2 Visualisation of D39 and WCH16 pathogenesis in live CD-1 mice

Mice were IN infected with either D39LUX or WCH16LUX cultures, as described in Section 2.4.3. At 1, 2 and 3 days post-challenge, animals were lightly anaesthetised with Nembutal and examined for luminescence using a 2 to 7 min exposure time on a Xenogen IVIS 100 Imaging System, as described in Section 2.4.6. Based on the presence of detectable photon emission levels using the IVIS 100 Imaging System, animals challenged with D39LUX were observed to establish colonisation in the nose and develop infection of the thoracic and abdominal cavities 2-3 days following challenge, possibly indicating the infiltration of pneumococci into the lungs, blood and liver. Mice exhibiting bacteraemia died within the subsequent 24 hr period. Images typical of D39LUX-infected mice 3 days post-challenge are shown in Figure 3.10.

Conversely, many WCH16LUX-infected mice appeared to develop infection of the upper thoracic cavity 2 days post-challenge. A signal from this region correlated with the presence of bacteria in the bloodstream when an aliquot of blood recovered from the mouse tail vein was plated on BA for enumeration. Generally, bacteraemia persisted at similar levels without causing mice to appear physically ill, or dropped to levels below the limit of detection of the IVIS 100 Imaging System, by 3 days post-challenge. Surviving mice at this time point were maintained for a further 4 days, to examine the outcome of disease. By day 7 post-challenge, 2 mice that exhibited bacteraemia at day 3 had died. Blood was recovered from dead mice and plated for CFU counts and confirmed that mice had died from pneumococcal infection, as they both exhibited high numbers of pneumococci in the bloodstream. This indicates that bacteraemia progresses to sepsis and death, or is resolved, in WCH16-infected mice. In addition, a bioluminescent signal was

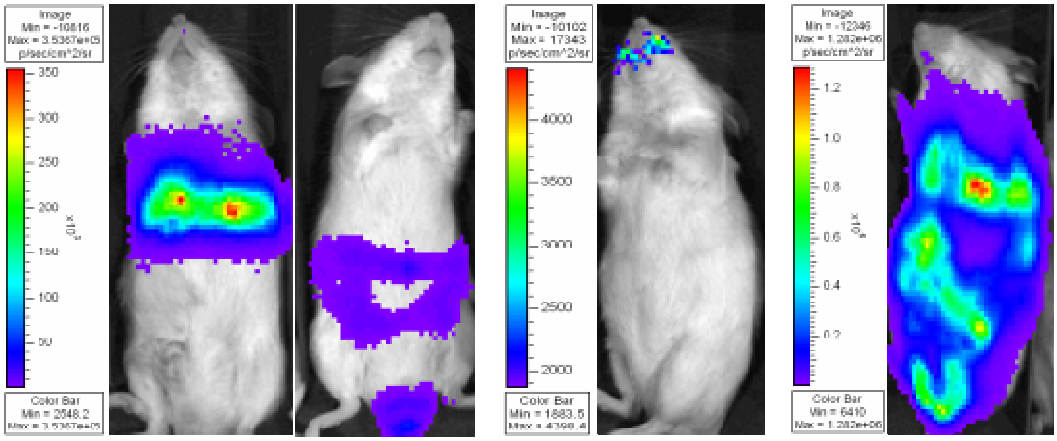


Figure 3.10: **Bioluminescent images of mice IN infected with D39**

3 days following IN infection with D39LUX, CD-1 mice were anaesthetised with Nembutal and monitored for bioluminescence using a Xenogen IVIS 100 Imaging System. Photographs of animals were overlayed with photon emission images. Photon intensity is relative to bacterial numbers. All mice shown here are typical of D39 IN-infected mice observed in these experiments. From the left: A mouse with lung infection; a mouse with infection of the lower abdominal cavity; nasopharyngeal carriage; a mouse exhibiting sepsis, close to death.

detected in the anterior head region of most mice by day 3 following dorsal imaging, but was not apparent in ventral images. It is possible pneumococci were present in the Olfactory lobes or the sinuses. The exact site of infection could be determined in future by sacrificing mice emitting a detectable signal from the area and enumerating bacteria present in the various tissues. Images of WCH16LUX-infected mice 3 days post-challenge are shown in Figure 3.11.

3.5.3 Determination of Ply production by, and haemolytic activity of, D39 and WCH16 cultures

Mice challenged IP with WCH16 bacteria often maintain a constant, sub-septic, level of bacteraemia for several days, whereas mice similarly infected with D39 quickly develop sepsis and die (Sections 3.2.1, 3.4.1 and 3.5.2). The absence of Ply has been previously shown to result in development of chronic bacteraemia instead of overwhelming sepsis (Benton *et al.*, 1995), so it is possible that differences in either specific haemolytic activity or overall Ply expression may contribute to the disparity in virulence between D39 and WCH16. To investigate this further, Ply production and haemolytic activity of each strain were examined. D39 and WCH16 were cultured in THY media to mid-exponential phase (A_{600} = 0.25) and WCLs were prepared as described in Section 2.8.1. Relative protein concentrations in the WCLs were estimated using Coomassie staining (Section 2.8.2). To determine the relative haemolytic ability of the two strains, haemolytic assays were performed for D39 and WCH16 WCLs (as described in Section 2.8.4), standardised according to the amount of protein in each WCL. Results from two biological replicate experiments were combined and are presented in Figure 3.12A. The relative amounts of Ply in the same D39 and WCH16 WCLs were determined by Western blot analysis, as described in Section 2.8.3, using antisera specific for the Ply

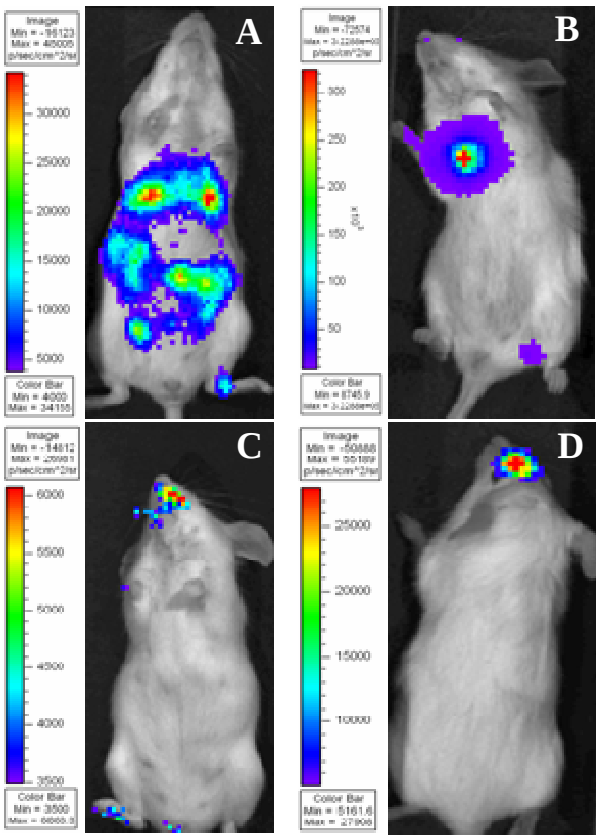


***Figure 3.11*: Bioluminescent images of mice IN infected with WCH16**

3 days following IN infection with WCH16LUX, CD-1 mice were anaesthetised with Nembutal and monitored for bioluminescence over a time course using a Xenogen IVIS 100 Imaging System. Photographs of animals were overlayed with photon emission images. Photon intensity is relative to bacterial numbers. **A)** one of the two mice that developed a high level of sepsis and lung infection and subsequently died **B)** a mouse with bacteria in the thoracic cavity **C)** a mouse with colonisation of the nasopharynx **D)** a mouse with bacteria in the upper skull, possibly indicating the development of meningitis.

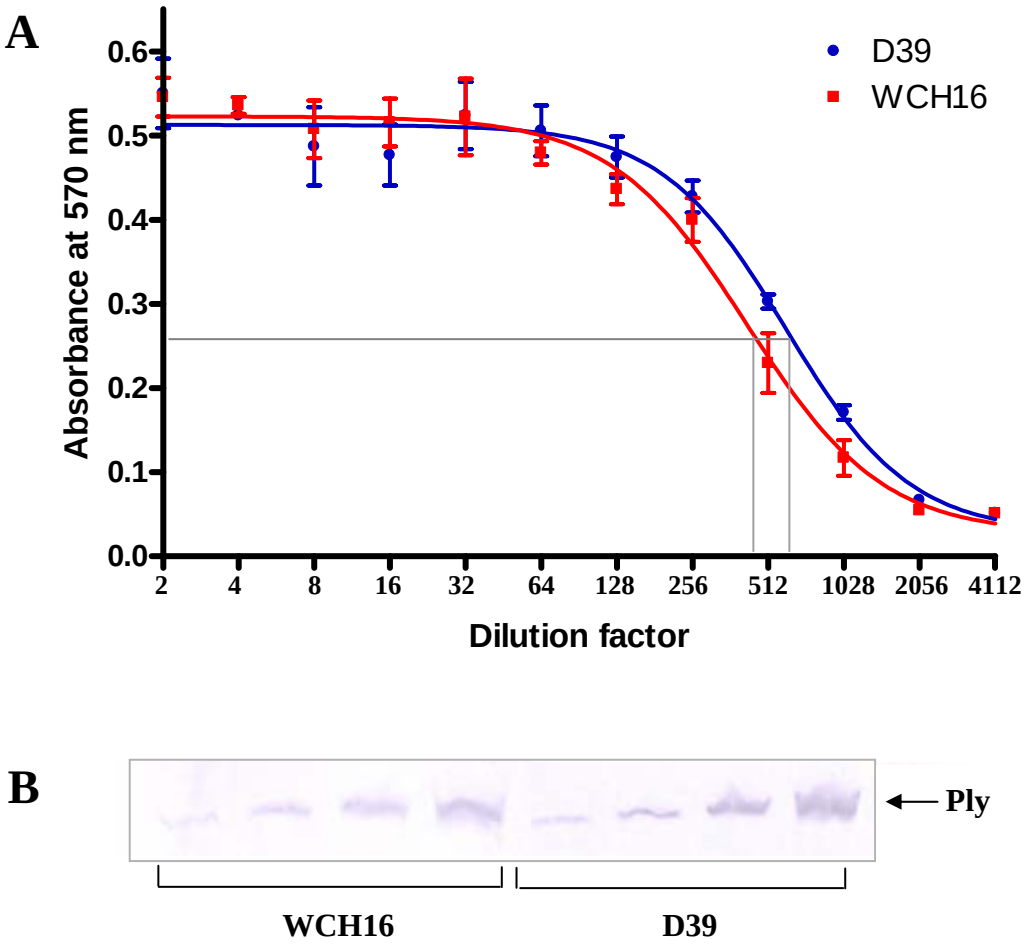


***Figure 3.12*: Haemolytic activity of, and Ply production by, D39 and WCH16 cultures**

A) WCLs prepared from D39 and WCH16 mid-exponential cultures were serially diluted in PBS, and haemolytic activity was determined using human RBCs, as described in Section 2.8.4. Data are mean ± SD from two biological replicate experiments. The dilution factor at which half of the RBCs were lysed for each strain is indicated by grey lines. **B)** Serial 2-fold dilutions of the same D39 and WCH16 lysates, were separated by SDS-PAGE, electroblotted onto nitrocellulose and reacted with mouse polyclonal antiserum specific for D39 Pdb (pneumolysin toxoid).

toxoid, Pdb (Figure 13.12B). Although D39 cultures appeared to produce slightly more Ply than WCH16 and, accordingly, exhibited a slightly higher haemolytic ability, there was not a significant difference between the two strains (<2-fold). Based on these results, it is unlikely that strain-specific differences in overall production or specific activity of Ply are responsible for the different kinetics of bacteraemia exhibited by D39- and WCH16-infected mice.

3.5.4 Survival of mice IN challenged with D39 or WCH16

In order to examine the relative virulence of D39 and WCH16 in CD-1 mice, IN-challenge survival studies were performed. Groups of 15 5-6 week old female CD-1 mice were IN challenged under Nembutal anaesthesia with either 2-3 $\times$ 10^7 D39 or WCH16 bacteria (Section 2.4.3), and deaths were recorded at 6 hr intervals over a period of 21 days. These results are shown in Figure 3.13. 1 mouse from the WCH16-challenged group did not recover from anaesthesia, and according there are only 14 deaths recorded for this group. For D39, the median survival time was 56 hr, and 2 animals were still alive at the end of the experiment indicating 87% mortality. The medium survival time for mice challenged with WCH16 was nearly twice that observed for D39 (107 hr), with 64% mortality. The survival times of D39- and WCH16- infected mice were compared using the Mann-Whitney test and were found to be significantly different ($P < 0.02$). Analysis using the Fisher Exact test found no significant difference between overall survival (2/15 and 5/14 mice, respectively). However, further survival experiments using different challenge doses may have detected a statistically significant difference in overall survival rate.

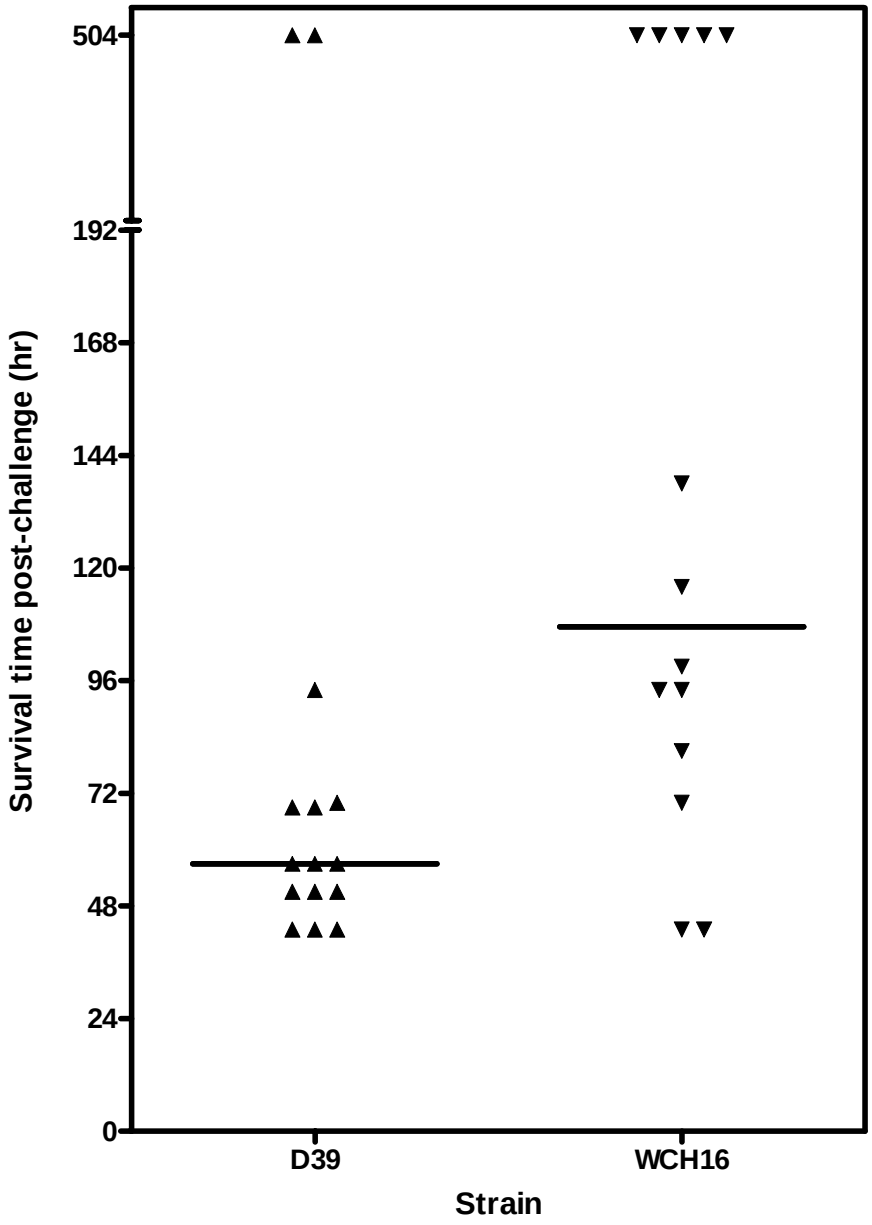


***Figure 3.13*: Survival time of mice IN-challenged with D39 or WCH16**

Groups of 15 CD-1 mice were challenged IN with 2-3 × 10^7 D39 or WCH16 bacteria under anaesthesia. 1 WCH16 mouse died during anaesthesia. The median survival time for each group is indicated by a horizontal bar.

3.6 RNA processing and quantitation

3.6.1 mRNA half-lives

To estimate the speed and efficiency needed to harvest and process pneumococcal samples recovered from mice with minimal mRNA degradation, several of the genes to be examined by real-time RT-PCR analysis were selected (*ply*, *psaA*, *cps2A* and *nanA*) and their mRNA half-lives relative to that of 16S rRNA were determined as follows. Pneumococci were cultured in THY until mid-exponential phase was reached (A_{600} = 0.3). Rifampicin (100 µg/ml), an antibiotic that binds to the core and holoenzyme of RNA polymerase (Yarbrough *et al.*, 1976), was added to the culture to prevent further transcription from occurring during the course of the experiment. The amount of rifampicin required to effectively halt transcription was determined empirically by using real-time RT-PCR to determine levels of 16S rRNA extracted over a period of 30 min from several cultures, each possessing different concentrations of rifampicin. A 1 ml aliquot was immediately removed and centrifuged at 15,500 × *g*, for 2 min at 4°C to pellet the bacteria. Nucleic acids were extracted from the pellet following the protocol described in Section 2.5.1. Further aliquots were taken at 2, 4, 8, 12, 16 and 20 min following the addition of rifampicin, and nucleic acids were extracted. All samples were ethanol-precipitated at -80°C for 12 hr. Following DNAse treatment, real-time RT-PCR was used to quantitate mRNA relative to 16S rRNA, and mRNA half-lives were determined by calculating the inverse slope of the line of best fit (Table 3.2). When maintained at room temperature over 20 min, the half-lives of the transcripts examined were relatively short, ranging from 3.6 min (*ply*) to 6.1 min (*nanA*). Experiments conducted in parallel, where cultures were incubated on ice over 20 min, indicated that maintaining pneumococcal cultures at a lower temperature following the addition of rifampicin increased the half-lives

mRNA	Half-life (min) at RT	R^2	Half-life (min) on ice	R^2
ply	3.6	0.973	13.5	0.874
psaA	4.2	0.981	12.9	0.878
cps2A	4.6	0.911	-	-
nanA	6.1	0.995	-	-

***Table 3.2*: Half-lives of *ply*, *psaA*, *cps2A* and *nanA* mRNAs**

100 µg/ml rifampicin was added to *S. pneumoniae* D39 THY cultures in mid-exponential growth phase. Cultures were either kept at RT or incubated on ice. Aliquots were taken at regular intervals, and RNA extracts were prepared using the acid phenol-cholorform method described in Chapter 2. Real-time RT-PCR was used to quantitate mRNA relative to 16S rRNA, and mRNA half-lives were determined by calculating the inverse slope of the line of best fit. R^2 is the coefficient of determination for the line of best fit, and indicates how well the time points fit the line.

of these transcripts by approximately 3 to 4-fold. This experiment reinforced the necessity for speed and low temperature during both the harvesting of bacteria from mice, and the processing of samples.

3.6.2 Optimisation of differential centrifugation

The process by which pneumococci are harvested from soft tissue such as the lungs and brains of mice yields a sample consisting of both bacterial cells and host tissue. Despite the use of the differential centrifugation method described by Ogunniyi *et al.* (2002) to separate host and pneumococcal cells, total RNA extracted from *in vivo* harvested samples was still found to contain a high percentage of eukaryotic RNA, as determined by comparing total RNA, as estimated by $A_{280/260}$ readings, and real-time RT-PCR using 16S-specific oligonucleotides and a standard curve generated from an *in vitro*-derived template of known concentration. Since the presence of eukaryotic RNA influences the efficiency of prokaryotic template amplification, it was desirable to lower the proportion of contaminating host cells prior to RNA extraction, since all ribonucleic acids are linearly amplified during downstream processes. To determine the effect of different centrifugation speeds on pneumococcal recovery from the lungs and brain of infected mice, D39 cultured in THY broth to mid-exponential phase was separated into five 1 ml aliquots. The brain and lungs of two CD-1 mice were obtained as described previously (Section 2.4.4), and homogenised separately in 2 ml sterile PBS. Each of the homogenates was combined with one pneumococcal culture aliquot. 2 ml sterile PBS was added to the remaining aliquot, as a control to determine maximum yield. Aliquots containing brain or lung homogenates were centrifuged at either 1000 × *g* or 1500 × *g* for 4 min at 4°C. Following centrifugation, the supernatants were removed and placed in a new 10 ml tube. Centrifugation was repeated to maximise host cell removal. All cultures

(including the PBS control culture) were subsequently centrifuged at 15,500 × *g* to pellet pneumococci, and pellets were resuspended in PBS. An aliquot was removed and diluted in PBS, then plated on BA to determine the bacterial yield following centrifugation at different speeds (Table 3.3). It was apparent that a higher initial centrifugation speed resulted in a significantly lower yield of pneumococci. This was particularly evident for pneumococci recovered from lung homogenates, with the bacterial yield decreasing by almost 50% when centrifuged at 1500 × *g* compared with samples centrifuged at 1000 × *g*.

To examine if a higher centrifugation speed reduced the percentage of eukaryotic cells in the supernatant, samples centrifuged at 1000 × *g* or 1500 × *g* were subsequently centrifuged at 15,500 × *g* and RNA was extracted from tissue/bacterial pellets using the acid-phenol method described previously, then DNase treated for 1 hr at 37°C. Brain and lung samples were enriched for prokaryotic mRNA using MicrobEnrich (Ambion), and then linearly amplified (SenseAmp, Genisphere). It has been estimated that 16S rRNA comprises approximately 27% of total RNA in a bacterial cell (Neidhardt *et al.*, 1996). Based on this assumption, real-time RT-PCR using 16S rRNA oligonucleotide primers was used to estimate the quantity of pneumococcal RNA recovered, which was then compared to the total RNA concentration estimated by A_{260} measurements (Table 3.4). For both lung and brain samples, the higher initial centrifugation speed resulted in a lower bacterial yield and a lower purity of prokaryotic RNA. That is, when centrifuged at 1500 × *g*, pneumococci were more readily pelleted than at 1000 × *g*, whereas the tissue was not. Therefore, tissue samples were centrifuged at 1000 × *g* for all subsequent studies.

	CFU recovered/ml homogenate	% recovery of pneumococci
No tissue	2.43×10^5	-
Lung		
$1000 \times g$	1.21×10^5	50
$1500 \times g$	6.38×10^4	26
Brain		
$1000 \times g$	8.63×10^4	36
$1500 \times g$	5.98×10^4	25

Table 3.3: **Pneumococcal recovery from host tissue following differential centrifugation**

S. pneumoniae THY cultures were combined with lung or brain tissue homogenates and then centrifuged at either $1000 \times g$ or $1500 \times g$ for 4 min at 4°C. Supernatants were removed and centrifuged again at the same speed. All cultures (including a culture prepared with PBS instead of homogenate) were centrifuged at 15,500 $\times$ g to pellet pneumococci, and pellets were resuspended in PBS. An aliquot was removed and grown on BA for enumeration. The yields from cultures containing homogenised tissue were compared to the control culture and presented as a % recovery of pneumococci.

	Total [RNA] ng/ul[a]	Estimated [bacterial RNA] ng/ul[b]	% bacterial RNA[c]
Lung			
1000 × *g*	1249	466	37
1500 × *g*	1015	225	22
Brain			
1000 × *g*	551	242	48
1500 × *g*	501	145	26

Table 3.4: **Efficiency of centrifugation at different speeds in separating pneumococci from host cells**

S. pneumoniae D39 THY cultures were combined with lung or brain tissue homogenates and differentially centrifuged twice at either 1000 × *g* or 1500 × *g*, as described in the text. All cultures were then centrifuged at 15,500 × *g* to pellet bacteria. RNA was extracted from pellets then enriched and amplified, as described in the text, and RNA was estimated by either absorbance readings or real-time RT-PCR. [a] Indicates total RNA determined by $A_{260/280}$ readings. [b] Indicates estimated [bacterial RNA] based on real-time RT-PCR quantitation of 16S rRNA compared to a standard curve performed with known concentrations of D39 total RNA, and adjusted on the assumption that 16S rRNA comprises 27% of total RNA in a prokaryotic cell (Neidhardt *et al.*, 1996). [c] Indicates the estimated percentage of total RNA in tissue homogenates following differential centrifugation that was prokaryotic RNA.

3.6.3 Enrichment of prokaryotic RNA and linear amplification

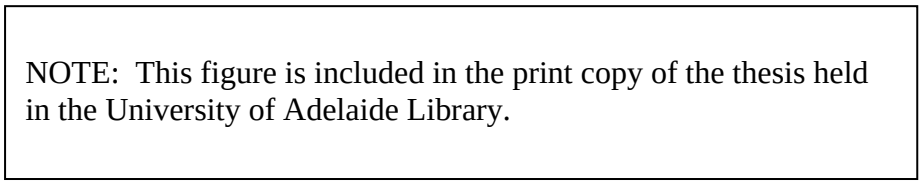
Eukaryotic mRNA, 18S rRNA and 28S rRNA were removed from *in vivo* RNA samples using MicrobEnrich (Ambion) according to the manufacturer's instructions. By removing eukaryotic RNAs, the relative proportion of prokaryotic RNA in a sample increases. Therefore, the efficiency of subsequent linear amplification reactions is also increased. Initially, RNA samples were purified by using RNAeasy clean-up columns (Qiagen). Samples were then enriched twice using a pull-down method which employed magnetic beads coated with capture oligonucleotides for polyadenylated mRNA, 18S rRNA and 28S rRNA to remove many other RNAs present in the sample; $A_{260/280}$ readings were obtained before and after each enrichment and RNA quantity was calculated. To test the viability of enrichment for bacterial RNA using the MicrobEnrich kit, lungs recovered from D39-infected mice were homogenised (Section 2.4.2 to 2.4.4) and RNA was extracted (as described in Section 2.5.1). 16 µg of the lung-derived RNA preparation was enriched. 25 µg of a control RNA sample (which was a mixture of 25 µg rat RNA and 2 µl *E. coli* RNA at 1 mg/ml, included in the MicrobEnrich kit) was enriched in tandem. Following the enrichment, the RNA quantity in the lung-derived sample decreased by 40% (returning a yield of 14.9 µg), which was a notable reduction in eukaryotic RNA using MicrobEnrich. 5.9 µg of control RNA was recovered following enrichment, indicating a 76% reduction in amount of RNA. Considering that bacterial RNA species comprised 7% of the original control RNA, and that MicrobEnrich does not remove small RNAs such as 5S rRNA and tRNA, the control reaction confirmed the kit was functioning efficiently. A second enrichment of both lung-derived RNA and control RNA did not result in a further reduction in RNA quantity (data not shown).

Previously, *in vivo* gene expression studies for bacteria such as *S. pneumoniae* had

been restricted by inherent difficulties encountered with harvesting sufficient numbers of bacteria from experimental animals and the concomitant low yields of bacterial mRNA. This is particularly problematic in niches where the bacteria exist at low levels, such as during asymptomatic carriage in the nasopharynx. This problem was circumvented by using a novel advanced RNA linear amplification kit, SenseAMP (Genisphere), which uses random primers to synthesise cDNA from prokaryotic RNA. A poly-T tail is then added to the 3' end of cDNA using terminal deoxynucleotidyl transferase. Linear amplification is driven by a T7 phage promoter, which is incorporated at the end of the synthetic poly-T tail (Figure 3.14). A second round of amplification was performed for all samples in order to obtain sufficient quantities of *in vivo*-derived RNA for analysis by real-time RT-PCR using LUX primers. To determine the integrity of two rounds of linear amplification, an RNA sample was prepared from *S. pneumoniae* D39 grown in THY broth to mid-exponential phase. The RNA sample was amplified twice using SenseAMP, according to the manufacturer's instructions. The original sample and the twice-amplified sample were analysed for several mRNAs (*pspA*, *ply*, *cps2A* and *piaA*) using real-time RT-PCR. The fold difference between the amount of mRNA present for each gene before amplification and after amplification was determined relative to the internal control 16S rRNA. Statistical analysis (Student's two-tailed *t*-test) of quadruplicate reactions showed that there was no significant difference between the relative proportion of a given mRNA species after the two rounds of amplification ($P < 0.05$) (Table 3.5). This analysis indicated that amplifying an RNA sample twice reasonably maintains the proportions of mRNAs and, therefore, is appropriate to use prior to mRNA analysis.

Figure 3.14 **Linear amplification using SenseAmp (Genisphere)**

This diagram depicts the steps involved in linear amplification using SenseAmp. **1)** RNA is primed using random oligonucleotides and cDNA is produced. **2)** First strand cDNA is tailed with dTTP using Terminal Deoxynucleotidyl Transferase. **3)** The T7 template is annealed to the 3' tail of the cDNA (via. a polyA sequence). Klenow enzyme fills in the 3' end of the first strand cDNA to produce a double-stranded T7 promoter. The T7 template contains a blocker to prevent second strand synthesis. **4)** Copies of the original RNA molecules are generated. Adapted from Genisphere SenseAmp protocol (2006).


NOTE: This figure is included in the print copy of the thesis held in the University of Adelaide Library.

Gene	Preservation of mRNA proportion after amplifications[a]	*P*-value[b]
pspA	1.3	NS
ply	1.1	NS
cps2A	1.3	NS
piaA	1.2	NS

Table 3.5: **Preservation of relative amounts of mRNA between linear amplifications using SenseAmp**

RNA was extracted from *S. pneumoniae* D39 grown in THY and amplified twice, as described in the text. The fold differences in the amount of mRNA present for *pspA, ply, cps2A* and *piaA* in the original RNA extract and after the two rounds of amplification were determined relative to the internal control 16S rRNA. [a] is the fold- difference in [mRNA] between the original and amplified RNA samples and indicates the magnitude of the bias that two amplifications using SenseAmp (Genisphere) conferred to an RNA sample, where 1.0 is no bias. [b] indicates the *P*-value, which was calculated using a two-tailed Student's *t*-test, where NS denotes a value > 0.05. The *P*-value indicates whether the relative amounts of mRNA present after amplification were statistically different to the relative amounts of mRNA present before amplification.

3.7 Discussion

The aim of the work in this chapter was to firstly establish an IN infection model in CD-1 mice for at least two *S. pneumoniae* strains, and secondly to optimise methods for isolation and amplification of bacterial mRNA in sufficient quantities and purity to enable comparison of relative expression of virulence genes in distinct niches.

Although *S. pneumoniae* is a strict human pathogen, several animals are capable of replicating certain aspects of its pathogenesis in the natural host. The chinchilla model is commonly used to study otitis media and, in some instances, nasopharyngeal carriage. Meningitis is often examined using a rabbit model. However, due to the handling, expense and availability considerations, the mouse model of infection is favoured when investigating pneumococcal colonisation, lung infection and sepsis. The pneumococcus is primarily a respiratory pathogen, with the development of invasive disease being largely incidental. Transmission occurs by the aerosol route. Therefore, an intranasal challenge route is considered to more accurately represent natural acquisition of the pneumococcus, than other commonly used challenge routes such as IP.

Wu *et al.* (1997) described a murine IN infection model, applicable to a number of different mouse strains. In the present study, this model was adapted to CD-1 mice, such that pneumococci can be harvested from several niches in numbers sufficient for RNA analysis. The time between euthanasia of each mouse and the snap-freezing of harvested bacterial pellets was approximately 15 to 20 min, which is 4 to 5 times the half-life at room temperature of the mRNA examined in this chapter. Maintaining bacterial cultures on ice over a time-course increased the half-lives of the transcripts to 13 to 14 min. This emphasised the need for a model where bacterial samples can be harvested, chilled and

processed quickly in order to maintain the integrity of the RNA, and speed was taken into consideration when optimising downstream processes such as differential centrifugation. The use of blood perfusion with ice-cold PBS prior to removal of the lungs and brain rapidly chilled these organs, and also ensures that pneumococci present in the lungs or brain samples possess minimal contamination with bacteria present in the blood. The efficiency of this process was demonstrated by the observation that mice with high levels of bacteraemia did not always contain detectable numbers of pneumococci in the lungs and brain after perfusion.

Stringent screening methods were employed for determining the presence of contaminating bacteria (such as oral streptococci) in the samples harvested from each niche, which is particularly important considering that visual recognition of contaminating bacteria is not always possible. A small proportion of CD-1 mice were found to possess *E. faecalis* in their lungs following infection with *S. pneumoniae*. Mice challenged IN with pneumococci were found to possess much higher numbers of enterococci in lungs than control mice challenged with PBS, indicating that pneumococcal infection augments the spread of *E. faecalis*. Although *E. faecalis* is not generally a respiratory pathogen, it may be opportunistic. Following infection with pneumococci, epithelial damage and decreased ciliary beating may reduce the clearance of enterococci that have been aspirated into the lungs incidentally. Another contributing factor could be the poor hygiene exhibited by ill mice. In addition to visual identification difficulties and contaminating 16S rRNA, it is possible that the presence of *E. faecalis* would influence the course of pneumococcal infection. As a consequence, samples harvested from mice possessing contaminants of any nature were excluded from further analysis. The isolation of contaminating bacteria that appears visually similar to *S. pneumoniae* when grown on BA and is resistant to

gentamicin, reinforces the requirement for stringent testing of bacteria isolated from laboratory animals during *in vivo* experimentation.

Strains D39 (a serotype 2 strain) and WCH16 (a serotype 6A strain) were chosen to examine mRNA expression changes. Both D39 and WCH16 were shown to effectively colonise the nasopharynx for up to 72 hr, and cause invasive disease in the CD-1 model described. D39 caused lung disease and sepsis, whereas WCH16 progressed to the blood and brain without establishing significant infection in the lungs. Additionally, WCH16 was able to persist at a moderate level in the bloodstream over 72 hr, and many mice infected with WCH16 also developed meningitis. To further investigate the pathogenesis of the two strains, CD-1 mice were IN-infected with either a D39 or WCH16 strain containing a plasmid constitutively expressing bacterial luciferase (Table 2.1). Using a Lux reporter, the same mouse can be imaged at several time-points during infection, conferring a significant advantage over models requiring mice to be euthanased at each time point in order to enumerate bacterial loads. This allows pathogenesis to be studied non-invasively and in real time, albeit at the expense of sensitivity (Francis *et al.*, 2000). Considering the benefits and limitations of both methods, using bioluminescence data and organ harvesting studies in tandem is likely to give a more comprehensive picture of pathogenesis than independently. Monitoring the mice over a 7-day period demonstrated that challenge of CD-1 mice with D39LUX established systemic infection followed by death within 24 hr of these niches being infected. In contrast, WCH16 generally caused what appeared to be infection of the brain and blood in the absence of lung infection, perhaps indicating translocation across the nasopharyngeal epithelia. This correlated with the results obtained by dissecting mice and enumerating bacteria present in these niches in Sections 3.2 and 3.4. Mice IN challenged with WCH16 had a mean survival time

approximately 50 hr longer than D39. The greater survival time of WCH16-infected mice may be largely attributable to WCH16 being less proficient in causing sepsis following establishment of bacteraemia than D39. This disparity between serotypes was also observed in real-time using a *lux* reporter, with WCH16LUX-infected mice exhibiting bacteraemia surviving several days and, in some cases, clearing the blood infection.

In contrast to D39 and WCH16, the serotype 4 strains examined (TIGR4 and WCH43) showed poor progression to bacteraemia by 72 hr, despite previous reports of TIGR4 virulence and high levels of sepsis in outbred MF1 mice (Ibrahim *et al.*, 2004; Ibrahim *et al.*, 2005). TIGR4 also showed inconsistent growth *in vitro*. Because of this, these strains were not examined further.

The separation of pneumococci from lung and brain homogenates has been refined in this chapter, allowing for greater efficiency during downstream processing. A higher initial centrifugation speed resulted in a lower yield of bacteria from the tissue suspension, and also a lower ratio of prokaryotic: eukaryotic RNA. These results indicated that an increased intial centrifugation speed did not result in the removal of a higher percentage of eukaryotic cells from the supernatant, but did cause a greater number of pneumococci to be pelleted and hence lost. Accordingly, initial centrifugation conditions were standardised at 1000 × *g* for 6 min. Following bacterial differential centrifugation, pneumococcal RNA recovered from bacteria from the lungs and brain was enriched and amplified. All RNA is tailed and amplified using SenseAmp. Therefore, it was desirable to increase the efficiency of linear amplification for pneumococcal RNA by decreasing the proportion of contaminating eukaryotic RNA. MicrobEnrich, a kit using a pull-down method with magnetic beads coated with capture oligonucleotides for polyadenylated mRNA, 18S

rRNA and 28S rRNA, was employed to reduce eukaryotic RNAs present in the sample. While a single enrichment was found to reduce $A_{280/600}$ readings compared to the original sample, subsequent enrichments did not, indicating that one enrichment was sufficient to remove the majority of eukaryotic RNA species in the pre-amplification sample. Subsequent linear amplification using SenseAmp was shown to preserve the relative 16S rRNA: mRNA ratio such that any fold differences observed between niches for a particular mRNA species, or between species within a niche, could be considered to be a reasonable approximation of real levels and not an artefact of biased amplification. Using the above procedures, sufficient mRNA could be obtained even from *in vivo* niches where pneumococci persist in low numbers to perform quantitative RNA assays.

In this chapter, a CD-1 IN challenge model and downstream RNA extraction and processing techniques are described, establishing conditions whereby *S. pneumoniae* RNA can be extracted in sufficient amounts to examine relative virulence gene expression *in vivo*, as will be described in the following chapters. Moreover, this model allows the transcriptional analysis of bacteria recovered from various *in vivo* niches in a single animal model, including pneumococci involved in nasopharyngeal colonisation, which has not been directly examined in the literature due to technical limitations.

CHAPTER 4 – Analysis of key virulence gene expression (for D39 and WCH16) *in vivo* by real-time RT-PCR

4.1 Introduction

S. pneumoniae is able to proliferate in a number of niches within its host. Most commonly, the pneumococcus exists asymptomatically in the nasopharynx. From this niche, it can translocate up the Eustachian tube into the middle ear and cause otitis media or be aspirated into the lungs and cause pneumonia. Bacteria can enter the bloodstream either from the lungs, or directly from the nasopharynx. Pneumococci can then cross the blood-brain barrier and proliferate in the brain causing meningitis (McCullers and Tuomanen, 2001). It is likely that many genes are regulated to enable the pneumococcus to survive in these varied environmental niches, but such regulation is poorly understood. Since transcription and translation are often coupled in prokaryotes (Campbell, 1996), using quantitative gene expression techniques such as real-time RT-PCR to assay mRNA levels may give an indication of relative protein levels and, therefore, reflect functional regulation. Previously, technical limitations have restricted quantitative experiments

examining pneumococcal gene expression *in vivo*, particularly during nasopharyngeal colonisation. In this chapter, the mRNA levels for specific pneumococcal virulence factors were assayed from resident bacteria in the nasopharynx, lungs, blood and brain using real-time RT-PCR. Two strains were examined; the serotype 2 strain D39 and the serotype 6A strain WCH16, both of which had been shown to reliably colonise and cause disease in a CD-1 IN infection model (Chapter 3).

4.2 Differential expression of D39 and WCH16 virulence genes *in vivo*

4.2.1 Recovery of bacteria from CD-1 mice

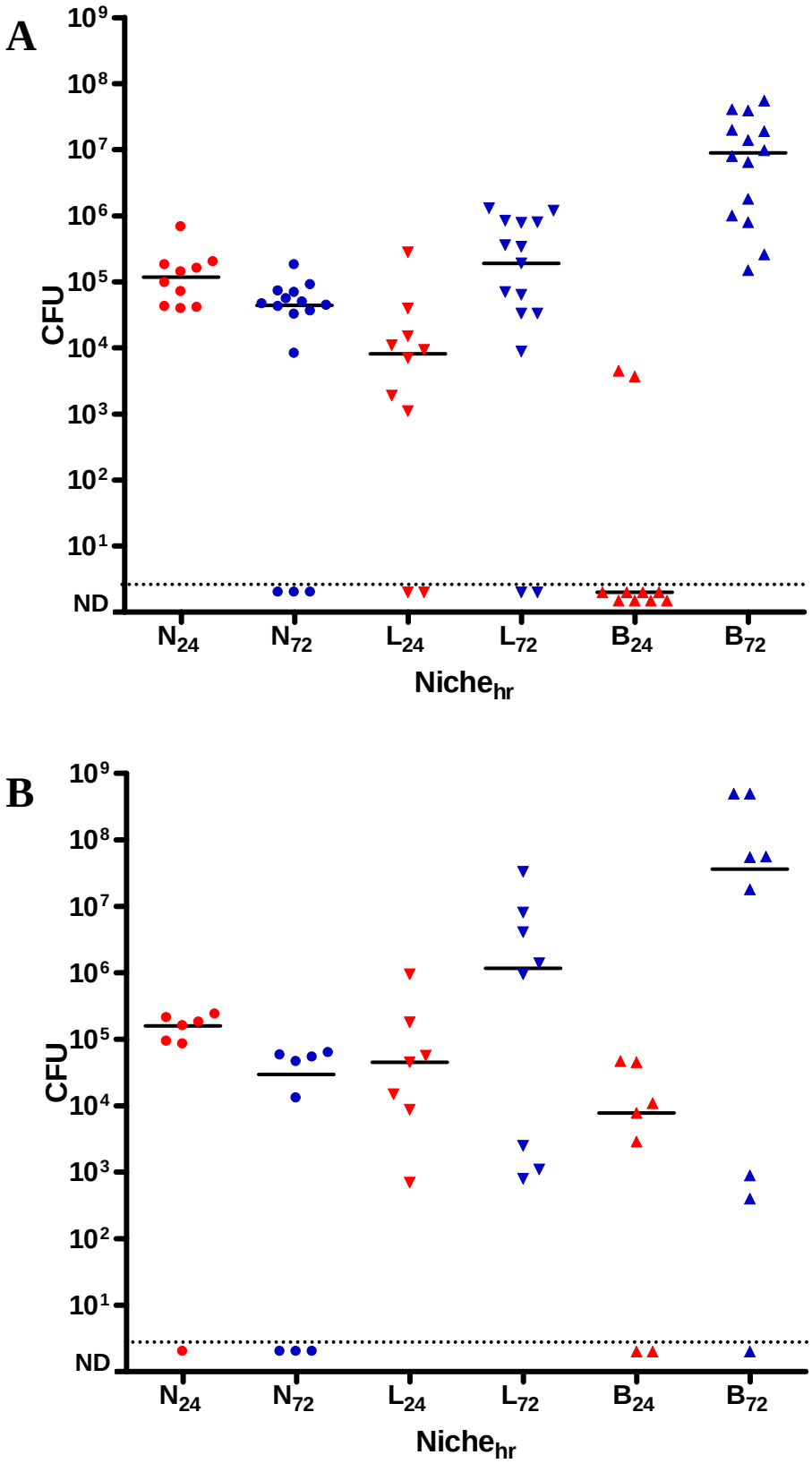
Using the model established in Chapter 3, D39 and WCH16 bacteria were recovered from various *in vivo* niches. CD-1 mice were anaesthetised and challenged with D39 or WCH16 as described in Sections 2.4.2 and 2.4.3. This experiment was performed twice for both D39 and WCH16. For the D39 experiments (D39.1 and D39.2) the challenge dose for each mouse was 1.15×10^7 and 2.8×10^7, respectively. In D39.1, 25 mice were challenged, and 10 mice were sacrificed at the 24 hr time point. In D39.2, 18 mice were challenged and seven were sacrificed at 24 hr. The remaining mice were euthanased at 72 hr post-challenge. The experiments performed in Chapter 3 indicated nasopharyngeal colonisation was established for both D39 and WCH16 by the latter time point and bacteria were also present in other niches, indicating disease. One mouse in D39.1, and four mice in D39.2, died prior to the 72 hr time point. The surviving animals appeared ill, and displayed typical symptoms of sepsis such as hunched appearance, lethargy, glazed eyes and poor hygiene. Bacteria were recovered from the nasopharynx,

lungs and blood of euthanased mice and prepared for RNA extraction (Section 2.4.4). Samples were also plated for CFU enumeration (Section 2.4.5). Typically, 10^6 to 10^8 bacteria/ml of blood were recovered from mice that were sacrificed at 72 hr. Almost all sacrificed mice were colonised with approximately 10^5 bacteria per nasopharynx and possessed pneumococci in their lungs at both time points, indicating the establishment of both carriage and lung disease. The CFU recovered from each of the niches for D39.1 and D39.2 are shown in Figures 4.1A and 4.1B, respectively.

For the WCH16 experiments (WCH16.1 and WCH16.2), 25 CD-1 mice were anaesthetised and challenged with 1.7×10^7 and 3×10^7 bacteria, respectively. At 24 hr and 72 hr post-challenge, bacteria were recovered from the nasopharynx, the lungs, the blood and the brain of 10 and 15 euthanased mice, respectively, and prepared for RNA extraction (Section 2.4.4). In addition to the three niches that were examined for D39-infected mice, the brains were also excised from mice challenge with WCH16, as this strain had been observed in Chapter 3 and by luminescence work in Chapter 4 to reach this niche rapidly following IN infection. In contrast to D39-infected animals, mice euthanased at 72 hr appeared relatively healthy, generally exhibiting none of the symptoms commonly associated with sepsis. Accordingly, only moderate numbers of bacteria were recovered from the bloodstream at this time point, and no mice died during the course of either experiment. All mice except one were colonised at a level of approximately 10^5 bacteria per nasopharynx in both experiments, which is consistent with that seen in the pilot experiment. Many mice also possessed low numbers of bacteria in the brain at both 24 and 72 hr post-challenge. Because all the mice that exhibited early meningitis also had bacteraemia, it is likely that pneumococci disseminated to the brain via the bloodstream. Alternatively, bacteria may have translocated from the nasopharynx to the brain by axonal

***Figure 4.1*: D39 pathogenesis during IN challenge experiments D39.1 and D39.2**

D39 was cultured in THY until mid-exponential phase and CD-1 mice were challenged IN. Mice were euthanased at 24 and 72 hr post-challenge, and nasopharyngeal washings (N), lung homogenates (L) and blood samples (B) were diluted and plated on BA for enumeration. Data are CFU per niche for nasal wash and lungs, and CFU/ml for blood. The time-point is indicated in subscript. **A)** and **B)** represent data obtained from the two independent experiments D39.1 and D39.2 (challenge doses 1.15×10^7 and 2.8×10^7 CFU respectively). The median for each time/niche is indicated by a bar, and the limit of detection (40 CFU) is shown by a dotted line. ND denotes no growth, i.e. <40 CFU.


A
B
CFU
ND
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
N_{24}
N_{72}
L_{24}
L_{72}
B_{24}
B_{72}
$Niche_{hr}$

transport through olfactory nerves (van Ginkel *et al.*, 2003). The CFU recovered from each of the niches for WCH16.1 and WCH16.2 are shown in Figures 4.2A and 4.2B respectively.

4.2.2 Extraction of *in-vivo* derived RNA

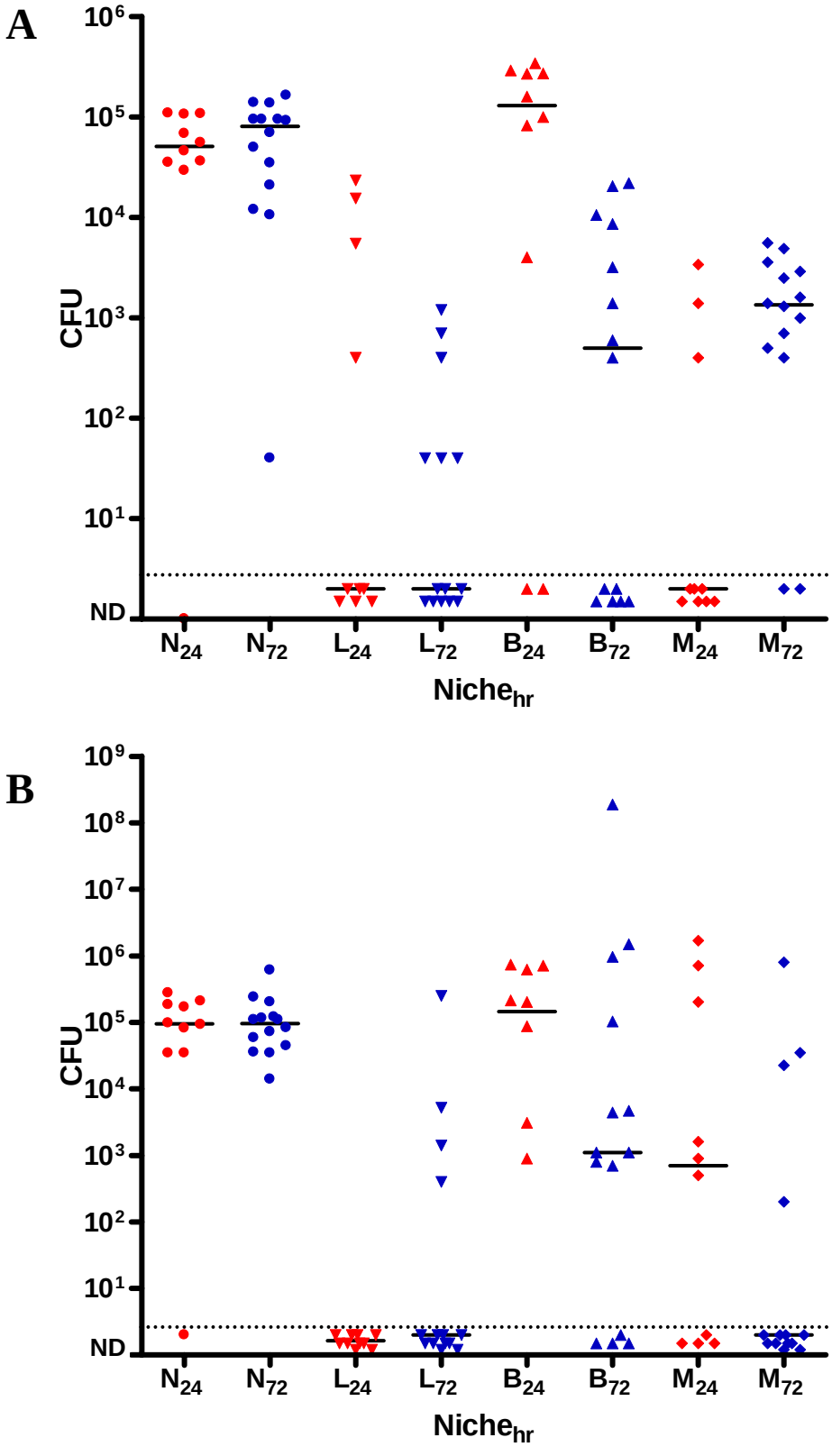
After harvesting the pneumococci from CD-1 mice as described in Section 4.2.1, RNA was extracted from the bacterial pellets using the acid-phenol method (Section 2.5.1), and treated with DNase to remove DNA. Four to five samples per niche at each time point were pooled, based on the absence of contaminating bacteria, the number of CFU harvested from the mouse, the amount of RNA present in the sample (determined by $A_{260/280}$ readings), and the absence of contaminating DNA (based on visual appearance on an agarose gel following PCR with and without reverse transcriptase using 16S rRNA primers). Subsequently, RNA samples obtained from the lungs and the brain were enriched for prokaryotic RNA using MicrobEnrich (Ambion), and linearly amplified twice for prokaryotic mRNA as described in Sections 2.5.2 and 2.5.3. Recombinant RNAsin RNAse inhibitor was added, and RNA was stored at –80 °C until use.

4.2.3 Preparation of RNA from *in vitro*-cultured pneumococci

For comparative purposes, an *in vitro* pneumococcal RNA sample was prepared against which all *in vivo*-recovered samples were compared. D39 or WCH16 pneumococci were cultured in THY to A_{600} = 0.25, a medium and optical density identical to the pneumococcal challenge cultures, and RNA samples were prepared as described in Section 2.5.1. The CFU/ml in the culture was determined retrospectively by plating dilutions on BA, and was found to be extremely similar to the CFU/ml of the challenge cultures. In this way, a comparison could be made between pneumococcal gene expression prior to mouse

***Figure 4.2*: WCH16 pathogenesis during IN challenge experiments WCH16.1 and WCH16.2**

WCH16 was cultured in THY until mid-exponential phase and CD-1 mice were challenged IN. Mice were euthanased at 24 and 72 hr post-challenge, and nasopharyngeal washings (N), lung homogenates (L), blood samples (B) and brain homogenates (M) were diluted and plated on BA for enumeration. Data are CFU per niche for nasal wash and lungs, and CFU/ml for blood. The time-point is indicated in subscript. **A)** and **B)** represent data obtained from the two independent experiments WCH16.1 and WCH16.2 (challenge doses 1.7×10^7 and 3×10^7 CFU respectively). The median for each time/niche is indicated by a bar, and the limit of detection (40 CFU) is shown by a dotted line. ND denotes no growth, i.e. < 40 CFU.


A
10^6
10^5
10^4
10^3
10^2
10^1
ND
CFU
N_{24}
N_{72}
L_{24}
L_{72}
B_{24}
B_{72}
M_{24}
M_{72}
$Niche_{hr}$
B
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
10^1
ND
CFU
N_{24}
N_{72}
L_{24}
L_{72}
B_{24}
B_{72}
M_{24}
M_{72}
$Niche_{hr}$

challenge, and several days post-challenge.

4.2.4 Quantification of mRNAs using real-time RT-PCR

In this study, the differential expression of well characterised virulence genes *cbpA, pspA, ply, psaA, cps2A, piaA, nanA and spxB* was examined. These genes were chosen based on their putative roles in virulence and, in some instances, potential of their proteins as vaccine candidates. The abundance of each transcript present in amplified RNA recovered from nasopharyngeal, lung and blood-borne pneumococci was quantitated by real-time RT-PCR (Section 2.6.2.1). Gene-specific LUX fluorogenic primer sets labelled with JOE were designed using the Invitrogen primer designer software, employing primers specific for 16S rRNA as an internal control (Table 2.2). The amplicons for the LUX primer pairs used in this study were between 81 and 166 bp (with most amplicon sizes being 90-110 bp) and the T_m for all LUX primers were similar. 16S rRNA was chosen for use as an internal control in these experiments from a group of several other housekeeping genes, as it could not be determined if other candidates would maintain the same expression level in the niches examined in this chapter and during the different conditions presented by disease. 16S rRNA is commonly used as a bacterial internal control in quantitative RNA assays, as it is a reliable estimate of defined amounts of RNA under different environmental conditions (Lion, 2001; Tasara and Stephan, 2007). For 16S rRNA, *ply*, *psaA*, *piaA*, *nanA* and *spxB*, the same LUX fluorogenic primers were used for analysis of D39 and WCH16 mRNAs, due to high nucleotide sequence homology between the strains for the target genes. Serotype- or strain-specific primers were designed for *cbpA*, *pspA* and *cpsA* (Table 2.2). Quantitative fold differences between niches for each transcript were determined using the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001). Amplification data for each gene were then standardised using values obtained for

the 16S rRNA control, and presented as a relative fold increase/decrease between niches (described in Section 2.6.2.2). Data for each independent experiment were analysed relative to the level of the respective transcript in *S. pneumoniae* D39 grown in THY broth (Figures 4.3 and 4.4). The fold-differences in relative mRNA between niches for each experiment are presented in Table 4.1 and 4.2. Likewise, data obtained from the two WCH16 experiments are presented as transcript levels in each niche relative to *in vitro* (Figures 4.5 and 4.6), and the fold-differences in relative mRNA levels between niches are presented in Tables 4.3 and 4.4.

The trends in gene expression for *cbpA*, *pspA*, *cps2A*, *piaA*, *ply*, *nanA* and *spxB* were reproducible at 72 h post-challenge between the two replicate experiments for D39. *cbpA* mRNA was detected at the highest levels in bacteria recovered from the nasopharynx and lungs, and at lower levels in bacteria from the blood. Similarly, the level of *pspA* mRNA was highest in bacteria harvested from the nasopharynx and blood, with lower levels detected from pneumococci recovered from the lungs. In contrast, *cps2A* was expressed at a relatively constant level in the nasopharynx, lungs and blood. The levels of both *nanA* and *spxB* transcripts were higher in bacteria recovered from the nasopharynx than the lungs or blood. However, *psaA* did not show a consistent difference in expression between niches. The combined data from both *in vivo* experiments and an *in vitro* sample are also depicted as adjusted mRNA concentration, by comparing the Ct values for each gene relative to that of 16S rRNA in the same sample (Figure 4.7). The results indicate that the mRNA of *spxB* was the most abundant *in vitro*, followed by that of *ply* and *cps2A*, with *nanA* mRNA being the least abundant. In the nasopharynx, the mRNA of *ply*, followed by that of *cps2A*, *pspA* and *psaA*, was highly abundant normalised against 16s rRNA, whereas *nanA* mRNA was the least abundant. In the lungs, however, the mRNA of *cps2A* was the

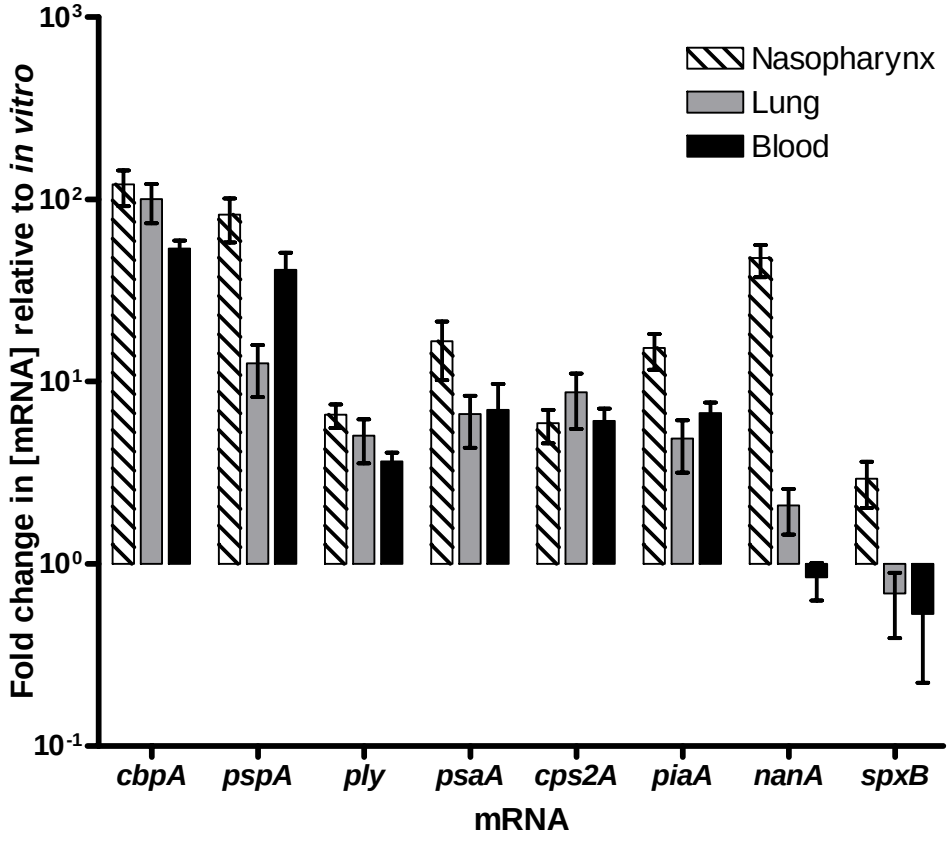


***Figure 4.3*: Expression of D39 genes *in vivo* by real-time RT-PCR in D39.1**

Following IN challenge with D39, mice were sacrificed at 72 hr post-challenge and RNA preparations were obtained from pneumococci in the nasopharynx, lungs and blood. Samples recovered from the same niche were pooled, as described in the text. Following RNA purifications and amplifications, mRNA was assayed by real time RT-PCR as described in the text. Data indicate the fold differences between mRNA present in different niches relative to pneumococci cultured *in vitro*. Error bars represent the SEM.

Gene	Fold difference		
	Nose/Lung	**Nose/Blood**	**Lung/Blood**
cbpA	1.2	2.2*	1.8*
pspA	6.5**	2.0*	0.3*
ply	1.3	1.8**	1.4
psaA	2.5	2.4*	1.0
cps2A	0.67	1.0	1.4
piaA	3.1**	2.3*	0.71
nanA	22.8**	56.6***	2.5*
spxB	4.2**	5.5**	1.3

***Table 4.1*: Relative amounts of mRNA present in different niches for D39.1**

This table indicates the relative fold differences between niches in experiment D39.1 (Figure 4.3). 16S rRNA was used as an internal control, and the quantitative fold increase or decrease for each transcript between niches was determined using the $2^{-\Delta\Delta Ct}$ method. The value represents the relative amount of mRNA in the first niche compared to the second. Data were analysed using Student's *t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

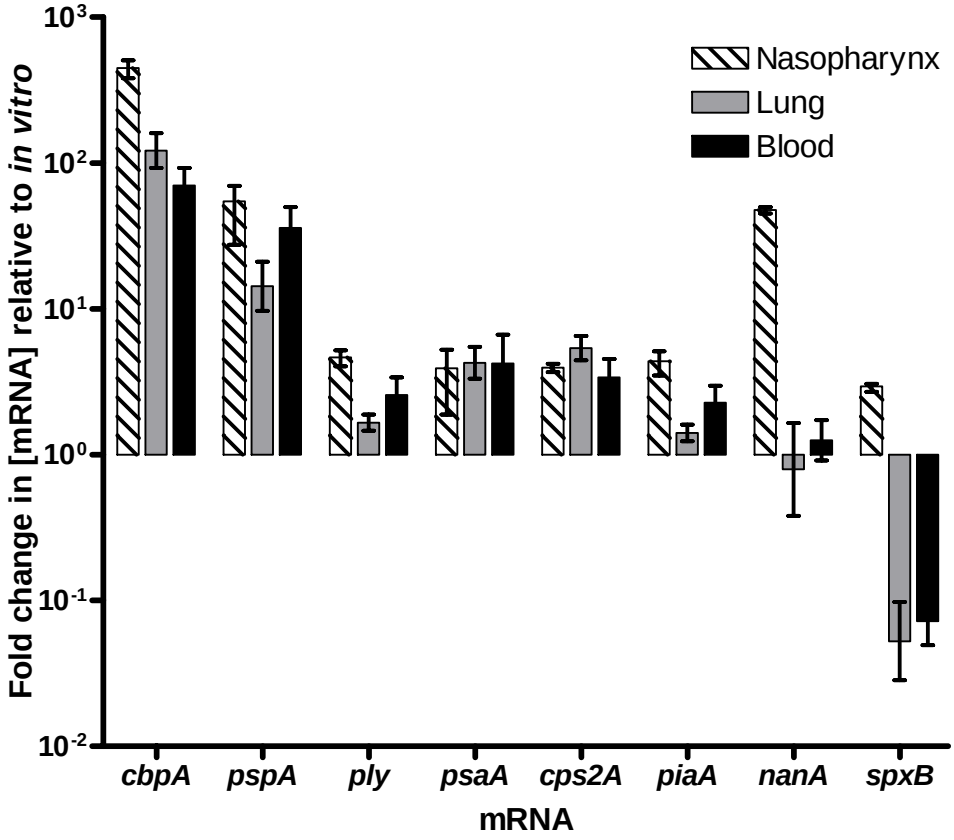


***Figure 4.4*: Expression of D39 genes *in vivo* by real-time RT-PCR in D39.2**

Following IN challenge with D39, mice were sacrificed at 72 hr post-challenge and RNA preparations were obtained from pneumococci in the nasopharynx, lungs and blood. Samples recovered from the same niche were pooled, as described in the text. Following RNA purifications and amplifications, mRNA was assayed by real time RT-PCR as described in the text. Data indicate the fold differences between mRNA present in different niches relative to pneumococci cultured *in vitro*. Error bars represent the SEM.

Gene	Fold difference		
	Nose/Lung	**Nose/Blood**	**Lung/Blood**
cbpA	3.7**	6.3***	1.7
pspA	3.8*	1.5	0.4*
ply	2.8***	1.5*	0.67
psaA	1.0	0.91	1.0
cps2A	0.71	1.2	1.6
piaA	3.1**	1.9*	0.63*
nanA	15.3**	11.1***	0.71
spxB	5.6*	4.1*	0.71

***Table 4.2*: Relative amounts of mRNA present in different niches for D39.2**

This table indicates the relative fold differences between niches in experiment D39.2 (Figure 4.4). 16S rRNA was used as an internal control, and the quantitative fold increase or decrease for each transcript between niches was determined using the $2^{-\Delta\Delta Ct}$ method. The value represents the relative amount of mRNA in the first niche compared to the second. Data were analysed using Student's *t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

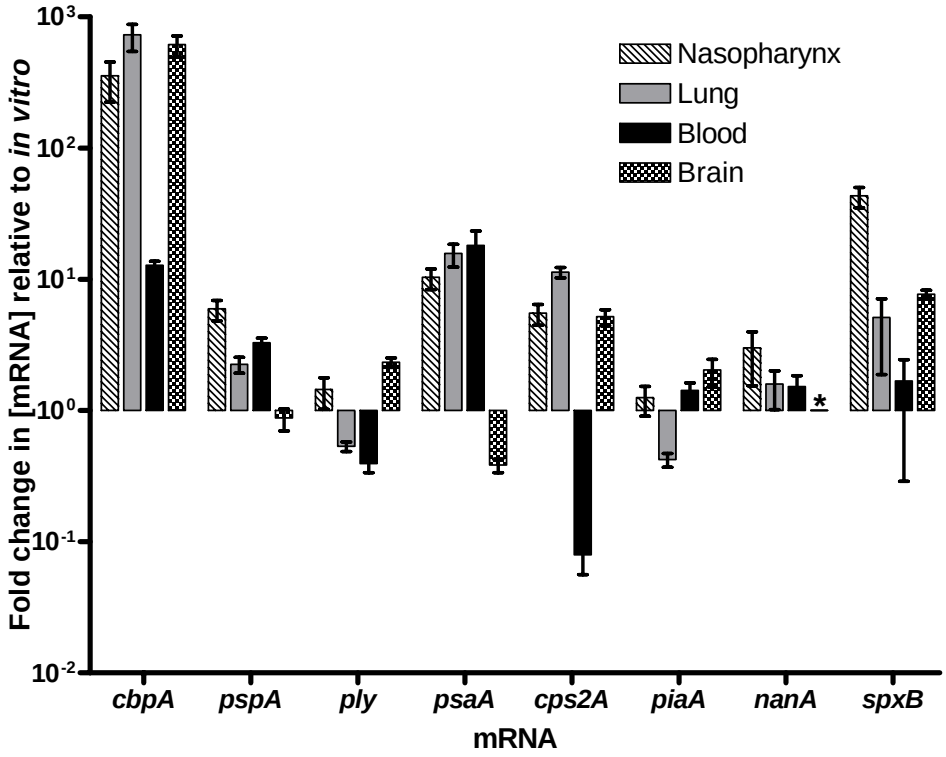


***Figure 4.5*: Expression of WCH16 genes *in vivo* by real-time RT-PCR in WCH16.1**

Following IN challenge with WCH16, mice were sacrificed at 72 hr post-challenge and RNA preparations were obtained from pneumococci in the nasopharynx, lungs, blood and brain. Samples recovered from the same niche were pooled, as described in the text. Following RNA purifications and amplifications, mRNA was assayed by real time RT-PCR as described in the text. Data indicate the fold differences between mRNA present in different niches relative to pneumococci cultured *in vitro*. Error bars represent the SEM. An asterisk indicates that data could not be obtained.

Gene	Fold difference					
	Nose/Lung	**Nose/Blood**	**Nose/ Brain**	**Lung/Blood**	**Lungs/Brain**	**Blood/Brain**
cbpA	0.5*	27.7**	1.0	56.8**	1.2	0.021***
pspA	2.6**	1.8*	6.0**	0.67*	2.6**	3.7***
ply	2.7*	3.7**	0.62**	1.4*	0.23***	0.17***
psaA	0.67	0.56	27.0***	0.83	41.0***	47.3**
cps2A	2.0**	69.7***	1.1	142.7***	2.2**	0.015***
piaA	3.0**	0.91	0.62	0.29**	0.21**	0.70
nanA	1.9	2.0	NA	1.0	NA	NA
spxB	8.5**	25.7***	5.6**	3.0	0.66	0.22**

***Table 4.3*: Relative amounts of mRNA present in different niches for WCH16.1**

This table indicates the relative fold differences between niches in experiment WCH16.1 (Figure 4.5). 16S rRNA was used as an internal control, and the quantitative fold increase or decrease for each transcript between niches was determined using the $2^{-\Delta\Delta Ct}$ method. The value represents the relative amount of mRNA in the first niche compared to the second. Data were analysed using Student's *t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. NA denotes data not available.

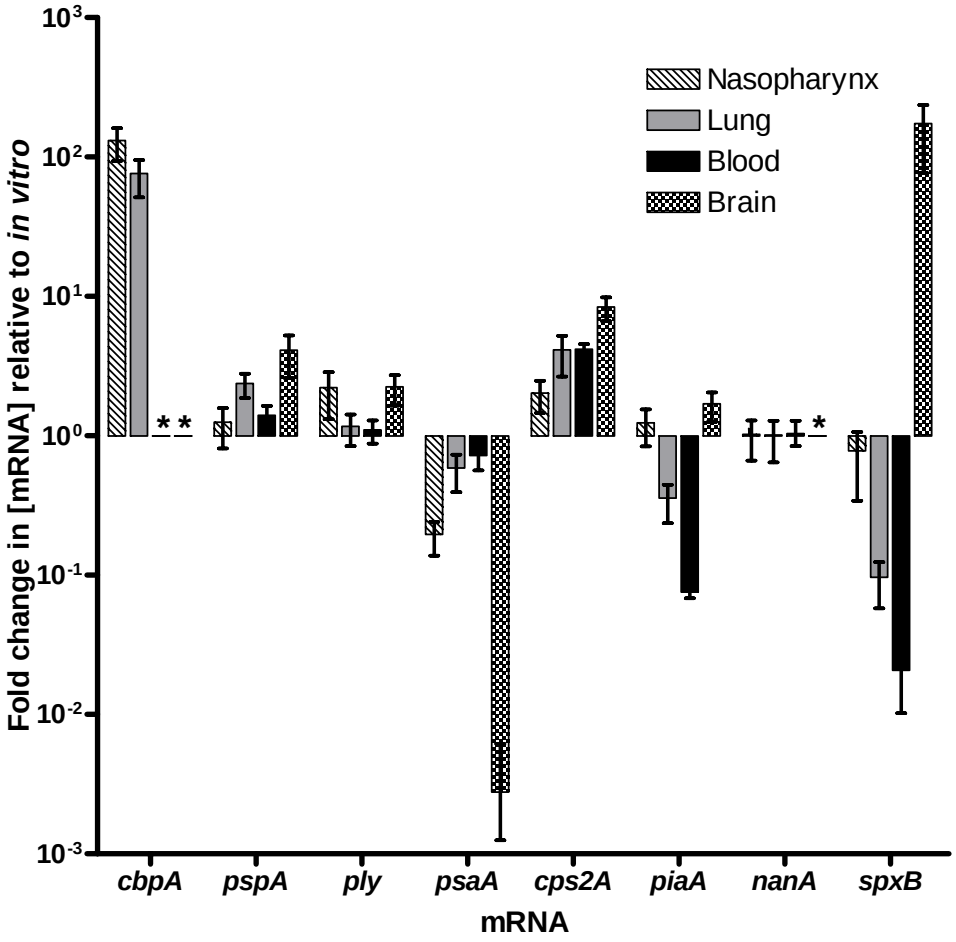


***Figure 4.6*: Expression of specific WCH16 genes *in vivo* by real-time RT-PCR in WCH16.2**

Following IN challenge with WCH16, mice were sacrificed at 72 hr post-challenge and RNA preparations were obtained from pneumococci in the nasopharynx, lungs, blood and brain. Samples recovered from the same niche were pooled, as described in the text. Following RNA purifications and amplifications, mRNA was assayed by real time RT-PCR as described in the text. Data represent the fold differences between mRNA present in different niches relative to pneumococci cultured *in vitro*. Error bars represent the SEM. An asterisk indicates that data could not be obtained.

Gene	Fold difference					
	Nose/Lung	**Nose/ Blood**	**Nose/Brain**	**Lung/ Blood**	**Lungs/Brain**	**Blood/Brain**
cbpA	1.7	NA	NA	NA	NA	NA
pspA	0.53*	0.91	0.31*	1.7*	0.59	0.34*
ply	1.8	2.0	1.0	1.0	0.53*	0.5*
psaA	0.33*	0.27**	705.2**	0.83	0.53**	2598***
cps2A	0.5	0.48**	0.24**	1.0	210*	0.5*
piaA	3.5*	16.3**	0.77	4.7**	0.21**	0.045**
nanA	1.0	1.0	NA	1.0	NA	NA
spxB	8.2*	37.5*	0.0045*	4.6*	.0007*	0.00015*

***Table 4.4*: Relative amounts of mRNA present in different niches for WCH16.2**

This table indicates the relative fold differences between niches in experiment WCH16.2 (Figure 4.6). 16S rRNA was used as an internal control, and the quantitative fold increase or decrease for each transcript between niches was determined using the $2^{-\Delta\Delta Ct}$ method. The value represents the relative amount of mRNA in the first niche compared to the second. Data were analysed using Student's *t*-test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$. NA denotes data not available.

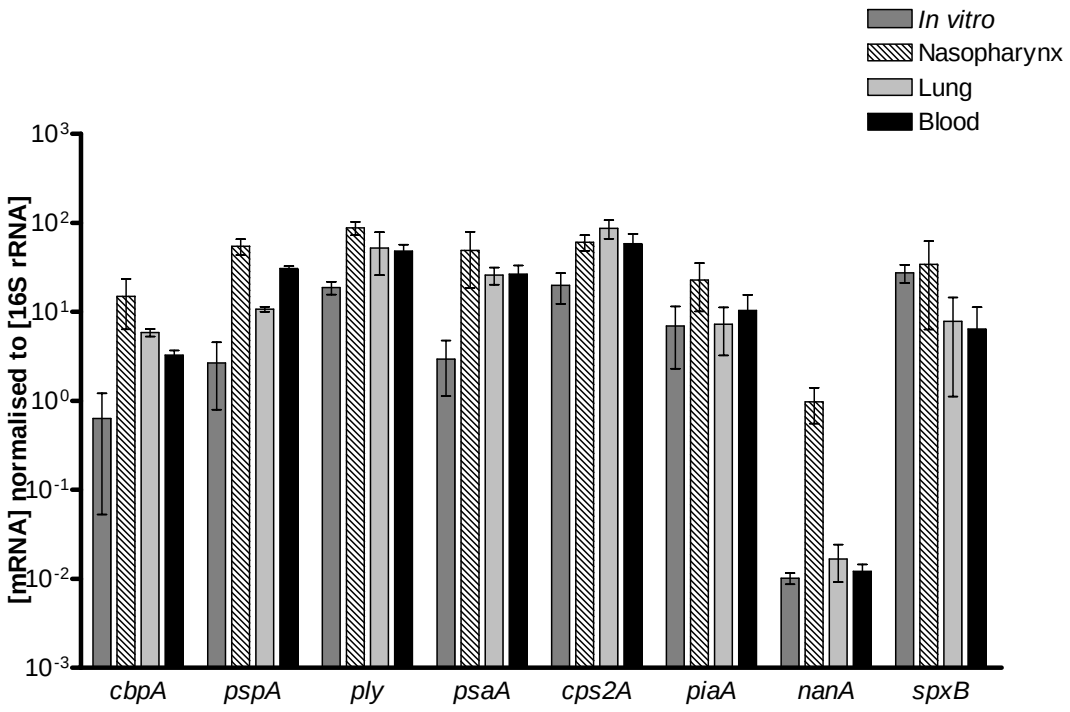


***Figure 4.7*: Expression of D39 genes *in vivo* by real-time RT-PCR**

Following IN challenge with D39, mice were euthanased at 72 hr post-challenge and RNA preparations were obtained from mice containing pneumococci in the nasopharynx, lungs and blood. Samples recovered from the same niche were pooled. Following RNA purifications and amplifications, mRNA was assayed by real time RT-PCR as described in the text. Ct data for each mRNA was normalised against 16S rRNA Ct values and adjusted as described in the text. Data from the two independent experiments (D39.1 and D39.2) were pooled, and are presented in the graph. The error bars represent the SEM.

most abundant, followed by that of *ply* and *psaA*. Again, *nanA* mRNA was the least abundant. In the blood, the mRNAs of *cps2A*, *ply*, *pspA*, *psaA* and *piaA* were present in high quantities, *cbpA* mRNA was present at a lower level, while *nanA* mRNA was the least abundant.

The WCH16 data from these two experiments indicate largely consistent trends in gene expression for *ply, psaA cbpA, nanA* and *spxB* at 72 hr post-challenge. *SpxB* mRNA levels were highest in bacteria recovered from the nasopharynx and the brains of mice, with substantially lower levels observed from bacteria recovered from the other niches. In contrast, the levels of *nanA* mRNA were expressed at a similar level in the nasopharynx, lungs, bloodstream and brain, and also in bacteria cultured *in vitro*. Levels of *cbpA* mRNA present in pneumococci from the blood and the brain in WCH16.2 and *nanA* mRNA harvested from the brain in both experiments were below the limit of detection for real-time RT-PCR, as indicated by a small cycle number difference observed between the sample and the no template control (NTC) for these transcripts. Despite the lack of data for these niches, it was still apparent that *cbpA* mRNA levels were detected at highest levels in bacteria recovered from the nasopharynx and lungs, compared to bacteria from the blood. In addition to Figures 4.5 and 4.6, which present data from each individual experiment, the combined data from both *in vivo* experiments and two *in vitro* samples are also presented as adjusted mRNA concentrations (Figure 4.8). Overall, the trends in gene expression observed for WCH16 were less consistent between experiments than for D39. This discrepancy could be due to the inconsistent level of bacterial infection observed for WCH16.1 and WCH16.2. A further WCH16 infection experiment needs to be conducted to clarify the results.

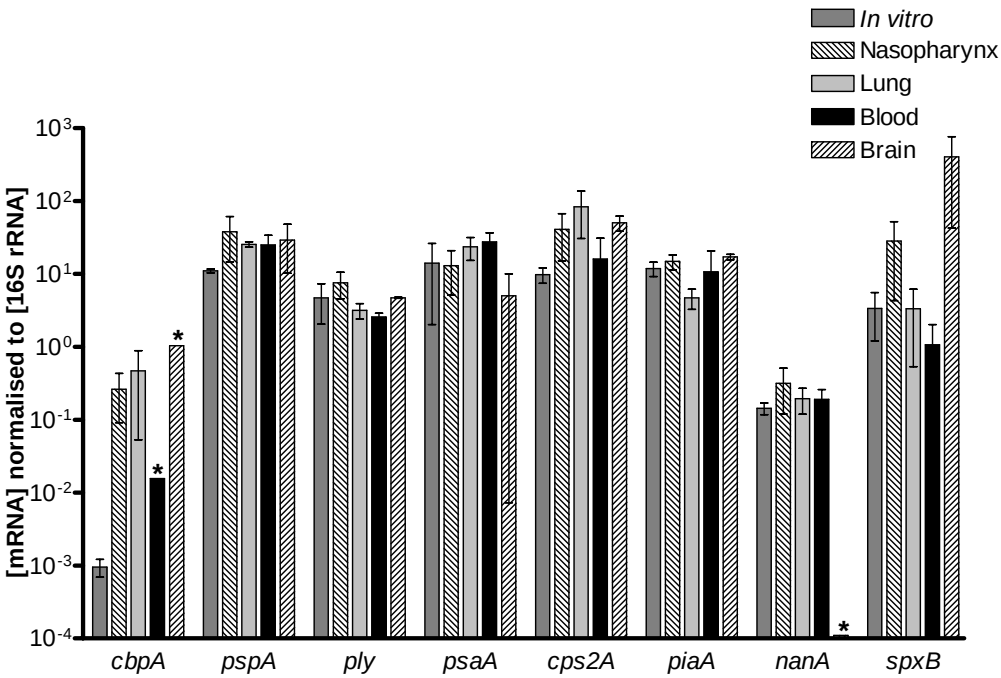


***Figure 4.8*: Expression of specific WCH16 genes *in vivo* by real-time RT-PCR**

Following IN challenge with WCH16, mice were euthanased at 72 hr post-challenge and RNA preparations were obtained from mice containing pneumococci in the nasopharynx, lungs, blood and brain. Samples recovered from the same niche were pooled. Following RNA purifications and amplifications, mRNA was assayed by real time RT-PCR as described in the text. Ct data for each mRNA was normalised against 16S rRNA Ct values and adjusted as described in the text. Data from the two independent experiments (WCH16.2 and WCH16.2) were pooled, and are presented in the graph. The error bars represent the SEM. An asterisk indicates that data could not be obtained for one or both experiments.

Niche-specific gene expression was also examined for bacteria recovered 24 hr post-challenge using the IN infection model described in Chapter 3 (data not shown). However, although data for some genes showed similar expression trends at this time point to those observed at 72 hr, the differential expression between niches was lower. The standard deviation between experiments was far greater for mice euthanased at the 24 hr time-point than at 72 hr. These issues can probably be attributed to the lower numbers of bacteria harvested from some niches at 24 hr, which may have led to inaccuracies in real-time RT-PCR quantitation. Small quantitation errors may be exacerbated for very low RNA samples, giving an exaggerated measure of up- or down-regulation. Additionally, it is likely that bacteria had been present in each niche for less time than pneumococci harvested at 72 hr and, therefore, may not have completely adjusted their transcriptional profiles, nor have elicited the same degree of host response as for the later time-point.

4.3 Discussion

S. pneumoniae can effectively colonise the nasopharynx and is also able to proliferate and cause disease in numerous other niches. The putative *in vivo* roles of several pneumococcal proteins involved in nasopharyngeal carriage or disease have been inferred using *in vitro* studies, as described in Chapter 1. The differential expression of several virulence genes between bacteria grown *in vitro* and bacteria harvested from a sepsis model has been previously reported (Ogunniyi *et al.*, 2002). However, at the commencement of this work, studies had not examined quantitative differences in specific virulence gene mRNA levels between niches of infection. The experiments presented in this chapter use the single, continuous infection model established in Chapter 3 to identify differential expression of key pneumococcal virulence genes during asymptomatic carriage

and disease. The genes chosen encode the virulence factors CbpA, PspA, Ply, PsaA, CpsA, NanA, PiaA and SpxB, all of which appear to play important roles in pneumococcal pathogenesis.

4.3.1 Gene expression during nasopharyngeal colonisation

Colonisation of the nasopharynx is considered a prerequisite for disease and provides a base niche from where pneumococci can progress to the lungs, bloodstream, middle ear or brain and cause disease. Adherence in the upper respiratory tract is considered to be facilitated by interactions between CbpA and hpIgR, an interaction that also promotes invasion of nasopharyngeal epithelial cells (Rosenow *et al.*, 1997; Zhang *et al.*, 2000). A second choline binding protein, PspA, interacts with the iron chelator apolactoferrin, inhibiting apolactoferrin-mediated killing of pneumococci in the nasopharynx and allowing carriage to be established (Shaper *et al.*, 2004). Both CbpA and PspA have been proposed as vaccine candidates due to their immunogenicity, contributions to multiple stages of pathogenesis, and exposure outside the pneumococcal capsule (Brooks-Walter *et al.*, 1999; Hammerschmidt *et al.*, 1997; McCool *et al.*, 2002; Ogunniyi *et al.*, 2001; Rosenow *et al.*, 1997; Shaper *et al.*, 2004; Talkington *et al.*, 1991; Tu *et al.*, 1999; Yother and Briles, 1992; Zhang *et al.*, 2000). In the experiments presented in this chapter, *cbpA* was expressed more highly in the nasopharynx and the lungs than in the bloodstream when *in vivo*-derived RNA extracts were subjected to real-time RT-PCR analysis. The *cbpA* mRNA levels were also dramatically higher in these niches compared to pneumococci cultured in THY broth, suggesting an increased requirement for CbpA in the nasopharynx and lungs than for *in vitro* growth. This was apparent for both D39 and WCH16, and is consistent with a role for CbpA in nasopharyngeal colonisation. The finding that *cbpA* mRNA is higher in colonising pneumococci than bacteria recovered from

the blood is also consistent with a recent study demonstrating that CbpA is not required for survival in the bloodstream (Orihuela *et al.*, 2004a). *pspA* mRNA levels were also elevated in colonising pneumococci compared to bacteria recovered from the lungs or cultured *in vitro* for D39 and one WCH16 experiment in the current study. These results agree with *in vitro* microarray data obtained by Orihuela *et al.* (2004b) that indicated *pspA* transcription is up-regulated in pneumococci co-incubated with Detroit-562 nasopharyngeal cells compared to when bacteria grown in tissue culture medium alone.

The pneumococcus possesses multiple proteins that are capable of reducing competing microflora in the nasopharynx, allowing it to effectively colonise this niche. The neuraminidase NanA and pyruvate oxidase SpxB are two such proteins. NanA reduces the carriage of other colonising bacteria by desialylating glycoconjugates on their surface (Shakhnovich *et al.*, 2002). It also facilitates pneumococcal adherence by modifying glycoconjugates on host epithelium, and has been shown to promote long-term carriage (King *et al.*, 2004; Tong *et al.*, 2000). SpxB catalyses the formation H_2O_2, which inhibits the growth of competing microflora such as *Haemophilus influenzae* and *Neisseria meningitidis*, and may also enhance pneumococcal adherence to the nasopharyngeal epithelium and progression to the lungs (Orihuela *et al.*, 2004a; Pericone *et al.*, 2000; Spellerberg *et al.*, 1996). In the D39 experiments conducted in this chapter, both *nanA* and *spxB* transcripts were present in highest amounts for pneumococci recovered from the nasopharynx rather than from the lungs or blood, which is consistent with their putative roles in carriage and transition to the lungs. These data are also consistent with a microarray study conducted by King *et al.* (2004) that associated increased *nanA* levels with transparent pneumococci (the colony opacity phenotype commonly isolated from the nasopharynx). Similarly, Pericone *et al*. (2000) reported that *spxB* is expressed at higher

levels in oxygen- and CO_2-rich environments, conditions which are prevalent in the nasopharynx. WCH16 data for *spxB* mRNA were similar to D39, with higher amounts present for colonising pneumococci than bacteria harvested from other niches. However, the levels of WCH16 *nanA* transcript remained relatively constant in all the niches examined and the *in vitro* sample, perhaps indicating that the role of *nanA* in establishing nasopharyngeal colonisation is less critical for this strain.

Carriage is a dynamic process, requiring a balance to be maintained between proliferation and evasion of the host's innate defences. Ply enhances disease and inflammation through its cytotoxic activity, activation of the complement pathway and a TLR-4-dependent inflammatory response, and ability to elicit the production of numerous pro-inflammatory cytokines (Boulnois *et al.*, 1991; Braun *et al.*, 1999; Cockeran *et al.*, 2002; Houldsworth *et al.*, 1994; Malley *et al.*, 2003; Paton *et al.*, 1984). Several studies have indicated that Ply is important in the development of sepsis, with mutants lacking Ply showing reduced replication and survival in the bloodstream of infected animals (Benton *et al.*, 1995; Berry *et al.*, 1989b; Berry *et al.*, 1999). Considering the roles of Ply in inflammation and disease, one might expect it to be expressed more highly in the lungs and the bloodstream. In fact, the data presented in this chapter indicated that *ply* mRNA level was higher in the nasopharynx than the other niches examined for both D39 and WCH16. This is an indication that even though Ply is important in invasive disease, it may also be required to promote carriage. The contribution of Ply to nasopharyngeal colonisation is unclear, with some groups reporting that carriage in a mouse model can occur in the absence of Ply with little attenuation (Rayner *et al.*, 1995; Rubins *et al.*, 1998), while findings from another study show that a Ply-negative mutant is cleared from the nasopharynx within 4 hr post-challenge (Kadioglu *et al.*, 2002). A recent study implicates

Ply in promoting the level of colonisation rather than the initial establishment of carriage, with a Ply insertion-duplication mutant of D39 colonising a similar number of mice compared to the WT D39, but at a lower level (Ogunniyi *et al*., 2007). The *ply* expression data presented in this chapter support a role for Ply in the nasopharynx. As Ply lacks an N-terminal signal sequence and its release from the pneumococcal cell most often occurs following LytA-mediated autolysis (Berry *et al.*, 1989a), it is also possible that levels of free Ply in the blood may be indirectly modulated by the extent of autolysis in that niche. Alternatively, Ply production may be regulated at a translational level.

In addition to *cbpA*, *pspA*, *nanA*, *spxB* and *ply*, the results in this chapter indicate that *piaA* mRNA is also present in higher abundance in the nasopharynx than in the other niches at 72 h following challenge for D39. A higher level of *piaA* mRNA was also observed for bacteria harvested from the nasopharynx of WCH16-infected mice rather than the lungs, although the results from bacteraemic pneumococci were inconclusive. This was surprising as PiaA has been shown to be important in sepsis and lung disease, with a *piaA*$^{-}$ strain exhibiting attenuated virulence in both models of infection (Brown *et al.*, 2001a). The results presented here may indicate an important role for of PiaA in the nasopharynx, where free iron levels are low. Mutagenesis studies investigating the effect of PiaA on nasopharyngeal colonisation would help to elucidate the requirement for PiaA in this niche.

4.3.2 Gene expression during lung disease

For D39 in particular, establishment of lung infection often precedes invasion of the bloodstream. *cbpA* mRNA levels for pneumococci recovered from the lungs were similar to that observed for colonising pneumococci, and higher than in bacteria from the

bloodstream for both strains examined. This was not surprising, as CbpA has been previously shown to bind cytokine-activated human lung epithelial cells and endothelial cells, implicating the protein in lung disease (Rosenow *et al.*, 1997). Interestingly, this study found levels of both *psaA* and *cpsA* mRNAs to be higher in the lungs than the nasopharynx for WCH16 but not for D39. However, infection of this niche was a rare occurrence during WCH16 experiments and only low numbers of bacteria were recovered from harvested lungs, despite the presence of pneumococci in both the bloodstream and brains of infected mice. It is possible that lung disease is not a typical symptom of WCH16 infection. Therefore, the real-time RT-PCR data obtained for WCH16 must be looked at with some reservations. Indeed, it is possible that pneumococci harvested from the lungs of WCH16-infected animals were passively aspirated from the nasopharynx and may not have resulted in pneumonia, making bacteria harvested from this niche more characteristic of colonising bacteria than bacteria involved in lung disease. Additionally, the very low bacterial yield from the lungs may have resulted in inaccuracies in subsequent real-time RT-PCR analysis.

4.3.3 Gene expression during sepsis

Pneumococci can enter the circulatory system and cause sepsis, either following the development of lung infection or by invasion from the nasopharyngeal epithelium into underlying tissues. The pneumococcus has several ways of preventing clearance by the host's innate immune response in the bloodstream. One such mechanism is the ability of PspA to reduce complement-mediated opsonisation, either by blocking the formation, or accelerating the dissociation, of the C3 convertase (Tu *et al.*, 1999). Both a PspA$^-$ mutant and pneumococci incubated with anti-PspA exhibit increased binding of C3 *in vitro*, and PspA$^-$ pneumococci are rapidly cleared from the blood following intravenous challenge,

compared to mice infected with the wild-type (Ren *et al.*, 2003; Ren *et al.*, 2004; Tu *et al.*, 1999). Although the highest amounts of *pspA* mRNA in the current study were recovered from bacteria in the nasopharyngeal washes for D39 and WCH16, *pspA* mRNA was also noticeably higher in the bloodstream than in the lungs for both D39 and WCH16.1, which supports the role of PspA in reducing complement-mediated clearance in the blood. This observation is also consistent with a study by Orihuela *et al.* (2004), which demonstrated that *pspA* transcription is up-regulated in the blood of infected mice compared to *in vitro* culture. Interestingly, the level of *pspA* mRNA in the blood compared to the *in vitro* culture was noticeably lower for WCH16 than for D39. Considering the role of PspA in resisting complement-mediated opsonisation (Tu *et al.*, 1999), it is possible that the lower level of *pspA* mRNA observed for WCH16 could result in greater bacterial clearance in the blood. This may contribute to the initial predisposition of WCH16 to cause only moderate-level bacteraemia in CD-1 mice after infiltration of the vascular system, whereas D39-infected mice develop sepsis (Chapter 3).

The CPS possesses strong antiphagocytic properties and has been shown to be essential for virulence (Austrian, 1981a; Briles *et al.*, 1998). Furthermore, immunoelectron microscopy has shown that opaque phase variants, which are preferentially recovered from the blood, possess more CPS than transparent colonies, underscoring the importance of CPS in this niche (Kim *et al.*, 1999). Despite this, no significant difference in *cps2A* mRNA was observed between niches for D39-infected mice at 72 hr post-challenge in the current study. Orihuela *et al.* (2004) observed similar results when they compared *S. pneumoniae* TIGR4 harvested from *in vivo* and *in vitro* by microarray analysis, with *cps4A* mRNA levels being comparable between pneumococci harvested from mouse blood and grown *in vitro*. In fact, they found *cps4A* expression was slightly up-regulated in bacteria

isolated from pneumococci co-cultured with Detroit-562 nasopharyngeal cells compared to the tissue culture media control. This was interesting, considering the importance of higher CPS levels in the bloodstream preventing phagocytosis, and lower CPS levels in the nasopharynx possibly facilitating the exposure of pneumococcal surface adhesins (Briles *et al.*, 1998). It is possible that the pneumococcal capsule is regulated at a post-transcriptional level, allowing rapid assembly when the pathogen is exposed to a new environment such as the bloodstream, in which the anti-phagocytic properties of the capsule are highly advantageous. These results are also consistent with previous work published from our laboratory that suggest that the level of encapsulation is modulated by autophosphorylation of CpsD (Morona *et al.*, 2000) (Morona *et al.*, 2003). However, a study by Ogunniyi *et al*. (2002) indicated that *cps2A* mRNA was present in higher levels in D39 recovered from the blood of IP-infected BALB/c mice than *S. pneumoniae* grown in THY broth. The reasons for the disparity between the results from Ogunniyi *et al*. (2002) and the current study are unclear, although the use of different mouse strains, infection models and RNA quantitation method (Ogunniyi *et al*. used relative quantitative RT-PCR) may be contributing factors.

4.3.4 Gene expression during early meningitis

In Section 3.4.1, WCH16 was shown to progress to the brain of some IN-infected CD-1 mice as early as 24 hr post-challenge, with low levels of pneumococci recovered from the majority of infected mice by 72 hr. This provided an opportunity to look at the relative expression of certain pneumococcal genes during early meningitis. Previously, CbpA has been implicated in transcytosis across brain microvascular endothelial cells, and has also been shown to be present in higher amounts in transparent pneumococci, the opacity phenotype associated with invasion of brain microvascular endothelial cells (King

et al., 2004; Ring *et al.*, 1998). Likewise, *cbpA* mRNA was present in higher amounts in pneumococci recovered from brain than from the bloodstream in this study. The level of *cbpA* mRNA was similar to that seen for the nasopharynx, where CbpA has been shown to play an important role as an adhesin. This may indicate that CbpA also has an important role in the brain, or perhaps that an increased level of CbpA is associated with the transparent phenotype, which is consistent with the putative role of CbpA in crossing the BBB (Ring *et al.*, 1998). Ply and H_2O_2 (the production of which is catalysed by SpxB) both contribute to the symptoms of meningitis by causing neuronal damage. Specifically, they damage brain cell mitochondria, which results in the liberation of apoptosis-inducing factors. They then effect the release of apoptosis-inducing factors from the cell by causing a Ca^{2+} influx or (in the case of Ply) by catalysing the formation of pores in the lipid bilayer (Bermpohl *et al.*, 2005; Braun *et al.*, 2002). The roles of these proteins in promoting early meningitis were supported by the mRNA data in this chapter, with elevated levels of *ply* and *spxB* transcripts observed for pneumococci harvested from the brains of WCH16-infected mice, compared to levels from the lungs and blood. Although Orihuela *et al.* (2004) found the transcription of *spxB* to be suppressed in the brain by microarray analysis, the fold-difference observed was relatively small. Furthermore, the meningitis model they employed involved direct intracisternal injection of TIGR4 with recovery of bacteria 4 hr post-challenge, which may be responsible for the disparity between their data and the results from this study.

4.3.5 Conclusion

The spread of the pneumococcus within a population occurs most readily by the aerosol route. Hence, there is minimal selective pressure for pneumococcal virulence factors that promote IPD alone. As the roles of various virulence factors have been

elucidated, it has become increasingly clear that many of those involved in promoting disease also are important in nasopharyngeal colonisation. In this chapter, levels of virulence gene expression in pneumococci extracted from different mouse niches were compared by real-time RT-PCR. This was the first time pneumococci harvested from a single animal model had been used for direct comparison of niche-specific mRNA levels. Moreover, the work in this chapter elucidated the expression patterns of several important pneumococcal virulence factors during carriage. The examination of transcriptional behaviour in the nasopharynx has been problematic prior to this study, due to the low number of bacteria present in this niche, and this chapter presents the first *in vivo* gene expression data for pneumococci during nasopharyngeal colonisation. The results from the real-time RT-PCR analysis of D39 and WCH16 mRNA indicated that several of the genes examined in this chapter appear important in multiple niches. For the most part, they appeared up-regulated in niches in which they had previously been shown to contribute to growth or infection in the literature. Furthermore, examining the gene expression of WCH16 allowed the unique opportunity to assay mRNA from the nasopharynx and the brain of infected mice simultaneously. Transparent pneumococci are better able to establish and maintain nasopharyngeal colonisation, and possess a far greater ability to invade and migrate across BMEC, than their opaque counterparts (Ring *et al.*, 1998; Weiser *et al.*, 1994). It was interesting to note that genes up-regulated in the nasopharynx of CD-1 mice (compared to the lungs and/or the blood) were also up-regulated in pneumococci harvested from the brain. This suggests that, despite several *in vitro* studies suggesting otherwise, there may be a link between opacity phenotype and the expression of *S. pneumoniae* virulence factors *in vivo*. To further examine pneumococcal behaviour during colonisation and disease, a global analysis of gene expression patterns in these various niches was performed in Chapter 5.

CHAPTER 5 – Global comparison of pneumococcal gene expression *in vivo* by microarray analysis

5.1 Introduction

While real-time RT-PCR is a sensitive technique to quantify relative amounts of specific transcripts *in vivo* (Chapter 4), a more global approach needs to be employed in order to identify novel or previously unconsidered genes that may play niche-specific roles in pathogenesis, and to gain a better understanding of pneumococcal behaviour *in vivo.* Several large-scale techniques have been used in the past to identify genes that are essential for virulence or that expressed more highly in certain niches and environmental conditions, such as differential fluorescence induction and signature-tagged mutagenesis (Hava and Camilli, 2002; Lau *et al.*, 2001; Marra *et al.*, 2002a; Polissi *et al.*, 1998). More recently, microarray analysis has been employed to examine pneumococcal gene expression both *in vitro* and *in vivo* (King *et al.*, 2004; Orihuela *et al.*, 2004b). However, microarray studies using *in vivo*-derived pneumococcal RNA have met with technical restrictions, resulting in data that are not completely comprehensive and must be interpreted with a degree of caution.

In this chapter, pneumococcal gene expression between different niches was assayed by RNA microarray analysis. Bacteria were recovered from all sites of interest using the IN model of infection, which simulated the natural route of pneumococcal acquisition and disease progression. These are the first microarray data generated using a single pneumococcal strain in a continuous infection model.

5.2 Comparison of gene expression in pneumococci involved in colonisation, lung infection and IPD by microarray analysis

5.2.1 Microarray analysis of D39 gene expression between niches

RNA samples harvested in Section 4.2.2 from D39.1 and D39.2 were amplified twice using SenseAmp (Genisphere), as described in Section 2.5.3. 3 µg of amplified RNA (as determined by $A_{260/280}$ readings) from two distinct niches were labelled with either Alexa 546 or Alexa 647 dyes (Genisphere). Microarray analyses reported in this chapter were performed using the Genisphere Array 900 MPX Kit, a commercial kit that is optimised for low quantities of RNA. This made it possible to use lower amounts of *in vivo*-derived RNA without a significant labelling bias. Labelled RNA samples were hybridised to microarray slides spotted with PCR products for each of the 2240 defined open reading frames in the *S. pneumoniae* TIGR4 genome (Bacterial Microarray Group). Additional spots were present on each array, consisting of 117 PCR products for open reading frames (ORFs) that were present in the R6 genome, but absent from the TIGR4 genome, or that exhibited low nucleotide sequence homology. Each ORF was represented twice per array slide. Arrays were read using an Axon 4000B Dual-wavelength Scanner

(Axon Instruments) and then normalised by adjusting the PMT 532 nm and 635 nm intensities until 16S rRNA ratios were approximately 1: 1. This method is described in Section 2.6.3.

5.2.2 Analysis of D39 microarray results

Two cDNA microarrays for each niche comparison were performed using biological replicates labelled with either Alexa 546 or Alexa 647 dyes. The raw data recovered from the microarrays were subjected to the following analytical criteria: Arrays were firstly analysed by GenePix Pro 4.0 using filtration algorithms and signal-to-noise ratios (based on the difference in signal intensity between the spots and the background). Genes for which the corresponding spots incurred errors, or were flagged "bad" or "not found" more than 25% of the time using GenePix Pro 4.0, were removed from further analysis. A cut-off level of $\log_2$ ratio 2-2.5 (4-fold to 6-fold difference in mRNA quantity between niches) was imposed for each array, and genes that exhibited an Alexa 546:Alexa 647 ratio that failed to reach this level were not analysed further. Two-tailed Student's *t*-test analysis (Microsoft Excel, 2003) was used to calculate the statistical significance of the differences in gene expression for each gene within arrays and between replicate biological experiments. Genes with high levels of significance ($P < 0.01$) and a minimum difference of 4-fold were considered up- or down-regulated. The results from this analysis are presented as fold-difference between niches in Table 5.1, classed according to their proposed functions, as listed on the TIGR Comprehensive Microbial Resource website. Using these criteria, 127 genes were identified as being differentially expressed between niches in a CD-1 mouse model. This is a conservative number and reflects both the stringency of analysis and the significant natural variation between biological replicates during *in vivo* experimentation.

***Table 5.1*: Differential *in vivo* expression of *S. pneumoniae* D39 genes**

Gene (Gene name, if available)	TIGR annotation	Fold increase or decrease								
		Nasal wash vs Lungs	*P*	Nasal wash vs Blood	*P*	Lungs vs Blood	*P*
Virulence factors										
bacteriocin (*blpU*)	Sp0041		2.5*	0.0123		5.3*	4.61E-04		5.5	9.00E-03
choline binding protein G (*cbpG*)	Sp0390		2.5*	4.61E-05		10.3	1.47E-04		3.2*+	0.0563
protective antigen A (*prtA*)	Sp0641		1.3*	0.0456		3.6*	1.56E-03		4.4*	7.50E-05
adherence and virulence protein A	Sp0966		NA			6.6	1.08E-04		4.5*+	3.21E-03
neuraminidase, putative	Sp1326	24.2		8.84E-06	23.8		0.010	5.6*+		0.0312
choline binding protein A (*cbpA*)	Sp2190	14.5		1.05E-06	22.0		2.41E-05	7.1*		3.70E-05
Ion-acquisition and binding										
Na/Pi cotransporter II-related protein (*phoU*)	Sp0496		3.3*	2.08E-03		4.9*+	8.00E-05		8.2	1.21E-04
v-type sodium ATP synthase, subunit D	Sp1315	9.5		4.41E-07	8.1*+		0.0115	4.2*+		6.81E-04
v-type sodium ATP synthase, subunit B (*ntpB*)	Sp1316	42.5		2.80E-08	16.0		1.70E-04	21.8+		0.0134
v-type sodium ATP synthase, subunit A	Sp1317	66.3		8.40E-08	6.4		1.58E-03	11.2*+		7.86E-03
v-type sodium ATP synthase, subunit E	Sp1320	14.8		3.16E-04	11.8+		1.57E-03	2.4*+		0.150
v-type sodium ATP synthase, subunit K (*ntpK*)	Sp1321	28.4		6.00E-04	10.7		4.38E-05	2.8*+		0.192
v-type sodium ATP synthase, subunit I (*ntpI*)	Sp1322	62.6		2.90E-05	53.1		3.34E-04	20.7+		7.74E-07
sodium:solute symporter family protein	Sp1328	34.3		3.20E-05	12.9		1.09E-05	20.5*+		0.0131
non-heme iron-containing ferritin	Sp1572		2.6*	1.02E-03		1.3*	0.581		5.6	4.40E-05
cation efflux system protein	Sp1857		1.6*	1.58E-03		12.0	2.32E-07		5.5*	1.24E-05
iron-compound ABC transporter, ATP-binding protein	Sp1871		3.6*	1.79E-03		12.4	1.66E-05		6.3*	8.19E-03
iron-compound ABC transporter, iron-compound-binding protein	Sp1872		1.7*	1.06E-03		13.0	5.90E-05		3.6*	4.64E-03

Table 5.1 *continued*

Gene (Gene name, if available)	TIGR annotation	Fold increase or decrease								
		Nasal wash vs	Lungs	*P*	Nasal wash vs	Blood	*P*	Lungs vs	Blood	*P*
hypoxanthine-guanine phosphoribosyltransferase (*hpt*)	Sp0012		4.0*	1.51E-03		8.8	1.08E-07		12.7	9.72E-05
transcriptional regulator (*mutR*)	Sp0141		4.7	2.47E-06		4.3*+	6.07E-08		4.9+	0.0127
transcriptional regulator, MerR family	Sp0501		2.2*	1.51E-03		4.7	6.35E-06		6.6*	9.94E-03
ATP-binding protein (*amiE*)	Sp1888		3.2*	2.17E-08		9.3	5.73E-05		3.1*+	4.46E-05
permease protein (*amiD*)	Sp1889		2.3*	6.16E-07		8.5	6.15E-07		4.0*	1.24E-05
Competence										
competence factor transport protein (*comB*)	Sp0043		2.9*	5.12E-03		12.9	6.68E-07		4.5*	1.51E-04
response regulator (*comE*)	Sp2235		2.6*	7.67E-08		10.2	2.59E-05		4.8*	4.13E-05
Metabolism/cellular growth										
putative acyl carrier protein	Sp0038		2.1*	1.56E-03		9.2	2.39E-06		2.8*	2.84E-03
phosphoglycerate mutase family protein	Sp0240		3.1*	4.08E-03		5.1*	9.04E-05		6.6	4.00E-03
phosphatidate cytidylyltransferase	Sp0262		3.2*	3.21E-03		9.5	1.73E-04		5.9	2.01E-06
lactate oxidase, truncation (*lctO*)	Sp0712		4.8	1.50E-06		4.3*+	6.07E-08		2.6*+	0.0390
cell division ABC transporter, permease protein (*ftsX*)	Sp0757		2.6*	1.22E-06		4.1*	9.12E-06		7.1	7.16E-04
phosphoenolpyruvate carboxylase (*ppc*)	Sp1068		2.1*	4.11E-03		11.4	4.15E-06		4.7*	5.74E-04
macrolide-efflux protein	Sp1110		3.4*	3.91E-05		7.3	1.17E-05		4.8*	2.57E-06
6-phospho-β-galactosidase (*lacG*)	Sp1184	15.2		9.42E-06	9.6		5.90E-04	7.8+		6.05E-04
putative platelet activating factor	Sp1450	4.3*+		0.384		4.7*+	0.0275		6.0	4.05E-06
transketolase (*recP*)	Sp1615	180.7		1.50E-04	37.6		2.03E-03	6.1+		4.17E-06
L-ribulose 5-phosphate 4-epimerase (*araD*)	Sp2033	1.3*+		0.0683	156.0		1.67E-04		NA	
hexulose-6-phosphate isomerase	Sp2034		NA		63.9		1.21E-05	1.9*+		0.109

Table 5.1 *continued*

Gene (Gene name, if available)	TIGR annotation	Fold increase or decrease								
		Nasal wash vs	Lungs	*P*	Nasal wash vs	Blood	*P*	Lungs vs	Blood	*P*
hexulose-6-phosphate synthase	Sp2035	20.4+		1.51E-03	114.9		1.35E-03		NA	
glycerol kinase (*gplK*)	Sp2186	28.6+		2.66E-05	17.7		3.48E-03	1.9*+		0.142
replicative DNA helicase (*dnaC*)	Sp2203		1.5*	1.63E-03		7.1	2.26E-05		4.3*	0.0106
spspoJ protein	Sp2240		NA			2.0*+	0.454		7.0	2.32E-05
Sugar transport										
putative PTS system IIA component	Sp0645	6.1		4.24E-07		2.5*+	0.0890		1.8*+	0.0538
PTS system, lactose-specific IIBC components	Sp1185	6.8		1.59E-06	4.5*+		6.82E-04	2.1*+		6.23E-03
PTS system, nitrogen regulatory component IIA, putative	Sp1620	11.6		1.19E-05	4.6*+		6.82E-04	1.9*+		0.347
PTS system, IIA component	Sp2036	9.0		1.63E-06	90.5		6.85E-04	2.1*+		0.395
PTS system, IIB component	Sp2037	2.3*+		4.34E-04	41.2		3.66E-05		NA	
PTS system, membrane component, putative	Sp2038	1.8*+		0.178	28.9		6.44E-05	2.5*+		0.124
Transcription and translation										
tRNA(Ile)-lysidine synthetase (*tilS*)	Sp0011		2.8*	4.13E-06		13.5	3.00E-06		8.6	6.19E-05
ribosomal protein S8 (*rpsH*)	Sp0224		1.7*	4.38E-04		9.0	1.36E-05		3.8*	9.34E-06
prolyl-tRNA synthetase (*proS*)	Sp0264	2.1*+		0.437		8.6	1.95E-05		3.2*	8.15E-05
N utilization substance protein A (*nusA*)	Sp0553		1.6*	2.16E-04		6.5	6.38E-04		6.3	9.17E-07
ATP-dependent RNA helicase, DEAD/DEAH box family	Sp0761		3.7*	1.11E-03		8.9	1.29E-05		5.2*	2.66E-04
putative transcriptional regulator	Sp1131	8.4		6.19E-08	4.9*+		5.47E-03		1.6*+	0.695
transcription antiterminator (*lacT*)	Sp1187	11.2		1.92E-04	4.2*		1.03E-03	4.6*+		1.08E-03
putative ATP-dependent RNA helicase	Sp1586		4.8	2.40E-07		2.1*	0.119		4.7*	3.58E-05
transcriptional regulator	Sp1809		2.6*	2.40E-03		7.3	3.60E-04		7.2	8.59E-06

Table 5.1 *continued*

Gene (Gene name, if available)	TIGR annotation	Fold increase or decrease								
		Nasal wash vs	Lungs	*P*	Nasal wash vs	Blood	*P*	Lungs vs	Blood	*P*
transcriptional regulator, MerR family	Sp1856		2.1*	2.22E-04		8.0	3.18E-06		5.0	6.26E-07
transcription antitermination protein (*nusG*)	Sp2007		2.9*	9.96E-04		7.2	2.90E-04		6.0	4.88E-06
transcriptional regulator, BglG family	Sp2032	12.1		3.42E-05	34.4		7.41E-06	6.1		2.21E-03
transcriptional regulator, MarR family	Sp2062		2.2*	6.45E-04		3.1*+	0.175		5.7	2.21E-05
transcriptional regulator, authentic frameshift	Sp2123	12.4		4.31E-03	3.1*+		0.209	1.7*+		0.358
ribosomal protein L9 (*rplI*)	Sp2204		2.3*	5.52E-03		7.1	2.26E-05		3.7*	8.65E-05
Protein fate										
preprotein translocase, SecY subunit	Sp0230		3.4*	9.45E-04		8.6	2.60E-06		3.6*	0.0302
putative preprotein translocase, SecE subunit	Sp2008		2.0*	1.39E-04		4.9*	9.92E-05		8.4	6.01E-05
SOS repair										
MATE efflux family protein (*dinF*) , putative SOS repair	Sp1939		4.1	1.57E-05		5.9*	1.75E-03		7.9	9.85E-04
Cell wall and membrane										
glycosyl transferase	Sp0102		2.6*	1.48E-03		7.4	5.42E-07		4.0*	1.06E-04
beta-lactam resistance factor (*fibA*)	Sp0615		1.5*	0.250		7.9	8.19E-05		3.7*	4.56E-05
putative membrane protein	Sp0858		2.3*	6.64E-03		8.8	8.01E-07		6.6	7.6E-05
endo-ß-*N*-acetylglucosaminldase (*lytB*)	Sp0965		2.9*	0.104		9.4	2.90E-05		4.8*+	8.49E-04
peptidoglycan N-acetylglucosamine deacetylase A (*pgdA*)	Sp1479		NA			7.4	5.35E-05		3.0*+	3.30E-04
glycosyl transferase, authentic frameshift	Sp1769	62.9		1.34E-05	20.7*+		1.08E-03	4.5*+		1.39E-05
cell wall surface anchor family protein	Sp1772	13.4		2.22E-05	9.4		8.14E-05	4.2*+		0.257

Table 5.1 *continued*

Gene (Gene name, if available)	TIGR annotation	Fold increase or decrease								
		Nasal wash vs	Lungs	*P*	Nasal wash vs	Blood	*P*	Lungs vs	Blood	*P*
glycosyl transferase, putative	Sp1838		3.9	1.47E-07		1.7*+	0.148		11.4*+	8.54E-03
Transposon										
IS630-Sp1, transposase Orf2	Sp0016		3.7*	2.90E-05		9.1	2.15E-05		4.8*	2.58E-04
IS1381, transposase (*orfB*)	Sp1086		1.4*	5.52E-08		6.4	1.10E-11		6.0*	5.84E-04
IS1167, transposase, degenerate	Sp1101		4.4	9.09E-06		9.4	9.88E-05		7.5*	2.30E-03
IS66 family element, Orf3, degenerate	Sp1311		1.8*	9.06E-03		8.9	4.09E-07		3.1*	7.49E-03
IS1380-Sp1, transposase	Sp1337	27.6		4.81E-08	57.2		2.89E-04	9.0*+		0.0876
IS1380-Sp1, transposase	Sp1352	87.0		8.74E-09	36.4		5.41E-03	27.8		0.0106
IS1380-Sp1, transposase	Sp1418	68.7		2.10E-04	12.2		7.33E-06	9.0*+		0.171
IS1380-Spn1, transposase	Sp1439	17.7		2.46E-06	23.7+		6.52E-05	11.0		0.0140
transposase, IS630-Spn1 related, Orf2	Sp1496	4.7		4.78E-09	1.9*+		0.0301	1.3*+		0.759
IS1380-Sp1, transposase	Sp1503	17.6		1.34E-06	26.7		0.0105	8.5*+		2.45E-04
transposase, IS200 family	Sp1622	19.8		1.73E-05	15.0*+		6.41E-03	5.6*+		1.20E-03
transposase, IS1380-Spn1 related, truncation	Sp2089	12.4		1.77E-04	1.5*+		0.655		NA	
IS66 family element, Orf3, degenerate	Sp2211		1.4*	4.75E-03		6.9	4.22E-06		4.4*	1.34E-05
Unknown genes/ unknown function										
GTP-binding protein	Sp0004		NA			11.8	5.31E-06		9.2	1.77E-04
hypothetical protein	Sp0008		NA			4.5*+	0.0284		12.8	5.89E-04
conserved hypothetical protein	Sp0095		1.9*	4.43E-03	1.1*+		0.724		5.4	3.59E-07
hypothetical protein	Sp0144		3.6*	3.87E-03		11.2	2.44E-03		18.9	2.87E-05
acetyltransferase, GNAT family	Sp0204		NA			23.5	4.11E-05		5.5*+	0.0255
conserved hypothetical protein	Sp0239		5.0	2.08E-04		2.9*+	0.0122		1.3*+	0.228
hypothetical protein	Sp0455		1.5*	9.78E-08		10.4	7.67E-04		4.5*+	2.59E-04
conserved domain protein	Sp0500		2.6*	2.74E-03		9.6	1.99E-06		6.0*	5.21E-05

Table 5.1 *continued*

Gene (Gene name, if available)	TIGR annotation	Fold increase or decrease								
		Nasal wash vs	Lungs	*P*	Nasal wash vs	Blood	*P*	Lungs vs	Blood	*P*
acetyltransferase, GNAT family	Sp0590		2.8*	0.0383		10.1	9.67E-05		6.7*	2.31E-06
chorismate binding enzyme	Sp0655		2.9*	4.58E-05		7.1	5.61E-05		6.3	5.03E-08
hypothetical protein	Sp0777		2.8*	4.31E-04		8.2	1.40E-06		3.6*	7.84E-03
hypothetical protein	Sp0781		2.5*	3.79E-03		4.6*+	2.00E-04		7.4	5.19E-05
hypothetical protein	Sp0830	11.6		3.02E-07		3.1*	7.66E-03		3.5*	1.60E-05
pilin gene inverting-related protein	Sp0917		3.0*	0.0243		15.3	2.86E-05		7.2*	2.18E-03
acetyltransferase, GNAT family	Sp1023		1.4*	1.45E-03		7.7	1.16E-05		3.5*	1.88E-04
hypothetical protein	Sp1059	19.2		1.78E-05	4.1*+		0.144		1.1*+	0.786
acetyltransferase, GNAT family	Sp1082		1.5*	0.350		13.0	3.41E-05		5.1*	6.98E-06
aminotransferase, class-V	Sp1094		3.3*	3.65E-03		9.3	9.28E-05		3.5*	1.55E-03
hypothetical protein	Sp1139	15.2		9.42E-06	10.2		6.15E-03	2.9*+		0.212
conserved hypothetical protein	Sp1143	10.5		1.09E-05	3.4*+		0.0116	1.6*+		0.463
hypothetical protein	Sp1189	32.2		1.92E-04	6.3*+		8.79E-03	1.8*+		0.095
SAP domain protein	Sp1292	15.9		9.23E-05	10.1		9.98E-04	2.6*+		2.29E-03
conserved domain protein	Sp1363	3.6*+		0.256		9.9	1.71E-05		5.3*	1.27E-04
hypothetical protein	Sp1558		2.5*	4.35E-04		5.9	1.42E-10		5.0*	2.23E-05
conserved domain protein	Sp1612	35.8		1.20E-03	11.5		1.03E-06	6.2*+		5.5E-04
hypothetical protein	Sp1643	37.0		1.05E-03	1.9*+		0.0889		NA	
ylmH protein	Sp1662		1.6*	0.0107		6.6	4.15E-08		3.6*	2.94E-03
ROK family protein	Sp1675	30.5		9.93E-07	4.8*+		0.173	1.8*+		0.366
ABC transporter, permease protein	Sp1688	19.5		5.08E-07	5.6		0.0168	9.8		1.19E-03
conserved hypothetical protein	Sp1747		3.4*	3.66E-07		3.7*	1.98E-05		7.7	7.26E-05
hypothetical protein	Sp1862		3.0*+	0.485		6.1*	3.06E-04		8.7	2.76E-05
acetyltransferase, GNAT family	Sp1943		2.8*	2.23E-04		8.7	1.50E-08		5.7	1.05E-03
CBS domain protein	Sp1963	2.8*+		0.456		6.2*	6.51E-05		7.6	3.17E-04
conserved hypothetical protein	Sp2031	19.2*+		3.57E-03	131		9.73E-05		2.3*+	0.126
conserved hypothetical protein	Sp2059	1.7*		0.488		6.8	7.05E-09		5.2	1.17E-05

Table 5.1 *continued*

Gene (Gene name, if available)	TIGR annotation	Fold increase or decrease								
		Nasal wash vs Lungs		***P***	**Nasal wash vs Blood**		***P***	**Lungs vs Blood**		***P***
glycosyl hydrolase-related protein	Sp2141	8.9		1.59E-03	11.9		1.47E-04	4.6*+		0.0281
ROK family protein	Sp2142	7.0		1.91E-04	19.8		5.58E-05	5.2*+		3.73E-03
conserved hypothetical protein	Sp2160	16.2		1.33E-06	4.6*		0.0876	2.2*+		0.445
hypothetical protein	Sp2185	9.3		2.11E-03	18.3		1.93E-05	12.0		1.92E-05
conserved hypothetical protein	Sp2202		1.7*	6.93E-04		6.5	5.47E-08		5.6*	2.50E-03

RNA was extracted from *in vivo*-derived pneumococci, enriched for prokaryotic RNA and amplified, as described in the text. Microarray experiments were then performed comparing pneumococci harvested from different niches using Genisphere MPX900, and analysed using Genepix Pro 4.0. Data were analysed as described in Section 5.2.2 and Student's *t* test analysis was used to calculate the statistical significance of the differences in gene expression for each gene within arrays and between replicate biological experiments. Figures shown in grey did not pass analysis criteria, due to data for that gene being below the cut-off threshold value for one, or both, experiments (*) or due to error or flag tags, as determined by Genepix Pro 4.0 (+). NA indicates that data could not be obtained, as the majority of spots were tagged as errors (hence, did not return a value).

A dye reversal experiment was performed to determine if there was a significant dye labelling bias using the Genisphere Array 900 MPX Kit. This consisted of performing microarray labelling and hybridisation in duplicate, labelling an RNA sample with Alexa 546 dye in one array experiment, and Alexa 647 in the other. The dye reversal was performed using nasopharyngeal and blood samples from D39.1. Analysis of one microarray slide identified a total of 245 genes that exhibited a significant fold-difference in gene expression between the niches. 205 (84%) of the identified genes were similarly up- or down- regulated on the dye reversal array. An opposing fold increase or decrease was observed for 12 (5%) genes, while 28 (11%) could not be compared due to error or flag tags by Genepix Pro 4.0. These results indicated that labelling bias using the labelling method provided by the Genisphere Array 900 MPX Kit with Alexa 546 and Alexa 647 was minimal.

Analysis of the microarray data identified *in vivo* differential expression of a large number of genes involved in fatty acid, energy or sugar metabolism. Genes involved in sugar metabolism were largely up-regulated in the nasopharynx compared to other niches, whereas genes with a role in fatty acid metabolism were expressed more highly in the blood. *lacG* and genes belonging to sucrose phosphotransferase systems (PTS) appeared up-regulated in the nasopharynx compared to either the blood or lungs. Likewise, several transposases exhibited higher expression in the nasopharynx than other niches. Although the gene encoding the major pneumococcal neuraminidase *nanA* was not identified in these experiments due to the corresponding array spots falling below the cutt-off criteria, a putative neuraminidase encoded by Sp1326 was up-regulated in lungs, and even more so in the nasopharynx, compared to the blood. Conversely, levels of competence factors *comB*

and *comE* mRNA were found to be higher in the blood than the nasopharynx, as were levels of *dinF* transcript which encodes a protein thought to be involved in SOS DNA repair. *amiD* and *amiE* transcripts were also present in higher amounts in pneumococci recovered from the blood than the nasopharynx. These two proteins encode transmembrane components of the Ami oligopeptide ABC transporter, which has previously been hypothesised to be involved in sensing environmental changes, indirectly resulting in the regulation of certain pneumococcal genes (Claverys *et al.*, 2000).

The microarray results for the virulence factors examined by real-time RT-PCR analysis in Chapter 4 are shown in Table 5.2. For many of these genes, the corresponding spot intensities on the microarrays performed in this chapter were unable to be accurately determined, either falling below the cut-off points, incurring an error when analysed by GenePix Pro 4.0, or possessing a *P*-value > 0.01. However, spots corresponding to *cbpA* did pass the analysis criteria, indicating the amount of *cbpA* transcript present in nasopharyngeal pneumococci was 22-fold higher than for bacteria recovered from the blood and 14.5- fold higher than for bacteria recovered from the lungs, which was consistent with the higher level of *cbpA* mRNA observed in the nasopharynx by real-time RT-PCR. Interestingly, although a difference in *nanA* quantity was not identified by microarray analysis, a putative neuraminidase gene (Sp1326) was up-regulated 24-fold higher in bacteria harvested from the nasopharynx compared to other niches, suggesting a potential role for this neuraminidase in carriage as well.

5.2.3 Preliminary results from WCH16 microarray analysis

Microarray analysis was also used to examine the differential expression of WCH16 genes in different niches in an IN mouse infection model. Nucleic acid samples

Gene	TIGR annotation	Fold increase or decrease								
		Nasal wash	**Lungs**	***P***	**Nasal wash**	**Blood**	***P***	**Lungs**	**Blood**	***P***
cbpA	Sp2190	14.5		1.05E-06	22.0		2.41E-05	7.1*		3.70E-05
pspA	Spr0121		NA		1.1*		0.922	2.7*		0.222
ply	Sp1923		2.2*	2.62E-04		1.2*	0.871		3.6*	4.56E-05
psaA	Sp1650	1.5*		1.79E-06		4.6*	2.45E-03		2.5*	4.18E-03
cps4A	Sp0346		2.0*	0.0289	1.1*+		0.948		NA	
piaA	Sp1032	1.9*+		0.0292		2.4*+	2.80E-03		2.3*+	1.31E-03
nanA	Sp1963	1.9*		2.07E-04	1.9*		0.353		4.5*+	3.19E-04
spxB	Sp0730		NA		1.2*		0.847		NA	

Table 5.2: **Differential expression of *S. pneumoniae* D39 virulence genes, as determined by microarray analysis**

RNA was extracted from *in vivo*-derived pneumococci, enriched for prokaryotic RNA and amplified using Genisphere SenseAmp. Microarrays were then performed comparing pneumococci harvested from different niches using Genisphere MPX900, and analysed as described in the text for the combined data from two independent biological experiments. Figures shown in grey did not pass analysis criteria, due to data for that gene being below the cut-off threshold value for one, or both, experiments (*) or due to more than 25% of spots being tagged "error" or "flag", as determined by Genepix Pro 4.0 (+). NA indicates that data could not be obtained, as the majority of spots were tagged as errors (hence, did not return a value).

recovered from *in vivo*-derived pneumococci in experiments WCH16.1 and WCH16.2 (Chapter 4) were amplified, labelled and used in microarray hybridisation experiments as described for D39 in Sections 5.2.1 and 5.2.2. Only the nasopharyngeal and blood samples could be compared by microarray due to poor RNA integrity for samples recovered from the lungs, and time constraints prohibited the recovery of fresh RNA from WCH16-infected mice to conduct further array analyses. Genes that exhibited different levels of expression between bacteria recovered from the nasopharynx and blood are listed in Table 5.3. In contrast to D39, there were only a few genes that were identified in both WCH16.1 and WCH16.2 arrays as being differentially expressed between niches. The different magnitude of disease observed in the replicate animal experiments may have contributed to this disparity (Figure 4.5). Furthermore, the use of a PCR-product array based on the genomic sequence of TIGR4 and R6 (a derivative of D39) to analyse the unrelated serotype 6A strain WCH16, may have resulted in reduced affinity for WCH16 genes with variant sequences. Nevertheless, many of the genes that exhibited niche-dependent expression for WCH16 were also identified in the D39 microarray experiments (Section 5.2.2). These are marked with an asterisk in Table 5.3. Several genes involved in competence were markedly up-regulated in the blood compared to the nasopharynx, including *comB* and *comE* which were found to be regulated similarly in this niche as for D39. *comX1* and *cgiA* were also identified in these WCH16 experiments as exhibiting increased expression in the blood, but were not differentially expressed in D39. However, although *comX1* showed similar expression in the nasopharynx and blood for D39.2 and hence didn't pass the analysis criteria, it was markedly up-regulated in the blood for D39.1 demonstrating a 9.9-fold increase in this niche.

Once again, the specific virulence genes examined in Chapter 4 did not pass the

Table 5.3: **Differential expression of *S. pneumoniae* WCH16 genes as determined by microarray analysis**

RNA was extracted from *in vivo*-derived WCH16 pneumococci, enriched for prokaryotic RNA and amplified as described in the text. Microarray analyses were performed comparing pneumococci harvested from the nasopharynx and blood niches using Genisphere MPX900, and analysed using Genepix Pro 4.0. Genes for which the corresponding spots incurred errors or were flagged more than 25% of the time using GenePix Pro 4.0 were removed from further analysis. A cut-off level of $\log_2$ ratio 2-2.5 (4- fold to 6- fold difference in mRNA quantity between niches) was imposed for each array and Student's *t* test analysis (Microsoft Excel) was used to calculate the statistical significance of the differences in gene expression for each gene within arrays and between replicate biological experiments. An asterisk indicates that these genes were similarly identified in D39 arrays.

Gene (Gene name, if available)	TIGR annotation	Fold increase or decrease		
		Nasal wash	Blood	*P*
Virulence factors				
adhesion lipoprotein (*lmb*)	Sp1002	6.0		7.37E-06
Ion-acquisition and binding				
iron-compound ABC transporter, ATP-binding protein*	Sp1871		11.8	4.44E-03
iron-compound ABC transporter, iron-compound-binding protein[D]	Sp1872		17.0	8.58E-04
Competence				
transcriptional regulator (*comX1*)	Sp0014		7.3	3.60E-05
competence factor transport protein (*comB*)*	Sp0043		11.1	1.23E-05
competence protein (*cglA*)	Sp2053		9.7	6.17E-04
response regulator (*comE*)*	Sp2235		11.0	8.03E-03
Metabolism/cellular growth				
alcohol dehydrogenase, iron-containing	Sp2026		13.3	2.42E-05
hexulose-6-phosphate isomerase, putative*	Sp2034	12.4		4.47E-04
hexulose-6-phosphate synthase, putative*	Sp2035	13.1		5.32E-04
Sugar transport				
L-ribulose 5-phosphate 4-epimerase, putative (*araD*)*	Sp2036	7.2		9.56E-04
PTS system, membrane component, putative*	Sp2038	6.5		1.59E-05
Cell wall and membrane				
glycosyl hydrolase, family 1	Sp2021		5.8	1.54E-04
Transposon				
IS3-Spn1, transposase, authentic point mutation	Sp0850		6.1	1.01E-04
Unknown genes/ unknown function				
hypothetical protein*	Sp0144	7.4		7.40E-04
conserved hypothetical protein	Sp0146	5.5		1.39E-04
conserved hypothetical protein	Sp0627		6.3	5.45E-03
oxidoreductase, Gfo/Idh/MocA family	Sp1686		6.3	1.29E-03
conserved hypothetical protein*	Sp2031	11.6		1.09E-04

analysis criteria that were set (Table 5.4). The large *P*-values obtained are indicative of the variation between biological experiments also observed by real-time RT-PCR in Chapter 4. In contrast to the D39 array data described earlier in this chapter, and also the differential expression for *cbpA* between the nasopharynx and blood observed by real-time RT-PCR (Chapter 4), WCH16 did not appear to up-regulate the expression of *cbpA* in the nasopharynx by microarray analysis in this study. Serogroup 6 pneumococci encode *cbpA* belonging to a different clade than D39 *cbpA* (Iannelli *et al.*, 2002). Therefore, variations in nucleotide sequence between WCH16 *cbpA* and the TIGR4 *cbpA*, with which the microarray slides were spotted, may account for the discrepant results. Additionally, *cpsA* (which is highly conserved between many pneumococcal serotypes (Morona *et al.*, 1999)) demonstrated a 3.8-fold increase in expression in the bloodstream compared to the nasopharynx of infected mice. Further biological experiments for WCH16 would need to be conducted in order to clarify the preliminary results reported in this section.

5.3 Discussion

The transcriptional changes that allow the pneumococcus to adapt to various host environments are poorly understood. This chapter describes the use of microarray analysis to directly compare gene expression at different sites in CD-1 mice following IN infection (as established in Chapter 3). This has allowed comparison of gene expression profiles possessed by pneumococci asymptomatically colonising the nasopharynx and bacteria that had progressed to cause lung disease and IPD. This is the first report of such *in vivo* studies using a single pneumococcal strain and a continuous infection model.

Gene	TIGR annotation	Fold increase or decrease		
		Nasal wash	Blood	*P*
cbpA	Sp2190		2.2*	0.292
pspA	Spr0121		1.1*	0.759
ply	Sp1923	2.0*		9.27E-03
psaA	Sp1650		3.5*	0.313E-03
cps4A	Sp0346		3.8*+	0.148
piaA	Sp1032		2.0*+	0.0829
nanA	Sp1963	1.5*		0.429
spxB	Sp0730	1.1*+		0.938

***Table 5.4*: Differential expression of *S. pneumoniae* WCH16 virulence genes as determined by microarray analysis**

RNA was extracted from *in vivo*-derived WCH16 pneumococci, enriched for prokaryotic RNA and amplified using Genisphere SenseAmp. Microarray were then performed comparing pneumococci harvested from different niches using Genisphere MPX900, and analysed as described in the text. Figures shown in grey did not pass analysis criteria, due to data for that gene being below the cut-off threshold value for one, or both, experiments (*) or due to more than 25% of spots being tagged "error" or "flag", as determined by Genepix Pro 4.0 (+). NA indicates that data could not be obtained, as the majority of spots were tagged as errors (hence, did not return a value).

5.3.1 Global *in vivo* gene expression analysis of *S. pneumoniae* using a single mouse model and challenge strain

Due to the difficulty in recovering sufficient bacteria from various *in vivo* niches, studies examining global pneumococcal gene expression have been largely performed *in vitro*. However, *in vitro* studies cannot entirely simulate an *in vivo* environment. Perhaps because of this, DFI failed to detect promoter inductions for most of the intensively studied pneumococcal virulence genes under different *in vitro* conditions (Marra *et al.*, 2002a). Furthermore, only minor changes in gene expression profiles between opaque and transparent phenotypes were reported by King *et al.* (2004), despite multiple groups observing a strong association between opacity phenotype and *in vivo* niche (Cundell *et al.*, 1995b; Ring *et al.*, 1998; Weiser *et al.*, 1994; Weiser and Kapoor, 1999). Although *nanA* mRNA was present in higher amounts in transparent pneumococci, albeit at the modest level of 2.1-fold for 6B strains, other virulence factors such as CPS and CbpA were expressed similarly in both phenotype variants. The small number of genes that exhibited opacity phenotype-specific gene expression, and the lack of promoter induction observed by DFI for many key virulence factors, indicates that there may not be a 'switch' that alters the expression of many genes simultaneously and that other *in vivo* factors, such as unspecified environmental conditions and host immune factors, may be crucial in inducing the transcriptional changes that allow pneumococci to efficiently adapt to different niches during pathogenesis.

The D39 microarray data in this chapter identified differential expression of genes involved in sugar uptake and metabolism, fatty acid and energy metabolism, cellular growth, and ion acquisition. In general, gene expression paralleled their putative roles in pneumococcal virulence and pathogenesis. Genes that contribute to sugar metabolism

were largely up-regulated in the nasopharynx compared to other niches. King *et al.* (2004) also identified several sugar metabolism genes that were expressed more highly in transparent pneumococci (the opacity phenotype associated with pneumococci in the nasopharynx) than opaque. Enzymes involved in sugar metabolism may promote carriage by cleaving both bacterial and host glycoproteins, thus reducing clearance or enhancing adherence in the nasopharynx. Several pneumococcal surface proteins were up-regulated in the nasopharynx for D39, such as a cell wall surface anchor family protein (Sp1772) and the well-characterised CBP, CbpA (Sp2190), which is a putative adhesin in this niche. CbpA was also identified by STM as being important for lung infection (Hava and Camilli, 2002) and was observed by Orihuela *et al.* (2004) to be up-regulated following intimate contact with nasopharyngeal epithelial cells, supporting the results of this work. Two other CBPs were identified by microarray analysis in this chapter. *lytB* encodes a CBP that has been shown to play a role in cell division (Garcia *et al.*, 2000), and displayed almost a 10-fold up-regulation in the blood compared to the nasopharynx, despite previous studies indicating that a *lytB* mutant was attenuated in carriage but not sepsis (Gosink *et al.*, 2000). *cbpG* (a serine protease) was also expressed more highly in the bloodstream than the nasopharynx, which is consistent with microarray results from Orihuela *et al.* (2004) and previous mutagenesis studies by Gosink *et al.* (2000) implicating CbpG in sepsis.

Although microarray studies performed by Orihuela *et al.* (2004) found no regulation of competence genes in the blood or pneumococci co-cultured with nasopharyngeal epithelial cells compared to baseline controls (bacteria grown in C+Y media or tissue culture media, respectively), data from the current study indicate that both *comB* and *comE* transcripts are up-regulated for D39 and WCH16 in the blood relative to the nasopharynx *in vivo*. This supports previous reports indicating that competence-related

genes undergo increased transcription during exponential growth such as during the development of sepsis (Claverys and Havarstein, 2002). Preliminary microarray data from the current study indicated that competence genes *comX1* and *cgiA* are also induced in the blood for WCH16. D39 results also suggested that pneumococci harvested from the blood expressed a higher amount of mRNA for the putative SOS repair gene, *dinF*. *dinF* is the third gene in the competence-induced *cin-recA* operon (Mortier-Barriere *et al.*, 1998; Pearce *et al.*, 1995), and the microarray data indicating an up-regulation of competence genes *comB* and *comE* in the blood are consistent with the increase in *dinF* mRNA observed in this niche for D39.

In Chapter 4, D39 *cps2A* was found to be expressed similarly in all niches when examined by real-time RT-PCR. Likewise, *cps2A* was not identified as being differentially expressed by the D39 microarray hybridisations performed in this chapter. This was surprising considering both the essential requirement for CPS in virulence and the difference in CPS quantities between opacity phase variants (Austrian, 1981a; King *et al.*, 2004), and may suggest that CPS is not regulated at the transcriptional level (Morona *et al.*, 2000). However, several other genes involved in cell wall modification were found to be differentially expressed *in vivo*. Peptidoglycan N-acetylglucosamine deacetylase A (*pgdA*) was up-regulated 7.4-fold in the blood compared to the nasopharynx. PgdA deacetylases peptidoglycan, which provides resistance against degradation by lysozyme in mucosal secretions (Vollmer and Tomasz, 2000). Mutagenesis studies have also shown PgdA$^-$ pneumococci to possess reduced virulence in an IP infection model, suggesting that it may be a virulence determinant (Vollmer and Tomasz, 2002), and results from the current study indeed support a role for it in the blood. Genes with roles in fatty acid metabolism were also up-regulated in the blood. Changes in the lipid composition of the cell membrane

occur in response to environmental conditions such as oxidative stress and temperature in *Lactobacillus helveticus* (Guerzoni *et al.*, 2001). Also, changes in membrane fluidity and fatty acid composition have been associated with pneumococcal opacity phenotype (Aricha *et al.*, 2004; Saluja and Weiser, 1995). The study presented in this chapter raises the possibility that niche-dependent alterations in membrane lipid composition also occur *in vivo*.

In contrast to D39, WCH16 *cpsA* was up-regulated 3.8-fold in the bloodstream compared to the nasopharynx of infected mice. The fold regulation was below the pre-determined cut-off criteria, and the variation between spot intensity resulted in this value not being significant when analysed by Student's *t*-test (two-tailed). This was consistent with expression trends observed for *cpsA* by real-time RT-PCR, which also indicated substantial variation in expression level between WCH16.1 and WCH16.2. WCH16 and D39 exhibit different kinetics of pathogenesis. WCH16 establishes bacteraemia in the absence of significant lung infection following IN challenge, and maintains a sub-septic level of bacteraemia for at least 72 hr. Many WCH16-infected mice also develop meningitis within 72 hr. In contrast, mice IN-infected with D39 develop bacteraemia a few days after challenge which rapidly progresses to sepsis and death of the animal, usually within 24 hr of pneumococci first infiltrating the blood (Chapter 3). CPS has been shown to be essential to pneumococcal virulence (Austrian, 1981a), so it is surprising that WCH16 appeared to up-regulate *cps2A* expression in the bloodstream compared to in the nasopharynx, whereas D39 did not. However, due to the disparities between biological experiments, further experiments are needed to confirm the niche-specific differential expression of WCH16 *cpsA*.

5.3.2 Comparison of D39 microarray data with other published studies

Several studies have been published that identify genes essential for virulence or expressed more highly in certain niches and environmental conditions. Some of the major studies are presented in Table 5.5.

Genes that exhibited differential expression between niches in the current study were compared against other global virulence gene studies in the literature (Table 5.5). Of the various STM, DFI and microarray studies reported, an STM study by Hava and Camilli (2002), a DFI study by Marra *et al*. (2002) and a microarray study by Orihuela *et al*. (2004) were chosen to compare with the microarrays performed in this chapter. These studies represented a cross-section of currently published methods for examination of virulence genes on a global level. While all three methodologies have contributed to elucidating the relevance and expression of *S. pneumoniae* virulence genes on a global scale, each has met with limitations. STM uses transposons tagged with a unique DNA sequence marker to construct a bank of insertion mutants. The tag sequences from a pool of these mutants are amplified prior to co-infection of the relevant animal, and amplified again following the recovery of bacteria from the host. Hybridisation of tags to arrays comprising DNA from the pool of mutants indicates the presence or absence of each STM mutant following infection and, therefore, whether that particular gene is essential for virulence. In the study published by Hava and Camilli (2002), 6149 mutants were screened for abrogated virulence in an IN challenge lung infection model. However, as STM is based on the generation of random mutants, screening is not comprehensive. This may have been partly responsible for relatively few of the known virulence factors previously reported in the literature, such as genes of the CPS locus, PspA and CbpA, being identified by the three *S. pneumoniae* STM studies (Table 5.5). Also, while STM studies identify genes whose

Year	Author	Screening method	Strain (Serotype)
1998	Polissi *et al.*	STM	G54 (19F)
2001	Lau *et al.*	STM	0160993 (3)
2002	Marra *et al.*	DFI	D39 (2)
2002	Hava and Camilli	STM	TIGR4 (4)
2004	King *et al.*	cDNA microarray	6A-O (6A), 6A-T (6A), 6B-O (6A), 6B-T (6B)
2004	Orihuela *et al.*	cDNA microarray	T4X (4), T4R (4, un-encapsulated), D39 (2)
2006	Oggioni *et al.*	Real-time RT-PCR	TIGR4 (4)

***Table 5.5*: Published studies examining genes required for colonisation or invasive disease**

This table lists the major studies in the literature that identify genes essential to virulence or expressed more highly in certain niches and environmental conditions, the type of experiment conducted, and the *S. pneumoniae* strain and serotype used. Shaded cells indicate the studies that were cross-referenced with results from this chapter.

products are important for virulence, they do not give an indication of their regulation. Specific promoter activity can be monitored by using a fluorescent or luminescent reporter protein. The DFI studies performed by Marra *et al.* (2002) examined D39 strains expressing *gfp* under the control of various pneumococcal promoters. They examined promoter activity in a mouse lung infection model, a gerbil otitis media model, and a mouse IP chamber implant model (requiring the surgical insertion of a chamber containing bacteria into the peritoneal cavity). Additionally, promoter activity under a variety of *in vitro* conditions that simulated different aspects of niches in the host were examined; low iron, high osmolarity, carbon dioxide, growth on blood agar and growth at different temperatures. The half-life of wild-type GFP is 26 hr (although derivatives exist with half-lives of approximately 6 hr), so DFI is limited to examining promoter induction *in vitro* or following direct challenge of a niche and is unable to report transcriptional fluctuations in real time (Corish and Tyler-Smith, 1999). More recently, DNA microarray analysis has been used to complement existing DFI gene expression studies. Orihuela *et al.* (2004) performed microarray studies examining the regulation of *S. pneumoniae* genes in several models compared to a baseline control. The study identified genes that were up- or down-regulated in the bloodstream of BALB/cJ mice following IN challenge with a D39 derivative, or in the brains of New Zealand white rabbit 4 hr after intracisternal infection with a TIGR4 derivative, compared to growth in C+Y media. To simulate nasopharyngeal colonisation, an un-encapsulated derivative of TIGR4 was co-cultured with Detroit-562 epithelial cells. RNA was extracted from the recovered bacteria and compared with that extracted from bacteria grown in tissue culture media. The authors raised several considerations in their discussion regarding their use of a different *S. pneumoniae* strain and disease model to examine each aspect of pathogenesis; carriage, meningitis and bacteraemia. Specifically, there is likely to be strain-related gene regulation *in vivo*, and

also host factors and responses that are specific to each disease model. Therefore, the comparisons that can be made regarding differential gene expression between niches are limited.

33% of the genes reported in this chapter as exhibiting significant differences in expression between niches were also identified by at least one other study. However, only 8% of identified genes exhibited changes that could be directly compared to other studies by cross-referencing (e.g. pneumococci were examined in the same niche in both studies) and were found similarly regulated. These genes are shown in Table 5.6. Direct comparisons in gene expression between studies were not always possible due to the different criteria used, and different niches or conditions examined, in the various publications. Moreover, none of the studies in the literature (or the current study reported in this chapter) were completely comprehensive. Genes that appeared to be important to carriage or disease in this study and others, but were unable to be directly compared because of the aforementioned reasons, are listed in Table 5.7. Orihuela *et al.* (2004) performed a similar comparison between their work and the literature, and likewise found their data contained few genes that had previously been identified in other studies. Perhaps a reason for this disparity is the use of different *S. pneumoniae* strains in the studies, particularly considering that there may be strain-dependent differences in gene expression *in vivo*. Additionally, many of the studies examined different aspects of disease, and thus used different infection models. Another consideration is that the work presented in this chapter directly compares gene expression between niches. Thus, genes that are similarly up-regulated in all niches examined compared to growth in un-inducing conditions such as *in vitro*, were not identified. Such genes are likely to be important to multiple aspects of virulence, but not *in vitro* growth.

***Table 5.6*: Comparison of differentially expressed and essential virulence genes reported in the literature with genes identified in this study: genes exhibiting similarity**

Gene (gene name, if available)	TIGR annotation	Regulation in this work[a]	Paper identified in[b]
competence factor transport protein (*comB*)	Sp0043	0.078 (N/B)	Marra (+3.7, blood) Orihuela (-48.8, CSF)
hypothetical protein	Sp0144	0.090 (N/B) 0.053 (L/B)	Marra (lung infection) Orihuela (+13.0, blood)
conserved hypothetical protein	Sp0239	0.20 (N/L)	Orihuela (+3.1, ECC)
choline binding protein G	Sp0390	0.097 (N/B)	Orihuela (+1.7, blood)
protective antigen A (*prtA*)	Sp0641	0.28 (N/B) 0.23 (L/B)	Marra (+2.6, blood)
aminotransferase, class-V	Sp1094	0.11 (N/B)	Marra (+1.9, blood agar; +1.9 otitis media; +4.8, CO_2)
6-phospho-beta-galactosidase (*lacG*)	Sp1184	15.2 (N/L) 9.6 (N/B)	Orihuela (+6.5, ECC)
hypothetical protein	Sp1558	0.17 (N/B)	Marra (+, blood)
putative ATP-dependent RNA helicase	Sp1586	0.21 (N/L)	Orihuela (+3.1, ECC)
ROK family protein	Sp1675	30.5 (N/L)	King (+4.64, transparent)
ATP-binding protein (*amiE*)	Sp1888	0.11 (N/B)	Orihuela (+2.4, blood; +2.9, CSF)
MATE efflux family protein DinF	Sp1939	0.24 (N/L) 0.13 (L/B)	Hava (essential in lung)
choline binding protein A (*cbpA*)	Sp2190	22.0 (N/B) 14.5 (N/L) 7.1 (L/B)	Hava (essential in lung) Orihuela (+2.3, ECC)

[a] Indicates the difference in mRNA between niches as determined by microarray analysis in this study (niches that were compared are in parentheses).

[b] Results from the following experiments: Hava and Camilli, 2002 (niche in which gene is essential); King *et al.*, 2004 (fold-difference in gene transcript between opacity phenotypes); Marra *et al.*, 2002 (promoter induction ratio under specified *in vitro* condition or *in vivo* niche); Orihuela *et al.*, 2004 (fold-difference in gene transcript in a specific niche compared to a baseline control). These studies are described Section 5.3.2. CO_2 refers to carbon dioxide shift, ECC refers to an epithelial cell contact model, and CSF refers to cerebrospinal fluid. + and – indicate up- and down-regulation of gene expression under the specified condition, respectively.

***Table 5.7*: Comparison of differentially expressed and essential virulence genes identified in the literature and this study: genes that were identified in multiple studies, but in different conditions or niches**

Gene (gene name, if available)	TIGR annotation	Regulation in this work[a]	Paper identified in[b]
GTP-binding protein	Sp0004	11.8 (N/B) 9.2 (L/B)	Marra (+1.1, blood)
bacteriocin (*blpU*)	Sp0041	0.18 (L/B)	Orihuela (-3.1, CSF)
conserved hypothetical protein	Sp0095	0.19 (L/B)	Hava (essential in lung)
glycosyl transferase	Sp0102	0.14 (N/B)	Hava (essential in lung)
transcriptional regulator (*mutR*)	Sp0141	0.21 (N/L)	Orihuela (+3.0, blood)
phosphoglycerate mutase family protein	Sp0240	0.15 (L/B)	Hava (essential in lung)
transcriptional regulator, MerR family	Sp0501	0.21 (N/B)	Orihuela (-5.7, CSF)
beta-lactam resistance factor	Sp0615	0.13 (N/B)	Orihuela (+4.0, ECC)
putative PTS system IIA component	Sp0645	6.1 (N/L)	Hava (essential in lung)
chorismate binding enzyme	Sp0655	0.14 (N/B) 0.16 (L/B)	Hava (essential in lung)
endo-ß-*N*-acetylglucosaminldase (*lytB*)	Sp0965	0.11 (L/B)	Marra (+7.96, static temperature shift) Orihuela (+3.5, ECC)
acetyltransferase, GNAT family macrolide-efflux protein	Sp1023 Sp1110	0.13 (N/B) 0.14 (N/B)	Hava (essential in lung) Orihuela (-4.9, CSF)
SAP domain protein	Sp1292	15.9 (N/L) 10.1 (N/B)	Hava (essential in lung)
IS66 family element, Orf3, degenerate	Sp1311	0.11 (N/B)	Orihuela (-27.4, CSF)

***Table 5.7* continued**

Gene	TIGR annotation	Regulation in this work[a]	Paper identified in[b]
v-type sodium ATP synthase, subunit K (*ntpK*)	Sp1321	28.4 (N/L) 10.7 (N/B)	Hava (essential in lung)
sodium: solute symporter family protein	Sp1328	34.3 (N/L) 12.9 (N/B)	Hava (essential in lung)
IS1380-Spn1, transposase M	Sp1439	17.7 (N/L)	Marra (lung infection)
peptidoglycan N-acetylglucosamine deacetylase A (*pgdA*)	Sp1479	0.14 (N/B)	Marra (+2.30, otitis media) Orihuela (-3.2, CSF)
non-heme iron-containing ferritin	Sp1572	0.18 (L/B)	Orihuela (-8.4, blood)
conserved hypothetical protein	Sp1143	10.5 (N/L)	Hava (essential in lung)
nitroreductase family protein	Sp1710	0.20 (L/B)	Orihuela (+2.2, CSF)
cell wall surface anchor family protein	Sp1772	13.4 (N/L)	Hava (essential in lung)
transcriptional regulator, MerR family	Sp1856	0.13 (N/B) 0.20 (L/B)	Hava (essential in lung)
response regulator (*comE*)	Sp2235	0.098 (N/B)	Orihuela (-15.0, CSF)
spspoJ protein	Sp2240	0.14 (L/B)	Marra (lung infection)

[a] Indicates the difference in mRNA between niches as determined by microarray analysis in this study (niches that were compared are in parentheses).

[b] Results from the following experiments: Hava and Camilli, 2002 (niche in which gene is essential); King *et al.*, 2004 (fold-difference in gene transcript between opacity phenotypes); Marra *et al.*, 2002 (promoter induction ratio under specified *in vitro* condition or *in vivo* niche); Orihuela *et al.*, 2004 (fold-difference in gene transcript in a specific niche compared to a baseline control). These studies are described in Section 5.3.2. CO_2 refers to carbon dioxide shift, ECC refers to an epithelial cell contact model, and CSF refers to cerebrospinal fluid. + and – indicate up- and down-regulation of gene expression under the specified condition, respectively.

5.3.3 Considerations and conclusion

In this chapter, some of the limitations faced by other microarray studies were addressed. Primarily, a single model of infection was used to examine pneumococci involved in colonisation, lung disease and IPD. This satisfies the considerations raised by Orihuela *et al.* (2004) pertaining to the use of different animal models and pneumococcal strains to recover sufficient bacteria for microarray analysis. In their study, bacteraemia was examined for a D39 derivative following intratracheal infection of BABLB/cJ mice, while a TIGR4 derivative was used to induce meningitis in rabbits by intracisternal injection and then harvested 4 hr post-infection to examine pneumococcal behaviour in the brain. The limited overlap between genes identified by the three STM studies in the literature, each of which examined a different strain, is likely to be partly due to STM screenings not being completely comprehensive, as not all genes in each genome would have been tested. It may also suggest strain-specific roles and requirements for many pneumococcal genes. Furthermore, transcription regulation by two component signal transduction systems HK/RR04 and HK/RR09 response regulators RR04 and RR09 have both been shown to exhibit serotype-specific differences in regulatory activities (Hendriksen *et al.*, 2007; McCluskey *et al.*, 2004). Therefore, studies using different pneumococcal serotypes to compare multiple facets of disease must be viewed with these compounding considerations in mind. In the present work, bacterial expression in the nasopharynx, lungs and blood were examined following IN challenge under anaesthesia, and subsequent infection of blood and brain occurred by normal pathogenic routes. Bacteria were harvested 72 hr post-challenge, a time-point at which pneumococci were considered to be adapted to each specific niche and had presumably been present for a sufficient duration to allow the generation of a host response. As pneumococci had entered

the bloodstream and brain by natural progression of disease rather than following direct challenge of these niches, the possibility that high inocula created unnatural levels of infection or host responses was reduced.

However, the use of the continuous infection model was not without its own drawbacks. The main limitation was inconsistent bacterial levels in both the lungs and blood at 72 hr following IN infection of mice, which is unavoidable in a continuous IN infection model due to animal-to-animal variation. Additionally, the genes that failed to meet the analysis criteria described in Section 5.2.2 were not examined. Conducting further array experiments might generate reliable data for these genes and provide a more comprehensive indication of comparative *in vivo* gene expression for the two serotypes examined. Such experiments could not be conducted during this study because of time limitations. It is possible that, due to the sensitivity limitations of microarray and the many algorithms and restrictions that are necessary to analyse microarray data, transcripts present in low abundance or that exhibit only minor fluctuations in expression between niches, may not be identifiable using this technique. As indicated by the real-time RT-PCR results reported in Chapter 4, genes such as *pspA* may be up-regulated significantly in both colonising and bacteraemic pneumococci compared to *in vitro*, indicating an importance in colonisation and systemic disease. However, the difference in expression between niches may not be large enough to detect by microarray analysis. Therefore, the use of more sensitive RNA quantitation techniques such as real-time RT-PCR is essential to corroborate microarray data. Despite these considerations, the study described in this chapter is the first global transcriptional analysis of pneumococci involved in colonisation versus invasive disease. Moreover, bacteria were recovered from a continuous mouse model. The microarray experiments identified niches in which metabolism, transcription

and translation, cell wall and membrane synthesis and competence genes are up-regulated. This chapter also identifies some genes that have also been reported by other studies as having niche-specific importance and, therefore, are likely to be relevant to several pneumococcal serotypes.

CHAPTER 6 – Final discussion

6.1 Summary of this work

S. pneumoniae is a major human pathogen, and is responsible for significant morbidity and mortality in both developed and developing countries. It is adept at establishing and maintaining nasopharyngeal colonisation, with 5-95% of healthy children under five years of age carrying at least one serotype at any time depending on age, geographical area, genetic background, and socio-economic conditions (Bogaert *et al.*, 2004a; Crook *et al.*, 2004). Occasionally, perhaps due to the host possessing a compromised or immature immune system, the pneumococcus translocates from this niche to other sites within the host, where it can cause diseases such as otitis media, meningitis, sepsis and pneumonia. Worldwide, pneumococcal diseases are estimated to be responsible for almost one million deaths of children under five each year, most of which occur in developing countries (World Health Organization, 2007). *S. pneumoniae* also causes a significant disease burden in developed countries, with approximately seven million causes of otitis media being attributed to pneumococci annually in the USA, and one in 500 children suffering IPD before two years of age in Australia (Dowell *et al.*, 1999; Mackenzie *et al.*, 2005). The mechanism of regulation of virulence factors that enable the pneumococcus to be both an asymptomatic commensal and highly invasive pathogen is

poorly understood. Although the differential expression of several virulence genes between bacteria grown *in vitro* and harvested from a sepsis model has been previously described (Ogunniyi *et al.*, 2002), at the commencement of this work, no studies had examined quantitative differences in *S. pneumoniae* virulence gene mRNA levels between different host niches during disease pathogenesis. Furthermore, pneumococcal virulence gene expression during nasopharyngeal carriage had not been investigated. This thesis aimed to examine the transcriptional patterns of *S. pneumoniae* virulence genes *in vivo*, and hence gain a better understanding of pneumococcal behaviour during carriage and disease. Specifically, this required the establishment of an animal infection model from which reasonable numbers of bacteria could be harvested from multiple sites free from contamination with other bacteria, and the optimisation of RNA extraction and amplification procedures. The animal model needed to be appropriate for several different *S. pneumoniae* strains to allow the examination of strain-specific expression patterns. Real-time RT-PCR and microarray analyses were used to examine the transcriptional profiles of *S. pneumoniae* recovered from different niches in this model, for specific virulence factors and on a genomic scale, respectively.

6.1.1 Recovery of pneumococci from an IN infection model and RNA extraction and modifications

Previously, the inability to recover reasonable and consistent numbers of bacteria from different *in vivo* sites within the one animal species had necessitated the use of multiple invasive infection models, none of which had permitted the examination of pneumococcal gene expression during carriage.

In Chapter 3, an IN infection model for CD-1 mice was described (adapted from

Wu *et al.*, 1997) whereby pneumococci could be harvested from distinct anatomical sites, namely the nasopharynx, lungs, blood and brain of a single mouse with negligible cross-contamination from other host niches, other microbial flora, or host cells. Mice exhibiting sepsis possess high numbers of bacteria in the bloodstream. However, potential cross-contamination of tissue samples with pneumococci circulating in the blood has often been overlooked in other studies that have enumerated bacteria in host tissue, such as the lungs. In the current model, this problem was circumvented by removing the blood by PBS perfusion prior to organ harvesting. This thesis aimed to examine pneumococcal gene expression during colonisation and disease progression using real-time RT-PCR and microarray analysis. Previous studies had encountered difficulties due to the limited amount of mRNA that could be harvested from *in vivo* sites. This was overcome by tailoring the procedure by which pneumococci are harvested from mice to optimise yield of bacteria, to minimise RNA degradation, and by linearly amplifying the extracted RNA prior to use in assays (Chapter 3). Furthermore, host tissue contamination from lung and brain samples was reduced by differential centrifugation prior to RNA extraction, followed by the enrichment of RNA samples for prokaryotic RNA species. This enabled downstream linear amplification procedures to be conducted with greater efficiency.

The infection model described here overcomes many of the complications raised by Orihuela *et al.* (2004) regarding the mRNA studies they performed. Their study utilised several different animal, cell culture and challenge models to simulate infection *in vivo* in order to recover sufficient bacteria for transcriptional analysis. A D39 intratracheal challenge model was used to examine bacteraemia, whereas meningitis was modelled by intracisternal injection of a TIGR4 derivative. Nasopharyngeal carriage was simulated *in vitro* by co-culturing an un-encapsulated TIGR4 derivative with Detroit-562

nasopharyngeal cells. The main advantage of the IN infection model and RNA processing described in Chapter 3 is that gene expression in the nasopharynx, lungs, blood and brain could be examined using a single pneumococcal strain and a continuous infection model. This overcomes the potential interpretational complications imposed by strain- and animal model-specific transcriptional regulation. Furthermore, an IN infection model simulates the normal route of acquisition of *S. pneumoniae*, and progression to the blood and brain. This also avoids introducing unnaturally high levels of bacteria directly into these niches, which is a consideration when using challenge models such as IP and intracisternal injection, and allows for natural host responses to be generated. As such, this model appears well suited to the examination of immune responses to *S. pneumoniae* infection *in vivo*.

6.1.2 D39 and WCH16 possess different pathogenesis profiles

In the CD-1 IN infection model established in Chapter 3, both D39 (a well studied serotype 2 laboratory strain originally isolated by Avery in 1916 [Avery *et al*., 1944]) and WCH16 (a more recent serotype 6A clinical isolate) were shown to persist in the nasopharynx for 72 hr and progress to other niches. By enumerating levels of pneumococci in specific sites using this model, performing tail bleeds and monitoring disease progression in individual mice using luminescent D39 and WCH16 derivatives, two distinct pathogenesis patterns emerged (Figure 6.1). D39 pathogenesis is characterised by the development of lung disease followed by infiltration of the blood by pneumococci (Chapter 3 and 4). Mice exhibiting bacteraemia quickly develop sepsis and often die within 24 hr after the establishment of bacteraemia. WCH16 is also virulent and able to cause invasive disease, usually progressing to the bloodstream and brain within 48 hr post-challenge. However, this often occurs in the absence of detectable lung disease, which

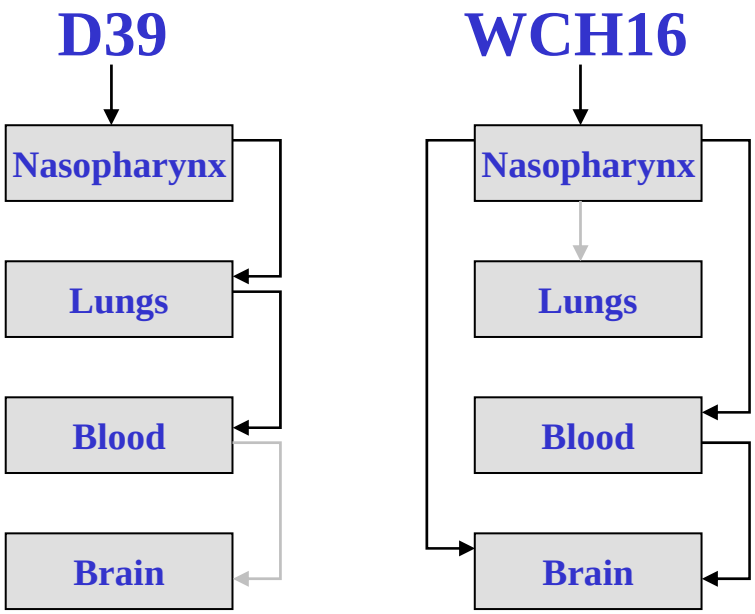


***Figure 6.1*: Distinct pathogenicity profiles of D39 and WCH16**

The pathogenesis of D39 and WCH16 infection in CD-1 mice after IN challenge, as determined in Chapter 3. Bold arrows indicate the preferred progression of infection for each strain. Grey arrows represent secondary routes of pathogenesis.

perhaps suggests that the preferred route for WCH16 to infiltrate the bloodstream is by translocating across the nasopharyngeal epithelium. A further disparity between the two strains is that mice challenged IP with approximately 2×10^7 WCH16 bacteria often maintain a constant, sub-septic, level of bacteraemia for several days and have double the median survival time of mice similarly infected with D39 (Chapter 3). The reasons for the different outcomes of bacteraemia were not elucidated in this work.

6.1.3 D39 and WCH16 genes that exhibit contrasting *in vivo* regulation

The different pathogenicity profiles observed for D39 and WCH16 in Chapter 3 raises the possibility that pneumococci might exhibit strain- or serotype- specific virulence gene regulation patterns *in vivo*, which may influence the course of disease. This appeared to be the case for several virulence factors examined by real-time RT-PCR in Chapter 4, as depicted in Figure 6.2. This figure summarises the *in vivo* niches in which specific pneumococcal virulence factors are up-regulated, and so perhaps are more likely to be important for virulence in that niche. An interesting discrepancy between the strains was the marked up-regulation of D39 *nanA* in the nasopharynx compared to other niches, but in WCH16 there was little *in vivo* transcriptional regulation relative to *in vitro* levels. NanA is thought to contribute to carriage by both desialylating the cell surfaces of competing microflora and modifying host cell glycoconjugate receptors, providing a competitive advantage for the pneumococcus and promoting adherence to the nasopharyngeal epithelium (King *et al.*, 2004; Shakhnovich *et al.*, 2002). Hence, it might be expected that a lack of transcriptional regulation for WCH16 *nanA* during pathogenesis would be reflected by attenuated colonisation compared to D39, which exhibits a marked up-regulation of *nanA* in the nasopharynx. However, in this study both D39 and WCH16 were able to establish similar levels of colonisation in CD-1 mice for at least 72 hr after IN

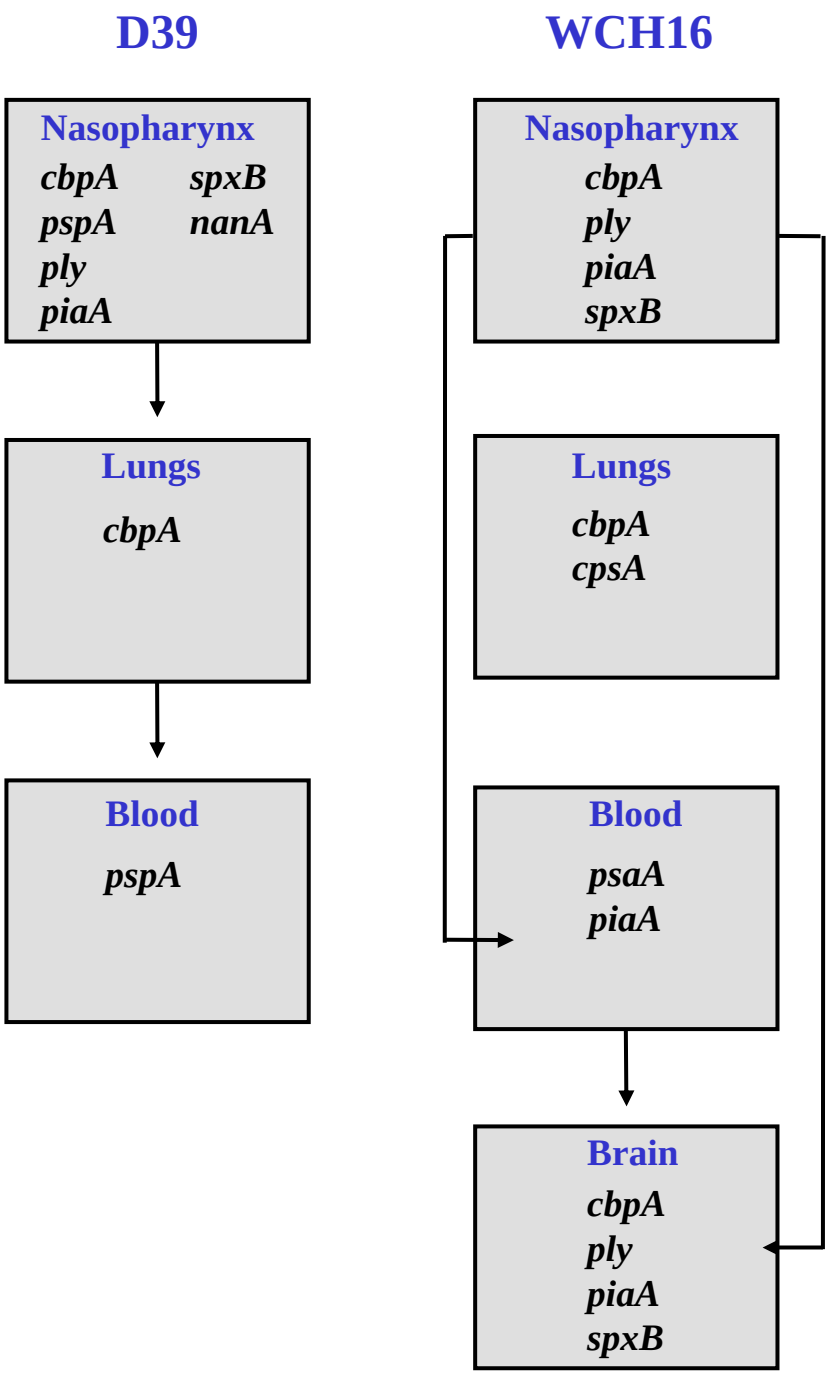


Table 6.2*: Importance of specific *S. pneumoniae* virulence factors *in vivo

Genes that were found to be up-regulated in specific *in vivo* niches by real-time RT-PCR in this study, relative to other niches and *in vitro*. Arrows indicate the preferred progression of disease for each strain.

challenge, based on bacterial numbers present in nasopharyngeal washings. Of course, it is possible that increased levels of NanA promote tighter pneumococcal adherence to the nasopharyngeal epithelium and that these bacteria may not be eluted by washing with a buffer (Briles *et al.*, 2005). This possibility was not examined in the current work, but could be determined in the future by enumerating bacteria in the nasopharyngeal tissue. PsaA also appears to exhibit different expression patterns during D39 and WCH16 infections. PsaA-negative mutants have been shown in other studies to exhibit greatly attenuated colonisation, lung disease and bacteraemia in mice, and also possess defective oxidative stress responses (Marra *et al.*, 2002b; McAllister *et al.*, 2004; Tseng *et al.*, 2002). This work suggests that PsaA plays a larger role during WCH16 sepsis than carriage, as it is more highly expressed in the blood than in other niches. *In vivo* expression was constitutive during D39 infection, which may indicate that PsaA contributes to both colonisation and invasive disease for D39, either in its primary role as a component of a Mn^{2+} transporter or in other undefined roles.

The work in thesis clearly indicates that *in vivo* transcriptional regulation of some virulence factors is strain-specific. It is also possible that strain-specific expression levels of certain genes such as *ply* may be partly responsible for corresponding differences in pathogenicity between strains. This highlights the importance of examining several *S. pneumoniae* strains and serotypes before drawing generalised conclusions about the contributions of specific proteins to virulence. This may be of particular importance when considering candidates for inclusion in protein-based pneumococcal vaccines, which need to provide broad protection against carriage and invasive disease caused by a wide spectrum of strains/serotypes.

6.1.4 D39 and WCH16 genes that exhibit similar *in vivo* regulation

This study found that several important virulence factors exhibited similar *in vivo* expression patterns for both D39 and WCH16, which may indicate the essential requirement of these factors for colonisation and virulence in multiple serotypes (Chapters 4 and 5). Expression of the putative nasopharyngeal adhesin CbpA is noticeably up-regulated during carriage for both D39 and WCH16, which supports its ascribed roles in carriage (Rosenow *et al.*, 1997; Zhang *et al.*, 2000). Likewise, D39 and WCH16 bacteria recovered from the nasopharynx express higher amounts of *spxB* transcript than bacteria recovered from other niches. The increased expression of *cbpA* and *spxB* in the nasopharynx is likely to help colonisation of this site by promoting adherence to the epithelium and suppressing the growth of competing microflora (Pericone *et al.*, 2000; Rosenow *et al.*, 1997; Spellerberg *et al.*, 1996).

Interestingly, both D39 and WCH16 express higher levels of *ply* in the nasopharynx than the lungs or bloodstream - niches where the cytotoxin is considered to be crucial to disease progression and development (Berry *et al.*, 1992; Cockeran *et al.*, 2002; Feldman *et al.*, 1990; Houldsworth *et al.*, 1994; Paton *et al.*, 1984; Steinfort *et al.*, 1989). This finding reinforces the notion that IPD is incidental to carriage, with pneumococci experiencing little selective pressure in the blood. Moreover, bacteraemia is counterproductive to the spread of *S. pneumoniae* in the community, and it is likely that the low incidence of IPD relative to the numbers of *S. pneumoniae* carriers has allowed strains that are capable of causing severe morbidity and mortality to persist within populations rather than being eliminated due to negative selection. With this in mind, it is unsurprising that many pneumococcal virulence factors involved in disease have also been implicated as being important for carriage. PspA is one such virulence factor, capable of promoting both

carriage and bacteraemia by inhibiting apolactoferrin-mediated killing of pneumococci in the nasopharynx, and also reducing complement-mediated opsonophagocytosis in the blood (Shaper *et al.*, 2004; Tu *et al.*, 1999). The real-time RT-PCR results in this study support roles for PspA in the nasopharynx and the blood for D39, with increased expression observed in both these niches, relative to the lungs and *in vitro*. Likewise, microarray data for D39 and WCH16 indicated no significant difference in *pspA* expression between bacteria recovered from the nasopharynx and blood. In addition, several virulence factors appear to be important in both carriage and meningitis. This is discussed in Section 6.1.5.

6.1.5 Bacteria recovered from the nasopharynx and brain exhibit similar expression of some key virulence genes

Pneumococci can be visually separated into two distinct opacity phenotypes, based on colony morphology when viewed on transparent agar (Weiser *et al.*, 1994). Despite there being few *in vitro* transcriptional differences between opaque and transparent variants reported, increased protein levels of several virulence factors such as CbpA and PspA have been associated with a specific opacity phenotype (Kim and Weiser, 1998; King *et al.*, 2004; Rosenow *et al.*, 1997). Furthermore, the transparent phenotype has been associated with carriage and bacteria crossing the BBB, whereas bacteria in the blood are predominantly of the opaque phenotype (Cundell *et al.*, 1995b; Ring *et al.*, 1998; Weiser *et al.*, 1994; Weiser and Kapoor, 1999). The current study compared pneumococci recovered from the nasopharynx and the brain, and clearly demonstrated that WCH16 pneumococci involved in colonisation and early meningitis exhibit similar expression profiles for key virulence genes, as summarised in Figure 6.2. Specifically, *cbpA*, *ply*, *piaA* and *spxB* were found to be up-regulated in both these niches compared to bacteria recovered from the

bloodstream and/or lungs. Moreover, microarray analysis indicated that genes with roles in fatty acid metabolism are up-regulated in the blood, which supports previous studies by other groups associating membrane fluidity and fatty acid composition with pneumococcal opacity phenotype (Aricha *et al.*, 2004; Saluja and Weiser, 1995). While these observations are not direct evidence supporting the existence of an 'opacity phenotype switch', they do suggest there may be a link between transcriptional changes, opacity phenotype and *in vivo* niche.

6.1.6 Global microarray analysis identifies differentially expressed genes during carriage or disease

Microarray analysis of D39 *in vivo*-derived pneumococci identified genes that exhibit niche-specific expression that were not specifically examined by real-time RT-PCR (Chapter 5). An Array 900 MPX Kit (Genisphere) was used for microarray experiments performed in this study, which is optimised for smaller quantities of RNA. Of particular interest, the microarray analysis found that mRNAs for competence genes *comB* and *comE* are clearly up-regulated in the blood compared to the nasopharynx for both D39 and WCH16. *comX1* and *cglA* are also up-regulated in this niche for WCH16. These results are consistent with the findings from other groups indicating increased transcription of competence genes occurs during exponential growth (Claverys and Havarstein, 2002). The expression of *dinF* (a gene in the competence-induced *cin-recA* operon (Mortier-Barriere *et al.*, 1998; Pearce *et al.*, 1995)) parallels that of competence genes, indicating a role for both competence and DNA repair during sepsis. These findings disagree with a recent study by Oggioni *et al.* (2006), which reported TIGR4 competence genes to be repressed in the blood. This may indicate competence has serotype-specific importance. The CBPs *lytB*, encoding a cell wall hydrolase essential for daughter cell separation (Garcia *et al.*,

1998), and *cbpG*, encoding a putative serine protease (Gosink *et al.*, 2000), are also expressed more highly during sepsis than carriage for D39, with pneumococci recovered from the blood possessing approximately 10-fold more of both transcripts (Chapter 5). The finding for *cbpG* strongly supports microarray data from Orihuela *et al*. (2004) and mutagenesis studies performed by Gosink *et al*. (2000), which found *cbpG* to be induced in the blood and necessary for full virulence in this niche. Conversely, the absence of LytB has previously been shown to have no effect on sepsis in an IP infection model (Gosink *et al.*, 2000). However, those experiments used a serotype 4 *S. pneumoniae* strain, and so it is possible that LytB has a role in sepsis for D39, but not for the serotype 4 strain used by Gosink *et al*. (2000).

Microarray analysis also identified several genes with unknown functions exhibiting large fold-differences in expression between colonising bacteria and bacteria involved in bacteraemia, thus indicating that they may contribute to virulence. In particular, hypothetical proteins encoded by Sp0144 and Sp1558 exhibit significantly elevated expression in the blood compared to the nasopharynx, and have been similarly identified by either DFI or other microarray studies (Marra *et al.*, 2002a; Orihuela *et al.*, 2004b). Additionally, a putative neuraminidase encoded by Sp1326 was up-regulated in the respiratory tract compared to the blood. The differential expression of these genes could be confirmed using real-time RT-PCR, and it may also be interesting to examine their role in further by mutagenesis studies.

6.1.7 Conclusion

An understanding of how the pneumococcus responds transcriptionally to different environments in the host has been sought for many years, but it has hitherto remained

elusive due to technical constraints. Although other global *S. pneumoniae* virulence gene discovery and expression studies have been reported in the literature (Hava and Camilli, 2002; Lau *et al.*, 2001; Marra *et al.*, 2002a; Polissi *et al.*, 1998), knowledge of differential niche-specific *in vivo* gene expression is limited. The work in this thesis has examined pneumococcal gene expression in a mouse model on both a specific and genomic level, and has determined *in vivo* transcriptional patterns. This is the first work that has examined multiple facets of *S. pneumoniae* infection using bacteria recovered from a single mouse model, and therefore is novel and complements existing studies in the literature. It reflects pneumococcal pathogenesis better than other studies which have required use of direct niche-specific challenge models and multiple *S. pneumoniae* strains to simulate human infection. Moreover, it suggests the existence of strain-specific gene expression for several key virulence factors, which may have implications for the development of protein-based pneumococcal vaccines. Similar gene expression profiles for pneumococci during carriage and early meningitis also allude to a link between transcriptional regulation, opacity phenotype, and host niche.

6.2 Future directions

Due to time constraints and technical complications, replicate microarrays using samples recovered from distinct biological experiments could not be completed for all niche comparisons during this study. In addition, the WCH16 data presented in Chapter 5 were inconclusive due to considerable variation between experiments. Replicate biological experiments and corresponding micorarray experiments comparing WCH16 expression *in vivo* need to be completed to clarify the results. Furthermore, selected genes that were found to be differentially expressed by microarray analysis should be validated by real-

time RT-PCR using specific oligonucleotides. This is particularly so for the competence genes *comB* and *comE,* which were not found to be up-regulated in the blood compared to Detroit-562 epithelial cells by Orihuela *et al.* (2004), but were found to be induced during sepsis in the current study for two strains of different serotype. Further analysis is also needed for the genes encoding CBPs *cbpG* and *lytB.* LytB demonstrated increased transcription in the bloodstream of infected mice for D39, despite studies by other groups using a serotype 4 strain *lytB*$^{-}$ mutant finding no attenuation in sepsis compared to the WT (Gosink *et al.*, 2000). Therefore, mutagenesis studies examining the role of *lytB* in D39 virulence would help elucidate the role of this CBP in sepsis and indicate whether it is a strain-dependent virulence determinant. A better understanding of the disparities in transcriptional profiles between strains is important.

It is important to note that none of the studies examining global pneumococcal gene expression *in vivo* using techniques such as STM and microarray analysis have been entirely comprehensive, including the microarray data presented in Chapter 5 of this thesis (Hava and Camilli, 2002; King *et al.*, 2004; Lau *et al.*, 2001; Orihuela *et al.*, 2004b; Polissi *et al.*, 1998). Nevertheless, the existing studies can be viewed in parallel with each other, suggesting that a combination of approaches needs to be employed to gain a complete picture of pneumococcal regulatory behaviour *in vivo*. The use of bioluminescence could be extended to examine *in vivo* promoter activity, by constructing a plasmid possessing a *lux* cassette under the control of specific promoters. Lux would be the preferred reporter for such a system, as the Lux proteins require less time to produce a signal than GFP (which undergoes significant post-translational modifications, requiring over 4 hr to form the fluorophore) (Cody *et al.*, 1993; Heim *et al.*, 1994). Lux proteins also have a significantly shorter half-life than GFP, which would allow promoter kinetics to be

monitored in real time (Cody *et al.*, 1993; Tombolini and Jansson, 1998). From a host perspective, many symptoms of IPD are thought to be a result of cyclic interactions between *S. pneumoniae* and the host, leading to an uncontrolled inflammatory response. Using real-time RT-PCR to examine the host cytokine levels and pneumococcal gene expression simultaneously may give a clearer picture of both pneumococcal behaviour and the host's response. This may also elucidate host-pathogen responses that lead to the pneumococcus either being cleared from a niche, or progressing to invasive disease.

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Appendix

Journal Publications

LeMessurier, K.S., Ogunniyi, A.D. and Paton J.C. (2006) Differential expression of key pneumococcal virulence genes *in vivo*. *Microbiology* **152**: 305-311.

Ogunniyi, A.D.*, **LeMessurier, K.S.***, Graham, R.M., Watt, J.M., Briles, D.E., Stroeher, U.H. and Paton, J.C. (2007) Contributions of pneumolysin, pneumococcal surface protein A (PspA), and PspC to pathogenicity of Streptococcus pneumoniae D39 in a mouse model. *Infect. Immun.* **75**: 1843-1851 (* Authors contributed equally).

Mahdi, L.K., Ogunniyi, A.D., **LeMessurier, K.S.** and Paton, J.C. (2007) Virulence gene expression and innate immune responses during pathogenesis of pneumococcal infection in a murine model (submitted).

Poster presentations

LeMessurier, K.S., Ogunniyi, A.D. and Paton J.C. Differential expression of key pneumococcal virulence genes *in vivo*. 7th European Meeting on the Molecular Biology of the Pneumococcus, 8-11 May 2005, Braunschweig, Germany.

LeMessurier, K.S., Ogunniyi, A.D. and Paton J.C. Differential expression of pneumococcal virulence genes *in vivo*. Abstract P05.06, p 198. 5th International Symposium on Pneumococci and Pneumococcal Diseases, 2-6 April 2006, Alice Springs, Australia.

LeMessurier, K.S., Ogunniyi, A.D. and Paton, J.C. (2006) Differential expression of key pneumococcal virulence genes *in vivo*
Microbiology v. 152, pp. 305-311, February 2006