Studies of the Two-Component Signal Transduction System RR/HK06 in *Streptococcus pneumoniae*

THE UNIVERSITY OF ADELAIDE
AUSTRALIA

Alistair James Standish, B Biotech (Hons)

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy from the University of Adelaide

September 2006

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

ABSTRACT

Streptococcus pneumoniae (the pneumococcus) is a major human pathogen responsible for significant morbidity and mortality worldwide. Pneumococcal disease, which can include both invasive conditions such as pneumonia, bacteremia and meningitis, as well as less severe conditions such as otitis media, is almost invariably preceded by asymptomatic colonisation of the nasopharynx. To successfully adapt to the different ecological niches it encounters, the pneumococcus is likely to rely on the co-ordinated regulation of key virulence factors. As is the case for many other prokaryotes, this is likely to occur through two-component signal transduction systems (TCSTSs). TCSTSs comprise a histidine kinase (HK) and response regulator (RR). They respond to environmental stimuli, and regulate gene expression by interacting with the transcription machinery. Thirteen complete TCSTSs have been identified in *S. pneumoniae*, along with a lone RR. This study focused on one of these systems, designated RR/HK06.

In order to study this system, in-frame deletion mutants of *hk06* (D39Δ*hk06*) and *rr06* (D39Δ*rr06*) were constructed in *S. pneumoniae* D39. Western immunoblot and real time RT-PCR analysis showed that expression of the major virulence factor and protective antigen choline binding protein A (CbpA) was increased (approximately 5-fold) in D39Δ*hk06* but decreased (approximately 3-fold) in D39Δ*rr06*, compared to the wild-type D39. This suggested *cbpA* expression is regulated by RR/HK06. Furthermore, binding of RR06 to DNA upstream of the *cbpA* start codon was demonstrated by solid phase binding assays, confirming this regulation. Over-expression of the system showed that RR/HK06 activates expression of *cbpA* in D39. However, an in-frame deletion mutant in both *hk06* and *rr06* (D39Δ*rr/hk06*) produced similar levels of *cbpA* mRNA as the D39 wild-type.

Over-expression and mutation of *rr/hk06* in 3 other *S. pneumoniae* strains showed that RR/HK06 regulates the expression of *cbpA* across all 4 pneumococcal strains tested, albeit with some differences. Most RRs are active in the phosphorylated form, as illustrated by the fact that mutations in the cognate HK result in a reduction in regulated gene expression. Thus, the increased expression of *cbpA* in D39Δ*hk06* was unexpected and prompted further investigation. Amino acid substitutions in D39 HK06 led to the hypothesis that RR06 may activate *cbpA* expression in the non-phosphorylated form, as a substitution thought to specifically abrogate the phosphatase activity of HK06 led to levels of *cbpA* expression similar to that seen in the wild-type D39. However, further biochemical analysis is needed to confirm this.

Studies into the system's role in the virulence of *S. pneumoniae* showed that RR/HK06 is important for the ability of the pneumococcus to adhere to epithelial cells *in vitro* and to survive and proliferate in an *in vivo* model. Both D39Δ*hk06* and D39Δ*rr06* exhibited reduced adherence to human epithelial cells, even though D39Δ*hk06* showed increased levels of CbpA, a known pneumococcal adhesin. These findings clearly implicate additional RR/HK06-regulated factors in adherence to epithelial cells of human origin. *In vivo* experiments in mice showed that D39Δ*rr06* had an increased capacity to colonise the nasopharynx and cause disease compared to the parental strain, while D39Δ*hk06* was unable to persist in the lungs and blood. However, a strain deficient in CbpA showed no significant differences relative to the wild-type in its ability to colonise the nasopharynx or translocate to the lungs and blood. These data clearly indicated that other, as yet uncharacterized RR/HK06-regulated factors play a significant role in both colonisation and invasive disease, at least in the mouse model.

In order to identify other RR/HK06-regulated genes, microarray analysis was undertaken to investigate changes in gene expression when RR06 was over-expressed in both D39 and TIGR4 *S. pneumoniae*. *cbpA* and its co-transcribed upstream gene showed

substantial increases in expression when RR06 was over-expressed in both strains. However, there were no other similarities between the genes regulated by RR/HK06 in the two strains, suggesting that regulation varies between *S. pneumoniae* strains. In D39, RR06 over-expression decreased expression of numerous factors including the major virulence factor *pspA*, and another pneumococcal TCSTS *vncRS*. Further investigation of *pspA* regulation by RR/HK06 showed that the factor appeared to be regulated in a different manner to that seen for *cbpA*.

DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

The author acknowledges that copyright of published works contained in this thesis (as listed below) resides with the copyright holder/s of those works.

.....

Alistair James Standish

Published work included in this thesis:

Standish, AJ, Stroeher, UH and Paton JC. (2005). The two-component regulatory transduction system regulates expression of *cbpA* in Streptococcus pneumoniae. *Proc Natl Acad Sci U S A.* **102:**7701-6.

ACKNOWLEDGEMENTS

I would first of all like to thank my supervisor Prof. James Paton for the endless support and encouragement he has provided over the past (almost) 5 years during both honours and my PhD. Your door was always open, no matter how busy you were. I would also like to acknowledge my co-supervisor, Dr. Uwe Stroeher, not only for his input into almost every aspect of the work described in this thesis, but also for being a great friend and helping me to remain sane over the past few years (.....well nearly).

To all other members of the pneumo lab, CHEERS! I would particularly like to thank Jan for always being there when I needed an extra set of hands, and for putting up with my cleanliness (or lack thereof) in our office. Thanks for the lifts home too! To Judy and Dave OOOOO, thank you for always being open to discussions on my work (and everything else in the universe) and for your help with animal experiments over the years. Hopefully it won't be too quiet in the lab now!

I would also like to acknowledge all others who have been a part of the lab in my time – Adrienne, Bec, Damien, David M, Emma, Jeng, Hui, Katie, Kim, Lauren, Layla, Maggie, Marcin, Richard, Rikki, Stu, Tony, Trisha and Ursula. You have all helped to make this lab such a great place to work in. Thanks also to the members of the Morona Lab and the entire School of Molecular and Biomedical Sciences for all your support and friendship. Thanks to Mark at the Microarray facility for his help with analysis, to Paloma Lopez for kindly providing the vector pLS1RGFP and to Jackie Hung for supplying me with *L. lactis*.

A special mention must also go to all the students who have gone through both the Paton and Morona labs in my time here. Thank you for being such great friends and for helping (along with beer and numerous other alcoholic beverages) to making everything

appear better on a Friday night (yes, even with all those undergrads!). Thanks also to Marcin for his critical reading of this thesis.

A special thank you must go to Kim LeMessurier and Kerrie May. I really don't know how I would have got through the past 7 and a half years without you both. I wish you both the best of luck in your futures, and hope they are all that you dream them to be. You both deserve it. Keep in touch.

Finally, I would like to thank my family and friends. Yes, I am no longer a student (seriously, no joke)! It must be my shout....To Mum and Dad, thanks for all your support (as always), sausage rolls and wine over the past few years. I am forever grateful. And, finally, Lucy. All of this is nothing without you. Thank you for putting up with me. I can't wait for the next adventure of our lives together to begin. I love you.

ABBREVIATIONS USED IN THESIS

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

A_{600}	Absorbance at 600 nm
aa	Amino acid
Asp	Aspartate
Amp	Ampicillin
BSA	Bovine serum albumin
C3	Complement C3
C3b	Complement C3b
C5	Complement C5
C-terminus	Carboxy terminus
Cml	Chloramphenicol
CbpA	Choline binding protein A
CFU	Colony forming units
CSP	Competence Stimulating Peptide
CXC	Cysteine (C)-X-C motif
DEPC	Diethyl Pyrocarbonate
DIG	Digoxigenin
DMEM	Dulbecco's modified essential medium
DOC	Deoxycholate
EDTA	Ethylene diamine tetraacetic acid
EMEM	Eagle's modified essential medium
EMS	Electrophoretic mobility shift
erm	Erythromycin resistance gene
Ery	Erythromycin
FCS	Foetal calf (bovine) serum
g	Gravity units
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Gent	Gentamicin
His	Histidine
HIV	Human immunodeficiency virus
HK	Histidine kinase
h	Hour(s)
Ig	Immunoglobulin
IL	Interleukin
i.n.	Intranasal
i.p.	Intraperitoneal
IPTG	Isopropyl-β-D-thiogalactoside
Kan	Kanamycin
LB	Luria Bertani broth

LD50	50% lethal dose
LytA	Autolysin A
Min	Minute(s)
MQ	Milli Q
N-terminus	Amino terminus
Ni-NTA	Nickel nitrolotriacetic acid
ORF	Open reading frame
PAF	Platelet activating factor
PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
pIgR	Polymeric immunoglobulin receptor
Ply	Pneumolysin
PS	Polysaccharide
PsaA	Pneumococcal surface antigen A
PspA	Pneumococcal surface protein A
^{R}	Resistant
RR	Response regulator
RS	Restriction site
RT	Room temperature
RT-PCR	Reverse-transcription polymerase chain reaction
^{S}	Sensitive
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	Standard error
Sec	Second
sIgA	Secretory immunoglobulin A
SPB	Solid phase binding
TCSTS	Two-component signal transduction system
THY	Todd-Hewitt broth supplemented with yeast extract
TTBS	Tween-Tris buffered saline
U	Units
WCL	Whole cell lysate
WT	Wild type
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galacto-pyranoside

CHAPTER ONE – INTRODUCTION

1.1 *Streptococcus pneumoniae*

Streptococcus pneumoniae, commonly known as the pneumococcus, is a major cause of morbidity and mortality worldwide. This gram-positive pathogen is responsible for diseases ranging from life-threatening pneumonia, bacteremia and meningitis to the less serious but highly prevalent otitis media and sinusitis. The incidence of pneumococcal disease is greatest in children under the age of 5, the elderly, and in people with predisposing conditions, particularly AIDS (Jedrzejas, 2001). It is estimated that the pathogen is responsible for approximately 1 million deaths in the under-5 age group each year, with the vast majority of these being in developing countries (WHO [http://www.who.int/mediacentre/factsheets/fs178/en], 2004). However, *S. pneumoniae* also causes problems in developed countries, such as the US, where it accounts for over 6 million cases of disease annually, the most prevalent of which is otitis media (Jedrzejas, 2001). This leads to a massive impact on health budgets, with estimates for the US alone in excess of $2 billion per annum (Klein, 2000). The ever-increasing rates of resistance of pneumococci to antibiotics, along with the real and perceived deficiencies of the current generation of vaccines, mean that the pneumococcus is still a major focus of research, with considerable effort directed at the identification of novel targets for both antimicrobial agents and vaccines.

1.2 Pathogenesis of disease

The pathogenesis of pneumococcal disease begins with asymptomatic colonisation of the nasopharynx. This is thought to be essential not only for the facilitation of disease, but also for the transmission of the bacterium, particularly in view of the lack of any animal or environmental reservoirs. Carriage is widespread throughout both developed and developing countries, although the rates vary widely between 5 and 95% depending on factors such as age, geographic location and socio-economic status (Crook *et al.*, 2004). Colonisation does not always lead to disease, with asymptomatic carriage being the prevalent state and typically lasting from three to six months depending on serotype (Gray *et al.*, 1980). Transmission most commonly occurs via asymptomatic carriers rather than through infected individuals (Briles *et al.*, 2000b), and is dependent on both the frequency and intimacy of the contact between the carrier and the susceptible individual (Riley & Douglas, 1981). Colonisation of healthy adults generally leads to the production of serotype-specific antibodies, resulting in clearance of pneumococci and possible (limited term) protection from the same serotype (Musher *et al.*, 1997).

In a proportion of individuals (approximately 15% in one paediatric study [Gray *et al.*, 1983]), the pneumococcus is able to penetrate host defences, and asymptomatic colonisation of the nasopharynx progresses to invasion of the ear resulting in otitis media, the lung causing pneumonia, the blood producing bacteremia and/or brain resulting in meningitis. Such infection typically follows recent acquisition of the invasive strain, rather than following extended carriage (Gray *et al.*, 1980). While this switch from carriage to disease is the watershed event in pneumococcal pathogenesis, the molecular events responsible are poorly understood. It is clear, however, that the translocation to a new niche results in a substantial change in the micro-environment to which the pneumococcus is exposed, and is associated with alteration in gene expression profiles, as has been seen in a number of recent studies (Ogunniyi *et al.*, 2002; Orihuela *et al.*, 2004b; LeMessurier *et*

al., 2006). A number of host factors have also been implicated in the progression from colonisation to invasive disease. In particular, decreased host immunity, due to smoking or viral infections such as HIV, is thought to have a marked effect on both the translocation of pneumococci to the lungs and subsequent invasion of the blood (Janoff *et al.*, 1993; Nourti *et al.*, 2000; Zimmerman, 2001). The pneumococcus may then spread from the blood to the brain causing meningitis, which is fatal in 30% of cases (Briles *et al.*, 1996).

1.3 Virulence Factors

Pathogenesis of pneumococcal disease is presumed to require the coordinated expression of a series of virulence-related genes that allow the pathogen to respond to the hostile environment of the host. While the capsule is recognised as the major virulence factor of the organism, there are several other factors that contribute to the virulence of *S. pneumoniae*. The first release of a pneumococcal genome sequence (http://www.tigr.org/) led to a massive leap forward in the identification of key virulence genes. Additionally, a number of recent studies using signature-tagged mutagenesis have identified a multitude of factors important in pneumococcal virulence (Polissi *et al.*, 1998; Lau *et al.*, 2001; Hava & Camilli, 2002). The majority of virulence factors identified to date are present on the cell surface (**Figure 1.1**), and are involved in a range of functions involved in all aspects of pneumococcal infection (AlonsoDeVelasco *et al.*, 1995; Briles *et al.*, 1998). Four virulence factors that have been studied extensively are described in detail below.

1.3.1 Polysaccharide Capsule

The polysaccharide capsule (PS) forms the outermost layer of *S. pneumoniae*. To date, a total of 90 structurally and serologically distinct capsular PS types have been recognised (Henrichsen, 1995), and chemical structures for approximately 60% of these have been determined (reviewed by van Dam *et al.*, [1990]). Recognized as the *sine qua non* of pneumococcal virulence (Austrian, 1981), the PS capsule has been a major target

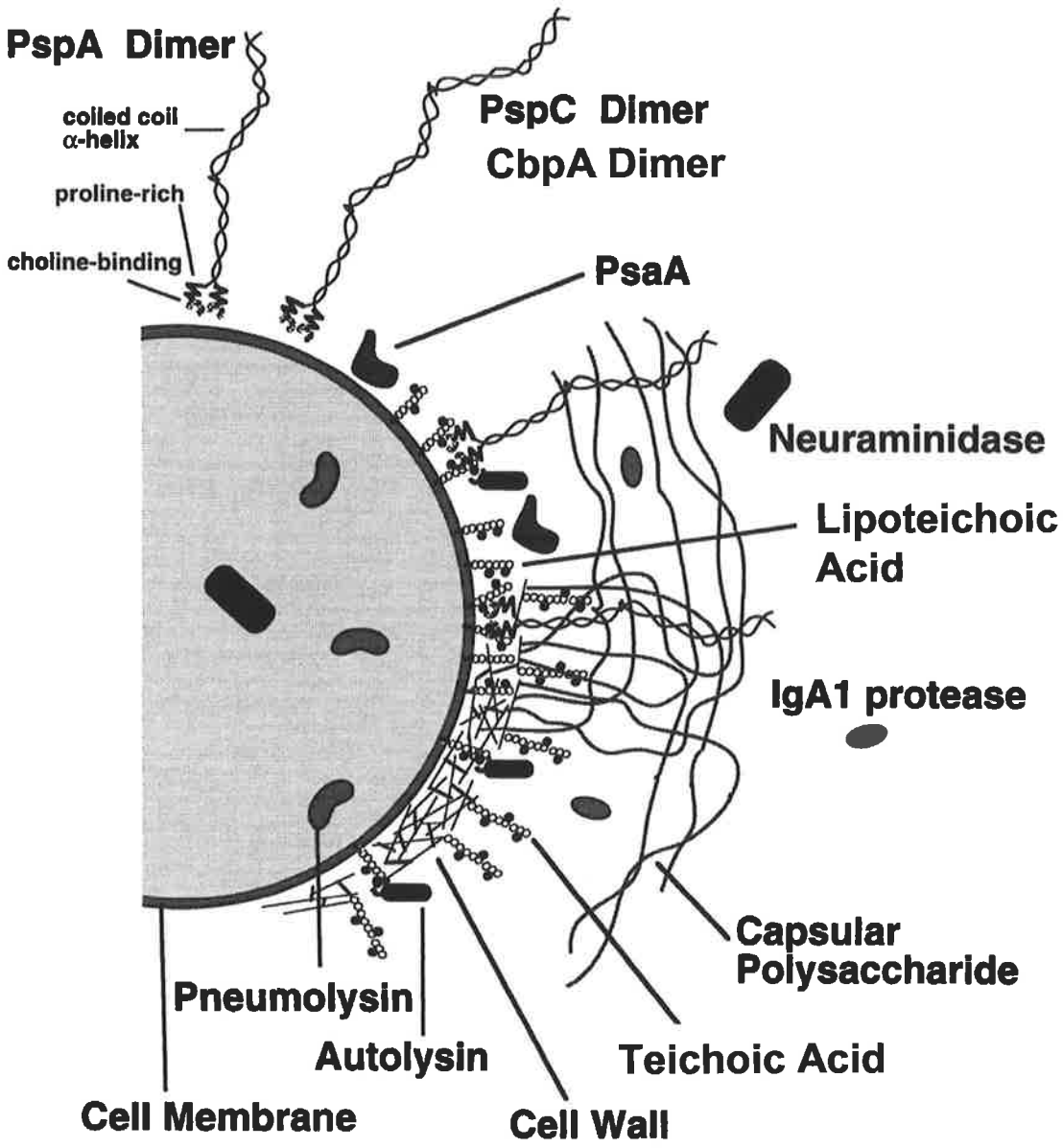

Figure 1.1. Schematic diagram of the pneumococcal surface.

Major surface components, along with a number of important virulence factors are shown, including pneumolysin, choline binding protein A (CbpA) and pneumococcal surface protein A (PspA). Adapted from Briles *et al.* (1998).

for the development of vaccines against the pneumococcus. Although there are reports of non-encapsulated strains associated with superficial infections such as conjunctivitis (Martin *et al.*, 2003), all clinical *S. pneumoniae* isolates from other sterile sites are encapsulated, and spontaneous non-encapsultated derivatives of such strains are almost completely avirulent. An early study demonstrated that enzymic depolymerisation of the capsular PS of a type 3 pneumococcous increased its 50% lethal dose (LD_{50}) more than 10^5-fold (Avery & Dubos, 1931). In recent times, studies have confirmed the importance of the capsule to pneumococcal virulence using defined mutations in genes essential for capsular PS synthesis (Hardy *et al.*, 2001; Magee & Yother, 2001; Bender & Yother, 2001; Morona *et al.*, 2004).

While not playing a role in adherence, invasion, or inflammation in the host (Tuomanen *et al.*, 1987), the PS capsule increases virulence by enabling the pneumococcus to avoid phagocytosis by resident macrophages (Thore *et al.*, 1985). The reasons behind this appear to be multifaceted. The majority of capsular PS serotypes are highly charged at physiological pH, and this may directly impede interactions with phagocytes (Lee *et al.*, 1991). Additionally, the capsule forms an inert shield which appears to prevent either the Fc region of IgG or iC3b (fixed to deeper cell surface structures) from interacting with receptors on phagocytotic cells (Winkelstein, 1981; Musher, 1992). A recent study has also suggested that the capsule may reduce the total amount of complement deposited on the bacterial surface (Abeyta *et al.*, 2003).

1.3.2 Pneumolysin

Other than the capsule, pneumolysin (Ply) is perhaps the best characterised factor associated with the virulence of *S. pneumoniae*. Produced by virtually all clinical isolates, early experiments in which the *ply* gene was insertionally inactivated showed that its mutation reduced pneumococcal virulence in both intranasal and systemic challenge models (Berry *et al.*, 1989). The mutation of *ply* led to an increase in LD_{50}, and survival

time after challenge of mice when compared with an otherwise isogenic parent strain. More recent studies have both confirmed and expanded upon these findings illustrating that Ply has a role in lung infection (Rubins *et al.*, 1995), bacteremia (Benton *et al.*, 1995), and meningitis (Wellmer *et al.*, 2002). Studies of its role in nasopharyngeal colonisation have yielded varying results, with Kadioglu *et al.* (2002) reporting that a *ply* mutant of the type 2 strain D39 was unable to colonise. However, a recent study using type 23F and type 6A *S. pneumoniae* backgrounds found that *ply*-negative mutants actually colonised at higher levels than the wildtype (wt) (van Rossum *et al.*, 2005). Nevertheless, these studies illustrate that Ply is a multi-functional virulence protein and appears to play a role in pathogenesis in multiple host niches.

A number of factors explain the contribution Ply makes to the virulence of *S. pneumoniae.* Ply belongs to a family of thiol-activated cytolysins, which share a common mode of action. These toxins bind cholesterol in target cell membranes, resulting in insertion into the membrane and oligomerisation to form large transmembrane pores, leading to cell lysis. Thus, the toxin can lyse any eukaryotic cell with cholesterol in its membrane. The toxin is cytoplasmic and released either by LytA-mediated autolysis or another unknown mechanism (Balachandran *et al.*, 2001). As well as it cytotoxic effects, Ply also the ability to activate the classical complement pathway in the absence of specific antibody (Paton *et al.*, 1984), and at sublytic levels can stimulate cells of the immune system to produce cytokines, resulting in modulation of the immune response against the pneumococcus (Houldsworth *et al.*, 1994; Baba *et al.*, 2002). Furthermore, Ply contributes to progression of pneumococcal disease by interfering with phagocyte recruitment into the lungs (Maus *et al.*, 2004), blocking proliferative and antibody-generating responses of B lymphocytes (Ferrante *et al.*, 1984), reducing ciliary beating (Feldman *et al.*, 1990) and increasing lung permeability (Maus *et al.*, 2004).

1.3.3 Choline binding proteins

As discussed above the capsule is the outermost component of the bacterial surface, overlying the cell wall and plasma membrane (**Figure 1.1**). The cell wall consists of a triple-layered peptidoglycan backbone to which the capsular PS is anchored (AlonsoDeVelasco *et al.*, 1995). Attached to this glycan backbone and the plasma membrane are teichoic and lipoteichoic acids, respectively. The phosphorylcholine moieties attached to these molecules are an important component of the cell surface, acting as docking sites for a class of proteins known as the choline binding proteins.

The choline binding proteins are a family of surface binding proteins comprising an N-terminal α-helical domain, a proline-rich domain, and a choline-binding region (**Figure 1.2**) (Brooks-Walter *et al.*, 1999). These proteins are important for a wide number of functions, such as cell lysis (LytA), inactivation of complement (Pneumococcal surface protein A; PspA) and adherance of pneumococci to epithelial cells (Choline binding protein A; CbpA) (Jedrzejas, 2001). A number of these are discussed in more detail below.

1.3.3.1 Choline Binding Protein A

CbpA (also known as PspC, and SpsA) is present in 75% of all clinical isolates and has received particular attention as a possible vaccine candidate (see Section 1.4) (Brooks-Walter *et al.*, 1999). The CbpA proteins of 43 pneumococcal strains have been divided into 11 groups on the basis of DNA homology (Iannelli *et al.*, 2002), with the well studied type 2 *S. pneumoniae* D39 CbpA belonging to group 3. Rosenow *et al.* (1997) originally isolated the protein through its ability to bind choline, and subsequently showed that pneumococci deficient in CbpA were severely attenuated in their ability to colonise the nasopharynx, as seen in an infant rat model. Later studies of mutants not expressing CbpA showed reduced virulence in mouse models of pneumonia, and nasal colonisation compared to the wt (Balachandran *et al.*, 2002; Orihuela *et al.*, 2004a). Iannelli *et al.* (2004) suggested that CbpA may also have an effect on sepsis, although similar studies

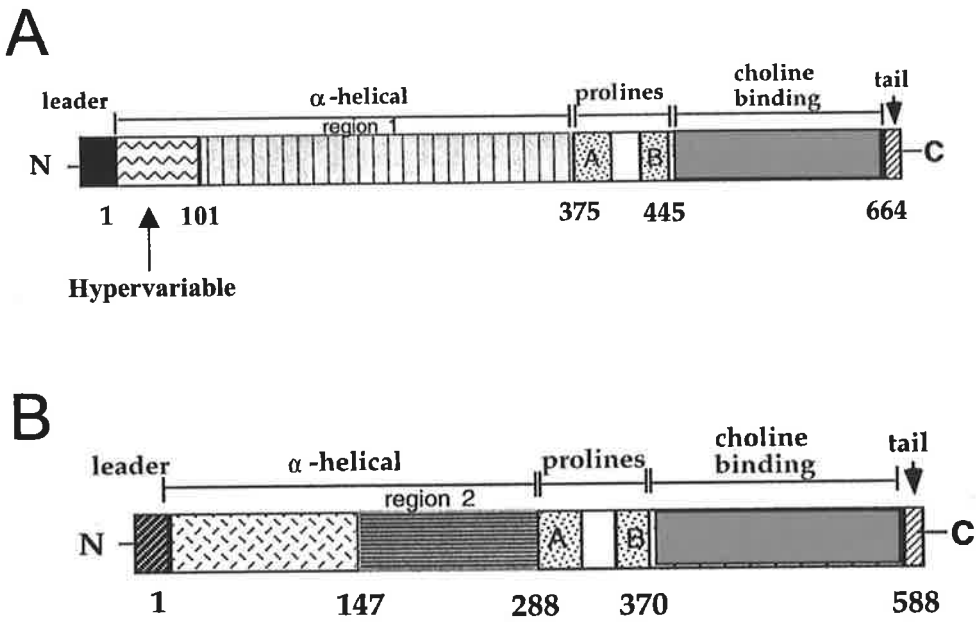

Figure 1.2. Schematic respresentation of choline binding proteins.

Schematic representation of (A) CbpA and (B) PspA showing the distinct domains of the molecules. Numbers represent amino acid residues. Adapted from Brooks-Walter *et al.* (1999).

undertaken by Orihuela *et al.* (2004) and Berry & Paton (2000) using the same *S. pneumoniae* strain suggested that it is of little importance in systemic disease. Additionally, Orihuela *et al.* (2004) recently showed that the protein also plays a role in the ability of the pathogen to cross the blood brain barrier. Thus, CbpA appears to be important for multiple facets of pneumococcal virulence.

CbpA is a major adhesin for the pneumococcus, with CbpA-deficient mutants showing significant reductions in adherence to lung and nasopharyngeal epithelial cells (Rosenow *et al.*, 1997; Zhang *et al.*, 2000; Elm *et al.*, 2004). In the lungs, adherence is thought to be mediated by the binding of teichoic acids to the platelet activating factor (PAF) receptor (Cundell & Tuomanen, 1994; Cundell *et al.*, 1995), although the ability of CbpA to bind complement component C3 may also play a role (Cheng *et al.*, 2000; Smith & Hostetter, 2000). In the nasopharynx, the binding of CbpA to the polymeric Ig receptor (pIgR) is thought to be responsible for the adherence and invasion phase of infection (Zhang *et al.*, 2000; Lu *et al.*, 2003). Binding of pneumococci to nasopharyngeal cells (Detroit 562) *in vitro* is inhibited by antibodies against pIgR, but not by antibodies against PAF (Zhang *et al.*, 2000).

CbpA has also been shown to bind to the secretory component of sIgA (Hammerschmidt *et al.*, 1997), and is able to inhibit complement activation by binding C3 and factor H (Smith & Hostetter, 2000; Duthy *et al.*, 2002). This inhibition is suspected to play a role in invasive infections such as pneumonia and bacteremia. Recent research in our laboratory has illustrated that CbpA may also contribute to virulence through its ability to modulate the innate immune response. Mutants deficient in CbpA showed increased levels of interleukin(IL)-8 induction, an important pro-inflammatory cytokine, in epithelial cells in comparison to the wt (Graham, 2005). Thus, CbpA may have an inhibitory effect on the CXC chemokine response of these cells.

1.3.3.2 Pneumococcal Surface Protein A

Another choline binding protein, PspA, is expressed in all clinically important pneumococcal serotypes (Crain *et al.*, 1990), and has received much attention as a possible vaccine candidate. Indeed, even though the N-terminal domain of PspA is highly variable between different serotypes (Crain *et al.*, 1990), it is highly cross-reactive, with Briles *et al.* (2000) showing that immunisation with a PspA molecule from one type produces antibodies that are cross-reactive against all PspA types tested.

McDaniel *et al.* (1987) first showed the importance of PspA to pneumococcal virulence by showing that mutation of PspA led to a 10-fold greater clearance from the blood. Subsequent studies confirmed this, by demonstrating that challenge with a PspA-deficent strain rather than the parent strain led to substantial increases in median survival time and rate in both systemic and intranasal models of infection (Briles *et al.*, 1988; Berry & Paton, 2000; Balachandran *et al.*, 2002) The protein is of critical importance to pneumococcal virulence through its ability to interfere with the complement factor C3 (Tu *et al.*, 1999). Recent studies have shown that C3 deposition on PspA-negative pneumococci is markedly increased compared to the parent strain, and that antibodies against PspA increase C3 deposition on wt pneumococci (Ren *et al.*, 2004a; Ren *et al.*, 2004b; Yuste *et al.*, 2005). Thus, it would appear that PspA prevents deposition of the opsonising C3b onto the bacterial surface, enabling *S. pneumoniae* to avoid complement mediated opsonophagocytosis. PspA has also been shown to bind human lactoferrin, an iron-sequestering glycoprotein present in mucosal secretions (Hammerschmidt *et al.*, 1999; Hakansson *et al.*, 2001). This sequestering of lactoferrin has been shown to protect the pneumococcus against killing by the iron depleted form of lactoferrin, apolactoferrin (Shaper *et al.*, 2004). In this way, PspA may contribute to colonisation by allowing increased survival at mucosal surfaces.

1.4 Current methods for treatment and prevention

1.4.1 Antibiotics

Since mid last century, penicillin has been the mainstay of treatment for *S. pneumoniae*. However, acquisition of resistance to penicillin in *S. pneumoniae* was demonstrated in the laboratory even before its widespread clinical use (Austrian, 1981), and recent data from the USA show that approximately 21% of all isolates exhibit resistance to penicillin, with the figure closer to 32% in isolates from pre-school children (Jones *et al.*, 2003; Brown, 2004). However, resistance is much higher in developing countries where overuse of antibiotics has taken place, with rates of penicillin resistance as high as 70% (Marton *et al.*, 1991). With the reduction in efficacy of penicillin, reliance has fallen on other classes of antibiotics, resulting in increasing rates of resistance to these drugs as well. In the USA resistance of pneumococci to more than one class of antibiotic has risen from 6.4% in 1992 to 28% in 2001 (Mera *et al.*, 2005); while in Asia up to 63% of isolates are resistant to three classes of antimicrobials including penicillin (Okeke *et al.*, 2005). Since pneumococci are naturally competent, the pathogen is easily able to acquire the genes required for drug resistance from other colonising pneumococci and/or commensal streptococci (Reichmann *et al.*, 1997). The rate at which *S. pneumoniae* is acquiring resistance to multiple classes of antibiotics, and the global spread of highly resistant clones, will ultimately lead to pneumococcal infections which are almost untreatable (Klugman, 1990; McGee, 2000).

1.4.2 Polyvalent PS vaccines

Due to the increasing difficulties associated with the use of antibiotics, other approaches to the management of pneumococcal disease have been investigated. The capsule is one of the most important pneumococcal virulence factors and is the dominant surface antigen (see Section 1.2). Accordingly, this has been the major target for the

development of vaccines against the pneumococcus. The early discovery that antibodies to the capsular PS are highly protective in a serotype specific manner has led to the development of polyvalent PS and PS-protein conjugate vaccines.

Polyvalent pneumococcal PS vaccines were first released in the 1970s, with the current formulation containing PS purified from 23 of the 90 serotypes discovered to date. This vaccine covers approximately 90% of the disease causing serotypes across the USA and Europe (Paton, 2004). However, due to the structural and antigenic differences between the capsular types, antibody against one serotype does not provide protection against another. The serotypes causing disease vary widely both temporally and geographically, resulting in vaccine coverage <60% in parts of Asia (Lee *et al.*, 1991). The lack of serotype prevalence data for many developing countries results in uncertain vaccine coverage. While the vaccine is protective in healthy adults, the efficacy is much lower in groups at high risk of pneumococcal infection, such as young children. Polysaccharides are T-cell-independent antigens and so are poorly immunogenic in children under 5 years of age (Douglas *et al.*, 1983).

1.4.3 Polyvalent PS-protein conjugate vaccine

The issue of poor immunogenicity has been addressed with the development of PS-protein conjugate vaccines. Conjugation to a protein carrier confers T-cell dependence, resulting in strong boostable antibody responses and immunological memory. For this reason, these vaccines have been highly efficacious in the young. The introduction of Prevenar®, a 7-valent conjugate PS vaccine, in 2000 has led to significant reductions in invasive disease in the USA both in the young, and, as a result of herd immunity, also in the elderly (Black *et al.*, 2004; Lexau *et al.*, 2005).

However, the protection elicited by the vaccine is still serotype-specific and because of high production costs, the number of types covered has been reduced to only 7. As a result this vaccine provides protection against a much more limited serotype range.

Furthermore, studies of nasopharyngeal colonisation during trials of the conjugate vaccine, showed that while carriage of vaccine types were reduced, the vacated niche was promptly occupied by non-vaccine serotypes potentially capable of causing disease in humans (Obaro *et al.*, 1996; Mbelle *et al.*, 1999). The same trend has also been seen after the introduction of the Prevenar® in the US (Huang *et al.*, 2005). A Finnish trial demonstrated that while the vaccine reduced the incidence of otitis media caused by vaccine types, this was largely off-set by increased incidence of otitis media attributed to non-vaccine serotypes (33% increase) or *Haemophilus influenzae* (11% increase) (Eskola *et al.*, 2001). It is likely that such replacement carriage and disease may be more pronounced in populations with pre-existing high rates of carriage of non-vaccine types. This has been seen in a trial of the impact of the vaccine in a group of Australian Aboriginal children. Whilst the carriage of vaccine types decreased from 45% to 13%, carriage of non-vaccine types increased from 32% to 69%, resulting in an essentially unaltered carriage rate of approximately 80% (A. Leach, unpublished). The effects of the vaccine on the incidence of invasive disease and otitis media have shown almost identical trends (A. Leach, unpublished). Thus, it seems likely that the widespread use of conjugate vaccines such as Prevanar® may simply result in alteration of the serotype distribution of pneumococcal disease rather than reducing its overall impact.

1.4.4 Protein vaccines

In view of the limitations of PS-based vaccines, a number of pneumococcal proteins are being investigated for their capacity to elicit protective immunity. Such vaccines would be expected to be highly immunogenic in the very young and the cost of production would also be much lower compared to conjugate vaccines, meaning that widespread use would be possible throughout the developing world. Current protein candidates for use in such a vaccine include (amongst others) PspA, Ply, and CbpA. All are present in a high percentage of clinical isolates, and have been shown to be effective in

eliciting protection in animal models (Paton *et al.*, 1983; Alexander *et al.*, 1994; Briles *et al.*, 2000a; Ogunniyi *et al.*, 2000). Since there is either minimal antigenic variation in these proteins, or at least broadly cross reactive epitopes, any one antigen is capable of eliciting protection against the majority of *S. pneumoniae* strains and serotypes. Furthermore, any such vaccine would likely be composed of a combination of the aforementioned proteins, maximising its protective efficacy and strain coverage (Paton, 2004).

1.5 Virulence gene regulation

The regulation of virulence factors is controlled by a number of discrete regulatory cascades that are likely to be crucial for the ability of pneumococci to multiply in humans and cause disease. In recent times, a number of proteins have been identified as regulators of factors important in the pathogenesis of the pneumococcus. RegM, a homologue of the staphylococcal catabolite control protein CcpA, is the first protein encoded by a gene outside of the capsule locus to be implicated in the regulation of capsular gene expression (Giammarinaro & Paton, 2002). Another protein, the heat-induced repressor CtsR is involved in the regulation of the pneumococcal *clp* genes, a set of genes involved in stress response, stationary phase adaptive responses, and virulence in a number of bacterial species (Chastanet *et al.*, 2001). PsaR, has also recently been implicated in the regulation of the manganese transporter PsaA, along with a number of other genes, suggesting it may be a global regulator of gene expression in *S. pneumoniae* (Johnston *et al.*, 2006).

Pneumococci have also been shown to exhibit spontaneous, reversible phase variation in colony phenotype, with the two phases referred to as "transparent" and "opaque". The transparent phenotype is generally associated with the presence of more surface choline, more CbpA and less capsule, and has been shown to establish dense and stable colonisation in the nasopharynx in an infant rat model (Weiser *et al.*, 1994; Rosenow *et al.*, 1997). The opaque phenotype, on the other hand, is more suited to survival in the

bloodstream, and is thought to produce higher levels of capsule, less cell surface choline and more PspA than transparent phase variants (Rosenow *et al.*, 1997; Kim & Weiser, 1998; Kim *et al.*, 1999). While phase variation appears to be a random event, the ability of pneumcocci to regulate the expression of virulence factors in response to varying external stimuli is anything but random.

Upon infecting the host, pathogens must express specific gene products to persist and proliferate in a given niche and to avoid host defences. During the course of infection with *S. pneumoniae,* the host environment changes markedly, meaning that the regulation of virulence factors is likely to have a large impact on the success or failure of this process. In fact, it has recently been demonstrated that known virulence-associated genes of *S. pneumoniae,* including *cbpA*, *pspA*, *ply*, and capsule genes, are differentially expressed *in vivo* (Ogunniyi *et al.*, 2002; Orihuela *et al.*, 2004b; LeMessurier *et al.*, 2006). In addition, Marra *et al.* (2002) used differential fluorescence induction (DFI) technology to identify factors involved in pathogenesis, as well as illustrating that a number of these identified genes were differentially expressed in varying *in vitro* and *in vivo* conditions. Given the evidence that a number of genes are differentially regulated in dissimilar conditions, the next step is to determine how this is achieved by the pathogen.

1.6 Two-Component Signal Transduction Systems

1.6.1 Introduction

Two-Component Signal Transduction Systems (TCSTSs) control the expression of genes in a wide range of bacterial species in response to external stimuli (reviewed in Hoch, 2000). These signalling systems regulate wide-ranging processes such as chemotaxis, surface adhesion, and phosphate uptake. The typical TCSTS consists of two proteins, a histidine kinase (HK) and a response regulator (RR). The HK, usually present in the cell membrane, acts as a sensor protein responding to external stimuli, while the RR

responds to the HK and binds to DNA, modulating gene expression. As shown in **Figure 1.3**, a signal acts to stimulate the HK to autophosphorylate a conserved histidine residue. The HK then transfers this phopshoryl group to a conserved aspartate on the RR. This leads to conformational changes in the RR, thus enabling the protein to mediate changes in gene expression by acting as a transcription factor, either activating or repressing gene expression (Robinson *et al.*, 2000; Stock *et al.*, 2000).

1.6.2 Histidine kinases

The histidine kinases (HKs) make up a novel family of protein kinases (for a recent review see Khorchid & Ikura, [2005]). While the larger group of Ser/Thr/Tyr kinases directly transfer phosphate from ATP to the protein substrate, HKs first autophosphorylate, before transferring the phosphate to the protein substrate, namely the RR. Moreover, in most TCSTSs, there is a one to one relationship between the HK and the RR. This is in contrast to the classical protein kinase amplification cascades, where one protein kinase acts to phosphorylate multiple targets (Robinson *et al.*, 2000).

The sensor kinases are generally composed of a diverse N-terminal sensing domain and a highly conserved C-terminal kinase core. The N-terminal domain generally contains two or more transmembrane segments, resulting in the localization of the HK to the cytoplasmic membrane as seen with EnvZ of *Escherichia coli* (Forst *et al.*, 1987). Little, if any, sequence similarity is shared between sensing domains, thus supporting the idea that they have been designed for specific ligand/stimulus interactions. The kinase core is the unifying structure of HKs, commonly containing a dimerisation domain, and a catalytic domain. The core is made up of four or five amino acid motifs or boxes known as the H, N, G_1, F, and G_2 boxes (Stock *et al.*, 1989; Parkinson & Kofoid, 1992; Alex & Simon, 1994) (**Figure 1.4**). In most HKs, the H box is part of the dimerisation domain and contains the conserved His substrate where autophosphorylation takes place. The N, G_1, F, and G_2 motifs are found in the catalytic domain, and are responsible for the binding of ATP for

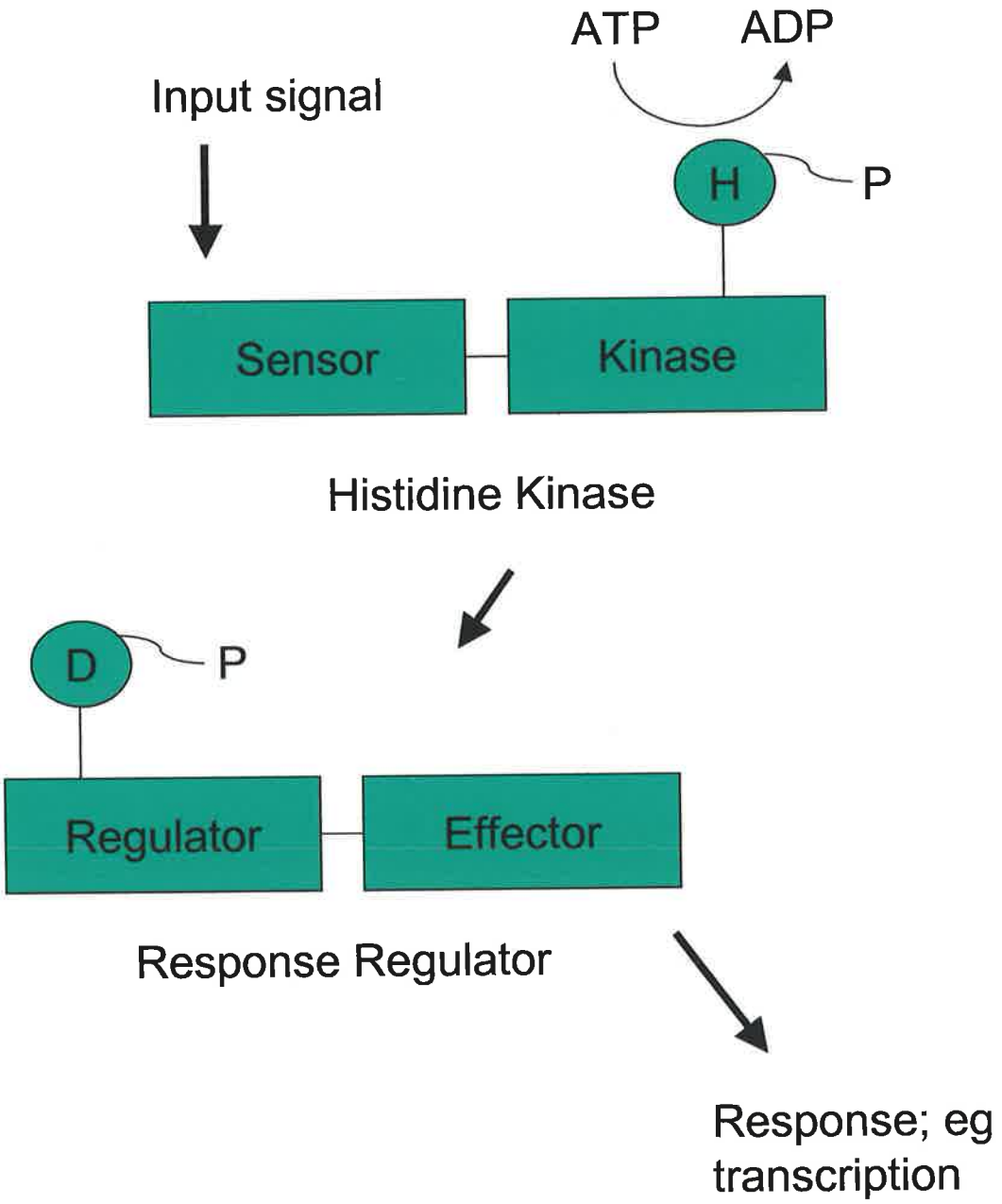

Figure 1.3. Schematic diagram of two component signal transduction.

Having sensed external stimuli, the histidine kinase is autophosphorylated, and subsequently transfers this phosphate to a response regulator protein. The phosphorylation of the response regulator results in a conformational change, leading to the regulation of gene expression, either by acitivation or repression. Adapted from Skerker *et al.* (2005).

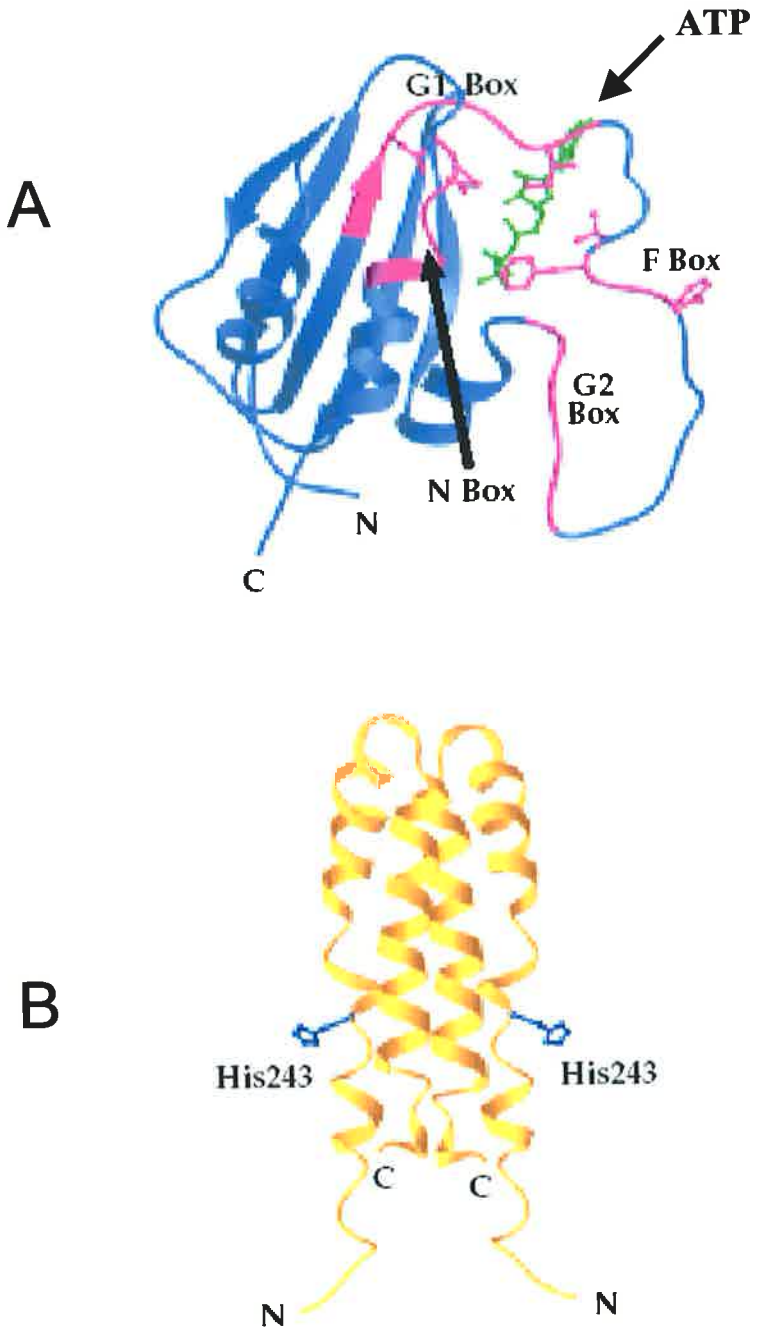

Figure 1.4. The histidine kinase core.

Nuclear magnetic resonance structure of the *E. coli* EnvZ histidine kinase. **A**; the catalytic domain containing N, G1, F and G2 boxes, shown bound to ATP. **B**; the dimerisation domain, where the conserved H box is found (Stock *et al.*, 2000).

use in the autophosphorylation reaction. This autophosphorylation of the kinase takes place as a trans-autophosphorylation reaction between homodimers whereby one HK monomer catalyzes the phosphorylation of the conserved His residue in the second (Perraud *et al.*, 1999; Stock *et al.*, 2000). Many HK also possess phosphatase activity, enabling them to dephosphorylate the associated RR, limiting the duration of the activated state (Robinson *et al.*, 2000).

Perhaps the least studied aspect of the HK is the region joining the above-mentioned domains, appropriately named the linker (or HAMP) domain. Responsible for linking the transmembrane sensing domains to the kinase core, they are made up of a transmembrane helix and a cytoplasmic linker (Robinson *et al.*, 2000). Varying in length from 40 to >180 amino acids, studies have indicated that this domain is critical for proper signal transduction (Park & Inouye, 1997).

1.6.3 Response regulators

Response regulators (RRs) generally consist of a conserved N-terminal regulatory domain, containing the Asp that is phosphorylated, and a variable C-terminal effector domain. The majority are transcription factors with DNA-binding C-terminal effector domains. RRs are thought to exist primarily in two conformations, corresponding to the active and inactive states. The inactive conformation is favoured in the unphosphorylated protein, whereas phosphorylation shifts the equlibrium toward the active state. When the regulatory domain becomes phosphorylated, a conformational change occurs in the C-terminal half of this domain, facilitating protein-protein interactions, such as the interaction of the RR with the RNA polymerase holoenzyme complex. The conformational change has also been seen to initiate the binding of the RR to DNA in a number of systems (Buckler *et al.*, 2000; Stock *et al.*, 2000).

The phosphotransfer from the HK to the RR is thought, in a large part, to be catalysed by the RR. The RR can also catalyze auto-dephosphorylation, thus effectively

limiting the lifetime of the activated state (Perraud *et al.*, 1999; Stock *et al.*, 2000). Interestingly, RRs can autophosphorylate independently of the HK through interaction with small molecular weight molecules such as acetyl phosphate, carbamoyl phosphate, imidazole phosphate, and phosphoamidate which act as phosphodonors (Feng *et al.*, 1992; Lukat *et al.*, 1992). This means that activation of a system can occur without the sensing of a signal through the HK, providing a possible mechanism for response to altered metabolic states.

RRs have been divided into families based on sequence similarity. These families have historically been named after their respective founder members including CheY, NtrC, and OmpR. CheY is an example of a single domain response regulator while the other families are multi-domain proteins. Easily the largest, the OmpR family is characterized by its effector domain, which is made up of three α-helices flanked on either side by anti-parallel β sheets and two C-terminal β sheets, forming the so called wing structure (**Figure 1.5**) (Martinez-Hackert & Stock, 1997). Despite structural similarity, different members of the family have different modes of action. For instance, OmpR interacts with the α subunit of RNA polymerase, whereas PhoB interacts with σ^{70} (Stock *et al.*, 2000). The family represented by the nitrogen regulatory protein NtrC is the most structurally and functionally complex RR subgroup. NtrC itself activates the σ^{54}-holoenzyme form of RNA polymerase, by forming octamers and binding DNA (Kustu *et al.*, 1989; Wyman *et al.*, 1997).

The high level of sequence conservation between the C-terminal kinase core of the HK and the regulatory domain of the RR would appear to expose TCSTSs to the danger of non-specific cross-reactions. Indeed a number of studies have indicated that non-cognate pairs can interact *in vitro* (Ninfa *et al.*, 1988; Fisher *et al.*, 1995). However, in recent times, Skerker *et al.* (2005) showed that while non-cognate pairs do interact, these interactions are generally of much lower affinity than cognate pairs, and are due to excessive *in vitro*

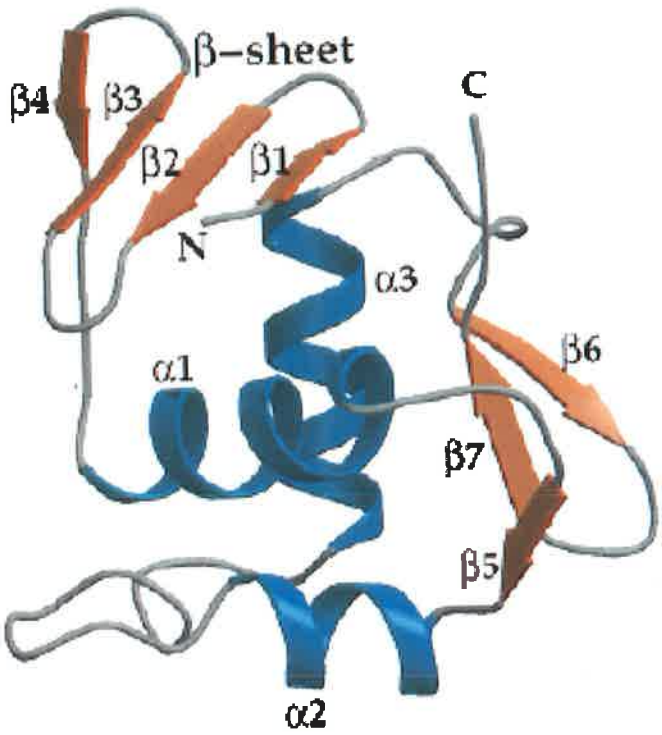

Figure 1.5. Structure of the DNA binding domain of OmpR.

Ribbon structure of OmpR effector domain illustrating three α-helices flanked on either side by anti-parallel β sheets and two C-terminal β sheets, forming the so called wing structure. Adapted from Martinez-Hackert & Stock (1997).

incubation times or a high concentration of reaction components. Thus, such interactions may be of little significance within the cell.

1.6.4 Model systems

One of the best described systems is the EnvZ-OmpR system from *E. coli*. This is a typical TCSTS, much like that depicted in **Figure 1.3**. By responding to changes in osmolarity in the medium, the HK EnvZ regulates the phosphorylation state of the RR, OmpR, which is responsible for controlling the expression levels of outer membrane porin proteins OmpF and OmpC. OmpR has been shown to bind to a number of different sites upstream of these genes, with different sites favoured depending on the phosphorylation state of the RR (Harlocker *et al.*, 1995; Cai & Inouye, 2002).

However, not all systems are quite as simple. A number comprise HKs containing a His-containing phosphotransfer domain (Hpt). This domain is approximately 120 amino acids in length and contains a His residue able to take part in phosphoryl transfer reactions. The domains do not display any kinase or phosphatase activity (Tsuzuki *et al.*, 1995), but serve the role of simply passing the signal onto the RR. CheA from *E. coli* is an example of one such HK. Interestingly CheY, the RR phosphorylated by CheA, is an example of a RR which does not have a DNA binding domain, but rather has a role in chemotaxis by associating with its effector protein FliM, a component of the flagellar motor (Welch *et al.*, 1993).

Another much-studied system is the sporulation control system from *Bacillus subtilis*. This system has created much interest as it is a multi-component TCSTS, commonly known as a phosphorelay. One of its major differences from the more typical systems, such as EnvZ/OmpR, is that this TCSTS involves the use of multiple HKs for the sensing of signals, passing this onto the final RR Spo0A via another RR Spo0F and the Hpt Spo0B. Spo0A then binds to a consensus sequence to activate a number of genes involved in sporulation (Burbulys *et al.*, 1991; Hoch, 1993; Msadek, 1999; Jiang *et al.*, 2000). The

systems described above are just a few of the model TCSTSs which have been studied extensively in prokaryotes, providing some insight into the complex mechanisms used by bacteria to regulate genes in response to changes in external stimuli.

1.6.5 TCSTSs as anti-microbial targets

The increasing resistance of bacterial pathogens to antibiotics has resulted in a desperate need for the development of novel targets for anti-microbials. As many TCSTSs are shown to regulate virulence factors of pathogens (Dziejman & Mekalanos, 1995; Beier & Gross, 2006), these systems could provide a common target for development of novel anti-microbials. TCSTSs are attractive targets for anti-microbial therapy for a number of reasons. One of these is their abundance in prokaryotes, and the relative lack of them in eukaryotes. The fact that TCSTSs share homology in key functional domains provides hope that both HK and RR inhibitors could be used as broad-spectrum antibiotics for the 21st century. Possible targets for blockade could include the domains responsible for the auto-phosphorylation signal, auto-phosphorylation of the HK, interaction of the phosphorylated HK and the RR, and binding to the regulated gene promoter (Barrett & Hoch, 1998; Stock *et al.*, 2000).

Roychoudhury *et al.* (1993) have already reported the discovery of inhibitors of a TCSTS regulator of alginate synthesis in *Pseudomonas aeruginosa* . The compounds were developed to target the HK phosphorylation/de-phosphorylation reaction, along with the ability of RR to bind DNA. Another example of an inhibitor of virulence gene regulation is a small molecule recently described by Hung *et al.* (2005), which is able to prevent the expression of two factors critical for the virulence of *Vibrio cholerae* through its effect on transcriptional regulator ToxT.

1.6.6 Pneumococcal TCSTSs

The release of the pneumococcal genome sequence towards the end of the last century prompted two separate studies of TCSTSs in *S. pneumoniae* (Lange *et al.*, 1999; Throup *et al.*, 2000). Through the use of homology searches, both groups identified a total of 13 TCSTSs, comprising a HK and RR, along with one lone RR (see **Table 1.1**). A number of these had been previously identified and investigated including ComDE (Havarstein *et al.*, 1995) and CiaRH (Guenzi *et al.*, 1994), but the majority were largely uncharacterised. Mutants were constructed in the various systems in order to investigate their affects on virulence. Interestingly, both groups were unable to delete the RR of one system. This system, known commonly as YycFG or VicRK, shows homology to another reported essential TCSTS from *Bacillus subtilis* (YycFG) (Fabret & Hoch, 1998).

In vivo studies performed on the remaining systems provided interesting results. Lange *et al.* (1999) used a bacteremic mouse model, and investigated the effect of mutations in the RRs in serotype 22 and serotype 3 backgrounds. None of the mutants tested were hindered in their ability to cause death, with all infected mice dying within 2 to 3 days. This would suggest that none of the systems have a major effect on the ability of *S. pneumoniae* to cause systemic disease, although it should be noted that this model bypassed the natural route of infection. Conversely, Throup *et al.* (2000) used a respiratory tract infection (RTI) model where mice were infected intranasally. At 48 hours, mutants in eight of the systems in a serotype 3 background showed significant defects in their ability to cause lung infection. In a later study, Sebert *et al.* (2002) tested the ability of number of the mutants described by Throup *et al.* (2000), and showed that only one, CiaRH, is deficient in its ability to establish and maintain nasopharyngeal colonisation in an infant rat model. Together these studies suggest that a number of pneumococcal TCSTSs do indeed play a role in pathogenesis, and are likely to regulate genes contributing to the virulence of

Table 1.1. Two-Component Signal Transduction Systems in *Streptococcus pneumoniae*.

Two-Component Signal Transduction System			Roles in *S. pneumoniae*
A	B	C	
TCS01	480		Role in lung infection (Throup *et al.*, 2000).
TCS02	492	YycFG, VicRK, MicAB	Only essential pneumococcal TCSTS (Lange *et al.*, 1999; Throup *et al.*, 2000); Regulates virulence factor PspA and PcsB (Ng *et al.*, 2003; Ng *et al.*, 2005).
TCS03	474		No role in lung infection (Throup *et al.*, 2000).
TCS04	481	PnpSR	*In vitro* growth attenuation (Lange *et al.*, 1999); Regulates PsaA in TIGR4 (McCluskey *et al.*, 2004); Role in lung and blood infection (Throup *et al.*, 2000; McCluskey *et al.*, 2004).
CiaRH	494		Regulates virulence factor HtrA; Role in colonisation (Sebert *et al.*, 2002), lung infection (Throup *et al.*, 2000) and competence (Guenzi *et al.*, 1994).
TCS06	478	RR/HK06	Under investigation here.
TCS07	539		Role in lung infection (Throup *et al.*, 2000).
TCS08	484		Role in lung infection (Throup *et al.*, 2000).
TCS09	488		Different effects on virulence in different strains (Blue & Mitchell, 2003)
TCS10	491	VncRS	Thought to be involved in tolerance to vancomycin (Novak *et al.*, 1999), but since been challenged (Robertson *et al.*, 2002; Haas *et al.*, 2004); Role in lung infection (Throup *et al.*, 2000).
TCS11	479		No role in lung infection (Throup *et al.*, 2000).
ComDE	498		Regulates development of competence in response to CSP (Pestova *et al.*, 1996).
TCS13	486	BlpHR	Responds to peptide pheromone, BlpC, in order to regulate a set of bacteriocins (de Saizieu *et al.*, 2000).
	489	RitR	Lone RR; importance in lung disease; involved in iron homeostasis (Ulijasz *et al.*, 2004).

A. Designation by Lange *et al.* (1999).
B. Designation by Throup *et al.* (2000).
C. Other names used in literature.

S. pneumoniae. However, the original studies made no attempts to look at the specific regulation that these systems exert.

The ability to take up DNA by *S. pneumoniae* is regulated by perhaps the most studied pneumococcal TCSTS, ComDE. The HK of the system, ComD, responds to a peptide pheromone, Competence Stimulating Peptide (CSP) encoded by the gene *comC*. This results in the cognate RR, ComE, regulating genes which encode the machinery for the uptake and recombination of DNA into the pneumococcal genome (Pestova *et al.*, 1996; Cheng *et al.*, 1997; Peterson *et al.*, 2004).

As previously mentioned, the RR of YycFG is essential for the viability of this organism. Several researchers have investigated this system and suggested that it has an effect on competence under oxygen limitation (Echenique & Trombe, 2001), and that mutation of the gene encoding the HK results in reduced virulence, as seen by a lower recovery from lungs and blood compared to the wt following intranasal infection (Kadioglu *et al.*, 2003). Investigation into genes regulated by the system have shown that the essential nature of the RR is due to its regulation of *pcsB*, which encodes an extracellular protein required for murein biosynthesis (Ng *et al.*, 2003). Other genes thought to be regulated by the system include genes involved in the regulation of the fatty acid biosynthesis pathway and in determining fatty acid chain lengths of membrane lipids (Mohedano *et al.*, 2005). A recent study has identified that the major pneumococcal virulence factor and protective antigen, PspA, also appears to be directly regulated by this system (Ng *et al.*, 2005).

A number of studies have indicated that the pneumococcal TCSTSs are likely to have diverse effects, in terms of both virulence and regulation, depending on strain. Blue & Mitchell (2003) demonstrated that mutations in RR/HK09 had varied effects on virulence depending on background strain, while a later study investigating RR/HK04, found the system regulated different genes in three seperate strains (McCluskey *et al.*, 2004). For

example, this latter study showed that the protective antigen *psaA* was regulated in TIGR4 (serotype 4), but not in the two other strains tested. The reasons for this divergent regulation are still unclear, and the subject of ongoing investigations.

Another system that has been investigated is CiaRH, which appears to have an effect on competence, and has also been associated with resistance to the ß-lactam antibiotic cefotaxime (Guenzi *et al.*, 1994; Echenique *et al.*, 2000). In recent times, research has also shown that this system regulates the expression of the protease/chaperone *htrA*, which has since been implicated as a major virulence factor of the pneumococcus (Sebert *et al.*, 2002; Ibrahim *et al.*, 2004a; Ibrahim *et al.*, 2004b).

Investigations into other pneumococcal TCSTSs have also been conducted. The orphan RR, entitled RitR, appears to play a role in maintaining iron homeostasis in *S. pneumoniae* (Ulijasz *et al.*, 2004), while BlpHR is similar to ComDE, responding to the peptide BlpC to regulate genes involved in bacteriocin production (de Saizieu *et al.*, 2000; Reichmann & Hakenbeck, 2000). The VncRS system was originally hypothesised to be involved in tolerance to vancomycin (Novak *et al.*, 1999). However, recent studies have contradicted this, suggesting that the presence of erythromycin in growth media was the cause of this result (Robertson *et al.*, 2002; Haas *et al.*, 2004).

Notwithstanding the above, more research is needed to understand the true contributions of these systems to the ability of the pneumococcus to colonise the human nasopharynx and/or cause invasive disease. For many TCSTSs we still do not know the genes that are regulated, nor the environmental stimuli to which they respond. It is hoped that a clearer understanding of pneumococcal TCSTSs will provide greater insight into the complex pathogenic process of this important human pathogen.

1.7 Pneumococcal TCSTS RR/HK06

The system RR/HK06 (also referred to as 478) is a typical TCSTS belonging to the EnvZ/OmpR family of systems (Throup *et al.*, 2000). The genes encoding the system are found in the pneumococcal genome adjacent to those for the heat shock associated protein ClpL, and the major virulence factor CbpA. Throup *et al.* (2000) identified RR/HK06 as one of eight pneumococcal TCSTS to have an effect on disease pathogenesis in a RTI model. This model used a mutant in the entire system in a serotype 3 strain, and showed that the effect on virulence was not apparent until 48 hours after intranasal challenge, suggesting that the system played a role in the later stages of infection. In contrast, Lange *et al.* (1999) showed that the system had no effect in a bacteremia model, although as discussed previously in Section 1.6.5, all TCSTS mutants tested appeared to perform as well as the wt in this particular virulence model (Lange *et al.*, 1999). Additionally, Sebert *et al.* (2002) reported that a mutant in HK06 in a serotype 3 background colonised to the same extent as the wt in an infant rat model.

1.8 Aims of this thesis

This study describes the first concentrated effort to decipher the role of RR/HK06 in the pneumococcus. At the beginning of this study, no RR/HK06-regulated factors had been identified. Thus, one of the aims of this project was to identify genes controlled by RR/HK06 through the use of Western blotting, real time RT-PCR and microarray analysis. While this would likely assist in a more complete understanding of the role RR/HK06 plays, such research would also be expected to lead to the identification of the DNA binding site of RR06. Elucidation of the specific binding site would in turn present the possibility of the identification of further genes co-controlled by RR/HK06, through a search for homologous regions in the *S. pneumoniae* genome.

A second aim was to investigate the systems contribution to pathogenesis through the use of both *in vitro* and *in vivo* models. The *in vivo* model commonly used in the laboratory provides advantages over others as it investigates the progression of pneumococcal disease, from asymptomatic colonisation of the nasopharynx to invasive disease of the lungs and blood. A greater understanding of the role of RR/HK06 will lead to a more complete understanding of pneumococcal pathogenesis, and the methods by which the pneumococcus adapts to the varied environmental niches in the body.

CHAPTER TWO – MATERIALS AND METHODS

2.1 Bacterial strains and cloning vectors

The strains and plasmids used in this study are listed in **Table 2.1**.

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

S. pneumoniae strains	Description	Source/Reference
D39	Capsular serotype 2 (NTCC 7466)	(Avery *et al.*, 1944)
D39*rr06*$^-$	EryR, *rr06* insertion-duplication mutant of D39	(Standish, 2002)
D39Δ*rr06*	*rr06* deletion mutant of D39	This study
D39Δ*hk06*	*hk06* deletion mutant of D39	This study
D39Δ*rr/hk06*	*rr/hk06* deletion mutant of D39	This study
D39CbpA$^-$	EryR, *cbpA* insertion-duplication mutant of D39	(Berry & Paton, 2000)
TIGR4	Capsular serotype 4	(Bricker & Camilli, 1999)
TIGR4*hk06:erm*	EryR, *hk06* insertion replacement of TIGR4	This study
TIGR4*rr06:erm*	EryR, *rr06* insertion replacement of TIGR4	This study
TIGR4*rr/hk06:erm*	EryR, *rr/hk06* insertion replacement of TIGR4	This study
TIGR4Δ*rr06*	*rr06* deletion mutant of TIGR4	This study

TIGR4*cbpA:erm*	EryR, *cbpA* insertion replacement of TIGR4	This study
R6	Unencapsulated derivative of D39	(Hoskins *et al.*, 2001)
D39 $RR06_{D51E}$	Asp 51 to Glu 51 of RR06 in D39	This study
D39 $RR06_{D57N}$	Asp 51 to Asn 51 of RR06 in D39	This study
D39 $HK06_{H242Y}$	His 242 to Tyr 242 of HK06 in D39	This study
D39 $HK06_{H242R}$	His 242 to Arg 242 of HK06 in D39	This study
D39 $HK06_{S241D}$	Ser 241 to Asp 241 of HK06 in D39	This study
D39:pControl	pLS1RGFP in D39	This study
D39:p*hk06*	*hk06* over-expressed in D39 using pLS1RGFP	This study
D39:p*rr06*	*rr06* over-expressed in D39 using pLS1RGFP	This study
D39:p*rr/hk06*	*rr/hk06* over-expressed in D39 using pLS1RGFP	This study
D39Δ*rr06*:pControl	pLS1RGFP in D39Δ*rr06*	This study
D39Δ*rr06*:p*hk06*	*hk06* over-expressed in D39Δ*rr06* using pLS1RGFP	This study
D39Δ*rr/hk06*:pcontrol	pLS1RGFP in D39Δ*rr/hk06* using pLS1RGFP	This study
D39Δ*rr/hk06*:p*rr06*	*rr06* over-expressed in D39Δ*rr/hk06* using pLS1RGFP	This study
D39Δ*rr/hk06*:p$RR06_{D51E}$	$RR06_{D51E}$ over-expressed in D39Δ*rr/hk06* using pLS1RGFP	This study
D39Δ*rr/hk06*:p$RR06_{D51N}$	$RR06_{D51N}$ over-expressed in D39Δ*rr/hk06* using pLS1RGFP	This study
TIGR4:pControl	pLS1RGFP in TIGR4	This study
TIGR4:p*hk06*	*hk06* over-expressed in TIGR4 using pLS1RGFP	This study
TIGR4:p*rr06*	*rr06* over-expressed in TIGR4 using pLS1RGFP	This study
TIGR4:p*rr/hk06*	*rr/hk06* over-expressed in TIGR4 using pLS1RGFP	This study
WCH4861	Serotype 6A clinical isolate	Women's & Children's Hospital, Adelaide, SA
WCH4861:pControl	pLS1RGFP in WCH4861	This study
WCH4861:pOE*rr06*	*rr06* over-expressed in WCH 4861 using pLS1RGFP	This study
WCH4832	Serotype 17 clinical isolate	Women's & Children's Hospital, Adelaide, SA
WCH4832:pControl	pLS1RGFP in WCH4832	This study
WCH4832:p*rr06*	*rr06* over-expressed in WCH4832 using pLS1RGFP	This study
DP1617	StrepR, NovR, EryR derivative of RX-1	(Shoemaker *et al.*, 1979)

***E. coli* strains**	**Description**	**Source/Reference**
DH5α	*E. coli* K-12 derivative: F'/*endA1 hsdR17*(r_K-m_K+)*glnV44 thi-1 recA1 gyrA* (Nalr) *relA1*Δ (*lacIZYA-argF*)*U169 deoR*(ϕ80*dlac* Δ(*lacZ*)M15)	Bethesda Research Laboratory, Gaitherburg, MD, USA
M15	*E. coli* K-12 derivative: Nal^S, Str^S, Rif^S, F^-, *th*$^-$, *lac*$^-$, *ara*$^+$, *gal*$^+$, *mtl*$^-$, *recA*$^+$, *uvr*$^+$, *lon*$^+$	Qiagen, Hilden, Germany
DH5α:RR06	*rr06* cloned into pGEM-T Easy and transformed into DH5α	(Standish, 2002)
DH5α:RR13	*rr13* cloned into pGEM-T Easy and transformed into DH5α	(Standish, 2002)
DH5α:pGEM-T	pGEM-T Easy transformed into DH5α	(Standish, 2002)
M15:RR06	*rr06* cloned into pQE30 and transformed into M15	This study
M15:RR13	*rr13* cloned into pQE30 and transformed into M15	This study
SU101	Homo-dimerisation reporter strain, wt *lexA* operator sequence cloned upstream of a *lacZ* reporter	(Dmitrova *et al.*, 1998)
SU101:p658*rr06*	*rr06* cloned into p658 and transformed into SU101	This study
SU101:p658	p658 transformed into SU101	This study
SU101:pDP804	pDP804 transformed into SU101	This study

***L. lactis* strains**	**Description**	**Source/Reference**
MG1363	Subsp *cremoris*	(Gasson, 1983)
MG1363 : pLS1RGFP	pLS1RGFP electroporated in MG1363	This study

Plasmids	**Characteristics**	**Source/Reference**
pVA891	Cml^R, Ery^R	(Macrina *et al.*, 1983)
pACH74	pVA891 derivative containing polylinker from pALTER; Ery^r Cml^r	(Giammarinaro & Paton, 2002)
pGEM-T Easy	Cloning vector, Amp^R	Promega Biotech, Madison, Wis, US
pQE30	His_6-tag expression vector, Amp^R	Qiagen
pLS1RGFP	Maltose inducible over-expression vector, Ery^R, GFP	(Nieto *et al.*, 2000)
pDP804	LexA DNA binding domain fused to Jun Zipper, Amp^R	(Dmitrova *et al.*, 1998)
p658	pMS604 derivative containing polylinker pTrcHisA, LexA DNA binding domain, Tet^R	(Daines & Silver, 2000)

2.2 Growth Media

E. coli strains were grown in Luria-Bertani broth (LB) (10 g/l tryptone-peptone [Difco, MD., USA], 5 g/l Bacto yeast extract [Difco], 5 g/l NaCl, adjusted to pH 7.5 with 5 M NaOH), LB agar plates (LB with 15 g/l Bacto agar [Difco]), or Terrific broth (24 g/l yeast extract, 12 g/l tryptone, 0.4% [v/v] glycerol, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4). Where appropriate, ampicillin (Amp) or chloromphenicol (Cml), were added to the growth medium at concentrations of 50 μg/ml and 25 μg/ml, respectively. *E. coli* strains were preserved in LB broth supplemented with 30% glycerol at -80°C.

S. pneumoniae were routinely grown in Todd Hewitt broth (Oxoid, England) with 1% yeast extract (THY), or on blood agar plates (39 g/l Columbia base agar [Oxoid], 5% [v/v] defibrinated horse blood). Alternatively, cells were grown in serum broth (SB) (10% [v/v] donor horse serum in nutrient broth [10 g/l of peptone (Oxoid), 10 g/l of Lab Lemco powder (Oxoid) and 5 g/l of NaCl]). Where appropriate, erythromycin (Ery), gentamicin (Gent) and Streptomycin (Strep) were added to the growth medium at concentrations of 0.2 μg/ml, 5 μg/ml and 1 μg/ml, respectively.

To differentiate between opaque and transparent colony opacity phenotypes, pneumococci were grown on THY plates (THY broth with 1.5% [w/v] Bacto agar) supplemented with 6300 Units (U) of catalase per plate (Roche Diagnostics, Germany). After incubation at 37°C in 95% air/ 5% CO_2 for approximately 40 h, colony phenotype was determined using oblique, transmitted light, as described by Weiser *et al.* (1994).

For storage, pneumococci were grown in serum broth then stored at -80°C. Alternatively, for storage of opaque or transparent colonies, *S. pneumoniae* was grown in THY, then stored at -80°C after addition of 30% glycerol.

L. lactis strains were grown at 30°C with shaking in M17 broth (Oxoid). Strains containing plasmids were grown in 5 μg/ml Ery.

2.3 Chemicals, Reagents and Enzymes

Most chemicals used were AnalaR grade and were purchased from Ajax Chemicals (NSW, Australia). Tris was purchased from Progen Industries (QLD, Australia). Maleic acid, and sodium dodecyl sulphate, were purchased from Sigma Chemical Company (St. Louis, MD., USA). Acrylamide, ammonium persulphate and N,N,N'N'-tetramethyl-ethylene-diamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). Deoxyribonucleoside triphosphates, isopropyl-β-D-thiogalactoside (IPTG), and herring sperm DNA were purchased from Roche Diagnostics (Roche Diagnostics). 5-bromo-4-chloro-3-indoyl-β-D-galacto-pyranoside (X-gal) was purchased from Astral Scientific (NSW, Australia). Sodium deoxycholate (DOC), was purchased from BDH Biochemicals (Poole, England). All enzymes were purchased from Roche Diagnostics.

2.3.1 Antibiotics

Amp was purchased from CSL (Victoria, Australia); Ery was purchased from Roche Diagnostics; Gent, Tet and Strep were purchased from Sigma.

2.3.2 Oligonucleotides

The oligonucleotides used in this study are listed in **Table 2.2** and were purchased from Sigma Genosys (Castle Hill, NSW, Australia).

Table 2.2. Oligonucleotide primers.

Name	Sequence 5'→3'	Description/Location[a]
Sp7c	CGGGATCCTATGCGAATCAAACAC	nt 1991834-1991850 (*Bam*HI site is underlined)
Sp7d	CCCAAGCTTTGGAGTTCTGGTAGT	nt 1992348-1992334 (*Hin*dIII site is underlined)
O1Bsp7	CATCGATTTTGCTCATTACGACTT	nt 1990993-1991010 (*Xba*I site is underlined)
AS5	GCACCTGATGCTTCAAGATAGTAC	nt 1987671-1987694
AS6	TGTCGGGAAAACAGCTCTGGC	nt 1994684-1994663

AS10	atacccaacCATCTGCAACTAAAATGTTCATC	nt 1992300-1992321, end of tail nt[b] 1991689-1991680
AS11	tattggcagatgGTTGGGTATAAGATTAGCTTATG	nt 1991689-1991667, end of tail nt[b] 1992308-1992300
AS13	taaattccatTAGACCAACACCACCTAGAATTGC	nt 1991598-1991621, end of tail nt[b] 1990361-1990351
AS14	ggtgttggtctaATGGAATTTAAGATTAGGTTGTAG	nt 1990338-1990361, end of tail nt[b] 1991597-1991689
16sF	GGTGAGTAACGCGTAGGTAA	nt 15455-15474
16sR	ACGATCCGAAAACCTTCTTC	nt 15780-15761
CbpAFNEW	TCAACTAGATAGAAGAAAACATACCCA	nt 1989422-1989396
CbpARedit	TCCTGGTTTCAATGATCTTTTTTAAACTT	nt 1989236-1989265
CbpAprom1	CAGCTACACTAGCTACTCCAATAC	nt 1989574-1989597
CbpAprom2	ATGTAGATCATATCTTGTTTAGGACAG	nt 1989939-1989913
RTHis-Rev	ATCAAATGGAATTATAGTTTTCTCCC	nt 1990400-1990375
RtcbpA-For	GCAACAACTACACTAGCTACTCC	nt 1989571-1989597
AS1	GAGGGATCCATGAACATTTTAGTTGCAGATG	nt 1922319-1992298 (*Bam*H1 site is underlined)
AS2	TTATCAAAGCTTTAAGCTAATCTTATACCCAAC	nt 1991666-1991689 (*Hin*dIII site is underlined)
AS3	AAGAAGGCATGCATGAGAATATTTGTTTTAG	nt 469762-469780 (*Sph*I site is underlined)
AS4	AATTTAAAGCTTTTGATGGCCTCAGACAC	nt 470483-470469 (*Hin*dIII site is underlined)
AS61	ttgttcatgtaatcactccttcCTTGGTTAATAATTTAGGATTTTTTATCAT	nt 2114082-2114111[c], end of tail binds *ery*
AS60	cgggaggaaataattctatgagGGTATGGAATTTAAGATTAGCTTGTAG	nt 2112806-2112780[c], end of tail binds *ery*
AS52	cgggaggaaataattctatgagAAGATTAGCTTATGATAAAAAATCCTA	nt 2114759-2114781[c], end of tail binds *ery*
AS53	ttgttcatgtaatcactccttcCATCTCTCTCCCTTTCTATCACC	nt 2114122-2114097[c], end of tail binds *ery*
HKF	TTTTGCTTTTGTATCTCCCATATAC	nt 1991251-1991227
HKR	TTGCTCATTACGACTTTCCAAC	nt 1990993-1991016
RRF	CTCAGTCACAGGTATTTGATCTC	nt 1992066-1992044
RRR	TATGCTGAATCAACACTTTAGTAC	nt 1991833-1991857
AS62	GATTCAGAACGAGATAAGGCAAGG	nt 2111950-2111867[c]
AS64	GTTTAGTGGAAGAGTCTGAACTTG	nt 2111608-2111631[c]
AS91	ATCATTTAAGATTTTATCTGGTGCC	nt 65890-65915

AS92	AGTCATAGTAGCGACCAACTGCC	nt 66240 - 66217
AS93	CCAGCTGATTTAGCTACTGCTC	nt 3275451-327567
AS94	ATCAACAGTTGCATTTCCATTGGC	nt 327894-327918
AS95	GAAGATGAAGAGATGATCCGTGAG	nt 527354 – 527378
AS96	GTAGCGCTTGAAAATCGCGTCC	nt 527646 – 527668
AS97	GATTATGATTTAACGTCGCCGCG	nt 626055 – 626078
AS98	CATAGCTAATCTAGTACCAGAACC	nt 626255-626281
AS99	GGTGTTATCATTGCTACTCCTGAGC	nt 1312881 – 1312906
AS100	AAGTTATCTAAGCAAGTTTCAAGG	nt 1313195 --1313171
AS101	ATGGGAATCATCGCAAATGTATTCG	nt 1598730 – 1598755
AS102	TTACTTTCTACCTGTAAAGAATGAC	nt 1598883-1598858
AS103	GACCAATCGTGTTTTGGCACGAG	nt 1640492-1640469
AS104	GTAGTCAGATGGAACACGATAAGC	nt 1640190-1640216
AS105	GAGAATGAGAAACGAATCCTTAGC	nt 1990192-1990169
AS106	CAACAAGGTCAAGCCAATCAACTC	nt 1989854-1989877
AS77	CGCGGAGCATCATACGAGTTGAAAACC	nt 1990957-1990930
AS78	GGTTTTCAACTCGTATGATG CTCCGCG	nt 1990930-1990957
AS 85	CGCGGAGCATCAGAAGAGTTGAAAACC	nt 1990957-1990930
AS 86	GGTTTTCAACTCTTCTGATGCTCCGCG	nt 1990930-1990957
AS 87	CGCGGAGCAAACCATGAGTTGAAAACC	nt 1990957-1990930
AS 88	GGTTTTCAACTCATGGTTTGCTCCGCG	nt 1990930-1990957
AS 89	CACATGAGTTGAAACGCCCTTTAG	nt 1990947-1990923
AS 90	CTAAAGGGCGTTTCAACTCATGTG	nt 1990947-1990923
AS75	CTCATGGTACTGGAGTTAATGATG	nt 1992181-1992157
AS76	CATCATTAACTCCAGTACCATGAG	nt 1992157-1992181
AS79	CTCATGGTACTGAACTTAATGATG	nt 1992181-1992157
AS80	CATCATTAAGTTCAGTACCATGAG	nt 1992157-1992181

AS67	ACGGTAGCTAGCAGTAAAGGAGGTAGAGAGATG AACATTTTAG	nt1992326-1992306 (*Nhe*I site underlined)
AS68	ACGGTAGCTAGCTCATAAGCTAATCTTATACCCA ACATTTTT	nt 1991664-1991695 (*Nhe*I site underlined)
AS69	ACGGTAGCTAGCACCAAAGGAGGTTTAGCTATGA TAAAAAATC C TAAATTATTAACC	nt 1991675-1991641(*Nhe*I site underlined)
AS70	ACGGTAGCTAGCCTACAAGCTAATCTTAAATTCC ATACC	nt 1990338-1990360 (*Nhe*I site underlined)
CbpaeryF	ttgttcatgtaatcactccttCTTTTCTTTCGCTTTTTGATGCA	nt 2112069-2112090[c], end of tail binds *ery*
CbpaeryR	cgggaggaaataattctatgagTGTAGATGGCTATGGAGTCA ATGCC	nt 210055-211003[c], end of tail binds *ery*
GAPDH Fwd	TCCTTGGAGGCCATGTGGGCCAT	GAPDH mRNA (XM_033258) nt 206-228
GAPDH Rev	TGATGACATCAAGAAGGTGGTGAAG	GAPDH mRNA (XM_033258) complementary nt 445-421
IL-8 Fwd	GAAGGAACCATTCTCACTGTGTGTA	IL-8 mRNA (M28130) nt 75-99
IL-8 Rev	TTATGAATTCTCAGCCCTCTTCAAAAAC	IL-8 mRNA (M28130) complementary nt 402-375
AS55	agagaaggaattGATTATAGTTTTCTCCCTTATGAACAC	nt 1990364-1990391 end of tail nt[b] 1992272-1992283
AS56	ccgagtttactataatcAATTCCTTCTCTAATCATTTCCTCG TC	nt 1992272-1992298 end of tail nt[b] 1990391-1990375
J214	GAAGGAGTGATTACATGAACAA	Binds to 5' end of *ery* gene
J215	CTCATAGAATTATTTCCTCCCG	Complementary to 3' end of *ery* gene
PspATIGRf	GCAGAAGAATCTCCACAAGTTGTCG	nt 118513-118537[c]
PspATIGRr	TGTTGCTCTTTCCTAGCCTTCTC	nt 118857-118833[c]
RMAG7	GCTGCACCGATAGACAGACGC	nt 1985081-1985101

[a.] Location in genome sequence of *S. pneumoniae* R6 (Hoskins *et al.*, 2001); GenBank Accession Number AE007317.

[b.] Sequence location of 'tail' region, which does not bind to DNA in first PCR step of overlap extension PCR (this region is indicated by lower case in the sequence).

[c.] Location in genome sequence of *S. pneumoniae* TIGR4 (Bricker & Camilli, 1999); GenBank Accession Number NC_003028.

2.4 Serotyping of pneumococcal strains

The production of capsule by pneumococci was assessed by Quellung reaction, using diagnostic pneumococcal typing sera purchased from Statens Seruminstitut (Copenhagen, Denmark). Serum broth was inoculated with cells, and grown for 4 h at 37°C. 10µl of the serum broth was then mixed with 10µl of the typing sera and microscopic examination for capsular swelling confirmed the production of type-specific capsule.

2.5 Optochin Sensitivity

In order to verify bacteria were *S. pneumoniae*, strains were tested for optochin sensitivity (an intrinsic characteristic of *S. pneumoniae*) by plating on BA in the presence of an 5µg optochin disk (Oxoid) and incubating for approximately 16 h at 37°C in 95% air/ 5% CO_2.

2.6 Bacterial Transformation

2.6.1 Transformation of *E. coli*

2.6.1.1 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.

2.6.1.2 Transformation of *E. coli*

Transformation of *E. coli* involved adding ~1 μg DNA directly to competent DH5α or M15 cells, 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.

2.6.2 Transformation of *S. pneumoniae*

2.6.2.1 Preparation of competent cells

Pneumococcal cells were grown in c-CAT medium (10 g/l Bacto Casamino acids [Difco], 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.

2.6.2.2 Transformation of pneumococci

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 h. After incubation, cells were plated onto BA plates containing 0.2 μg/ml Ery, and incubated for approximately 16 h at 37°C in 95% air/ 5% CO_2.

2.6.2.3 Back transformation of pneumococci

500 µl of CTM-pH 7.8 and 25 ng CSP-1 were added to an aliquot of competent cells. The cells were then incubated at 37°C for 20 min before addition of 1–100 ng DNA and incubated at 32°C for 30 min then at 37°C for 2 h. The total volume of cells was split between 2 tubes and 5 µl Ery (0.01 mg/ml) was added to each tube before incubation at 37°C for 30 min. 50 µl Amp (25 mg/ml) was then added and each tube incubated for 4 h at 37°C. Cells were then pelleted by centrifugation and washed 2 times in CTM before finally being resuspended in 1 ml CTM. 100 µl of a 1/1000 dilution was then plated onto BA plates and incubated overnight at 37°C in the presence of 5% CO_2. Colonies were replica plated onto BA and EryBA plates to identify Ery-sensitive back-transformants.

2.6.3 Transformation of *L. lactis*

2.6.3.1 Electroporation of *L. lactis*

Plasmids were transferred to *L. lactis* by electroporation essentially as described by Lopez de Felipe *et al.* (1995). *L. lactis* were grown O/N at 30°C with agitation in M17 supplemented with 0.5% glucose, threonine (23.8 mg/ml) and 1 mM $MgSO_4$. The culture was diluted to approximately A_{600} = 0.1 and grown to A_{600} = 0.3. Cells were then centrifuged at 3880 × *g* for 10 min, washed once each in cold water, 50mM EDTA and 0.3 M sucrose, and then finally resuspended into 0.2ml of 0.3M sucrose. Plasmid DNA was then added (no more than 10µl), and the mix was transferred to a cold electroporation cuvette which was then electorporated under the following conditions: resistance 200Ω; capacitance 25 µF; voltage 2500 V; time constant 3.5-4.0 mseg. After electroporation, cells were transferred to M17GS (M17 + 5 µl 1M $MgSO_4$ + 0.5 % glucose with 1% sucrose), incubated for 2 h at 30°C and then plated on M17GS with Ery.

2.7 DNA isolation and manipulation

2.7.1 Agarose gel electrophoresis

DNA was electrophoresed through horizontal agarose gels (0.8-2% w/v agarose dissolved in Tris Borate EDTA (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 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 transillumination with short wavelength UV light and results recorded using Tracktel video camera (Mitsubishi video imaging system). Approximate sizes of visualised fragments were calculated by comparison of their mobility with that of DNA size markers. Markers used in this study were *Eco*RI-digested *Bacillus subtilis* bacteriophage SPP-1 DNA (Geneworks, Adelaide, SA., Australia), *Hin*dIII-restricted bacteriophage lambda DNA (Geneworks), or *Hpa*II-restricted pUC19 plasmid DNA (Geneworks). Approximate sizes of visualised fragments were calculated by comparison of their mobility with that of DNA size markers derived from *Eco*RI digested *Bacillus subtilis* bacteriophage SPP1 DNA (fragment sizes: 8.56, 7.43, 6.11, 4.90, 3.64, 2.80, 1.95, 1.88, 1.52, 1.41, 1.16, 0.99, 0.71, 0.49, 0.36, 0.08 kilobase pairs) or *Hpa*II digested pUC19 DNA (fragment sizes: 501, 404, 331, 242, 190, 147, 111, 110, 67, 34, 26 base pairs).

2.7.2 Pneumococcal Chromosomal DNA isolation

Pneumococcal chromosomal DNA was isolated using the Wizard Genomic DNA purification kit (Promega Life Sciences, WI USA), according to the manufacturer's instructions, with cells lysed by re-suspension in 200 µl 50 mM EDTA supplemented with 0.1% DOC.

2.7.3 Purification of Plasmid DNA

A 10 ml overnight culture of *E. coli*, *S. pneumoniae*, or *L. lactis* grown in the relevant media was pelleted by centrifugation at 2880 × *g* for 10 min. Plasmid DNA was isolated using the QIAprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. Plasmids from *L. lactis* were purified using an alternative Solution 1 containing 20% sucrose, 10mM Tris-HCl pH 8, 1mM EDTA, 50 mM NaCl and 30 mg/ml lysozyme.

2.7.4 Restriction endonuclease digestion of DNA

Restriction endonuclease digestions of DNA were carried out in a volume of 20 µl. Each reaction contained approximately 1 µg DNA, and 1-2 U of the restriction enzyme(s), in the appropriate reaction buffers according to the manufacturer's instructions (Roche Diagnostics). Reactions took place at the manufacturer's suggested temperature for 2-16 h. Restriction digests were analysed using agarose gel electrophoresis.

2.7.5 DNA ligation

Ligation reactions were performed using T4 DNA ligase (Roche diagnostics). Each reaction contained ~3 units of T4 DNA ligase (Roche Diagnostic), the manufacturer's 10 × ligation buffer, and approximately 500 ng each of the vector and insert DNA. The ligation proceeded at room temperature overnight. Alternatively, cloning PCR products into pGEM-T Easy was performed according to the manufacturer's instructions (Promega).

2.7.6 Polymerase Chain Reaction (PCR)

Standard PCR reactions were carried out on a thermal cycler (Hybaid, Middlesex, England), in a final volume of 50 µl, using *Taq* polymerase according to the manufacturer's instructions (Roche Diagnostics).

High fidelity PCR reactions were carried out on a thermal cycler (Hybaid), in a final volume of 50 µl, using the Expand™ Long Template PCR system (Roche

Diagnostics). Each reaction contained approximately 100 ng of the DNA template, 1-2 units of Expand™ DNA polymerase, 4 μM of each oligonucleotides primer, 200 μM of each dNTP in 1 × Expand PCR buffer 3. Reaction conditions comprised 30 cycles as follows: Denaturation at 94°C for 1 min; annealing at 60°C for 1 min; and extension at 68°C for various times depending on the length of the product (approximately 1 min for each kb of product).

Overlap extension PCR was carried out as described above using the Expand™ Long Template PCR system (Roche Diagnostics). The reaction conditions were as follows: Denaturation at 94°C for 2 min, followed by 2 cycles of denaturation at 92°C for 30 sec, annealing at 40°C for 1 min, and extension at 68°C for 7 min, and then 33 cycles of denturation at 92°C for 30 sec, annealing at 55°C for 1 min, extension at 68°C for 7 min, with a final extension at 68°C for 8 min.

2.7.7 Purification of PCR products

PCR products were purified using the Ultraclean PCR Clean-up DNA Purification Kit (Mol Biol Laboratories, Solana Beach, CA, USA) according to the manufacturer's instructions.

2.7.8 Isolation and purification of PCR fragments from agarose gels

DNA fragments of interest were excised from agarose gels and purified using the UltraClean GelSpin DNA purification kit (MolBiol) according to the manufacturer's instructions.

2.7.9 Cloning of PCR products

For cloning, PCR products were directly ligated into pGEM®-T Easy, as recommended by the manufacturer (Promega). Briefly, the ligation mixture consisted of the purified PCR product (Section 2.7.7), 50 ng pGEM®-T Easy vector DNA, 3 U T4 DNA

ligase and 1 × ligation buffer, in a total volume of 10–15 µl. The reaction mixture was incubated overnight at 4°C. Ligation mixtures were then transformed into *E. coli* DH5α as described in Section 2.6.1.

2.7.10 DNA sequencing and analysis

DNA sequencing reactions were carried out using the method described in the PRISM™ Dye Primer Cycle Sequencing Ready Reaction Kit Manual (Applied Biosystems, Foster City, CA., USA). Each reaction contained double stranded DNA template (50-200 ng), 4 µl Big Dye terminator mix, and 3.2 pmol of a single primer, made up to a final reaction volume of 20 µl with sterile water. Sequencing reactions were performed on a thermal cycler (Hybaid) under the following conditions: heat denaturation at 95°C for 30 sec, primer annealing and extension at 60°C for 4 min for 25 cycles. When the reaction was completed, the dye terminator labelled-DNA was precipitated with 80 µl of 75% (v/v) isopropanol and 20 µg of glycogen (Roche). The mixture was left at RT for 2 h, after which the precipitate was pelleted by centrifugation at 14,900 × *g* for 30 min. The pellet was subsequently washed with 250 µl of 70% (v/v) ethanol and centrifuged for a further 5 min at 14,900 × *g* at 4°C. The DNA pellets were then dried at 65°C for 10 min. Sequencing was performed by the Molecular Pathology Unit at the Institute of Medical and Veterinary Science, Adelaide, S.A., Australia using an Applied Biosystems 3700 DNA sequencing analyser. DNA sequence data were analysed using the DNAMAN program (Lynnon Biosoft®, Vaudreuil, Quebec, Canada), and BLAST searches were used to identify similarities with sequence data at the National Center for Biotechnology Information (Bethesda, MD, USA [www.ncbi.nlm.nih.gov/blast]) (Altschul *et al.*, 1997).

2.8 Protein analysis

2.8.1 SDS-PAGE

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. The SDS-PAGE molecular weight marker used were Broad Range Marker (Bio-Rad laboratories, CA, USA). Fragment sizes were 200, 112.5, 97.4, 66.2, 45, 31, 21.5, 14.4 and 6.5 kDa.

2.8.2 Preparation of Whole Cell Lysates (WCL) for SDS-PAGE analysis

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.

2.8.3 Western blotting

Proteins were separated by SDS-PAGE, and electroblotted onto nitrocellulose (Pall Life Sciences, MI., USA) at 300 mA for 1 hr, as described by (Towbin *et al.*, 1992). 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 was probed with specific polyclonal antisera at a dilution of 1/3,000 in TTBS overnight at room temperature with gentle agitation. The filter was washed 3 times with TTBS for 10 min, before the addition of blotting grade goat anti-mouse-AP conjugate (BioRad Laboratories) 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 with TTBS, and then equilibrated in 15 ml DIG buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 2 mins before addition of 45 µl DIG 4 (75 mg/ml nitroblue tetrazolium salt in 70% (v/v) dimethylformamide), and 35 µl DIG 5 (50 mg/ml 5-bromo-4-chloro-3-indolylphosphate toluidinium salt in dimethylformamide) in 10 ml DIG buffer 3. After the desired colour reaction had taken place, the reaction was stopped by the addition of TE. The membrane was then rinsed in water and dried.

2.8.4 Pro-Q Diamond phosphoprotein stain

Pro-Q Diamond phosphoprotein staining of SDS-PAGE was undertaken as described by Agrawal & Thelen (2005). Gels were first fixed by two 30 min washes in 50% (v/v) methanol and 10% (v/v) acetic acids, washed in deionised water, and stained in 3-fold diluted Pro-Q for 120 min. Destaining was then performed by several changes of 50 mM Sodium Acetate (pH 4.0) with 20% (v/v) acetonitrile. Gels were scanned on a Typhoon Trio (GE Healthcare Life Sciences, USA) with 532-nm excitation and 580-nm emission.

2.9 Protein purification

2.9.1 Expression of recombinant proteins

The response regulators RR06 and RR13 from *S. pneumoniae* D39 (serotype 2) were expressed as His_6-fusion proteins using the vector pQE30 (Qiagen), in the *E. coli* K12 expression strain M15 (Qiagen). Expression strains were grown in 100 ml Terrific broth overnight at 37°C with agitation in the presence of 50 µg/ml Amp. This starter culture was then diluted 1/10 into 2 × 500 ml LB or Terrific broth with 50 µg/ml Amp, and incubated at 37°C with agitation to A_{600} of 0.5. High-level expression of His_6-tagged proteins was

induced by the addition of 2 mM IPTG, and incubation at 37°C for 3 h with agitation. The culture was then centrifuged at 4°C for 8 min at 11,440 × *g*. The pellet was resuspended in 20 ml of lysis buffer (50 mM sodium-phosphate, 20 mM imidazole, 2 M NaCl, pH 8.0), and the cells were lysed using a French pressure cell (SLM Instruments) operated at 12,000 psi. The lysate was then centrifuged at 120,000 × *g* for 1 h at 4°C, to remove cellular debris.

2.9.2 Purification of RR06 and RR13 by Ni-NTA chromatography

Recombinant His_6-tagged RR06 and RR13 proteins were purified by Nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Qiagen). A 2 ml Ni-NTA bed was equilibrated with 20 ml lysis buffer. Lysate supernatant was treated with 20 mM β-mercaptoethanol, 1% (v/v) Triton-X100, DNase and RNase for 10 min on ice, and then loaded onto the column at a rate of 15 ml/h. After the entire supernatant had been loaded, the column was washed with 20 ml wash buffer (10 mM sodium-phosphate, 1 M NaCl, 0.5%(v/v) Triton-X100, pH 6.0). Bound His_6-tagged protein was eluted from the column with a 0-500 mM imidazole gradient in elution buffer (10 mM sodium-phosphate, pH 6.0). Fractions were collected and immediately placed on ice. 10 µl from each of the fractions was subjected to analysis by SDS-PAGE, as described in Section 2.8.1, to confirm the presence and purity of the desired protein. Fractions containing the protein were concentrated in 10 mM sodium-phosphate (pH 7.0), and stored in 50% glycerol at -20°C. Protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as a standard.

2.10 RNA Methods

2.10.1 RNA extraction from *S. pneumoniae*

Pneumococcal RNA was extracted using either the RiboPure bacteria kit following the manufacturers instructions (Ambion, Austin, TX, USA) or an adaptation of the acid/phenol method as described by Mortier-Barriere *et al.* (1998). Pneumococci were grown in THY to the specified A_{600}. Cells were pelleted by centrifugation at 4,000 × *g* for 10 min at 4°C. Cells were subsequently resuspended in 400 µl of pre-warmed acid-phenol/chloroform/isoamyl alcohol (125 : 24 : 1), pH 4·5 (Ambion) and incubated at 65°C for 5 min. 400 µl of pre-warmed NAES (50 mM sodium acetate, 10 mM EDTA, 1% [w/v] SDS, pH 5.1, treated with 0.5% [w/v] Diethyl Pyrocarbonate [DEPC]) was then added, mixed by inversion and incubated at 65°C for a further 5 min. The lysate was placed on ice for 1 min and centrifuged at 14,900 × *g* for 1 min at 4°C. The aqueous phase was removed and the phenol extraction was repeated twice more. The aqueous layer was subsequently mixed with 30 µl of 0.05% (w/v) DEPC-treated 3 M sodium acetate and 750 µl 100% ethanol, and then precipitated overnight at -70°C. Following centrifugation at 14,9000 × *g* for 30 min at 4°C, the pellet was washed with 300 µl 70% ethanol and centrifuged for 10 min at 14,900 × *g*. The pellet was then resuspended in 40 µl DEPC-treated water. RNase's were inhibited by the addition 20 U RNasin® Ribonuclease Inhibitor (Promega). Contaminating DNA was removed with RNase-free DNase I (Roche Diagnostics).

2.10.2 RNA extraction from eukaryotic cells

Whole cellular RNA was extracted using TRIZOL® reagent according to the manufacturer's instructions (Invitrogen, California, USA). Briefly, 1 ml TRIZOL® reagent was added directly to cell monolayers and the cells suspended by passing through a pipette several times. The homogenised samples were incubated for 5 min to permit the complete dissociation of nucleoprotein complexes, before the addition of 200 µl chloroform. The

mixture was agitated for 15 sec then centrifuged at 12,000 × *g* for 15 min. After centrifugation the aqueous phase containing the RNA was transferred to a clean reaction tube and RNA was precipitated by the addition of 500 µl isopropanol and incubation at RT for 10 min. RNA was pelleted by centrifugation at 12,000 × *g* for 10 min and the pellet washed with 1 ml 75% ethanol for 5 min at 7500 × *g*. The pellet was then air dried for 10 min and resuspended in 45 µl RNase-free water (Promega) at 58°C for 10 min to facilitate resuspension. RNA was reprecipitated by the addition of 1/10 volume sodium acetate (pH 4.8) and 2 volumes absolute ethanol and incubation at –80°C overnight. The following day, RNA was pelleted by centrifugation at 12,000 × *g* for 30 min at 4°C, washed in 75% (v/v) ethanol, and resuspended in nuclease-free water. RNasin® Ribonuclease Inhibitor was added to RNA, and contaminating DNA was digested with RQ1 RNase-free DNase, followed by DNase stop solution, according to the manufacturer's instructions (Promega).

2.10.3 Reverse transcription PCR (RT-PCR)

RT-PCR was carried out using the Access RT-PCR System (Promega) in accordance with the manufacturer's instructions. Each reaction was performed in a final volume of 20 µl, with each reaction containing 20 nmol of each oligonucleotide. RT-PCR amplification was performed using a Hybaid Thermocycler (Hybaid). The standard amplification conditions consisted of one reverse transcription cycle at 48°C for 45 min followed by 30 cycles, each consisting of 30 sec of denaturation at 95°C, 45 sec of annealing at the optimal temperature and 1 min of elongation at 68°C. The purity of all RNA isolated from *S. pneumoniae* was confirmed by subjecting them to RT-PCR analysis with or without reverse transcriptase, using 16S rRNA-specific primers (see Table 2.2). Similarly, the absence of DNA contamination in RNA preparations from eukaryotic cells was confirmed by RT-PCR analysis using oligonucleotides specific for the gene encoding the house-keeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table

2.2). The gene encoding GAPDH contains an intron such that mRNA template directs amplification of a 239-bp product, whereas chromosomal DNA template directs amplification of a 341-bp product. The absence of DNA contamination was determined by electrophoresis of 10 µl of each PCR product on a 2% (w/v) agarose gel.

2.10.4 Real Time RT-PCR

Real time RT-PCR was performed using the one-step access RT-PCR system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Each reaction was performed in a final volume of 20 µl, each reaction containing 20 nmol of each oligonucleotide, and 1/20,000 dilution of Sybr green I nucleic acid stain (Invitrogen). The quantitative RT-PCRs were performed on a Rotorgene RG-2000 (Corbett Research, Mortlake, NSW, Australia) and included the following steps: 45 min of reverse transcription at 48°C, followed by 2 min denaturation at 94°C, and then 40 cycles of amplification comprising 94°C for 30 sec, the optimum annealing temperature for 30 sec, and 72°C for 45 sec. Three replicates were performed for each sample, using 16S rRNA as an internal standard for RNA levels (Table 2.2). Results were calculated using the comparative cycle threshold ($2^{\Delta\Delta Ct}$) method (User Bulletin no. 2 [http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf]; Applied Biosystems), in which the amount of target mRNA is normalized relative to an internal control (16S rRNA). Data are expressed as relative changes in mRNA levels compared to 16S rRNA levels. Relative change standard deviations (SD) were initially determined as the $\sqrt{((SD\ sample)^2 + (SD\ 16S)^2)}$, and this was then applied to the formulas $SD+ = 2^{\Delta\Delta CT-SD} - 2^{\Delta\Delta CT}$ and $SD- = 2^{\Delta\Delta Ct} - 2^{\Delta\Delta CT+SD}$.

2.10.5 Microarray analysis

Total bacterial RNA was labelled and hybridised using the Genisphere Array 900 MPX Kit (Genisphere, Hatfield, PA USA), according to the manufacturer's instructions. 3 μg of each RNA preparation was used per microarray slide with hybridisation reactions carried out at 65°C. 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. The array was read using a GenePix 4000B scanner (Molecular Devices Sunnyvale, CA, USA), and images were acquired using GenePixPro6.0 (Axon). Results were analysed using R package and the program Spot (CSIRO Mathematical and Information Sciences, Image Analysis Group, North Ryde, NSW, Australia) and LimmaGUI (Bioinformatics, Walter and Eliza Hall Institute, Melbourne, Vic, Australia). Gene lists were prepared with a 2-fold cut-off and with *P* value < 0.05. The *P*-value was calculated from *t*-tests between the intensities of individual spots.

2.11 Electrophoresis Mobility Shift Assays (EMSA)

Promoter DNA targets for gel mobility shift assays were amplified by PCR from *S. pneumoniae* D39 chromosomal DNA. Approximately 10 pmol of PCR product was then labelled using DIG-11dideoxy UTP (ddUTP) and terminal transferase according to the manufacturer's instructions (Roche Diagnostics). Cell extracts were prepared by lysing overnight cultures using a French pressure cell operated at 12,000 psi. Extracts were stored in the presence of 50% glycerol at -80°C. Lysate containing specified protein concentrations were pre-incubated on ice with 100 μg herring sperm DNA, and 6μl of the specified binding buffer (with MQ H_2O up to 12 μl) for 20 min. The binding buffer used was TM-1 (25 mM Tris-HCl, pH 7.6; 100 mM KCl; 0.5 mM DTT; 5 mM $MgCl_2$; 0.5 mM EDTA; 10% [v/v] glycerol). Approximately 1 pmol of labelled DNA target was then

incubated with the mixture on ice for a further 20 min. After the addition of loading buffer (250 mM Tris-HCl pH 7.5, 40% [v/v] glycerol, 0.2% [v/v] bromophenol blue), the binding reactions were loaded onto a pre-run 4% non-denaturing PAGE containing 1 x TBE. Electrophoresis was performed at 100 V for approximately 2-3 h at 4°C. The DNA-protein complexes were then transferred onto Hybond-N^+ membrane (Amersham, Buckinghamshire, UK) at 400 mA for 30 min, and the DNA was then crosslinked for 3 min under short wavelength UV radiation. The membrane was washed for 1 min in DIG buffer 1 (100 mM malic acid, 150 mM NaCl, pH 7.5) and gently agitated for 30 min at RT in 100 ml of DIG buffer 2 (1% [w/v] Blocking Reagent in DIG buffer 1). The membrane was then incubated at RT for 30 min in 30 ml of 1/5000 dilution of anti-DIG-alkaline phosphatase (AP) conjugate (Roche Diagnostics) in DIG buffer 2, with gentle agitation. Following incubation, the membrane was washed twice with 100 ml of DIG buffer 1 for 15 min. The membrane was equilibrated for 2 min with 30 ml of DIG buffer 3. A substrate solution containing 10 ml of DIG buffer 3 , 45 µl DIG 4 and 35 µl DIG 5 was added and place in the dark until the desired colour reaction had taken place, at which time the reaction was stopped by the addition of TE. The membrane was then rinsed with water, and dried for storage.

2.12 Solid phase binding assay

Solid phase binding assays were undertaken using an adaptation of that described by Gabrielsen & Huet, (1993). Approximately 40 pmol of DNA was biotin end-labeled using biotin-ddUTP and terminal transferase, according to the manufacturer's instructions (Roche Diagnostics). Strepdavidin MagneSphere Paramagnetic Particles (Promega) were washed three times in 1 × CB buffer (1 M NaCl, 5 mM Tris-HCl pH 8.0, 0.5 mM EDTA). Labeled DNA was then bound to the beads in 1 × CB buffer with gentle agitation for 60 mins at room temperature followed by three washes in 1 × CB and three washes in TM-1.

The protein-DNA binding reaction was performed in a total volume of 100 µl, containing 50 µg of specified lysate, 100 µg of herring sperm DNA, and 0.6 mg of coated beads for 20 min on ice. After washing three times vigorously in binding buffer, bound proteins were eluted by boiling the beads for 5 min in lysis buffer and were analyzed by SDS-PAGE and Western blotting using anti-RR06 as described in Section 2.8.3.

2.13 DNAse I Footprinting

Footprinting was performed essentially as previously described by Yindeeyoungyeon & Schell (2000). 5' carboxyfluorescein (FAM) labelled PCR product was incubated with specified lysate as described for EMS assays as described in Section 2.11. Following this, DNase I (Roche Diagnostics) was added at the specified concentration and incubated for the specified time at 26°C. DNase I was diluted in D buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 5 mM $CaCl_2$ and 0.1 mg/ml BSA). Reactions were stopped by the addition of 0.5 M EDTA. DNA was extracted with phenol-chloroformisoamyl alcohol (25:24:1), and DNA fragments were further purified using a PCR clean up kit as described in Section 2.7.7. Samples were then dried under vacuum and resuspended in 3 µl of formamide. Fragments were subsequently separated on an ABI prism 310 Sequencer/Genetic analyser following addition of a suitable size standard.

2.14 LexA Bacterial Two Hybrid System

The LexA based repression assay was performed essentially as described by Dmitrova *et al.* (1998). Overnight cultures were diluted 1/20 in LB, and grown for 4 h at 37°C with agitation. β-galactosidase activity was determined as previously described by Miller (1972).

2.15 Eukaryotic cell culture

Eukaryotic cells were routinely grown and maintained in Falcon 75 cm^2 vented tissue culture flasks (Becton, Dickinson and Company, New Jersey, USA). Detroit 562 (human pharyngeal epithelial) cells (ATCC CCL-138) were grown in Eagle's modified essential medium (EMEM) supplemented with 10% (v/v) foetal calf serum (FCS) (Gibco BRL-Life Technologies, Grand Island, NY, USA), 1 mM sodium pyruvate (Trace), 100 IU/ml penicillin, and 100 µg/ml streptomycin (Gibco Life Technologies) at 37°C in the presence of 5% CO_2. A549 (human type II pneumocyte) cells (ATCC CCL-185) were grown in Dulbecco's modified essential medium (DMEM) (Trace Biosciences), supplemented with 10% (v/v) FCS, 100 IU/ml penicillin, and 100 µg/ml streptomycin, at 37°C. Upon reaching confluence, cell monolayers were detached by the addition of a solution of 0.25% (w/v) trypsin and 0.2% (w/v) EDTA in phosphate buffered saline (PBS) (0.137 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO4, 1.5 mM KH_2PO_4, pH 7.4) and flasks were reseeded at a ratio of 1:15 (cell suspension: media). Cells were stored in the appropriate growth medium supplemented with 20% (v/v) FCS and 10% (v/v) dimethylsufoxide (DMSO) at -80°C for long-term storage.

2.16 Infection of epithelial cell monolayers with *S. pneumoniae* D39

For chemokine assays, A549 cells or Detroit 562 cells were seeded in Falcon 6-well tissue culture trays (Becton, Dickinson and Company) and allowed to attach overnight. Cells were used at 90–100% confluence. Cells were washed twice with PBS, 1 ml of the appropriate culture medium (without antibiotics or FCS) was added to each well, and cells were left to incubate at 37°C in air for A549 cells, or at 37°C in 95% air/5% CO_2 for Detroit 562 cells for 4 h. *S. pneumoniae* from overnight plates was inoculated into 10 ml THY medium and grown to an A_{600} of 0.15. *S. pneumoniae* cultures were then pelleted at

2500 × *g* for 10 min in a benchtop centrifuge, and the pellets resuspended in 2 ml of the appropriate cell culture medium without FCS or antibiotics to remove residual THY before pelleting again. Pellets were then resuspended in 1.5 ml of cell culture medium and 100 µl of this suspension (approximately 5×10^7 CFU) was used to infect cell culture monolayers. 4 h control cells received 100 µl cell culture medium in place of *S. pneumoniae* culture, with the 0 h control receiving nothing. Cell monolayers were then incubated in air at 37°C for A549 cells, or 37°C in 95% air/5% CO_2 for Detroit 562 cells for 4 h, at which time the cell monolayer was lysed in 1 ml TRIZOL® Reagent for RNA extraction (Section 2.10.2). Samples were also collected at 0 h to determine baseline IL8 expression in A549 or Detroit 562 cells before stimulation with *S. pneumoniae*.

2.17 *In Vitro* Adherence Assay

Adherence assays were performed on A549 or Detroit 562 cells essentially as described by Talbot *et al.* (1996). *S. pneumoniae* cells from fresh overnight BA cultures were grown in SB to an A_{600} of 0.08 (approximately 10^8 CFU/ml). The bacteria were then diluted 20-fold in DMEM + 10% (v/v) FCS. Washed A549 monolayers were inoculated with 0.5 ml *S. pneumoniae* suspension and incubated for 2 h at 37°C. The culture fluid was then removed and the cells were washed 3 times with PBS to remove any unbound pneumococci. The cell monolayers were detached from the plate by treatment with 100 µl of 0.25% (v/v) trypsin, 0.02% (w/v) EDTA (pH 7.5). Cells were lysed by the addition of 400 µl of a 0.025% (w/v) Triton X-100 solution and 100 µl of each lysate (and serial dilutions thereof) were plated onto BA to quantify adherent pneumococci. Assays were performed in quadruplicate and viable counts were used to determine the precise dose for each strain. These were used to normalize the recovery of adherent pneumococci from each group. The resulting adherence of the mutants was expressed as a percentage of adherent

cells relative to wt. The significance of differences in adherence was analysed using Student's unpaired *t*-test (2-tailed).

2.18 Challenge of mice.

2.18.1 Growth of challenge strain

Strains of *S. pneumoniae* to be used for intranasal studies were grown in THY to an A_{600} of 0.25. The bacteria were then diluted to the appropriate concentration with THY so that 25 µl aliquots contained the required challenge dose. The actual dose administered was determined retrospectively by plating serial dilutions of the challenge inocula after administration to the mice.

2.18.2 Intranasal Challenge

Mice were anaesthetised prior to challenge by i.p. injection with Nembutal (pentobarbitone sodium, Rhone-Merieux) at a dose of 66 µg per 1g of body-weight. 25 µl of the desired pneumococcal strain at the required challenge dose was then introduced into the nares and involuntarily inhaled. Mice were monitored closely until they had regained consciousness.

2.18.3 Sacrifice of mice

Following intranasal challenge, five mice per group were sacrificed by carbon dioxide asphyxiation at 24, 48, and 96 h post-challenge. Blood was collected from each mouse directly from the heart, of which 40 µl was diluted in 160 µl of sterile PBS. After exposure of the trachea, the nasopharynx was washed with 1 ml of buffer (0.5% trypsin–0.02% EDTA in sterile PBS) by insertion of a 26-gauge needle sheathed in tubing into the tracheal end of the upper respiratory tract. Buffer was allowed to drip into the nasopharynx slowly and was collected from the nose. Following this, the entire nasopharynx was

removed and placed in 5 ml of sterile PBS. The heart was perfused with PBS to ensure removal of contaminating pneumococci from the blood, following which, lungs were entirely removed, rinsed twice in PBS, weighed, and placed in 2 ml of sterile PBS. The lungs and excised nasopharyngeal tissue were then homogenized with a CAT X120 homogenizer (CAT, M, Zipperer GmbH, Staufen, Germany) at 30,000 rpm for approximately 10 s. All blood, lung, and nasal washout and lysate samples were then serially diluted and plated on BA (supplemented with 5μg/ml of gentamicin to inhibit contaminating microflora) to determine the number of viable pneumococci. Differences in the levels of colonization were analyzed using Student's unpaired *t* test (two-tailed).

2.18.4 Immunisation Protocol

2.18.4.1 Antigen Preparation

Antigens were formulated with aluminium hydroxide adjuvant (Alum) at a final ratio of 100 μg antigen to 1 mg Alum adjuvant. Antigens were first diluted in sterile PBS, such that, after further addition of Alum, a final antigen concentration of 100 μg/ml was achieved. To the required amount of antigen, the appropriate amount of Alum was added drop-wise over a 5 min period, while agitating the mixture with a magnetic stirrer between additions. The antigen-adjuvant mixture was then agitated with a magnetic stirrer for a further 1 h.

2.18.4.2 Intraperitoneal Immunisation

Mice were immunised by intraperitoneal injection of 100 μl volumes containing 10 μg protein in 100 μg Alum. Three immunisations were performed, spaced 14 days apart.

2.18.4.3 Analysis of Sera by Western Blotting

His_6-RR06 was separated by SDS-PAGE, and transferred to nitrocellulose as described in Section 2.8.3. After transfer, 5 mm strips were cut from the membrane,

which were blocked, probed and washed as described in Section 2.8.3. Each strip was probed with the serum from a different mouse.

CHAPTER THREE – RR/HK06 AFFECTS *cbpA* EXPRESSION

3.1 Introduction

A number of recent studies have shown that genes important for the virulence of the pneumococcus, such as *cbpA*, *pspA* and *ply*, are differentially regulated during the disease process (Orihuela *et al.*, 2004; LeMessurier *et al.*, 2006). However, the exact mechanism by which this regulation is achieved is not understood. One possibility is through TCSTSs, thirteen of which have been identified in *S. pneumoniae* (Lange *et al.*, 1999; Throup *et al.*, 2000).

Many of these thirteen pneumococcal TCSTSs remain largely uncharacterised. Of these, one system, RR/HK06 (also known as 478), was of particular interest as the gene encoding the major virulence factor CbpA, is found in close proximity to the genes

encoding RR/HK06 (**Figure 3.1**). A number of prokaryotic TCSTSs, such as BlpRS from *S. pneumoniae*, regulate adjacent genes on the chromosome (de Saizieu *et al.*, 2000). Thus, in this Chapter, we investigated the possibility that RR/HK06 may regulate *cbpA*.

3.2 Results

3.2.1 Construction of *S. pneumoniae* D39 with deletions in *rr06* and *hk06*

In order to investigate the TCSTS RR/HK06, *S. pneumoniae* derivatives with mutations in *hk06* and *rr06* were constructed. Previously, an insertion duplication mutant in *rr06* (D39*rr06*$^-$) had been constructed (Standish, 2002). The process of insertion-duplication involves transformation with a suicide plasmid containing an internal fragment of the gene to be interrupted, in this case a 515-bp fragment of *rr06* (see **Figure 3.2**). A single cross-over event between the homologous segment in the plasmid and the chromosome integrates the plasmid into the target gene. However, insertion-duplication mutants have a number of drawbacks including the production of truncated gene products, which may possess partial activity, along with possible polar effects on downstream genes. As we were interested in the system's effect on expression of the neighboring gene *cbpA*, unmarked deletion mutants in both *hk06* and *rr06* were constructed in *S. pneumoniae* D39.

In order to construct these mutants in *hk06* and *rr06*, overlap-extension PCR mutagenesis was used (Ho *et al.*, 1989; Horton, 1993) (**Figure 3.3**). For *rr06*, separate 5' and 3' flanking fragments were generated by PCR with D39 DNA as the template and primer pairs AS6/AS10 and AS5/AS11. The two PCR products were then combined and subjected to a second round of amplification using primer pair AS5/AS6. This generated a 6339-bp linear DNA fragment comprising the *rr06* gene with flanking sequences, but with nt 23 - 639 of the *rr06* open reading frame (ORF) deleted. Nucleotides 73 - 1308 of the

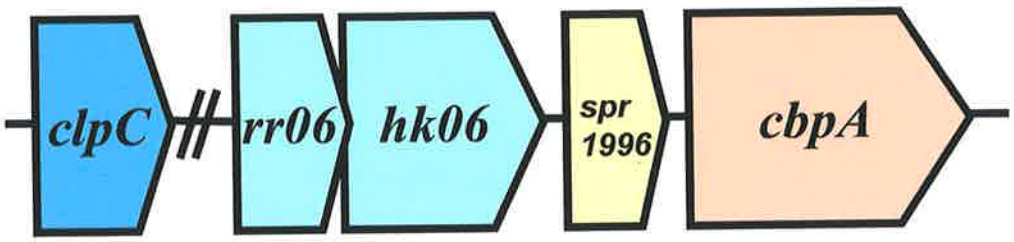

Figure 3.1. Diagram of R6 genome illustrating genes surrounding *rr06/hk06*.

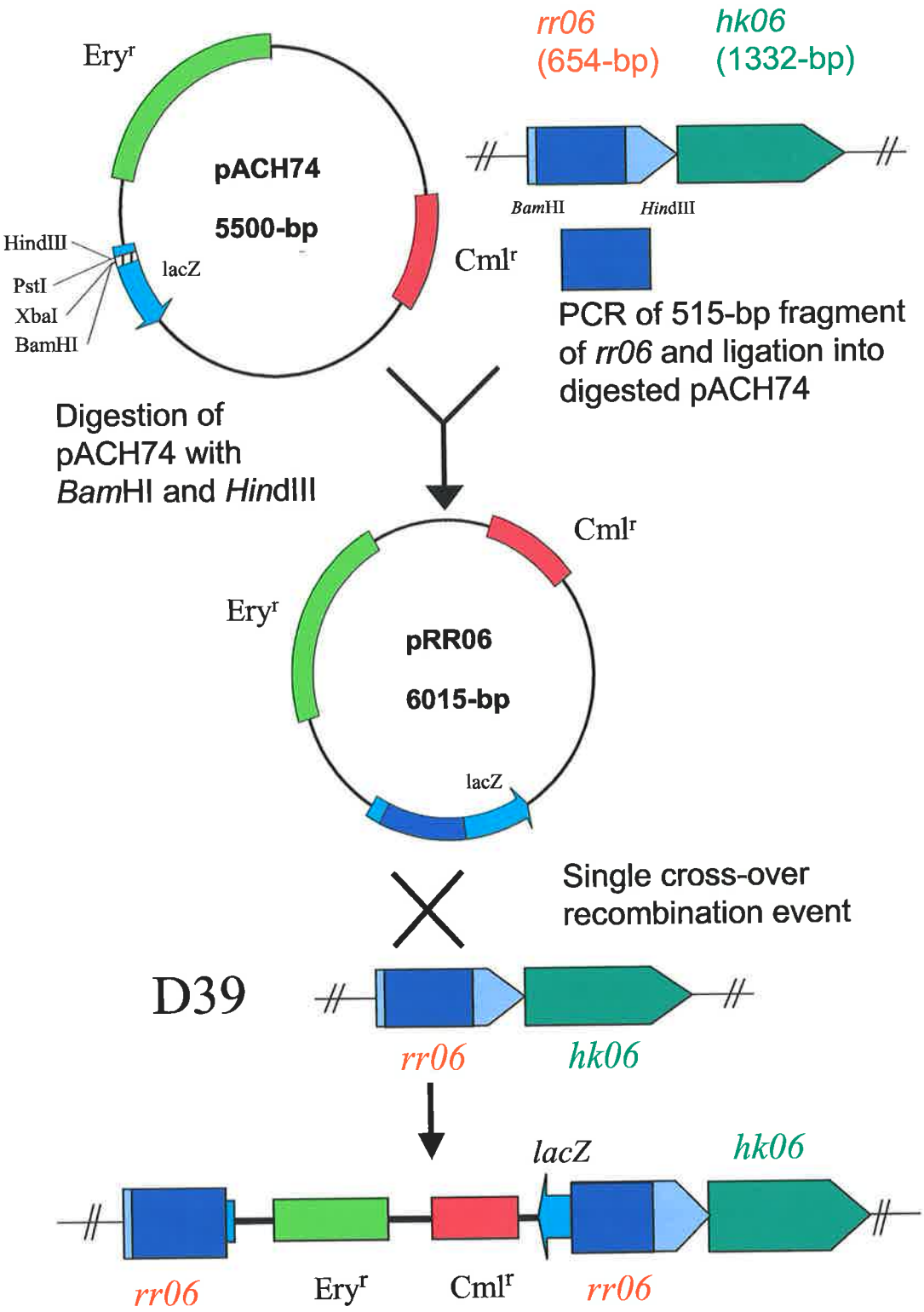

Figure 3.2. Insertion duplication mutagenesis of the *rr06* gene.

A 515-bp fragment of the *rr06* gene was obtained by PCR amplification and cloned into the *Bam*HI and *Hind*III sites of vector pACH74. When this plasmid is transformed into *S. pneumoniae*, a single cross over event occurs between the *rr06* fragment on the vector and the homologous region in the *rr06* gene. This results in integration of the vector into the chromosome and thus interruption of *rr06*.

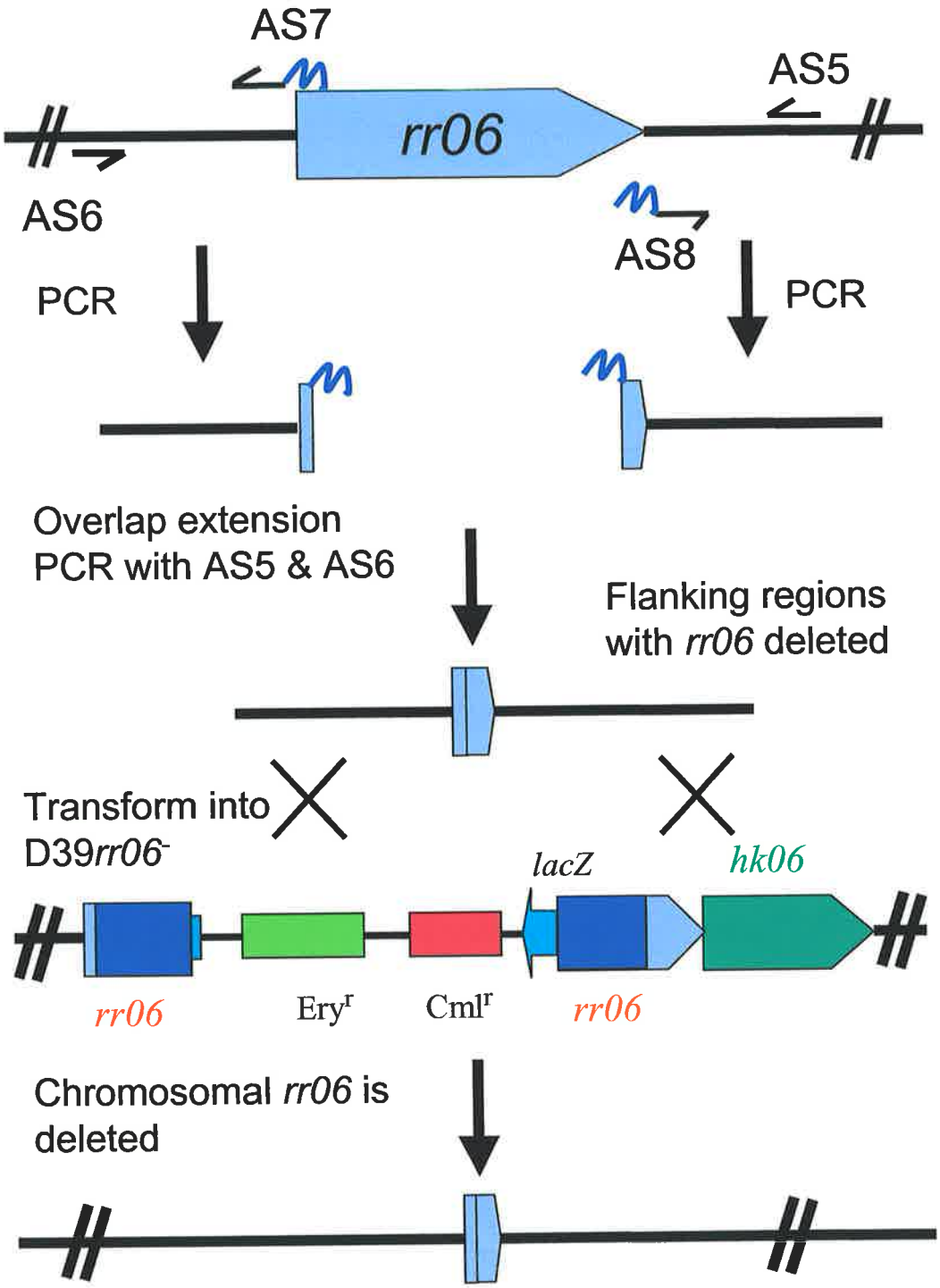

Figure 3.3. Deletion of *rr06* from the *S. pneumoniae* chromosome.

D39*rr06*- *S. pneumoniae* were transformed with a PCR product created by overlap extension PCR containing the flanking regions of *rr06* but without *rr06* itself. Homologous recombination of the flanking regions with those on the chromosome result in deletion of *rr06*.

hk06 gene were similarly deleted using AS5/AS14 and AS6/AS13 primers in round one, followed by a second round of amplification using primer pair AS5/AS6. These constructs were then used to transform the insertion-duplication mutant D39*rr06*$^-$ and homologous recombination (allelic replacement) between the PCR product and the flanking regions of *rr/hk06* resulted in the appropriate deletion. Transformants were selected for loss of Ery resistance by replica plating on BA and EryBA plates. Putative mutants were checked by PCR using flanking oligonucleotides (**Figure 3.4**) and sequence analysis (data not shown). These mutants were designated D39*Δrr06* and D39*Δhk06* respectively. Sensitivity of the strains to optichin was used in order to confirm the strains were *S. pneumoniae*, while Quellung reactions (Section 2.4) were performed to confirm that the mutants expressed a type 2 capsule.

3.2.2 Comparative growth rates of mutants

Since defects in the growth rate of the mutants would have a marked effect on their virulence, it was important to determine whether the mutations in *hk06* and *rr06* affected growth compared to the wt D39. Accordingly, growth rates in THY were followed over an eight hour period by measuring A_{600} (**Figure 3.5**). No significant difference was seen between the three strains.

3.2.3 Effect of *hk06* and *rr06* deletion on pneumococcal virulence protein expression

We have previously demonstrated that several well-characterised virulence genes of *S. pneumoniae*, including *ply*, *pspA*, *psaA* and *cbpA* are differentially expressed under varying environmental conditions (Ogunniyi *et al.*, 2002; LeMessurier *et al.*, 2006). Of these, *cbpA* (Section 1.3.3.1) was of particular interest, as it is located only 750 nt downstream of *rr/hk06*, and a number of prokaryotic TCSTS regulate adjacent genes on

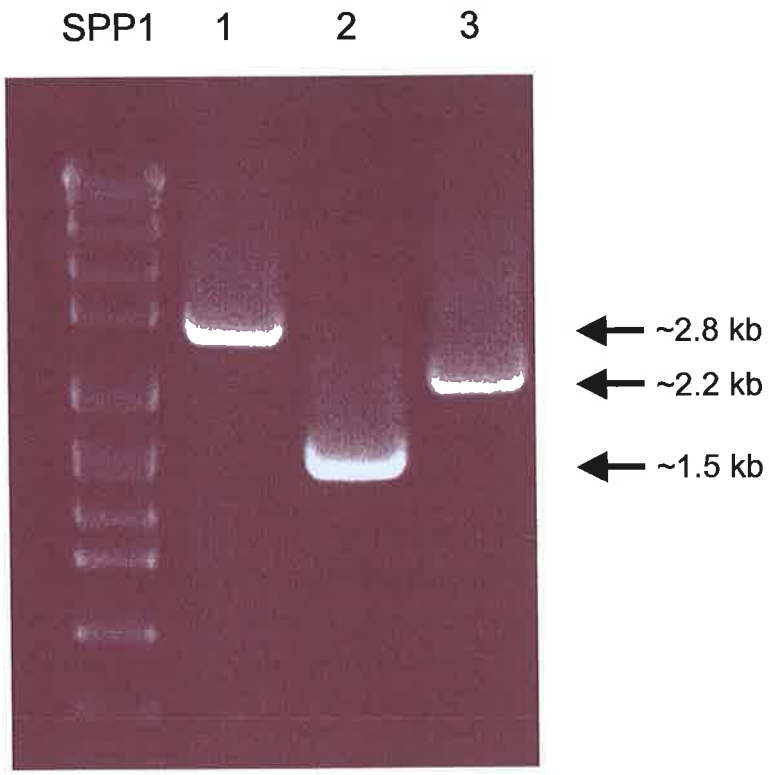

Figure 3.4. PCR analysis of D39Δ*hk06* and D39Δ*rr06*.

DNA from (1) D39, (2) D39Δ*hk06* and (3) D39Δ*rr06* were subjected to PCR analysis with oligonucleotides CbpAprom1 and AS 1 (Table 2.2). Sizes of products were estimated according to their mobilities relative to the SPP1 marker. WT D39 resulted in a fragment of approximately 2.8 kb. Both D39Δ*hk06* (1.5 kb),*and* D39Δ*rr06* (2.2 kb) resulted in bands of sizes consistent with deletion of the respective gene.

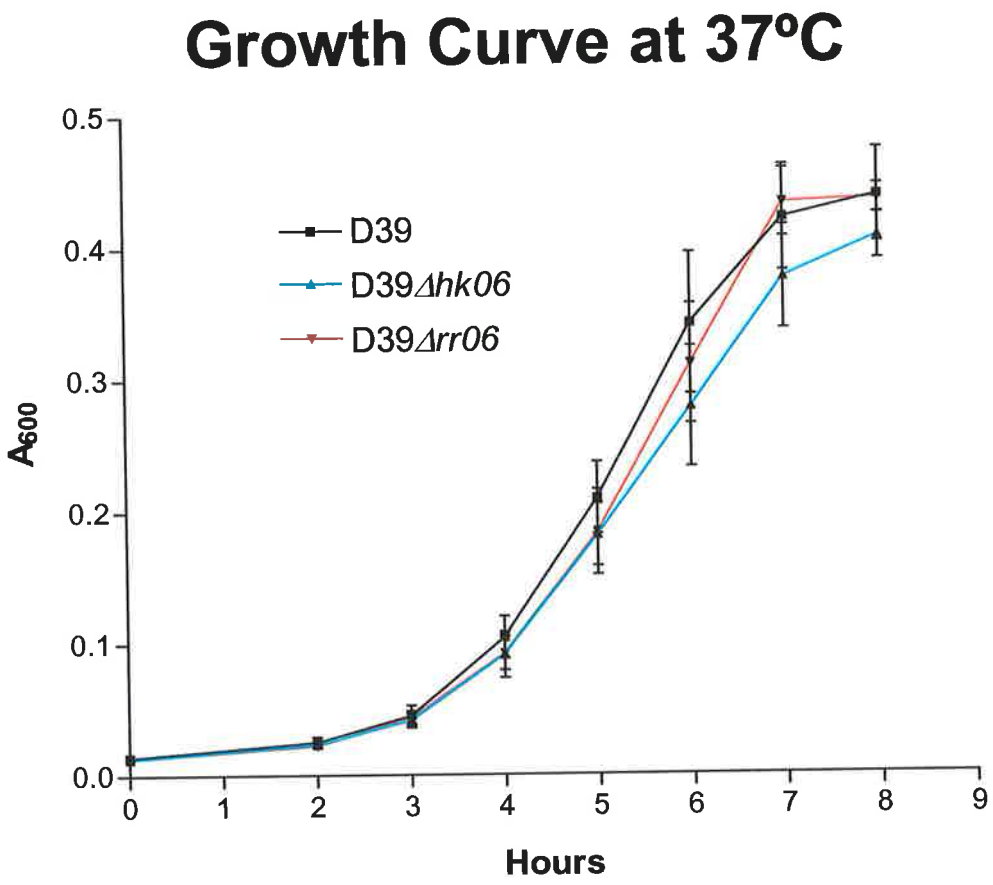

Figure 3.5. Growth curve for mutant D39 strains.

D39, D39Δ*hk06*, and D39Δ*rr06* were grown overnight on BA and inoculated into 10 ml THY broth. Culture density (A_{600}) was measured over the following 8 h. Mean ± standard error of 5 experiments are shown.

the chromosome including the *blpH/R* system from the pneumococcus (de Saizieu *et al.*, 2000).

Western immunoblot analysis with polyclonal murine antisera was used to compare the levels of CbpA, as well as those of Ply, PspA and PsaA in lysates of D39, D39*Δhk06* and D39*Δrr06*. Since a number of proteins are reported to be differentially expressed in opaque and transparent phase pneumococci (Rosenow *et al.*, 1997), all strains were confirmed to be in the transparent phase (Section 2.2) prior to Western immunoblot analysis. Strains were grown to identical culture densities (A_{600} = 0.35 in THY); equal numbers of cells were harvested, resuspended in lysis buffer, and then subjected to SDS-PAGE and Western blotting as described in Section 2.8.3. No differences were observed in the levels of PspA, PsaA and Ply in D39, D39*Δhk06* and D39*Δrr06* lysates (**Figures 3.6A, 3.6B and 3.6C**). However, as shown in **Figure 3.7A**, CbpA expression for strains grown in both THY and also in SB (grown to A_{600} = 0.09) appeared to be up-regulated in D39*Δhk06* and down-regulated in D39*Δrr06*, compared to the parent strain. The culture supernatants from each strain grown in THY were also tested for the presence of CbpA, and showed a similar result (**Figure 3.7B**), indicating that the differences seen in CbpA expression are not due to effects on anchorage of CbpA to the cell surface. The same changes in CbpA expression were also seen when the strains were in the opaque phase (data not shown).

3.2.4 Effect of D39Δ*hk06* and D39Δ*rr06* mutations on transcription of *cbpA*

In order to determine whether *cbpA* is transcriptionally coupled with *rr/hk06*, reverse-transcription PCR (RT-PCR) was performed using D39 mRNA as the template and oligonucleotides primers from within the *cbpA* and *hk06* genes (RTHis-Rev and RtcbpA-For; Table 2.2). While RT-PCR using oligonucleotides Sp7d and Sp7c (Table 2.2) produced the expected 515-bp internal fragment of *rr06* with both D39 DNA and RNA,

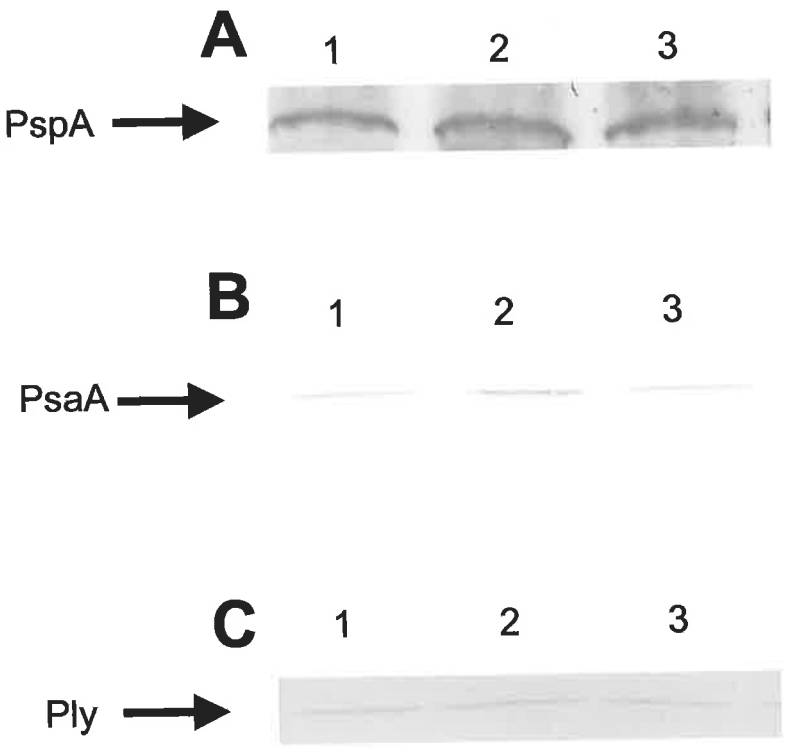

Figure 3.6. Western immunoblot analysis of virulence factors.

Proteins in lysates of (1) D39, (2) D39Δ*hk06* and (3) D39Δ*rr06* grown in THY to A_{600} of 0.35 were separated by SDS-PAGE, transferred onto nitrocellulose and probed with (A) anti-PspA, (B) anti-PsaA, and (C) anti-Ply as described in Section 2.8.3.

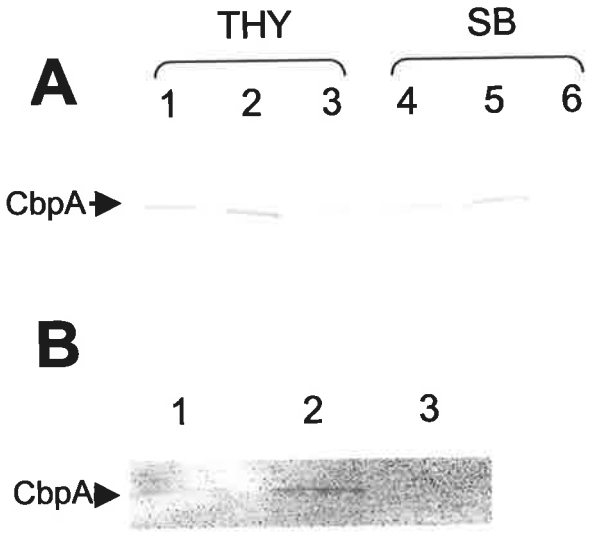

Figure 3.7. CbpA Western blot analysis.

A) Proteins in whole cell lysates of D39 (lanes 1 and 4), D39Δ*hk06* (lanes 2 and 5), and D39Δ*rr06* (lanes 3 and 6) (grown in THY or SB to an A_{600} of 0.35 and 0.09 respectively) were separated by SDS-PAGE, transferred onto nitrocellulose and probed with polyclonal murine anti-CbpA serum.

B) Proteins in the THY culture supernatants of D39 (lane 1), D39Δ*hk06* (lane 2), and D39Δ*rr06* (lane 3) were separated by SDS-PAGE, transferred onto nitrocellulose and probed with polyclonal murine anti-CbpA serum.

only DNA and not RNA resulted in the expected 829-bp product with RTHis-Rev and RtcbpA-For (**Figure 3.8**). This indicated that *cbpA* and *hk06* are transcribed separately. Indeed Throup *et al.* (2000) had previously reported that while 5 pneumococcal TCSTSs appeared to be co-transcribed with neighboring genes, *rr/hk06* and *cbpA* were not one such example.

We then used real time RT-PCR with *cbpA*-specific oligonucleotides (CbpAFNew and CbpAREdit; Table 2.2) to determine the relative levels of *cbpA* mRNA in cultures of D39, D39*Δhk06* and D39*Δrr06*. Strains were grown in THY to an A_{600} of 0.35 and RNA was isolated as described in Section 2.8.1. 16S rRNA levels (determined in parallel using primers 16sF and 16sR, Table 2.2) were used as an internal control. Real time RT-PCR was performed in quadruplicate on three independently isolated mRNA samples for each strain. In D39*Δhk06*, the *cbpA* mRNA level was up-regulated approximately 5-fold relative to that in D39 ($P < 0.01$), while in D39*Δrr06*, *cbpA* mRNA was down-regulated 3-fold compared to the wt parent ($P < 0.01$) (**Figure 3.9**). This correlates with the Western immunoblot data, and indicates that the regulation occurs at the level of transcription.

Real time RT-PCR using gene specific oligonucleotides RRF/RRR and HKF/HKR (Table 2.2) was also used to study possible autoregulatory effects by examining the levels of *rr06* and *hk06* mRNA in D39*Δhk06,* D39*Δrr06*, and in the wt D39. No significant differences in levels of *rr06* and *hk06* gene expression were observed (**Figures 3.10.A & 3.10.B**).

3.2.5 Construction of mutations in *hk06* and *rr06* in *S. pneumoniae* strain TIGR4

Research by McCluskey *et al.* (2004) has illustrated that the regulation of genes by pneumococcal TCSTSs may vary greatly across strains. Thus, in order to investigate whether regulation of CbpA by RR/HK06 occurs across strains, efforts were made to

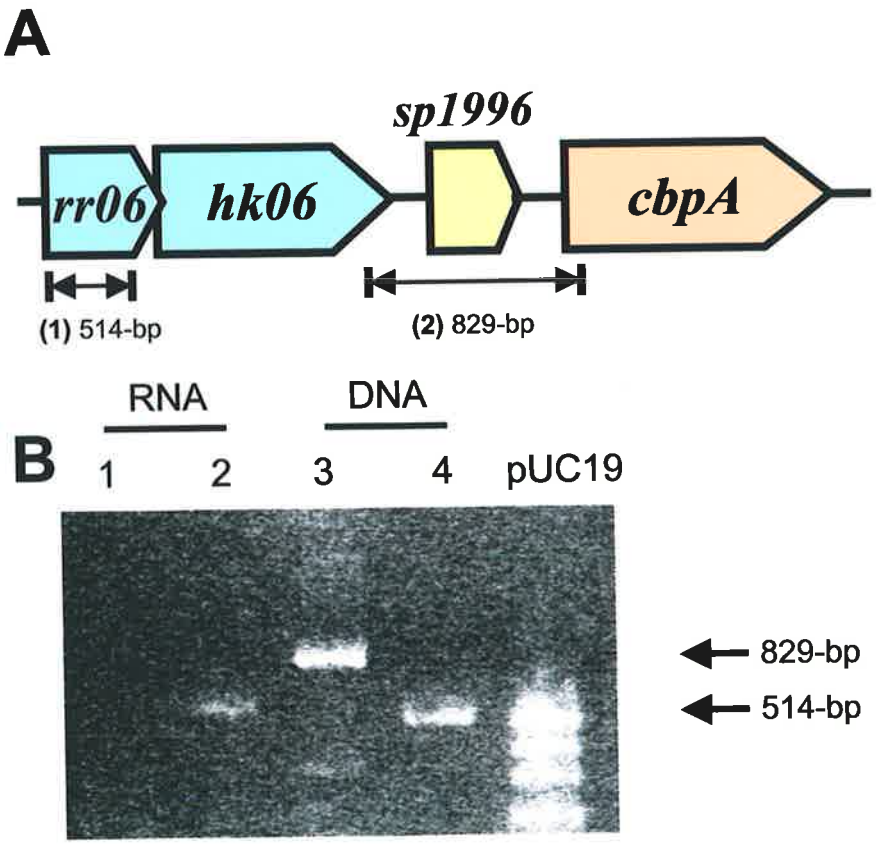

Figure 3.8. *cbpA* and *hk06* are not co-transcribed.

A) Schematic representation of products formed following PCR with oligonucleotides (1) Sp7c + Sp7d and (2) RT-cbpA-for +RT-hisrev.

B) RNA (lanes 1 and 2) and DNA (lanes 3 and 4) isolated from D39 *S. pneumoniae* as described in Sections 2.10.1 and 2.7.2 was subjected to RT-PCR with oligonucleotides RT-cbpa-for + RThis-rev (lanes 1 and 3) and Sp7c + Sp7d (lanes 2 and 4). Oligonucleotides Sp7c + Sp7d, which amplify a 514-bp region within *rr06*, produced products of expected size with both DNA and RNA. However, RT-cbpA-for + RT-hisrev, which amplifies a 829-bp region between *hk06* and *cbpA* only produced a band of expected size with DNA. This indicated that *cbpA* and *hk06* were not co-transcribed. Sizes of products were estimated according to mobilities relative to the pUC19 marker.

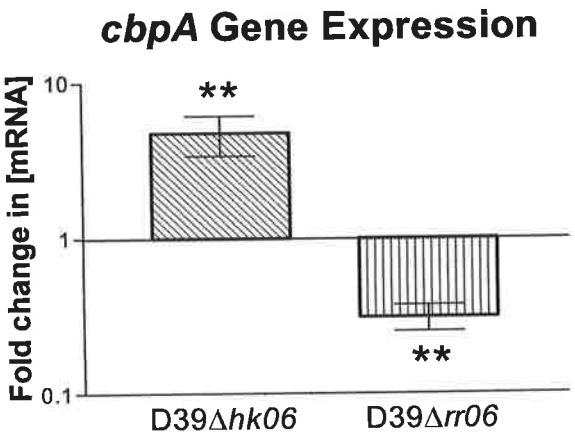

Figure 3.9. Real time RT-PCR analysis of *cbpA* mRNA.

RNA was isolated from D39, D39Δ*hk06*, D39Δ*rr06* and *cbpA* mRNA levels were compared using real time RT-PCR as described in the Section 2.10.4. Data shown are fold increase (± standard error) in [*cbpA* mRNA] relative to D39. ** Denotes significant difference relative to *cbpA* mRNA levels in D39 ($P < 0.01$), as determined by One-way ANOVA with a post-hoc Tukey test.

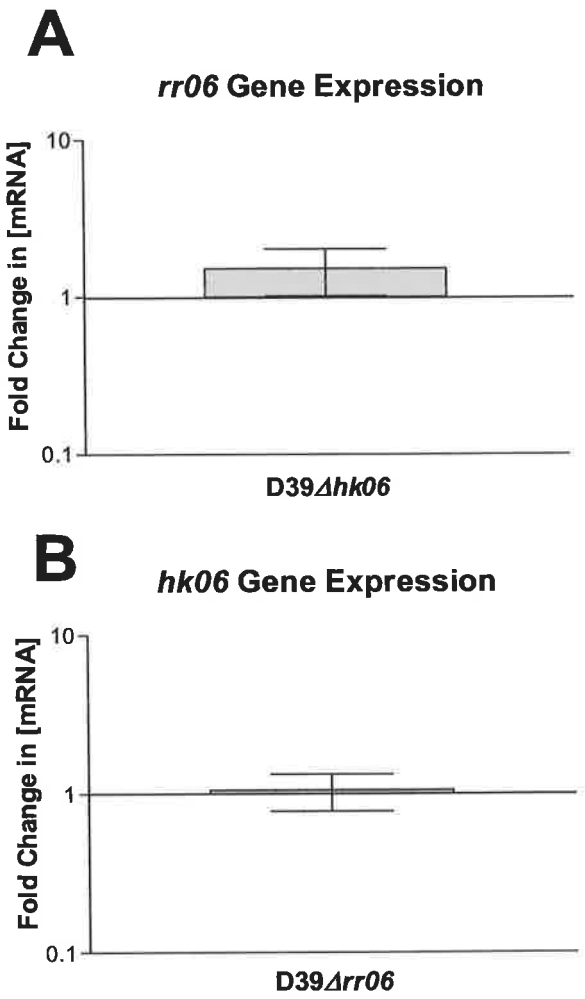

Figure 3.10. Real time RT-PCR analysis of *hk06* and *rr06* mRNA.

RNA was isolated from D39, D39Δ*hk06*, D39Δ*rr06* as described in Section 2.10.1. (A) *rr06* mRNA levels in D39 and D39Δ*hk06*, and (B) *hk06* mRNA levels in D39 and D39Δ*rr06* were compared using real time RT-PCR as described in Section 2.10.4. Data shown are fold increase (± standard error) in [mRNA] relative to D39.

construct in-frame deletion mutants in the serotype 4 strain TIGR4 (Bricker & Camilli, 1999). This involved a two-step process, with the first being construction of an Ery resistant strain which allows for subsequent selection of Ery sensitivity in order to identify unmarked in-frame deletion mutants. Initially, pRR06 (Table 2.1) was transformed into competent TIGR4, creating the insertion-duplication mutant TIGR4*rr06*$^-$. PCR using oligonucleotides m13r, which binds within the inserted suicide vector, and AS1 were used to confirm this mutation (**Figure 3.11**).

The second step was the construction of the in-frame deletion mutants. Using TIGR4 chromosomal DNA as template, overlap extension PCR was performed as described in Section 3.2.1 using the same oligonucleotide combinations. The PCR product obtained was then used to transform TIGR4*rr06*$^-$ competent cells. Unfortunately, great difficulty was encountered transforming TIGR4 *S. pneumoniae*. When overlap extension PCR products deleting either *hk06*, or *rr06* were transformed into TIGR4, replica plating on BA and EryBA did not identify any pneumococci which had lost resistance to Ery. The transformability of TIGR4*rr06*$^-$ competent cells was tested by transforming the cells with chromosomal DNA from *S. pneumoniae* DP1617 (Table 2.1), which contains genes encoding antibiotic resistance for both Ery and Strep. When DP1617 chromosomal DNA was transformed into competent TIGR4*rr06*$^-$ and TIGR4 *S. pneumoniae* (Section 2.6.2.2) no strep resistant colonies were obtained, indicating the transformation was not successful. pRR06 was previously transformed into TIGR4, suggesting that while efficiency was low, transformation of TIGR4 was possible. We thus continued attempts to transform overlap extension PCR products into TIGR4*rr06*$^-$, varying incubation times, and using both CSP-1 and CSP-2 in an attempt to improve transformation efficiency. An in-frame deletion mutant in *rr06* (TIGR4Δ*rr06*) was eventually obtained. TIGR4Δ*rr06* was confirmed by

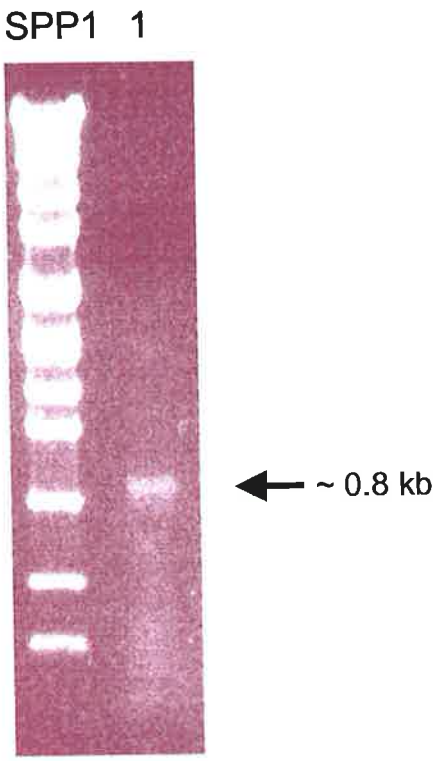

Figure 3.11. PCR analysis of TIGR4*rr06*⁻.

DNA from TIGR4*rr06*⁻ (lane 1) was subjected to PCR analysis with oligonucleotides AS1 and m13r (Table 2.2). The size of the product was estimated according to the mobility relative to the SPP1 marker. TIGR4*rr06*⁻ gave a band at approximately 800-bp which is consistent with insertion of pRR06 into the TIGR4 chromosome.

PCR (**Figure 3.12**), sequencing (data not shown), optichin sensitivity, and a Quellung reaction to confirm that it expressed a type 4 capsule.

Due to the inability to successfully construct an unmarked in-frame deletion mutant for *hk06* in TIGR4, mutants in *hk06* and *rr06* were constructed by replacement of the gene with an *erm* cartridge. Such mutants have a number of advantages over insertion-duplication. First of all there is no possibility of producing any active truncated products as the major portion of the ORF is deleted and then replaced with *erm*. Additionally, as only *erm* is being introduced and not an entire plasmid, no promoters or terminators are introduced, reducing the likelihood of polar effects.

The method used to construct these mutants is essentially the same as described previously for the in-frame deletion mutants in Section 3.2.1 and summarised in **Figure 3.13**. Overlap extension PCR was undertaken to produce DNA with the required gene deleted and replaced with an *erm* cartridge. Oligonucleotide combinations AS53/AS5 & AS54/AS6, and AS60/AS5 & AS61/AS6 were used for production of flanking DNA of *rr06,* and *hk06* respectively. The *erm* cassette was amplified from pVA891 (Table 2.1) using J214 and J215, and products were joined using overlap extension PCR with oligonucleotides AS5/AS6. PCR products were subsequently transformed into TIGR4. Mutants designated TIGR4*hk06:erm*, and TIGR4*rr06:erm* were confirmed by PCR (**Figure 3.14**) and sequencing (data not shown). Optichin sensitivity and Quellung reactions were utilised to demonstrate that these were *S. pneumoniae* with a type 4 capsule.

3.2.6 Comparative growth rates of mutants

To investigate any possible growth defects in the TIGR4 mutants, growth rates in THY were followed over a seven hour period by measuring A_{600} (**Figure 3.15**). While TIGR4*hk06:erm*, and TIGR4*rr06:erm* grew at essentially the same rate as the wt, TIGR4Δ*rr06* grew to a higher OD, perhaps suggesting a defect in autolysis. One

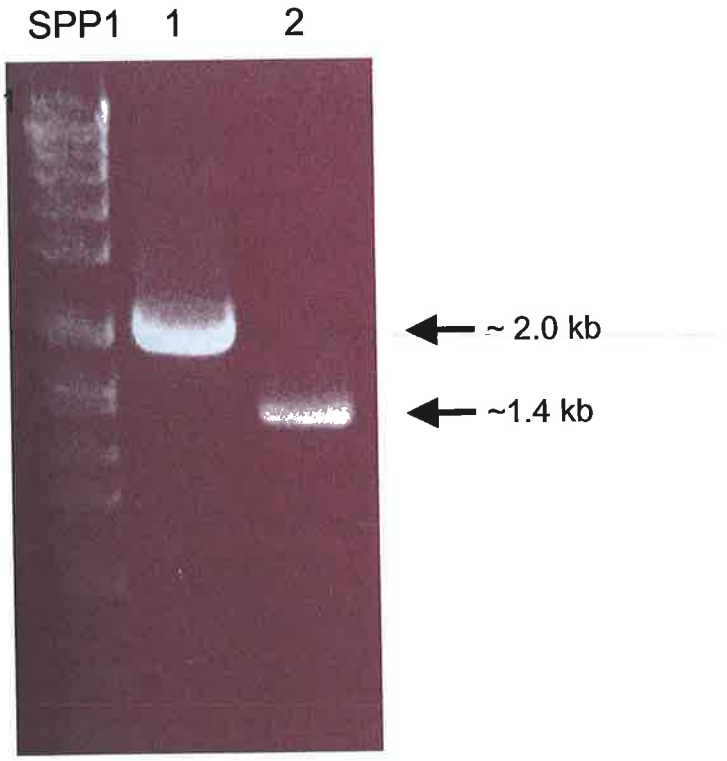

Figure 3.12. PCR analysis of TIGR4Δ*rr06*.

DNA from TIGR4 (lane 1) and TIGR4Δ*rr06* (lane 2) were subjected to PCR analysis with oligonucleotides AS1 and AS58 (Table 2.2). Sizes of products were estimated according to their mobilities relative to the SPP1 marker. TIGR4 gave a product of the expected 2.0 kb, while TIGR4Δ*rr06* gave a product at 1.4 kb, consistent with the deletion of *rr06*.

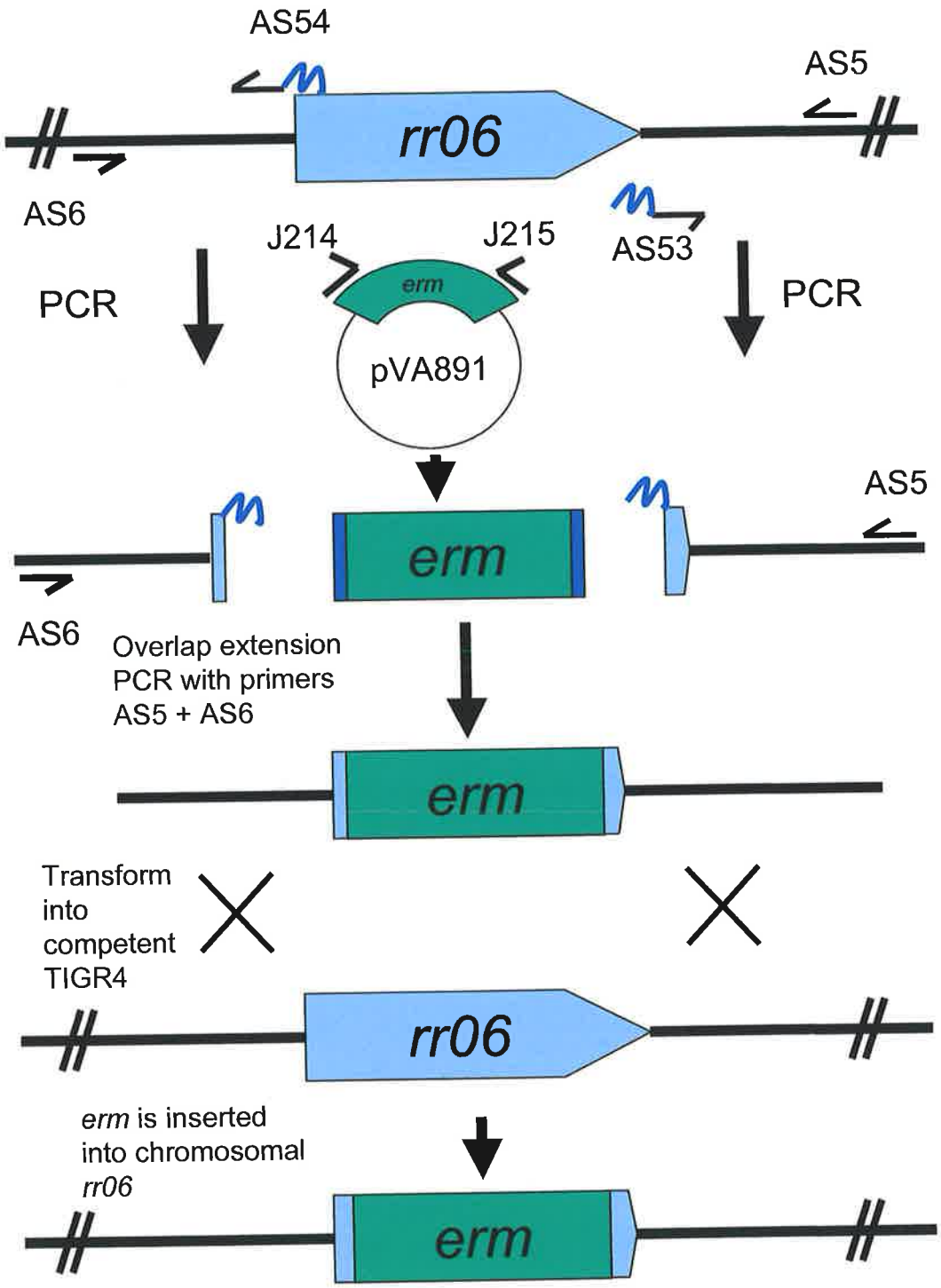

Figure 3.13. Creation of TIGR4*rr06:erm*.

A PCR product containing the *erm* gene from pVA891 flanked by regions of *rr06* is created by overlap extension PCR. Transformation of this PCR product into competent *S. pneumoniae* TIGR4 results in homologous recombination between flanking regions of *erm*, and thus insertion of *erm* into *rr06*.

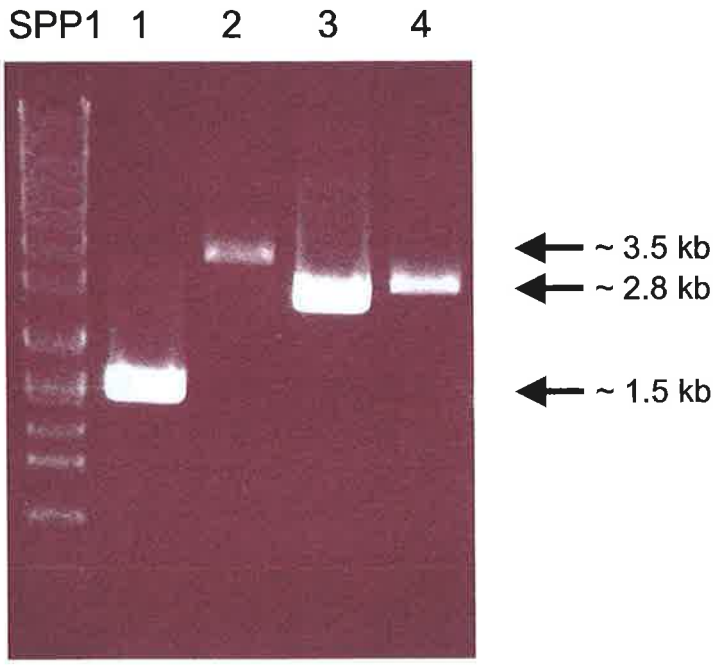

Figure 3.14. PCR analysis of TIGR4*hk06:erm*, and TIGR4*rr06:erm*.

DNA from TIGR4*hk06:erm* (lanes 1 and 2), and TIGR4*rr06:erm* (lanes 3 and 4) were subjected to PCR analysis with oligonucleotides J214 and AS23 (lanes 1, and 5) and J215 and AS12 (lanes 2, and 4) (Table 2.2). J214 and J215 bind to inserted *erm* gene while AS23 and AS12 bind to flanking regions of *rr/hk06*. TIGR4*hk06:erm* gave products of (1) 1.5-kb, and (2) 3.5-kb, TIGR4*rr06:erm* gave products of (3) 2.8-kb and (4) 2.8-kb. These products were all consistent with replacement of the respective gene(s) with *erm* gene.

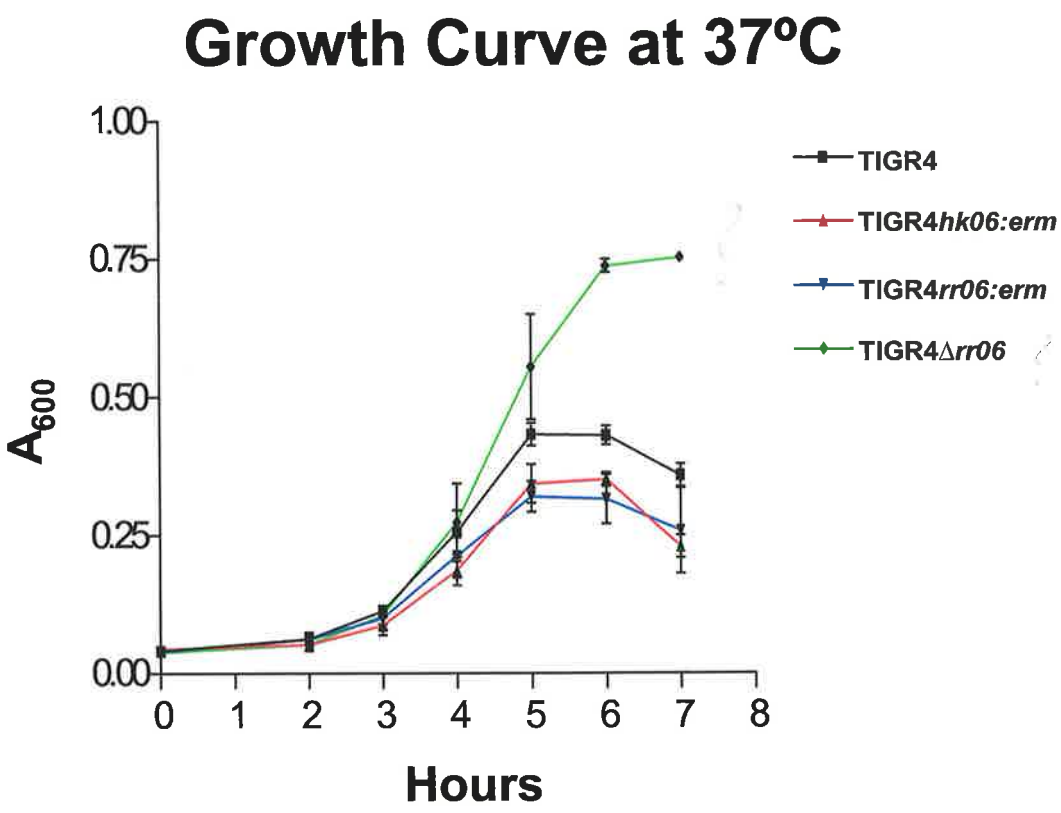

Figure 3.15. Growth curve for mutant TIGR4 strains.

TIGR4, TIGR4*hk06:erm,* TIGR4*rr06:erm*, and TIGR4Δ*rr06* were grown overnight on BA and inoculated into 10 ml THY broth. Culture density (A_{600}) was measured over the following 7 hrs. Mean ± standard error of 3 experiments are shown.

possibility is that the isolated mutant had an additional spontaneous mutation in the major autolysin gene *lytA*. Microarray analysis undertaken in Chapter Seven showed that over-expression of RR06 in TIGR4 did not result in changes in the expression of *lytA* or other genes encoding proteins associated with autolysis. Since only a single Δ*rr06* mutant was obtained, it was not possible to test an independent TIGR4Δ*rr06* mutant. Thus, care was taken when interpreting results from this strain in subsequent studies, with TIGR4*rr06:erm* considered a more reliable mutation of *rr06*.

3.2.7 RR/HK06 regulation of *cbpA* in TIGR4

The effect of mutations in RR/HK06 in TIGR4 on CbpA was investigated using both Western immunoblot to look at the level of translation and real time RT-PCR to look at the level of transcription, using similar methods to those described in Sections 3.2.3 and 3.2.4. Due to the reduced growth of the strains in comparison to D39, TIGR4 *S. pneumoniae* were grown to A_{600} = 0.25 in THY, and both whole cell lysates for Western blots, and RNA for real time RT-PCR were prepared as described in Sections 2.8.2 and 2.10.1. Western blot (**Figure 3.16A**) demonstrated that TIGR4*hk06:erm* exhibited an increased level of CbpA, while TIGR4*rr06:erm*, and TIGR4Δ*rr06* all showed a similar level of CbpA to the wt.

As the gene encoding *cbpA* varies markedly between D39 and TIGR4, new oligonucleotides were designed to measure the level of *cbpA* mRNA in TIGR4 (AS62 and AS64, Table 2.2). Real time RT-PCR was performed in quadruplicate on three independently isolated mRNA samples for each strain. While there was no statistically significant differences between any of the strains (using a One way ANOVA with a post-hoc Tuckey test), TIGR4*hk06:erm* consistently showed an approximate 2-fold increase in the level of *cbpA* mRNA (**Figure 3.16B**). Thus, there was an apparent difference in the

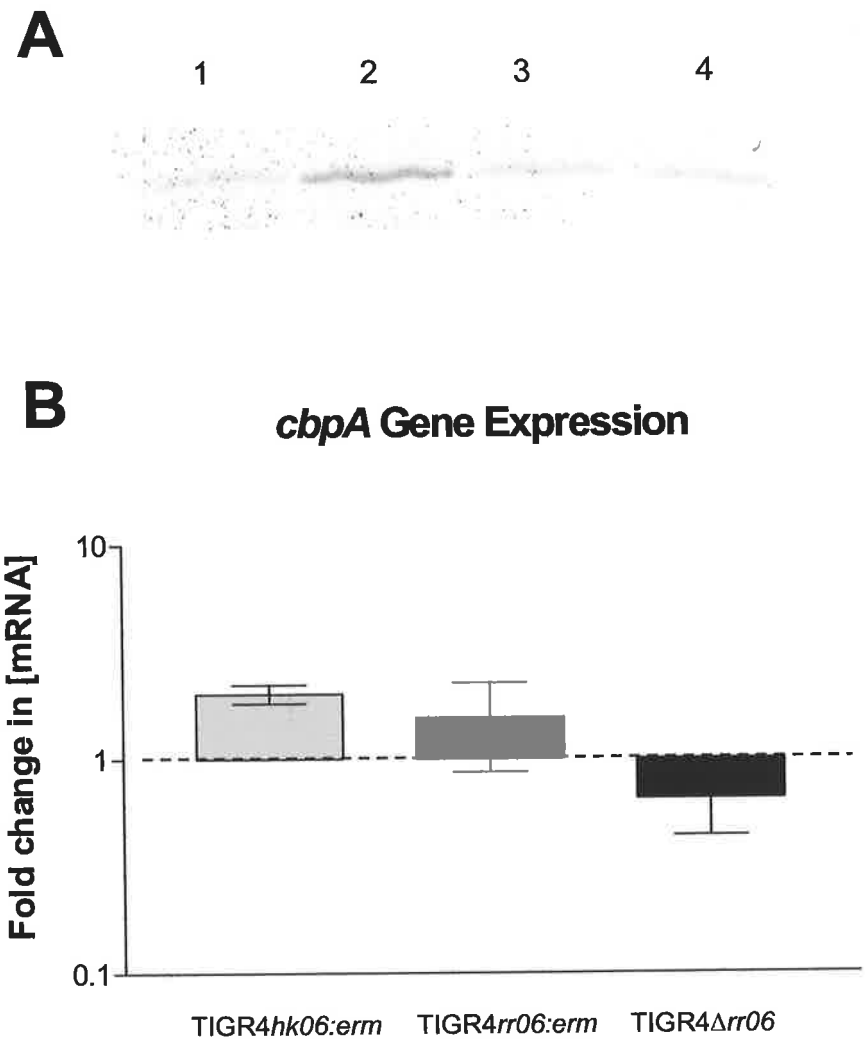

Figure 3.16. CbpA in TIGR4

A) Proteins in whole cell lysates of TIGR4 (lane 1), TIGR4*hk06:erm* (lane 2), TIGR4*rr06:erm* (lane 3) and TIGR4Δ*rr06* (lane 4) grown in THY to an A_{600} of 0.35 were separated by SDS-PAGE, transferred onto nitrocellulose and probed with polyclonal murine anti-CbpA serum.

B) RNA was isolated from TIGR4, TIGR4*hk06:erm*, TIGR4*rr06:erm*, and TIGR4Δ*rr06* and *cbpA* mRNA levels were compared using real time RT-PCR. Data shown are fold increase (± standard error) in [*cbpA* mRNA] relative to TIGR4.

effect of RR/HK06 on *cbpA* expression between D39 and TIGR4, with mutants in *rr06* in TIGR4 showing comparable levels to the wt rather than the reduced level seen in D39.

Further analysis using real time RT-PCR to investigate any possible auto-regulation by the system showed similar results to those seen in D39, with very little difference in the expression of *hk06* in TIGR4*rr06:erm* and TIGR4Δ*rr06* compared to the wt, and in the expression of *rr06* in TIGR4*hk06:erm* compared to the wt (**Figure 3.17A** and **3.17B**).

3.3 Discussion

Coordinated regulation of virulence genes is likely to be fundamental for the capacity of *S. pneumoniae* to asymptomatically colonize the nasopharynx, and to cause invasive disease in humans. In this Chapter, the role of the TCSTS RR/HK06 in the regulation of the important pneumococcal virulence factor CbpA was investigated. Using Western immunoblot analysis, differential expression of *cbpA* was demonstrated in the TCSTS mutants D39Δ*hk06* and D39Δ*rr06*, relative to the wt parent. RT-PCR was used to show that *cbpA* and *rr/hk06* are separate transcriptional units. Moreover, real time RT-PCR analysis indicated that the regulation of *cbpA* occurred at the level of transcription, and not through a post-transcriptional effect. This suggested that in D39, RR/HK06 exerts regulatory control over *cbpA*.

Confirmation of the above findings in the unrelated TIGR4 strain was complicated by the inability to construct un-marked in-frame deletion mutants of *hk06* and both genes together in this background. However, an insertion mutant TIGR4*hk06:erm* also exhibited upregulation of *cbpA* at both the protein and mRNA level (although this did not reach significance) compared to the wt. However, differences in *cbpA* expression were not observed for TIGR4*rr06:erm*, nor for the in-frame deletion mutant TIGR4Δ*rr06*. A difference in findings between the same two *S. pneumoniae* strains (D39 and TIGR4) has

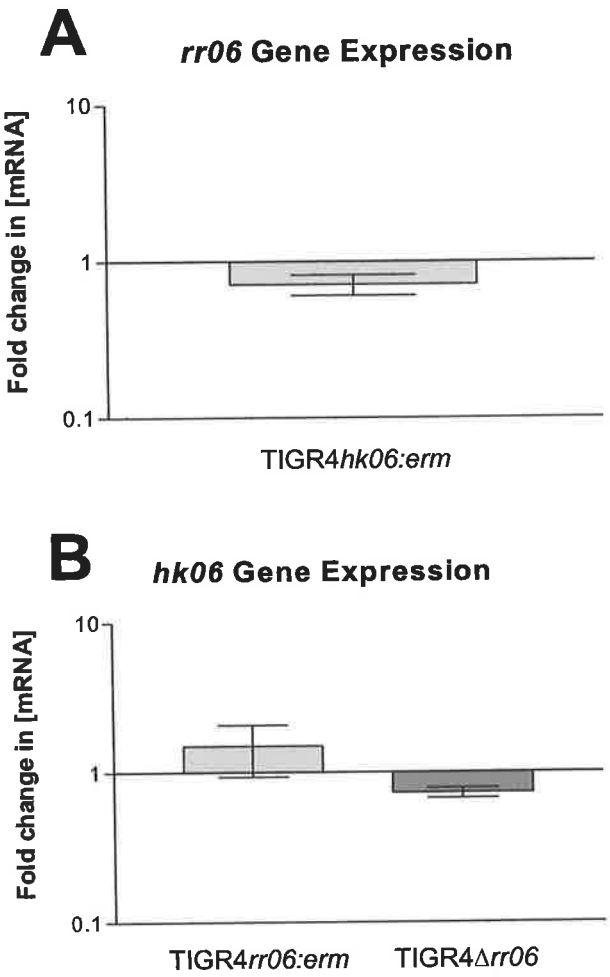

Figure 3.17. Real time RT-PCR analysis of *hk06* and *rr06* mRNA.

RNA was isolated from TIGR4, TIGR4*hk06:erm*, TIGR4*rr06:erm* and TIGR4Δ*rr06* as described in Section 2.10.1. **(A)** *rr06* mRNA levels in TIGR4 and TIGR4*hk06:erm*, and **(B)** *hk06* mRNA levels in TIGR4, TIGR4*rr06:erm* and TIGR4Δ*rr06* were compared using real time RT-PCR as described in Section 2.10.4. Data shown are fold increase (± standard error) in [mRNA] relative to TIGR4.

been reported with respect to the effects of mutations in a different TCSTS (TCS04) on expression of the Mn transporter PsaA (McCluskey *et al.*, 2004). Although *hk06* and *rr06* are highly conserved, as is the promoter region upstream of *cbpA*, TIGR4 and D39 vary substantially in genome sequence (Bruckner *et al.*, 2004), and hence may differ in some other regulatory element affecting *cbpA* expression.

There are a number of possible explanations for the up-regulation of *cbpA* seen for the *hk06* mutants. Firstly RR06 could be activated independently of HK06, perhaps through small molecular weight phosphodonors such as acetyl phosphate, or other non-cognate HKs. Systems such as CheY, PhoB/PhoR, and NtrB/NtrC from *E. coli* are examples of systems able to be activated by small molecular weight phosphodonors (Amemura *et al.*, 1990; Feng *et al.*, 1992; Lukat *et al.*, 1992). The higher level of CbpA is likely to occur due to HK06 possessing phosphatase as well as kinase activity, a trait commonly found amongst HK proteins. Thus, in non-inducing conditions, a higher level of the regulated gene would be produced in the absence of HK phosphatase activity. More RR06 would be phosphorylated through other intermediaries, leading to an increased level of *cbpA* transcription.

This increase might also be explained by RR06 binding and increasing *cbpA* expression when it is in the non-phosphorylated state. If HK06 is acting as a kinase, its deletion will lead to a higher level of non-phosphorylated RR06, increasing *cbpA* expression. Alternatively, RR06 may be required for repression of *cbpA*, although this does not account for the decrease in *cbpA* mRNA seen in D39Δ*rr06*. It is worth noting that Robertson *et al.* (2002) saw a similar increase with mutants in another pneumococcal TCSTS, VncRS. Mutation of VncS (HK) led to an increase in expression of *vex123*, while deletion of the cognate RR, VncR, resulted in levels similar to the wt.

In this Chapter, we have illustrated that a number of mutations in the pneumococcal TCSTS RR/HK06 result in differential expression of the major virulence factor CbpA. However, many questions remain unanswered. While this would appear to suggest that the system is involved in the regulation of *cbpA*, is this direct or indirect regulation? Does RR06 bind to the *cbpA* promoter region and act as a transcription factor? Alternatively, does RR/HK06 regulate another factor, which in turn affects *cbpA* expression? These questions are addressed in Chapter Four.

CHAPTER FOUR – BINDING OF RR06 TO THE *cbpA* PROMOTER REGION

4.1 Introduction

In Chapter Three, work was undertaken to investigate the possible regulation of the major pneumococcal virulence factor, *cbpA*, by the TCSTS RR/HK06. Mutants constructed in the system in two different pneumococcal strains resulted in differential expression of *cbpA*. Whilst the effects of the mutations on *cbpA* expression differed between the two strains, these data nevertheless suggest that RR/HK06 plays a role in the regulation of this virulence factor.

In the majority of TCSTSs, the phosphorylation of the RR by the HK, results in the regulation of gene expression through the binding of the RR to DNA. However, research completed in Chapter 3 did not allow us to determine whether the regulation of *cbpA* by RR/HK06 was a direct result of binding of RR06 to the *cbpA* promoter region, rather than an indirect effect of RR/HK06 on another intermediate regulatory factor.

Previous research (Standish, 2002; Standish *et al.*, 2005) (**Appendix Figure 3**) provided evidence for the binding of RR06 to the *cbpA* promoter region using an

electrophoretic mobility shift (EMS) assay with *E. coli* lysates over-expressing RR06. This Chapter describes further attempts to characterize this binding by attempting EMS assays with purified His_6-RR06, and solid phase binding (SPB) assays in order to illustrate the specificity of the interaction. Furthermore, DNase I Footprinting was also attempted to delineate the actual binding site of RR06, such that genome searches might be used identify additional regulated genes.

4.2 Results

4.2.1 Purification of RR06 and RR13

The first step in this process involved the expression of RR06 (and the unrelated RR13 as a control) as His_6-fusion proteins. The RR06 and RR13 ORFs were amplified with oligonucleotides AS1/AS2 and AS3/AS4 (Table 2.2) incorporating restriction endonuclease sites *Bam*HI/HindIII and *Sph*I/*Hin*dIII respectively. Following digestion, these 653-bp (*rr06*) and 737-bp (*rr13*) products were ligated into the *Bam*H1/*Hin*dIII and *Sph*I/*Hin*dIII sites of pQE30 (Table 2.1) respectively. The constructs were then transformed into the *E. coli* over-expression strain M15 (Table 2.1). Amp- and Kan-resistant transformants were selected and the presence of the construct was confirmed by sequencing (data not shown).

Over-expression of proteins was then performed as described in Section 2.9.1. His_6-RR06 and His_6-RR13 were then purified using Ni-NTA affinity chromatography (Section 2.9.2). Fractions of Ni-NTA purified eluent were analysed by SDS-PAGE and stained with Coomassie brilliant blue to assess the purity of His_6-RR06 and His_6-RR13. The purified proteins migrated with apparent molecular sizes of approximately 27 (His_6-RR06) and 31 kDa (His_6-RR13), consistent with their expected sizes of 25 and 28 kDa respectively, together with the addition of the N-terminal His_6 tag (**Figure 4.1**). Proteins were judged to be > 95% pure. Aliquots were stored with 50% glycerol at -80°C.

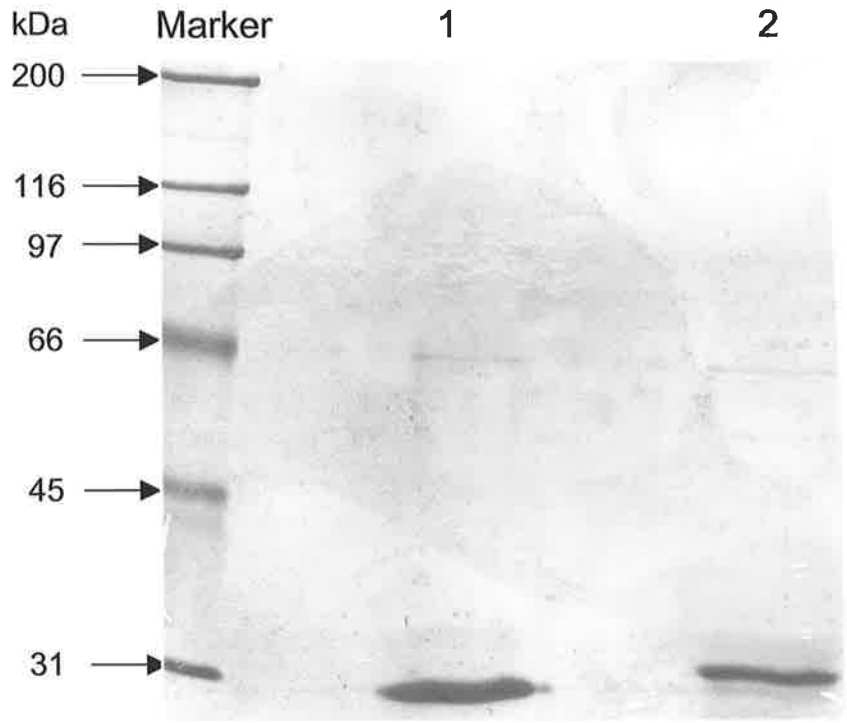

Figure 4.1. SDS–PAGE analysis of His_6-RR13 and His_6-RR06.

Ni-NTA-purified His_6-RR13 (lane 1) and His_6-RR06 (lane 2) were subjected to SDS-PAGE analysis and stained with Coomassie brilliant blue R-250 as described in Section 2.8.1.

4.2.2 Electrophoretic mobility shift assays

In order to investigate the binding of His_6-RR06 to the *cbpA* promoter region, oligonucleotides AS23 and cbpAprom2 (Table 2.2) were used to amplify a 377-bp DNA region corresponding to nt –299 to 78 relative to the *cbpA* initiation codon (nt 1989574-1989939 of the *S. pneumoniae* R6 genome [accession number AE007317]). This is the same DNA used in previous EMS assays with *E. coli* lysates over-expressing RR06 (Standish, 2002). The purified PCR product was then labeled with Dig-ddUTP as described in Section 2.11. Various amounts ranging from 1.5 μg to 7.5 μg of purified RR06 were then incubated with the labeled *cbpA* promoter fragment. Reaction mixtures were electrophoresed, transferred to a nylon filter and developed as described in Section 2.8.3. However, incubation of the DNA with various amounts of His_6-RR06 did not lead to a shift in the mobility of the *cbpA* promoter DNA (**Figure 4.2**). The maximum amount of His_6-RR06 used was approximately 7.5 μg. In contrast, 10 μg total protein from *E. coli* cell lysates over-expressing RR06 (DH5α:RR06; Table 2.1) enabled visualisation of a shift in the mobility of the DNA (**Figure 4.2**), as had been seen previously (Standish, 2002; Standish *et al.*, 2005) (**Appendix Figure 3**). Considering the over-expressed RR06 accounted for only a small fraction of the total cell lysate protein, 7.5 μg of His_6-RR06 should have been well in excess of that needed to obtain a shift in the mobility of the DNA.

It is possible that the stability of His_6-RR06 was an issue, with the activity of the protein being lost during the purification process. Thus, the protein was purified again, with care taken to ensure the protein was always kept at 4°C, and with the EMS assay performed directly following purification. However, there was still no shift in the mobility of the *cbpA* promoter DNA (data not shown). Alternatively, it was thought that the lack of binding may have been due to the requirement of RR06 to be phosphorylated prior to binding DNA. In prior experiments using *E. coli* lysates, RR06 may have been phosphorylated by either small molecular weight phosphodonors or non cognate HKs

Figure 4.2. Electrophoretic mobility shift assay

A 377-bp DNA fragment spanning the *cbpA* promoter was DIG-labeled (lane 1), and then incubated with His_6-RR06 (lanes 2-4) with 1.5 µg, 3.75 µg, 7.5 µg respectively, 7.5 µg of His_6-RR06 with 100-fold excess unlabelled competitor DNA (lane 5), 7.5 µg His6-RR13 (lane 6), 30 µg of DH5α:RR06 (lane 7), and 30 µg of DH5α:RR06 with 100-fold excess competitor DNA (lane 8). Protein-DNA complexes were separated by PAGE, transferred to nitrocellulose and labeled DNA was detected as described in Section 2.11.

present in the lysate, thereby enabling RR06 binding to the promoter region. Previous studies have shown that acetyl phosphate can phosphorylate RRs *in vitro*, thus we attempted EMS assays, following the incubation of various concentrations of acetyl phosphate up to 100 mM with the protein for 30 min. The binding assay was then undertaken as previously described. However, there was still no change in the mobility of the DNA (data not shown). It is feasible that the addition of the N-terminal His_6 may affect protein folding, resulting in a form of RR06 unable to bind DNA. The possibility also remains that during purification the activity of RR06 is lost. For these reasons, alternative methods were used to investigate the interaction between RR06 and the *cbpA* promoter DNA.

4.2.3 Immunisation of mice with His_6-RR06 and analysis of sera

In order to further investigate the binding of RR06 to the *cbpA* promoter region, solid phase binding (SPB) assays were undertaken. However, prior to this, anti-sera against RR06 was required. Serum was raised as described Section 2.17.4. Five male CD-1 mice (6 weeks) were immunized intraperitonealy (i.p.) with 10 μg of His_6-RR06 formulated with Alum, and boosted with 10 μg of the same antigen at 14 and 28 days following initial immunization. 7 days following final immunization, mice were exsanguinated by cardiac puncture and serum was collected. Serum from each mouse was subjected to Western immunoblot analysis using purified RR06 (**Figure 4.3**). Mouse 3 and 4 appeared to produce the highest titre sera against His_6-RR06, although all appeared to react with purified His_6-RR06 to some degree. Additional analysis probing whole cell lysates from D39, D39Δ*hk06* and D39Δ*rr06*, produced no band corresponding to the expected size of RR06 (data not shown). This indicated that RR06 is expressed at very low levels in *S. pneumoniae*, which was not necessarily a surprising result for a RR. When whole cell lysates from DH5α:pGEMT and DH5α:RR06 (Table 2.1) were probed with anti-RR06, the antiserum reacted strongly with a 25 kDa species (the expected size of RR06) in

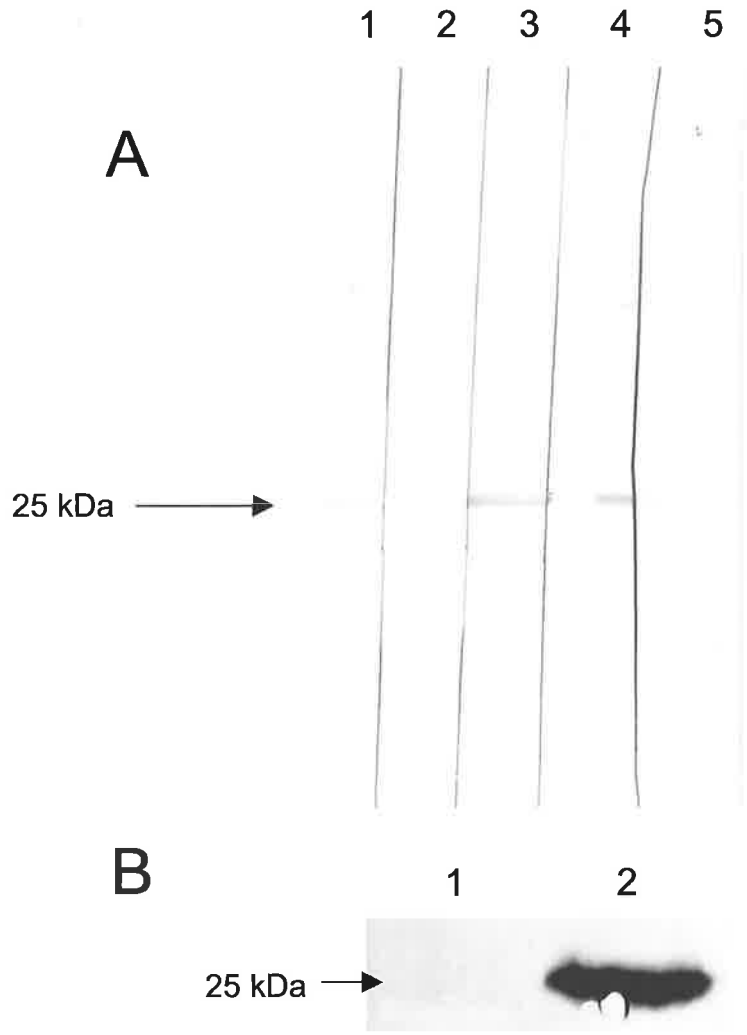

Figure 4.3. Western blot analysis of sera from mice immunised with RR06.

(A) 5 μg of His_6-RR06 was electrophoresed on an SDS-PAGE gel, transferred onto nitrocellulose and probed with sera collected from 5 individual mice (lanes 1 - 5) following three immunisations with His_6-RR06 as described in Section 2.17.4.

(B) Protein in lysates of DH5α:pGEM-T (lane 1) and DH5α:RR06 (lane 2) were seperated by SDS-PAGE, transferred onto nitrocellulose and probed with polyclonal murine anti-RR06 serum.

DH5α:RR06 crude lysate, but did not label any protein species in the crude lysate of DH5α:pGEMT (**Figure 4.3B**). This showed that the serum was specific for RR06.

4.2.4 Solid phase binding assay

To further investigate the specificity of RR06-*cbpA* promoter interaction, a SPB assay was undertaken. The *cbpA* promoter region, or an internal fragment of the 16S rRNA gene, was biotin-labeled and attached to Strepdavidin MagneSphere Paramagnetic Particles as described in Section 2.12. The beads were subsequently incubated with cell lysates of DH5α:pGEMT or DH5α:RR06 (Table 2.1). After washing in the presence of herring sperm competitor DNA, bound protein was eluted by boiling the beads in lysis buffer. These samples were then probed for presence of RR06 by western blot using anti-RR06 serum. The antiserum labeled a 25 kDa species in the eluate from the *cbpA* promoter-coated beads that had been incubated with the DH5α:RR06 lysate, but no such species was labeled in the eluate of beads incubated with DH5α:pGEMT (**Figure 4.4**). When an internal fragment of the 16S rRNA gene was coated onto the beads and incubated with either lysate as a further control, no RR06 binding was detected (**Figure 4.4**). These data confirmed the specificity of binding of RR06 to the *cbpA* promoter region.

4.2.5 DNase I Footprinting

While binding of RR06 to the *cbpA* promoter region had already been illustrated, efforts were made to delineate the actual binding site through the use of DNase I Footprinting. This was undertaken essentially as described by Yindeeyoungyeon *et al.* (2000). This method was seen to be advantageous as it did not involve the use of radioactivity, and removed the need to run sequencing gels as detection of binding was undertaken using an automated capillary DNA sequencer.

The promoter region of *cbpA* was PCR amplified using oligonucleotides AS23 and cbpAprom2 (Table 2.2), and the subsequent product was cloned into pGEM-T Easy as

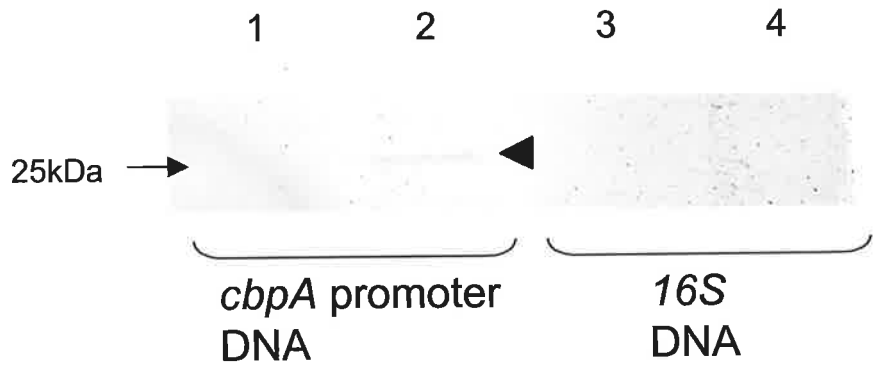

Figure 4.4. Solid phase binding assay

A 377-bp fragment spanning the *cbpA* promoter (lanes 1 and 2) or the *16S* rRNA gene (lanes 3 and 4) was biotin labelled, attached to streptavidin magnetic beads and then incubated with DH5:pGEM-T (lanes 1 and 3) or DH5:RR06 (lanes 2 and 4). Samples were subsequently separated by SDS-PAGE, transferred onto nitrocellulose and probed with polyclonal murine anti-RR06 serum. The black arrowhead indicates the 25 kDa immunoreactive species in lane 2.

described in Section 2.7.9. Correct insertion was determined by sequencing (data not shown). In order to obtain a fluorescently labelled product, a region was amplified out of pGEM-T Easy containing the *cbpA* promoter using m13f (5' FAM) and m13R (Table 2.2). The fluorescently labelled PCR product of 594-bp was subjected to digestion with various DNase I concentrations in the presence of *E. coli* whole cell lysates containing the over-expressed RR06 (DH5α:RR06) or the control lysate (DH5α:pGEM-T) as described in Section 2.13. Binding reactions were initially performed essentially as described previously for EMS and SPB assays (Sections 4.2.3 and 4.2.4). Following this, 6 different binding reactions incubated with 30 µg of either cell lysate were treated with DNase I (6.5µl) at concentrations of 3×10^{-5}, 1×10^{-5} and 6×10^{-6} U/µl for 1.5 and 3 min at 26°C. Following digestion, reactions were stopped by chilling on ice and adding 22.5 µl of 0.5 M EDTA, pH 8.0. DNA was then extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and DNA fragments were purified. Those samples incubated with the same *E. coli* cell lysate were combined using a PCR cleanup kit (Section 2.7.7). Samples were then vacuum dried and resuspended in 3 µl of formamide.

Fragments were separated on an ABI prism 310 Sequencer/genetic analyser following the addition of a size standard to allow determination of the size of the fragments. The result of incubation of DH5α:RR06 with the *cbpA* promoter DNA is illustrated in **Figure 4.5**. Unfortunately, no protection was evident after comparing this to the *cbpA* promoter DNA incubated with DH5α:pGEMT, as both produced the same digestion pattern. To overcome this problem, attempts were made with an increased amount of protein (up to 300µg). However, as the amount of protein was increased, the efficiency of DNA recovery was significantly reduced. Sequencing showed only one peak, corresponding to the full size product. Thus, it was not possible to delineate the exact bases required for the binding of RR06 to this region. Previous results from EMS assays did not produce a complete shift in the mobility of the DNA, which may suggest that the

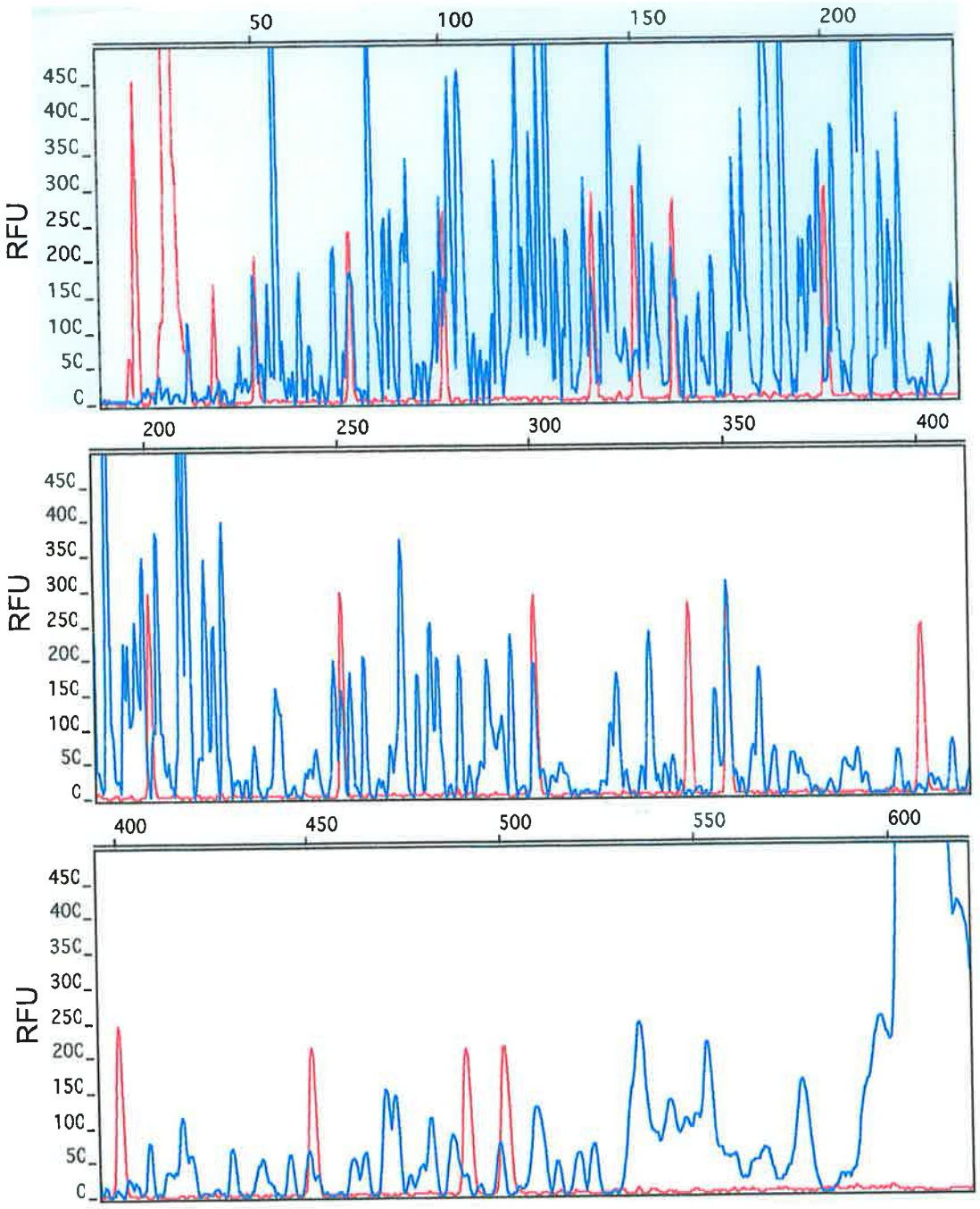

Figure 4.5. DNase I Footprinting.

A 594–bp fragment fluorescently labeled with FAM at one end was incubated with 30µg of DH5α:Control or DH5α:RR06 as described in Section 2.13. Protein:DNA complexes were then subjected to DNase I digestion, and purified DNA was analysed by an ABI prism 310 Sequencer. Data shown is only for DH5α:RR06 as both lysates provided the same digestion pattern. Approximate size of products was obtained according to the ROX marker added to the reaction (shown in red). RFU, relative fluorescence units. X-axis represents nucleotides.

conditions for the DNA:protein binding reactions were not ideal. Thus, it is likely that the DNA:protein interaction is not stable during DNase I digestion, resulting in a lack of the protection of the binding site of RR06.

4.2.6 Dimerisation of RR06

Dimerisation is important for the ability of a large number of RRs to bind to their respective consensus DNA sequences. For example, OmpR from *E. coli* binds to a total of four sites upstream of the regulated *ompF*, with each site bound only by OmpR dimers. Furthermore, oligomerisation between dimers bound at these sites is important for OmpR DNA binding (Harlocker *et al.*, 1995). Thus, effort was made to investigate the ability of RR06 to form homo-dimers.

To investigate this a LexA bacterial two-hybrid system was used essentially as described by Dmitrova *et al.* (1998). LexA is a member of the winged helix-turn-helix group of transcription factors, comprising an N-terminal DNA binding domain and a C-terminal dimerisation domain. The protein acts as a transcriptional repressor only when it is a dimer (Schnarr M & M, 1993). This property enables one to replace the C-terminal dimerisation domain with a protein of interest in order to study dimerisation. Oligonucleotides AS38 and AS39 were used to amplify RR06 ORF (653-bp) with *Sac*I and *Kpn*I restriction sites incorporated into the oligonucleotides. Following appropriate digestion, this was ligated into the *Sac*I-*Kpn*I sites of pSR658 (Table 2.1), transformed into *E. coli* DH5α, and transformants were selected on Tet and checked by sequencing (data not shown). This plasmid was designated p658:RR06, and comprised of RR06 fused to the N-terminal DNA binding domain of LexA. To investigate whether RR06 homo-dimerisation occurred, p658:RR06 was transformed into the *E. coli* reporter strain SU101. This strain contains a *lacZ* gene with a LexA operator sequence, enabling *lacZ* repression to be determined by assaying β–galactosidase activity. pSR658 was transformed into SU101 alone as a negative control, while another plasmid pDP804 (Table 2.1) was used as a

positive control. pDP804 contains a Jun zipper, which is known to form homo-dimers, fused to the N-terminal LexA DNA binding domain, and has previously been shown to bind and repress *lacZ* (Dmitrova *et al.*, 1998). In order to measure β-galactosidase activity, strains were grown overnight at 37°C with agitation in 10 ml LB. The original culture was diluted 1/20 in LB and grown for 4 h at 37°C with agitation. β-galactosidase activity was then determined as described in Section 2.14. p658 resulted in β-galactosidase activity of approximately 500 U while pDP804 led to a significant repression in β-galactosidase activity (approximately 30 U) illustrating the dimerisation of Jun zippers (**Figure 4.6**). The construct containing RR06 fused to the LexA DNA binding domain also exhibited significant repression to a level similar to that of pDP804 (**Figure 4.6**), indicating that RR06 is able to undergo dimerisation. This suggests that like OmpR, oligomerisation may be important for the ability of RR06 to bind DNA and regulate gene expression.

4.3 Discussion

In most prokaryotic TCSTSs, regulation occurs via binding of the RR to the promoter region of the regulated gene. This Chapter involved investigation of the binding of RR06 to the *cbpA* promoter region in order to determine whether the regulation of *cbpA* by RR/HK06 is indeed direct, and not a consequence of indirect regulation via another system. EMS and SPB assays, as well as DNase I footprinting, were used in order to study this interaction between RR06 and DNA.

Previously, we had shown binding of RR06 to a 377-bp DNA region corresponding to nt –299 to 78 relative to the *cbpA* initiation codon (nt 1989574-1989939 of the *S. pneumoniae* R6 genome [accession number AE007317]). In this study, we attempted to demonstrate similar binding using His_6-RR06. However, no shift was seen in the mobility of the DNA. In an attempt to phosphorylate RR06, acetyl phosphate was added but still did no shift was evident. A number of RRs, including YycF from *B. subtilis*, are not able to be

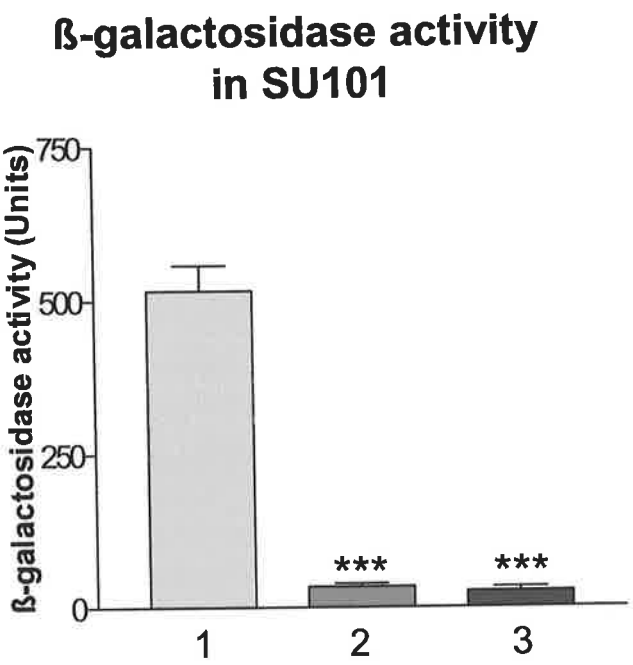

Figure 4.6. Measurement of ß-galactosidase activity

(1) p658, (2) pDP804 and (3) p658:RR06 were grown for 4h in *E. coli* strain SU101 and were subsequently analysed for ß-galactosidase activity as described in Section 2.14. *** $P < 0.001$, as determined by Student's two tailed *t* test, unpaired.

phosphorylated by acetyl phosphate (Fukuchi *et al.*, 2000). Further investigation using HPLC should enable differentiation between the phosphorylated and non-phosphorylated forms of RR06, and thus determine whether phosphorylation of RR06 by acetyl phosphate actually takes place. This would also identify whether this small compound may act as another input into the RR06 regulatory pathway.

The lack of shift seen in these EMS assays may have also been due to the loss of activity of RR06 during the purification process. Indeed, when using *E. coli* lysates over-expressing RR06 only fresh lysates were able to produce a shift in the mobility of the DNA. The purification process of His_6-RR06 takes up to 5 h from cell lysis to final purification, and it may be that during this time, the protein loses activity, resulting in an inability to bind to DNA. Another possibility is that addition of the His_6 tag to the N-terminus alters the folding of RR06, rendering the protein unable to undergo DNA:protein interactions.

To further investigate the specificity of RR06-*cbpA* promoter interaction, a SPB assay was undertaken. This method has been used previously to illustrate the DNA binding specificity of pneumococcal RR ComE (Ween *et al.*, 1999). SPB assays rely on the ability of target DNA coupled to a substrate to bind the RR, in this case RR06. The results indicate that a protein of approximately 25 kDa in the DH5α:RR06 lysate bound to immobilized *cbpA* DNA target, and that this protein reacted with anti-RR06. As a further control, this protein was not evident when the DH5α:RR06 lysate was incubated with another unrelated fragment of DNA. This indicated that the binding of RR06 to the *cbpA* promoter DNA was indeed specific, providing further evidence for the binding of RR06 to this region of DNA.

Attempts were then made to delineate the actual binding site of RR06. It was hoped that the identification of this site may have led to the identification of other candidate regulated genes through homology searches of the pneumococcal genome. In an attempt to

identify the site we undertook DNase I Footprinting, which relies on bound protein protecting the DNA from digestion by DNase I. However, no protected regions of DNA were evident. This was probably due to the strength of the DNA:protein interaction being sub-optimal. Indeed, during EMS assays with DH5α:RR06 lysate we were not always able to completely shift all the DNA (Standish *et al.*, 2005). Further experimentation using altered binding conditions may lead to a stronger interaction between RR06 and *cbpA* promoter DNA, and thus a greater chance of providing protection against DNase I digestion.

Many RRs, including the much studied *E. coli* OmpR, bind and regulate gene expression as oligomers (Harlocker *et al.*, 1995). To investigate whether RR06 is able to form dimers, a bacterial two-hybrid assay was used. This assay relies on the repression capabilities of *E. coli* LexA (Dmitrova *et al.*, 1998). RR06 repressed β-galactosidase activity to a similar extent as a control protein with a known dimerisation domain (Jun zippers) fused to the LexA DNA binding domain. Thus, this demonstrated that RR06 at least forms dimers, and that this capability may be important to its ability to bind DNA.

This Chapter shows that the *cbpA* promoter region is one possible binding site for RR06, providing further evidence for the regulation of *cbpA* by RR/HK06. The fact that RR06 is able to form dimers suggests that like other RRs which form oligomers, such as OmpR, its binding site may consist of a direct or inverted repeat. We were unable, however, to identify such a site in this 377-bp region. Additionally, BLAST searches have not identified regions with significant DNA homology elsewhere in the *S. pneumoniae* genome. This is not unexpected, since consensus sequences for RRs are often quite variable. For example, OmpR, the founder member of the family to which RR06 belongs, binds to a basic consensus sequence that varies markedly (Harlocker *et al.*, 1995; Throup *et al.*, 2000). Additionally, the pneumococcal RR, YycF has recently been shown to bind to a consensus sequence that is quite degenerate (Ng *et al.*, 2005). Thus, it is likely that to

successfully identify a RR06 binding consensus sequence we will need to identify further genes directly regulated by RR06.

CHAPTER FIVE – ROLE OF RR/HK06 IN VIRULENCE

5.1 Introduction

In Chapters Three and Four results showed that the major pneumococcal virulence factor CbpA was regulated by TCSTS RR/HK06. As described in Chapter One, CbpA is one of a number of pneumococcal proteins being investigated for possible inclusion in a protein-based vaccine. Moreover, a number of *in vivo* studies have demonstrated that CbpA plays numerous roles in pneumococcal pathogenesis; including nasopharyngeal colonisation, lung infection, and meningitis (Rosenow *et al.*, 1997; Balachandran *et al.*, 2002; Orihuela *et al.*, 2004). A recent study has suggested that CbpA may also play a role in sepsis (Iannelli *et al.*, 2004), although previous studies using the same serotype in this laboratory (Berry & Paton, 2000) along with work by Orihuela *et al.* (2004) suggested that CbpA is of little importance for the ability of the pathogen to cause systemic disease.

CbpA also contributes to the ability of *S. pneumoniae* to adhere to host cells, which may in part explain its impact on pneumococcal pathogenesis. Mutants deficient in CbpA have reduced adherence to both Detroit 562 (nasopharyngeal) and A549 (type II pneumocyte) cells (Rosenow *et al.*, 1997; Zhang *et al.*, 2000). Indeed, the interaction of *S.*

pneumoniae with Detroit 562 cells has been shown to be mediated through the ability of CbpA to interact with the pIgR (Zhang *et al.*, 2000). Additionally, recent studies in this laboratory using epithelial cells have suggested that CbpA may act to limit the release of the pro-inflammatory chemokine IL-8, with a mutant in *cbpA* producing a higher level of IL-8 release from epithelial cells than the wt (Graham, 2005).

Previous studies of the contribution of RR/HK06 to pathogenesis utilised a respiratory tract inoculation model, along with a systemic virulence model which bypassed the natural mode of infection (Lange *et al.*, 1999; Throup *et al.*, 2000). While the respiratory tract model showed that the system may play a role in lung infection, the other model suggested that the system was not important for systemic disease. Additionally, in recent times a HK06-deficient mutant has been shown to colonise and persist in the nasopharynx to a similar level as the wt in an infant rat model (Sebert *et al.*, 2002). In order to investigate the role of RR/HK06 in pathogenesis in more detail, this Chapter employed *in vitro* epithelial cell adherence assays and a murine intranasal *in vivo* model, which involves monitoring of the infection from colonisation of the nasopharynx to invasion of the lungs and blood.

5.2 Results

5.2.1 Adherence to epithelial cells

Previous studies had demonstrated that a mutant deficient in CbpA production has reduced adherence to both A549 (human type II pneumocytes) and Detroit 562 cells (human nasopharyngeal cells) (Rosenow *et al.*, 1997; Zhang *et al.*, 2000). Given the apparent role of RR/HK06 in regulation of *cbpA* expression, *in vitro* adherence assays were undertaken, essentially as described by Talbot *et al.* (1996) (Section 2.16). To ensure any differences seen where not due to opacity differences, all strains were confirmed to be in the transparent phase prior to the assays. Approximately 2×10^6 pneumococci were

incubated with confluent cell monolayers in 24-well plates, and the number of adherent bacteria were determined. Assays were performed in quadruplicate on at least two separate days. The mean adherence for each strain was expressed as a percentage of the respective wt strain on that day, after correction for minor differences in initial inoculum and growth rates, as described in Section 2.16. Adherence data were analysed statistically using an unpaired two-tailed Student's *t* test.

5.2.1.1 Adherence of D39, D39Δ*hk06*, D39Δ*rr06*, and D39*cbpA*⁻ to A549 and Detroit 562 cells.

The *in vitro* adherence of D39, D39*Δhk06*, and D39*Δrr06* to human lung (A549) and nasopharyngeal (Detroit 562) cell lines was investigated using the assay described above. For comparative purposes, a D39 *cbpA*⁻ insertion-duplication mutant previously constructed in this laboratory was also tested (Berry & Paton, 2000).

Total adherence of wt D39 to the A549 cells was approximately 4.4×10^4 CFU per well, but that of either D39*Δhk06* or D39*Δrr06* was approximately 75% lower ($P < 0.001$) (**Figure 5.1A**). The D39*cbpA*⁻ mutant exhibited a further significant decrease in adherence compared to the TCSTS mutants (D39*Δhk06* vs CbpA⁻, $P < 0.001$; D39*Δrr06* vs D39*cbpA*⁻, $P < 0.05$).

Adherence of D39 to Detroit 562 cells was approximately 3.1×10^4 CFU per well, showing no statistically significant difference compared to the adherence of D39 to A549 cells. Again, both the D39*Δhk06* and D39*Δrr06* mutants showed a statistically significant reduction in adherence ($P < 0.001$). D39*Δrr06* also exhibited significantly lower adherence than D39*Δhk06* ($P < 0.05$). The adherence of the D39*cbpA*⁻ mutant to Detroit 562 cells was approximately 75% lower than that of D39 ($P < 0.001$), but was not significantly different from that of either D39*Δhk06* or D39*Δrr06* (**Figure 5.1B**).

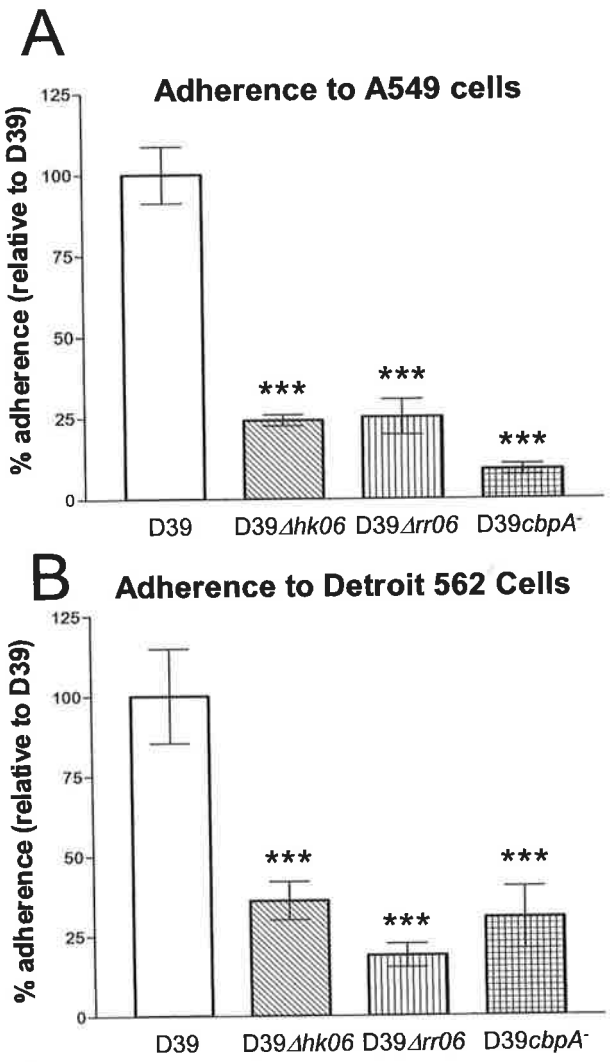

Figure 5.1. Adherence to (A) A549 (human lung) and (B) Detroit 562 (human nasopharyngeal) cells.

D39, D39Δ*hk06*, D39Δ*rr06*, and D39*cbpA*$^-$ were incubated with confluent cell monolayers for 2 h, after which adherent bacteria were enumerated. Data are presented as percentage adherence relative to that of wild-type D39 (mean ± standard error). Mean adherence of D39 to A549 and Detroit 562 cells (100%) was 4.4×10^4 CFU/well and 3.1×10^4 CFU/well, respectively. *** Denotes significantly different from the wild-type ($P < 0.001$) as determined by unpaired Student's *t*-test, two-tailed.

As the decreased level of adherence in D39Δ*hk06* did not appear to correlate with the level of CbpA as seen in THY and SB, we investigated the level of CbpA during the adherence assay. Following incubation for 2h with A549 cells, all pneumococci (both adherent and non-adherent) were removed, pelleted and re-suspended in lysis buffer. Additionally, pneumococci grown in tissue culture media alone were also investigated. Equal numbers of cells were then subjected to SDS-PAGE and Western immunoblotting. We saw similar changes in CbpA expression between D39 and its in-frame *hk06* and *rr06* deletion mutants, suggesting CbpA expression is not affected by either tissue culture medium or contact with epithelial cells (**Figure 5.2**).

5.2.1.2 Adherence of TIGR4, TIGR4*hk06:erm*, TIGR4*rr06:erm*, TIGR4Δ*rr06* and TIGR4*cbpA:erm* to A549 cells

As described above, mutations in RR/HK06 in *S. pneumoniae* D39 resulted in deficiencies in epithelial cell adherence. We wanted to investigate whether RR/HK06 played a role in epithelial cell adherence across strains. Thus, the *in vitro* adherence of *S. pneumoniae* TIGR4, TIGR4*hk06:erm*, TIGR4*rr06:erm*, and TIGR4Δ*rr06* to A549 cells was investigated. For comparative purposes, a TIGR4 mutant deficient in CbpA was constructed using the same methods as described in Section 3.2.5, replacing the majority of the *cbpA* gene with an Ery cassette. Oligonucleotide combinations cbpAerystart/RMAG7 and CbpAeryR/AS1 (Table 2.2) were used to produce *cbpA* flanking regions. The *erm* cassette was amplified using J214 and J215 from pVA891, and all products were joined using overlap extension PCR with oligonucleotides RMAG7/AS1. The resultant PCR product was transformed into TIGR4, with possible mutants identified by acquisition of resistance to Ery. Mutant TIGR4*cbpA:erm* was confirmed by PCR (**Figure 5.3A**), sequencing (data not shown) and Western immunoblot (**Figure 5.3B**).

Figure 5.2. CbpA Western blot analysis.

Proteins from lysates of D39 (lanes 1 & 2), D39Δ*hk06* (lanes 3 & 4) and D39Δ*rr06* (lanes 5 & 6) grown in tissue culture media with (lanes 1, 3 & 5) and without cells (lanes 2, 4 & 6) were separated by SDS-PAGE, transferred onto nitrocellulose and probed with mouse polyclonal anti-CbpA as described in Section 2.8.3. Lane 7 was a control with A549 cells only.

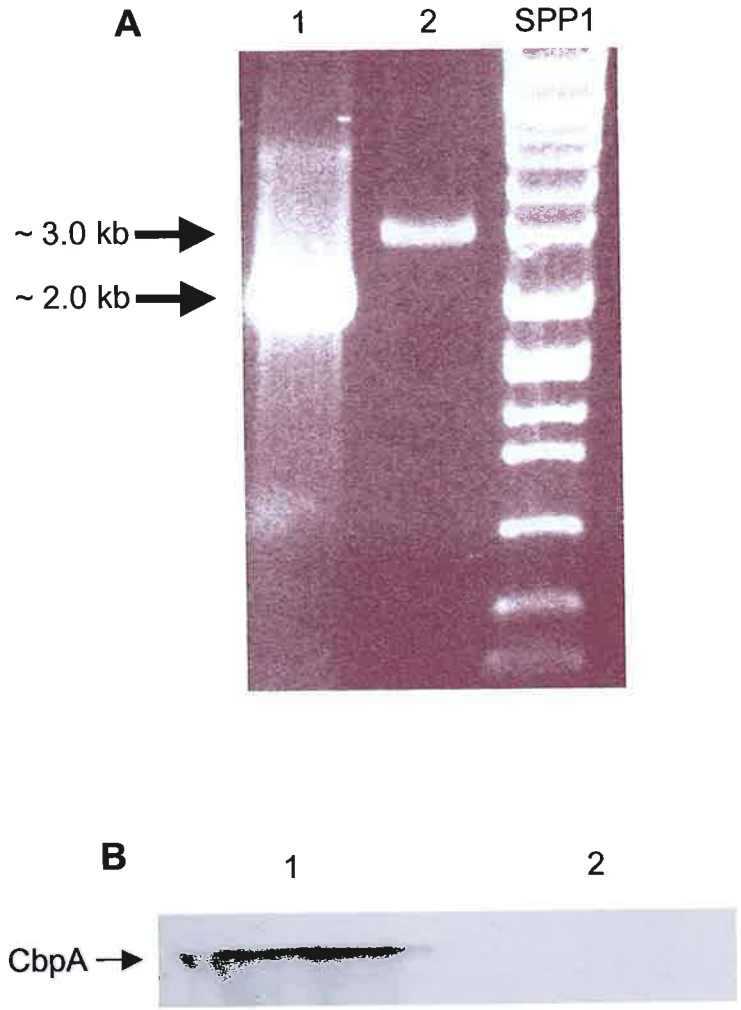

Figure 5.3. Analysis of TIGR4*cbpA:erm*

(A) DNA from TIGR4*cbpA:erm* was subjected to PCR analysis using oligonucleotides J215 & AS87 (lane 1) and J214 & RMAG7 (lane 2). Sizes of products were estimated according to their mobilities relative to the SPP1 marker. Lane 1 gave a product of approximately 2.0 kb, with lane 2 approximately 3.0 kb. These bands are consistent with the replacement of *cbpA* with the *erm* gene.

(B) Proteins in cell lysates of TIGR4 (lane 1) and TIGR4*cbpA:erm* (lane 2) were subjected to SDS-PAGE and Western immunoblot using anti-CbpA.

Investigation into adherence of TIGR4 and its isogenic mutants to A549 cells was undertaken as described above. Total adherence of wt TIGR4 to the A549 cells was approximately 1.1×10^5 CFU per well, significantly higher than that seen for D39 ($P < 0.01$). Only TIGR4*rr06:erm* showed a statistically significant loss of adherence compared to the wt ($P < 0.01$), although both TIGR4*hk06:erm* and TIGR4Δ*rr06* showed similar losses, and were not significantly different to the adherence of TIGR4*rr06:erm* (**Figure 5.4**). Interestingly, TIGR4*cbpA:erm* showed a similar level of adherence as the wt, suggesting CbpA does not play a significant role in epithelial cell adherence in TIGR4.

5.2.2 *In vivo* studies.

Given the reduced adherence of D39*Δhk06* and D39*Δrr06* to epithelial cells, along with the system's regulation of a known virulence factor, work was undertaken to investigate whether these results correlated with a role for RR/HK06 in nasopharyngeal colonisation and invasive disease. Thus, the impact of in-frame *hk06* and *rr06* deletions in D39 on the ability to colonise the nasopharynx, and to translocate to the lungs and blood were examined. As in the *in vitro* adherence assays described above, D39*cbpA*$^-$ was used for comparative purposes. The *in vivo* model employed was a modification of that of Wu *et al.* (1997) as developed in this laboratory by LeMessurier (2002) (Section 2.17.3). We used anaesthesia prior to intranasal inoculation as previous studies found that inoculation without anaesthesia led to inconsistent colonisation between mice inoculated with the same dose and strain. In order to inhibit growth of endogenous nasopharyngeal flora, serial dilutions of nasal washes and nasopharyngeal homogenate were plated on BA with 4 μg/ml Gent. *S. pneumoniae* are naturally resistant to low concentrations of Gent, and initial studies illustrated that this level of antibiotic had no impact on the viable counts of D39, D39*Δhk06*, D39*Δrr06* or D39*cbpA*$^-$. Additionally, D39*cbpA*$^-$ was plated on BA supplemented with Ery.

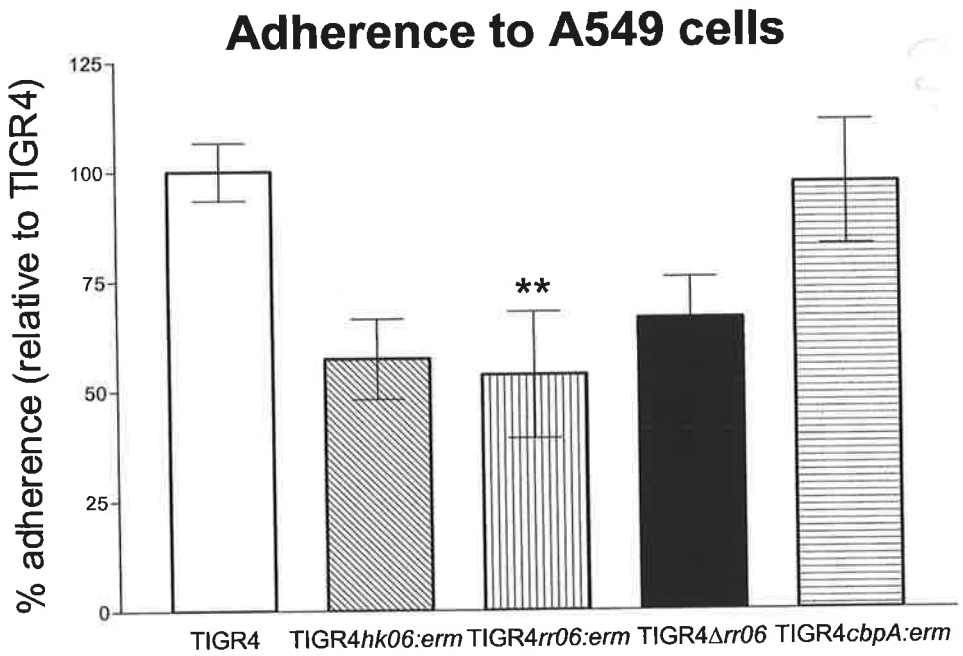

Figure 5.4. Adherence to A549 (human lung) cells.

TIGR4, TIGR4*hk06*, TIGR4*rr06*, TIGR4Δ*rr06*, and TIGR4*cbpA*$^-$ were incubated with confluent cell monolayers for 2 h, after which adherent bacteria were enumerated. Data are presented as percentage adherence relative to that of wild-type TIGR4 (mean ± standard error). Mean adherence of TIGR4 to A549 cells (100%) was 1.1×10^5 CFU/well. ** Denotes significantly different from the wild-type ($P < 0.01$) as determined by unpaired Student's *t*-test, two-tailed.

Groups of 18 CD-1 mice were inoculated intranasally following anaesthesia with D39, D39*Δhk06*, D39*Δrr06* or D39*cbpA*⁻ at a dose of approximately 2×10^7 CFU. At least 5 mice from each group were sacrificed after 24, 48 and 96 h, and the numbers of pneumococci in the nasopharynx, lungs, and blood were determined as described in Section 2.18.3. The results presented are pooled from two independent experiments. Whilst neither D39*Δhk06* nor D39*cbpA*⁻ appeared to show any significant difference in nasopharyngeal colonisation compared to the parent strain, D39*Δrr06* showed a statistically significant increase in number of pneumococci colonizing the nasopharynx at both 48 and 96 h ($P < 0.05$ and $P < 0.01$, respectively; Student's two-tailed unpaired *t* test) (**Figure 5.5**). Translocation to the lungs and blood at 96 h appeared to be severely affected in D39*Δhk06*, with only 1/16 mice showing evidence of bacteria in the lungs or blood in the two experiments (**Figure 5.6 & 5.7**). In contrast, 13 and 9 of the 16 mice infected with D39 had pneumococci in the lungs and blood, respectively ($P < 0.001$ and $P < 0.025$, Fisher Exact Test) (**Table 5.1**). At the same timepoint, however, D39*Δrr06* showed a significant increase in its ability to cause infection in the lung ($P < 0.05$, Student's two-tailed unpaired *t* test) (**Figure 5.6**). D39*cbpA*⁻ showed no differences relative to the wt in either its ability to colonise the nasopharynx, or translocate to the lungs and blood (**Figure 5.5, 5.6 & 5.7**).

5.2.3 Release of IL-8 by respiratory epithelial cells in response to WT D39, D39Δ*hk06*, D39Δ*rr06* and D39*cbpA*⁻.

Recent results from the laboratory (Graham, 2005), suggested that CbpA may play a role in modulating the release of the CXC chemokine IL-8 from respiratory epithelial (A549 and Detroit 562) cells. IL-8 is an important mediator of inflammation, responsible for the recruitment of neutrophils to the site of infection, and likely to play a vital role in

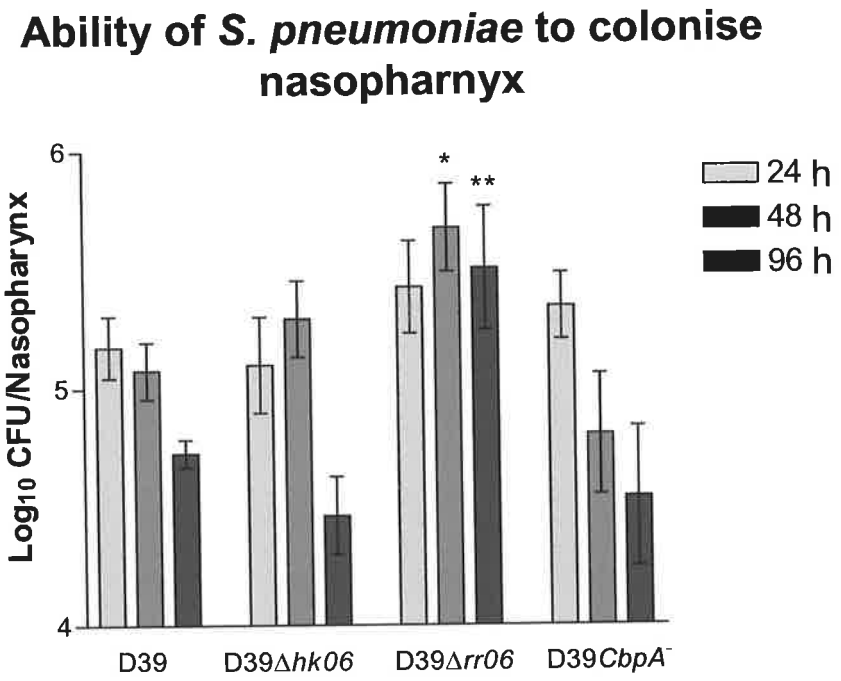

Figure 5.5. Numbers of *S. pneumoniae* in nasopharnynx (total log_{10}CFU) following intranasal challenge.

Groups of mice were challenged intranasally with the indicated *S. pneumoniae* strains and after 24, 48 and 96 h, at least five mice from each group were sacrificed and the sums of the *S. pneumoniae* in nasopharyngeal washings and nasopharyngeal homogenates were determined, as described in Section 2.17.3. Data represented are the mean ± SE of the log_{10} total CFU for the five mice. * represents $P < 0.05$ and ** represents $P < 0.01$ compared to D39, as determined by Student's two tailed unpaired *t* test on log-transformed CFU data.

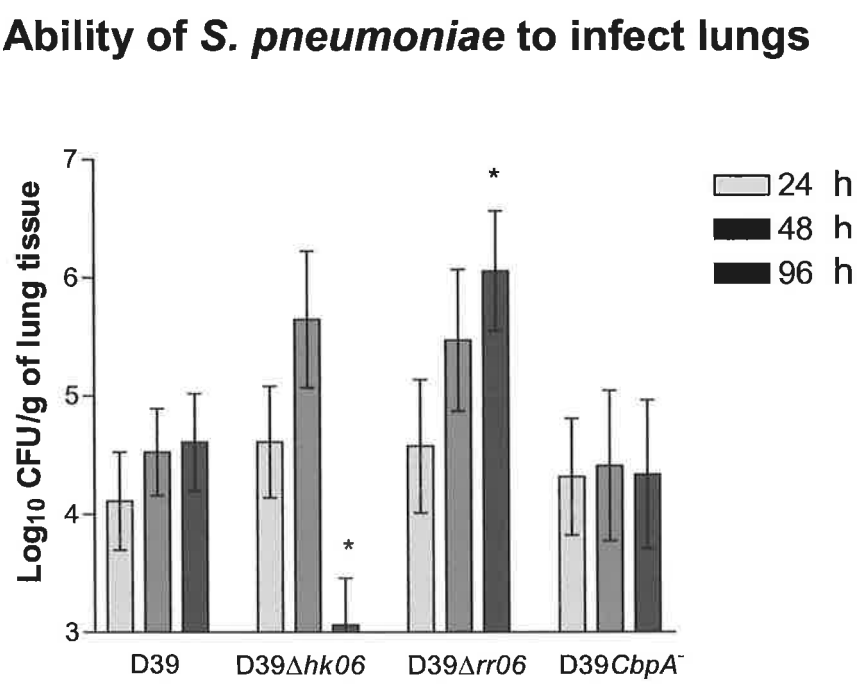

Figure 5.6. Numbers of *S. pneumoniae* in lungs (log_{10}CFU/g) following intranasal challenge.

Groups of mice were challenged intranasally with the indicated *S. pneumoniae* strains and after 24, 48 and 96 h, at least five mice from each group were sacrificed and the lungs were excised, homogenised and plated in order to determine number of pneumococci, as described in Section 2.17.3. Data represented are the mean ± SE of the log_{10} CFU/g for the five mice. * represents $P < 0.05$ when compared to D39 as determined by Student's two tailed unpaired *t* test on log-transformed CFU data.

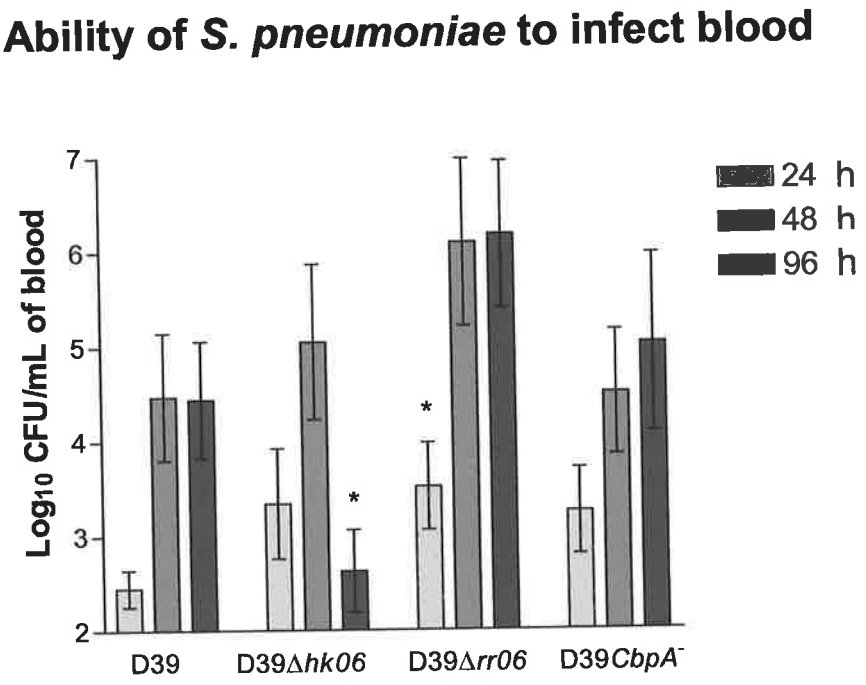

Figure 5.7. Numbers of *S. pneumoniae* in blood (log_{10}CFU/ml) following intranasal challenge.

Groups of mice were challenged intranasally with the indicated *S. pneumoniae* strains and after 24, 48 and 96 h, at least five mice from each group were sacrificed and numbers of pneumococci in blood were enumerated, as described in Section 2.17.3. Data represented are the mean ± SE of the log_{10} CFU/ml for the five mice. * represents $P < 0.05$ compared to D39 as determined by Student's two tailed unpaired *t* test on log- transformed CFU data.

TABLE 5.1 Number of mice from which pneumococci were recovered for each site 24 h, 48 h and 96 h post-intranasal infection.

Strain	Time point	Nasopharynx		Lung		Blood	
		No. of mice	Significance compared to D39, P^a	No. of mice	Significance compared to D39, P^a	No. of mice	Significance compared to D39, P^a
D39	24 h	10/10	-	6/10	-	2/10	-
	48 h	10/10	-	9/10	-	6/10	-
	96 h	16/16	-	13/16	-	9/16	-
D39*Δhk06*	24 h	10/10	NS	6/10	NS	3/10	NS
	48 h	10/10	NS	9/10	NS	6/10	NS
	96 h	13/13	NS	1/13	$P < 0.001$	1/13	$P < 0.025$
D39*Δrr06*	24 h	10/10	NS	6/10	NS	5/10	NS
	48 h	10/10	NS	8/10	NS	7/10	NS
	96 h	11/11	NS	9/11	NS	8/11	NS
D39*cbpA*$^-$	24 h	10/10	NS	8/10	NS	4/10	NS
	48 h	10/10	NS	5/10	NS	6/10	NS
	96 h	10/10	NS	5/10	NS	5/10	NS

NOTE. NS, not significant

[a] Using Fisher exact probability test.

determining the outcome of a pneumococcal infection. Thus, preliminary experiments were undertaken to investigate if RR/HK06 also modulates this release.

D39, D39Δ*hk06*, D39Δ*rr06* and D39*cbpA*$^-$ were tested for their ability to elicit an IL-8 response using a model developed in the laboratory by Graham (2005) as described in Section 2.15. A549 or Detroit 562 cells were infected with approximately 5 $\times 10^7$ CFU *S. pneumoniae* D39 or its isogenic mutants deficient in RR/HK06 or CbpA. Cellular RNA was extracted at 4 h and analysed for the presence of IL-8 mRNA by real time RT-PCR using gene-specific oligonucleotides (IL-8 Fwd and IL-8 Rev) (Table 2.2). The mRNA for the housekeeping gene GAPDH (determined in parallel using GAPDH Fwd and GAPDH Rev; Table 2.2) was used as an internal control. In experiments performed using both A549 and Detroit 562 cells, D39*cbpA*$^-$ resulted in an increased level of IL-8 mRNA compared to the wt, similar to results seen by Graham (2005) (**Figure 5.8A and 5.8B**). In contrast D39Δ*hk06*, and D39Δ*rr06* elicited levels of IL-8 mRNA comparable to the wt (**Figure 5.8A and 5.8B**). Thus, this suggested that RR/HK06 does not play a significant role in the induction of the CXC chemokine IL-8.

5.3 DISCUSSION

The ability of the pneumococcus to adhere to host epithelial cells is important for colonisation of the host, particularly in the nasopharynx where the pathogen can reside for extended periods without causing disease. Such asymptomatic colonisation is a precursor to invasive disease, but carriers are also the principal reservoir for *S. pneumoniae*, which is a human-specific pathogen. Previous studies have shown that CbpA mediates the adherence of pneumococci to A549 cells and Detroit 562 cells (Rosenow *et al.*, 1997; Zhang *et al.*, 2000). Indeed, in this study we also saw a dramatic decrease in the ability of pneumococci lacking CbpA to adhere to both cell types. Both D39Δ*hk06* and D39Δ*rr06* also showed a reduction in adherence to A549 as well as Detroit 562 cells.

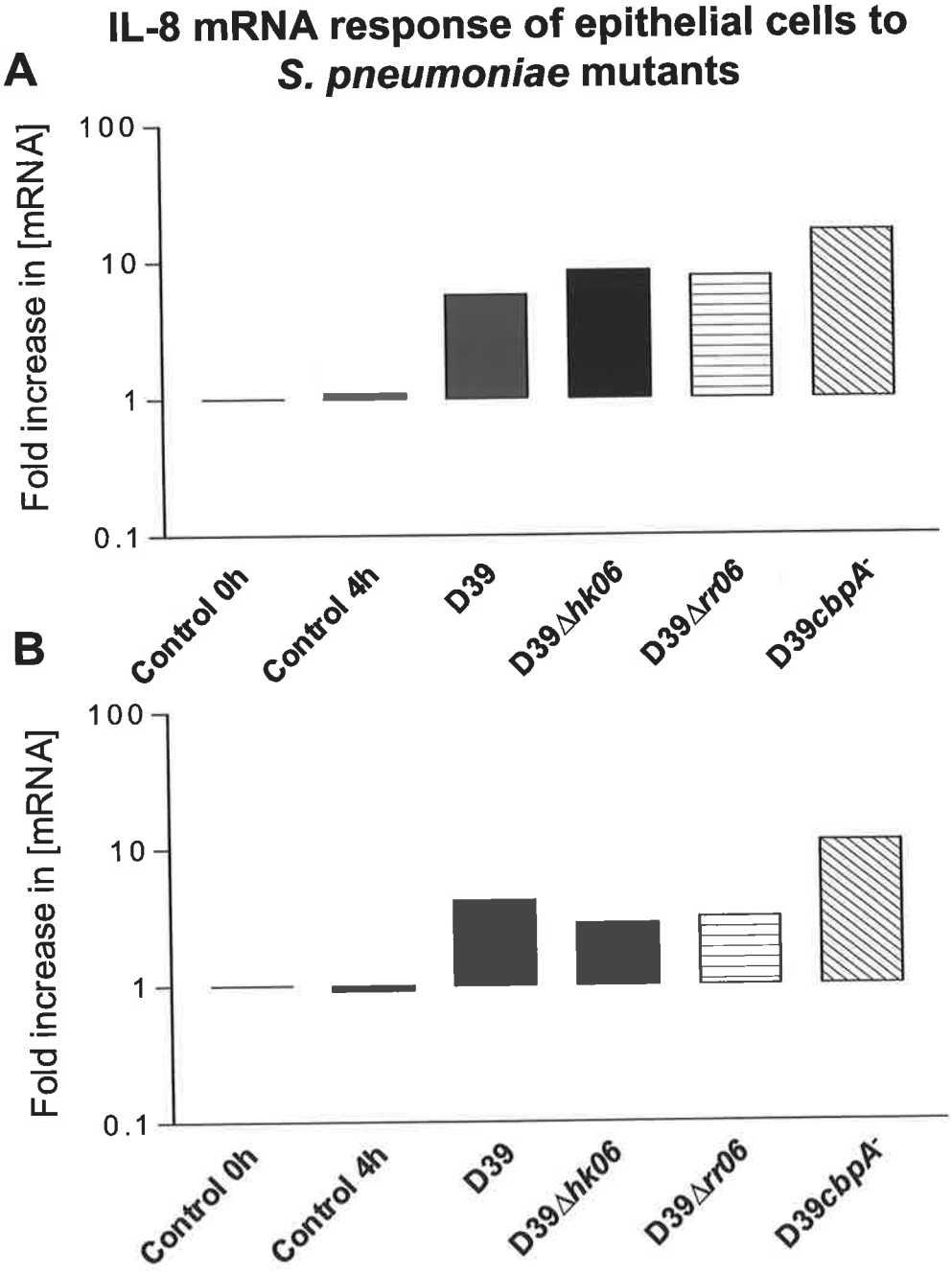

Figure 5.8. IL-8 mRNA response of epithelial cells to D39, D39Δ*hk06*, D39Δ*rr06*, and D39*cbpA*$^-$.

Confluent monolayers of (A) A549 or (B) Detroit 562 cells were incubated with approximately 5×10^7 CFU *S. pneumoniae* D39, D39Δ*hk06*, D39Δ*rr06* or D39*cbpA*$^-$ for 4 h before extraction of cellular RNA and analysis for IL-8 specific mRNA by real-time RT-PCR. Data presented are from individual experiments performed in quadruplicate.

The findings for D39Δ*hk06* were unexpected, given the increase in *cbpA* expression in this mutant. Additional Western immunoblot analysis confirmed that the relative expression of CbpA was unaffected by either tissue culture medium or contact with the eukaryotic cells. These findings clearly implicate additional, as yet uncharacterized, RR06/HK06-regulated factors in adherence to epithelial cells of human origin. Recent research by Orihuela *et al.* (2004) using *S. pneumoniae* TIGR4, demonstrated that pneumococci adhering to Detroit 562 cells exhibited an increased level of *cbpA* mRNA. Thus, further investigation comparing adherent D39, D39Δ*hk06* and D39Δ*rr06* may provide insight into not only reasons for the loss of adherence, but also a possible signal sensed by HK06.

Investigation of the role of RR/HK06 in epithelial cell adherence of TIGR4 gave similar results to D39, although only TIGR4*rr06:erm* reached statistical significance. Interestingly, a *cbpA erm* replacement mutant in TIGR4 (TIGR4*cbpA:erm*) did not exhibit any defect in adherence. No previous studies have investigated the ability of such a mutant in TIGR4 to adhere to epithelial cells, and these data suggest that CbpA may have varying importance for epithelial cell adherence in different strains. These results also suggested that, as with D39, differences in epithelial cell adherence are due to regulated factors other than CbpA.

The results from the *in vivo* experiments are not easy to reconcile with the *in vitro* adherence data. This is not surprising given the likelihood of differences in receptor expression between transformed human cell lines and mouse respiratory epithelium. Whilst CbpA is clearly important for *in vitro* adherence in D39, mutation of *cbpA* in this strain had no obvious effect on either colonization of the murine nasopharynx, or progression to pulmonary or systemic disease. This result is in contrast to previous studies which suggested that CbpA contributes to multiple stages of pneumococcal pathogenesis. These discrepancies are likely due to differences in animal models and pneumococcal strains. For instance, Rosenow *et al.* (1997) used an infant rat model to illustrate a deficiency in colonisation, Balchandran *et al.* (2002) used CBA/n mice, while our study used CD-1 mice. Additionally, both previous studies enumerated pneumococci in

nasopharnyx solely by plating nasal washes. Our model enumerated numbers of *S. pneumoniae* in the nasopharynx by undertaking a nasal wash, and also by excising and homogenizing the nasopharyngeal cavity to ensure all pneumococci residing in the nasopharynx were counted. Additionally, the use of anaesthesia in our model may have affected results, particularly in the nasopharynx, with anaesthesia likely to cause a greater level of aspiration of bacteria directly to the lungs. A recent study by Orihuela *et al.* (2004), while still illustrating a defect in colonisation by a strain deficient in CbpA, found that this was only present at 24 h, with wt levels at 48 h. This is in contrast to both Balchandran *et al.* (2002) and Rosenow *et al.* (1997), who saw differences in colonisation at 7 and 5 days post inoculation, respectively, and saw a much larger disparity between the strains. Thus, this may suggest that the importance of CbpA is strain dependent.

In contrast to D39*cbpA*$^-$, D39Δ*rr06* exhibited increased colonisation capacity, despite showing reduced adherence to epithelial cells. This strain was also present at higher levels in the lungs and blood, suggesting that this mutant is more virulent than the wt. Despite reduced *in vitro* adherence to nasopharyngeal epithelial cells, D39Δ*hk06* colonised the nasopharynx to a similar level as the wt, as was seen previously in an infant rat model by Sebert *et al.* (2002). However, D39Δ*hk06* was substantially attenuated in its capacity to invade the lungs and blood relative to the wt. As D39Δ*hk06* and D39Δ*rr06* showed different levels of CbpA, it is not surprising that they show differences in their ability to colonise and cause infection. The data clearly indicate that other as yet uncharacterized RR/HK06-regulated factors play a significant role in both colonisation and invasive disease, at least in the mouse model. Further investigation in TIGR4 is needed to determine whether these effects on pathogenesis are evident in different pneumococcal strains, or whether the system has strain specific effects, similar to that seen for TCS09 (Blue & Mitchell, 2003).

Recent research in our laboratory has illustrated that CbpA may play a role in modulating the release of IL-8, a pro-inflammatory chemokine important in the recruitment of neutrophils to the site of infection (Graham, 2005). While an increased level of IL-8 mRNA was evident in D39*cbpA*$^-$ relative to D39, as had been seen previously (Graham,

2005), both D39Δ*hk06* and D39Δ*rr06* produced IL-8 mRNA levels comparable to the wt. Thus, these results would suggest that differences seen in the *in vivo* model were not due to modulation of the release of IL-8. However, caution needs to be exercised when correlating *in vitro* with *in vivo* assays, as seen in the *in vitro* epithelial cell adherence assays described above.

This Chapter has demonstrated that RR/HK06 plays a significant role in epithelial cell adherence, as well as in pneumococcal pathogenesis. Interestingly, both effects appear unrelated to the regulation of *cbpA* by the system, suggesting that other as yet uncharacterised factors regulated by the system are likely to play a significant role in epithelial cell adherence, colonisation and invasive disease.

CHAPTER SIX – UNDERSTANDING THE REGULATION OF *cbpA* BY RR/HK06.

6.1 Introduction

Previous results, described in Chapters Three and Four, provided evidence for the regulation of the major pneumococcal virulence factor CbpA by the TCSTS RR/HK06. However, the differential levels of CbpA seen in D39Δ*hk06* and D39Δ*rr06*, at both the level of transcription and translation, proved difficult to explain. It was expected that mutations in both genes would led to similar changes in expression of regulated genes. The work described in this Chapter was aimed at improving our understanding of the regulation of *cbpA* by RR/HK06, using a combination of defined mutations, amino acid substitutions and over-expression of this TCSTS.

6.2 Results

6.2.1 Construction of D39Δ*rr/hk06* and TIGR4*rr/hk06:erm*

To further investigate the regulatory effect of RR/HK06 on *cbpA*, mutants in which the entire system was deleted were constructed in *S. pneumoniae* D39 and TIGR4. In D39, this involved the production of an in-frame deletion mutant of both *hk06* and *rr06* created

using the same method as described in Section 3.2.1. Oligonucleotide combinations AS55/AS5 and AS56/AS6 produced the regions flanking *rr/hk06*, and PCR using AS5/AS6 produced a 5074-bp linear DNA fragment with nt 49-1930 of *rr/hk06* deleted. This was then transformed into D39*rr06*⁻, and subsequent mutants were confirmed by PCR (**Figure 6.1**), and sequencing (data not shown). This mutant was designated D39Δ*rr/hk06*.

In TIGR4, a mutant was constructed in which the majority of *rr/hk06* was replaced with an *erm* cartridge as explained in Section 3.2.5. Oligonucleotide combinations AS53/AS6 and AS60/AS5 were used to amplify flanking regions, while J214/J215 directed amplification of the *erm* cartridge from pVA891. Overlap-extension PCR using AS5/AS6 combined the products to produce a 5747-bp product in which nt 4–1954 of *rr/hk06* were replaced with the *erm* cartridge. The PCR product was subsequently transformed into TIGR4, with possible mutants identified by acquisition of resistance to Ery. The successful mutant, designated TIGR4*rr/hk06:erm*, was confirmed by PCR (**Figure 6.2**), and sequencing (data not shown).

The effect of the loss of both *hk06* and *rr06* on *cbpA* expression was then investigated. Both Western immunoblot (**Figure 6.3A & 6.4A**) and real time RT-PCR (**Figure 6.3B & 6.4B**) using protein and RNA extracted from strains grown in THY to A_{600} = 0.25 showed that D39Δ*rr/hk06* and TIGR4*rr/hk06:erm* produced levels of CbpA similar to that seen in their respective parent strains.

6.2.2 Over-expression of *rr/hk06*

6.2.2.1 Construction and characterisation of p*hk06*, p*rr06*, and p*rr/hk06*

In recent times, over-expression of TCSTSs has proven to be a good model for identifying regulated genes, particularly when the environmental signal that activates the system is unknown (Ogura *et al.*, 2001). In order to over-express the RR/HK06 system, *hk06*, *rr06*, as well as both genes together were cloned into the maltose inducible expression vector pLS1RGFP (kindly provided by P. Lopez), which has previously been

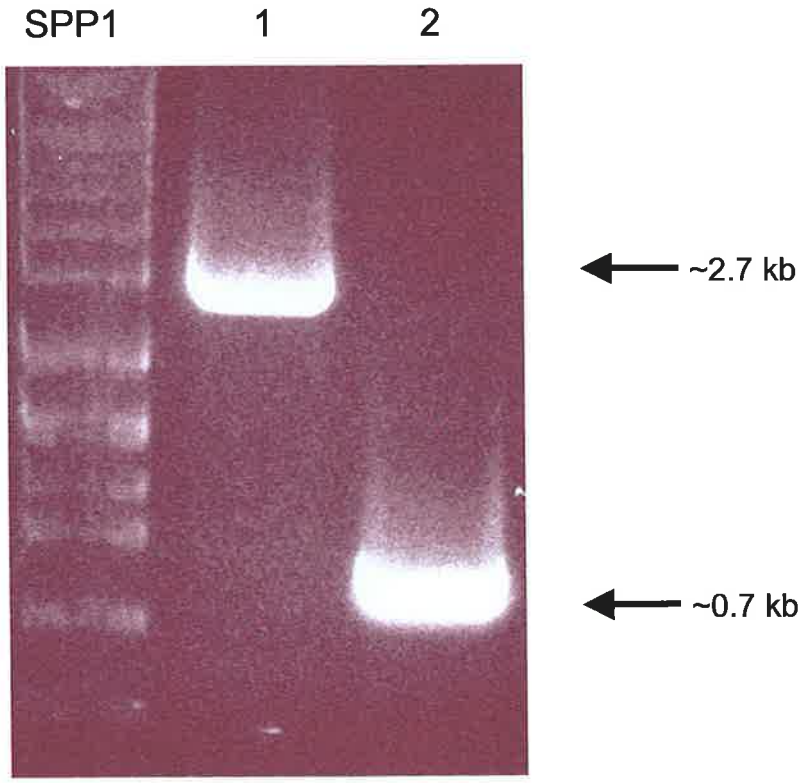

Figure 6.1. PCR analysis of D39Δ*rr/hk06*
DNA from D39 (lane 1) and D39Δ*rr/hk06* (lane 2) were subjected to PCR analysis with oligonucleotides AS23 and AS1 (Table 2.2). Sizes of products were estimated according to the mobilities relative to the SPP1 marker. D39 gave an approximate size of 2.7-kb, while D39Δ*rr/hk06* gave a size of approximately 0.7-kb consistent with the deletion of *rr06* and *hk06*.

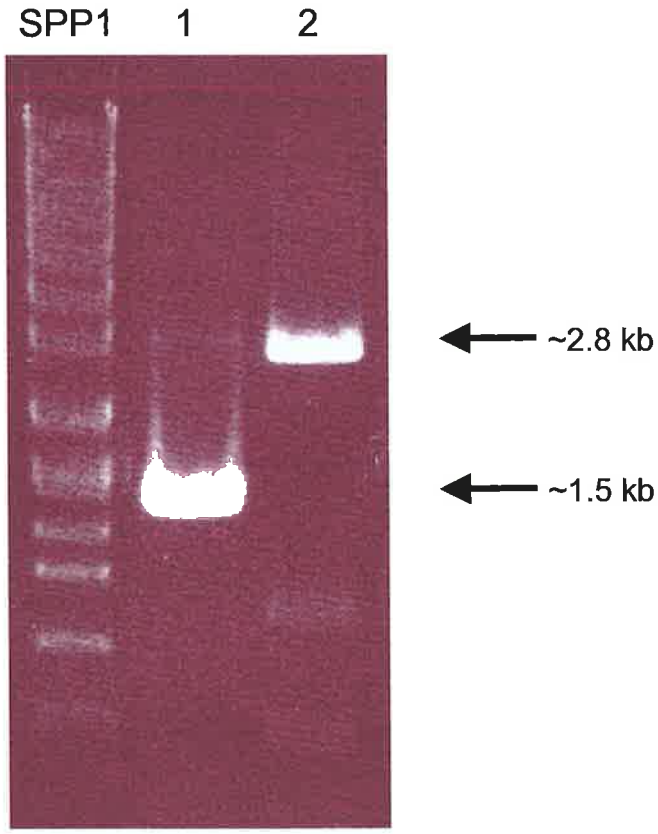

Figure 6.2. PCR analysis of TIGR4*rr/hk06:erm*

DNA from TIGR4*rr/hk06:erm* was subjected to PCR analysis with oligonucleotides J214 & AS23 (lane 1), and J215 & AS12 (lanes 2) (Table 2.2). J214 and J215 bind to inserted *erm* cartridge while AS23 and AS12 bind to 5' and 3' flanking regions of *rr/hk06*, respectively. TIGR4*rr/hk06:erm* gave products of (1) 1.5-kb, and (2) 2.8-kb. These products were consistent with replacement of the respective genes with the *erm* cartridge.

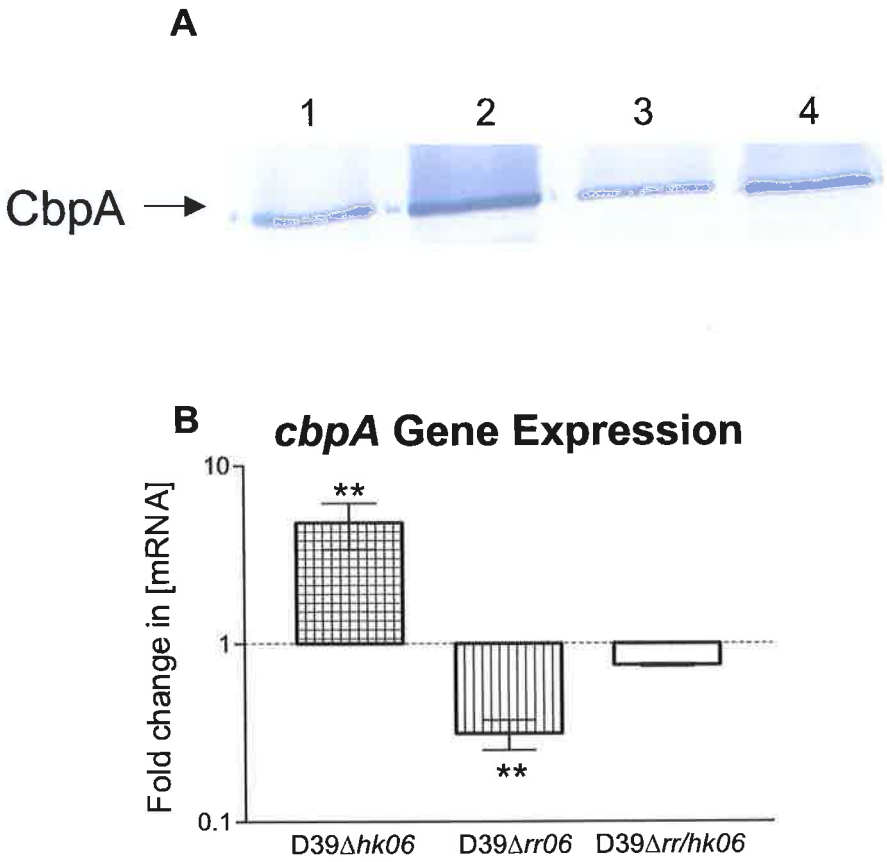

Figure 6.3. *cbpA* expression in D39Δ*rr/hk06*.

A) Proteins in lysates of D39 (lane 1), D39Δ*hk06* (lane 2), D39Δ*rr06* (lanes 3), and D39Δ*rr/hk06* (lane 4) grown in THY to an A_{600} of 0.35, were separated by SDS-PAGE, transferred onto nitrocellulose and probed with polyclonal murine anti-CbpA serum.

(A) RNA was isolated from D39 and D39Δ*rr/hk06,* and *cbpA* mRNA levels were compared using real time RT-PCR as described in Section 2.10.4. Data shown are fold increase (± standard error) in [*cbpA* mRNA] relative to D39. Data from real time RT-PCR investigating *cbpA* mRNA levels in D39Δ*hk06* and D39Δ*rr06* from Chapter Three are shown for comparative purposes. (** $P < 0.01$ as determined by One-way ANOVA with post-hoc Tuckey test).

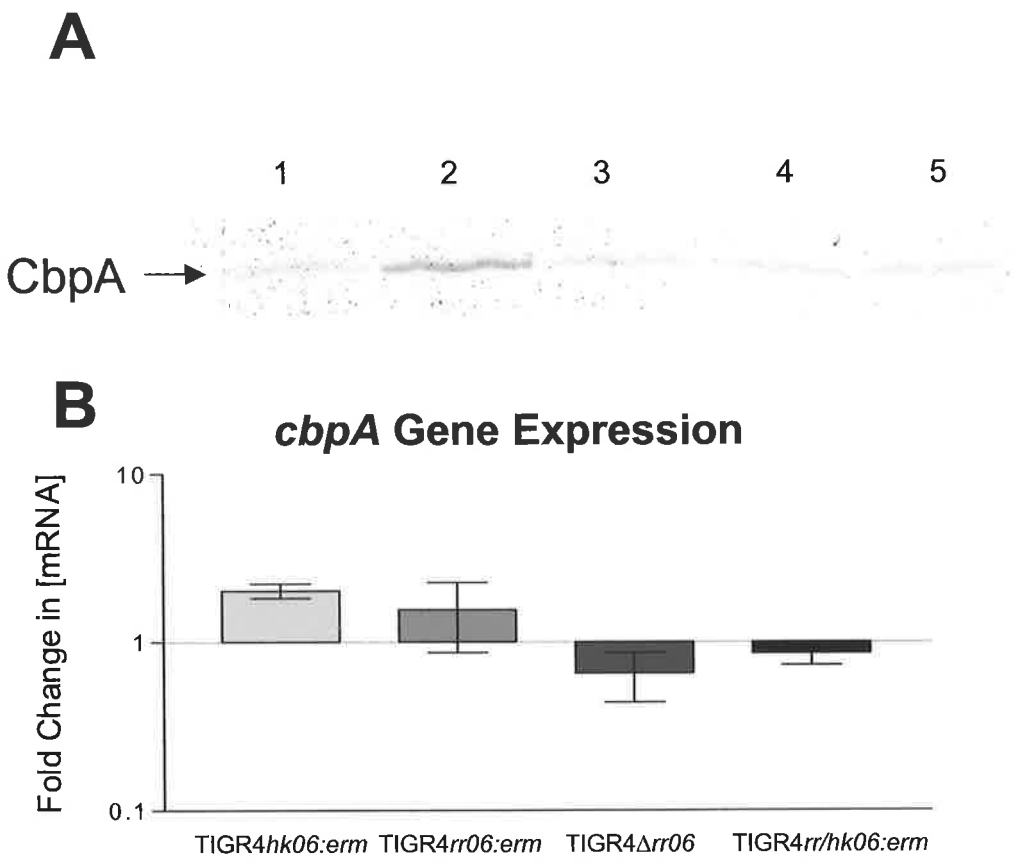

Figure 6.4 CbpA in TIGR4*rr/hk06:erm*

A) Proteins in lysates of TIGR4 (lane 1), TIGR4*hk06:erm* (lane 2), TIGR4*rr06:erm* (lane 3), TIGR4Δ*rr06* (lane 4) and TIGR4*rr/hk06:erm* (lane 5) grown in THY to an A_{600} of 0.25 were separated by SDS-PAGE, transferred onto nitrocellulose and probed with polyclonal murine anti-CbpA serum.

B) RNA was isolated from TIGR4 and TIGR4*rr/hk06:erm,* and *cbpA* mRNA levels were compared using real time RT-PCR as described in Section 2.10.4. Data shown are fold increase (± standard error) in [*cbpA* mRNA] relative to TIGR4. Data from real time RT-PCR investigating TIGR4*hk06:erm*, TIGR4*rr06:erm*, TIGR4Δ*rr06 cbpA* mRNA levels from Chapter Three are shown for comparative purposes.

used to successfully over-express the essential pneumococcal RR YycF (Mohedano *et al.*, 2005). Oligonucleotides AS69 and AS70, AS67 and AS68, and AS67 and AS70 were used to amplify *hk06*, *rr06* and *rr/hk06* genes along with the ribosome binding site before the respective start codon. PCR products were digested with *Nhe*I, and attempts were made to clone these products into the unique *Xba*I site of the vector pLS1RGFP. However, pLS1RGFP could not be digested with *Xba*I when the construct was isolated from *S. pneumoniae*. When the plasmid was electroporated into its other host strain, *Lactococcus lactis* MG1363 (Section 2.6.3), and subsequently isolated, pLS1RGFP could be partially digested with *Xba*I. The linearised plasmid was gel purified, and ligated with *Nhe*I digested PCR product. The ligation mix was then transformed into the un-encapsulated *S. pneumoniae* R6 and successful transformants were selected on Ery. Transformants were then checked for presence of the correct insert by sequencing using AS71 which binds at the start of the maltose inducible promoter P_M (data not shown). These plasmids were designated p*hk06*, p*rr06* and p*rr/hk06* respectively. These plasmids were subsequently transformed into *S. pneumoniae* D39 for further investigation.

In order to induce over-expression, D39:p*hk06*, D39:p*rr06*, D39:p*rr/hk06* along with D39 containing vector alone (D39:pControl) were grown in THY to A_{600} = 0.25, centrifuged at 4000 × *g* for 15 min at RT and then resuspended in THY supplemented with 0.8% maltose. After 30 min, strains were pelleted and RNA extracted as described in Section 2.10.1. Real time RT-PCR using *hk06*- and *rr06*- specific oligonucleotides (HKF, HKR & RRF,RRR; Table 2.2) demonstrated over-expression of *hk06*, *rr06* and *rr/hk06* from their respective plasmids (**Figure 6.5**). Equal numbers of cells harvested from the above experiment and resuspended in lysis buffer, were subjected to SDS-PAGE and Western blotting using polyclonal anti-RR06. An approximate 25kDa immunoreactive band corresponding to the expected size of RR06 was evident in cell lysates from D39:p*rr06* and D39:p*rr/hk06* but not in D39:pControl and D39:p*hk06* (**Figure 6.6**). These

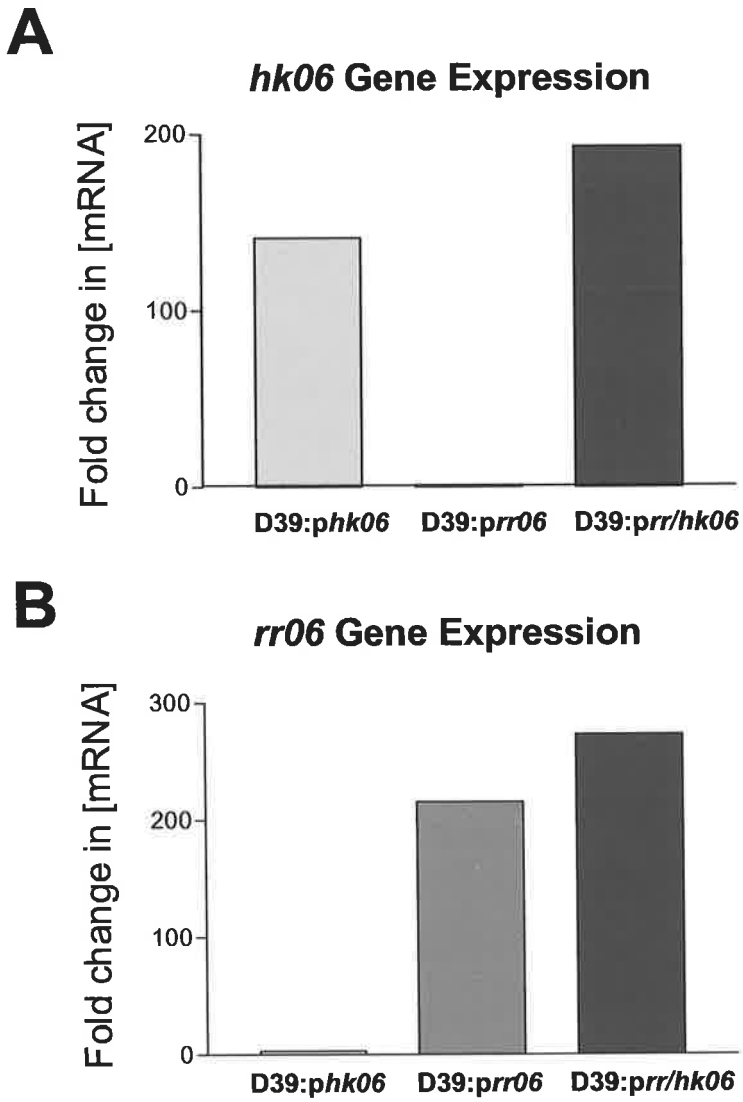

Figure 6.5. Real time RT-PCR analysis of over-expression constructs.

RNA was isolated from D39:pControl, D39:p*hk06*, D39:p*rr06* and D39:p*rr/hk06,* and *hk06* **(A)** and *rr06* **(B)** mRNA levels were compared using real time RT-PCR as described in Section 2.10.4. Data shown are fold increase in [mRNA] relative to D39:pControl.

Figure 6.6 Western immunoblot analysis of RR06 expression.

Proteins in lysates of (1) D39:pControl, (2) D39:p*hk06* and (3) D39:p*rr06* and (4) D39:p*rr/hk06* grown in THY as described in Section 6.2.2.1, were seperated by SDS-PAGE transferred onto nitrocellulose and probed with anti-RR06.

results provided evidence for successful over-expression at both the level of transcription and also translation for RR06. Over-expression of HK06 at the level of translation was unable to be confirmed as no purified HK06, and thus no anti-HK06, had been produced. It was assumed that as with RR06, the increase of *hk06* mRNA correlated with increased protein. Examination of the growth rate of the strains demonstrated that all strains grew at the same rate following maltose induction, indicating that over-expression was unlikely to be deleterious to the cell (**Figure 6.7**).

In order to determine when over-expression was maximal post induction, D39:pControl and D39:p*rr06* were grown in THY to A_{600} = 0.25 and then induced with 0.8% maltose. RNA was extracted from these strains at 0, 15, 30, 45, 60, 90 and 120 min post induction. Real time RT-PCR using oligonucleotides RRF & RRR (Table 2.2) was then used to examine over-expression at these timepoints. Over-expression was maximal for D39:p*rr06* at 15 and 30 minutes post maltose induction (**Figure 6.8**). This result was comparable to that described by Mohedano *et al.* (2005), who used the green fluorescent protein (GFP) expressed from the plasmid as a marker of over-expression of *yycF*. Interestingly, a much greater level of over-expression of *rr06* was evident (approximately 300-fold) than that seen by Mohedano *et al.* (2005), while there was an increase in expression of *rr06* (approximately 10-fold) prior to induction (**Figure 6.8**), likely due to differences in culture media used. For all further experiments, expression was induced for 30 min.

6.2.2.2 Effect of over-expression on CbpA levels

In Chapter Three, it was demonstrated that in-frame deletion mutants in both *hk06* and *rr06* resulted in differential expression of *cbpA* compared to wt *S. pneumoniae* D39. The levels of CbpA were therefore investigated in strains over-expressing HK06 and RR06. Equal numbers of cells from D39:pControl, D39:p*hk06*, D39:p*rr06* and D39:p*rr/hk06* grown as described above (Section 6.3.1) were subjected to SDS-PAGE and

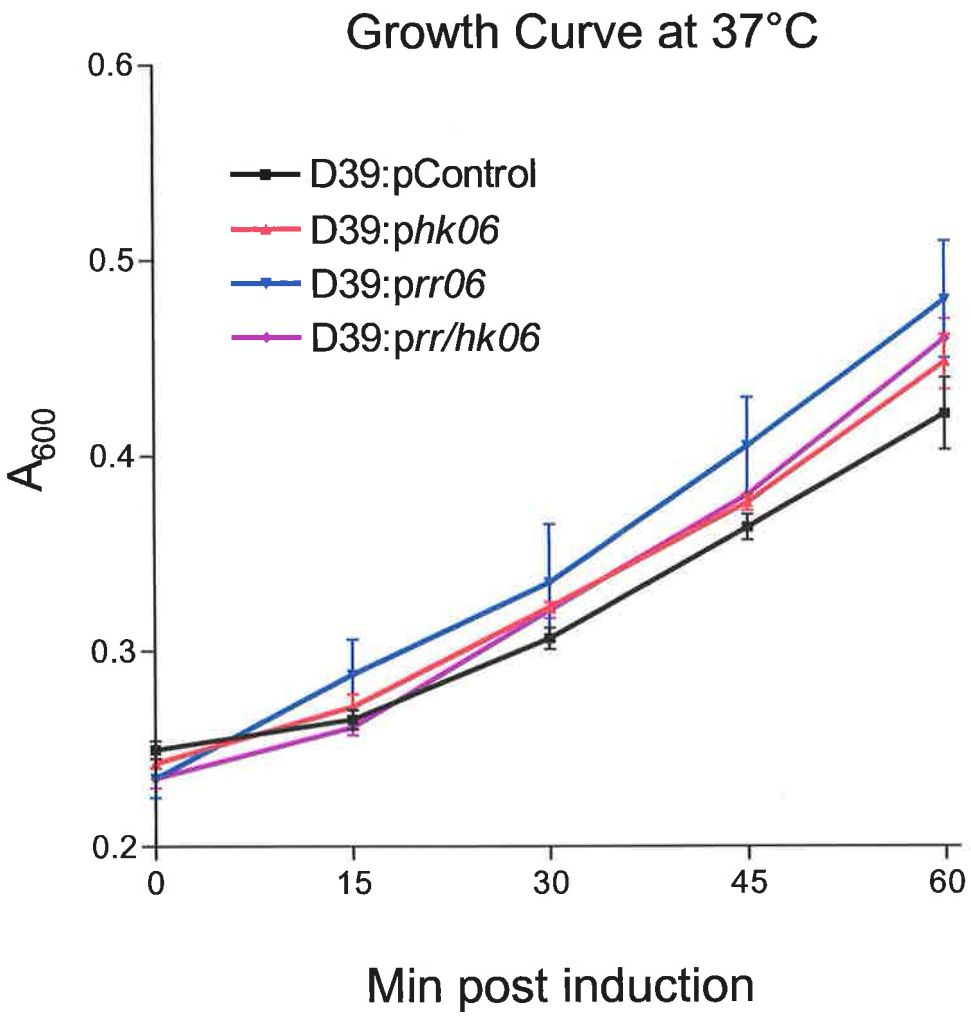

Figure 6.7 Growth following maltose induction.

Culture density (A_{600}) of D39:pControl, D39:p*hk06*, D39:p*rr06*, and D39:p*rr/hk06* was measured every 15 min following induction of over-expression. Mean ± standard error of 2 experiments are shown.

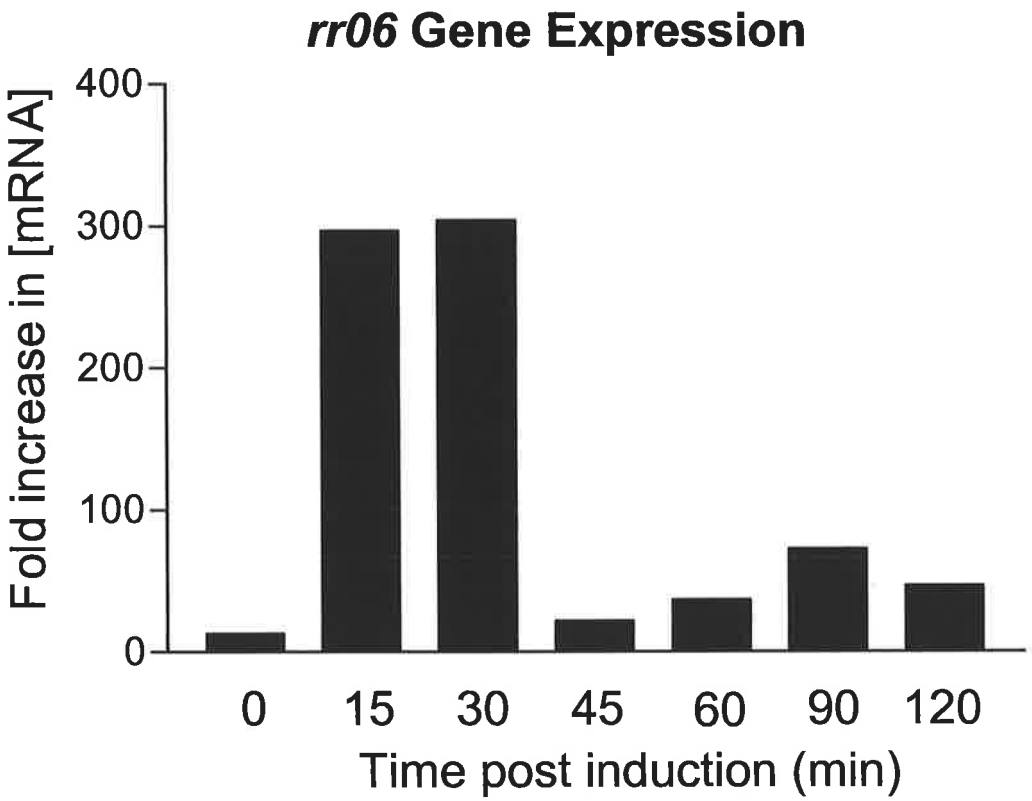

Figure 6.8. Levels of *rr06* mRNA following maltose induction.

RNA was isolated from D39:pControl and D39:p*rr06* harvested 0, 15, 30, 45, 60, 90 and 120 min post maltose induction and *rr06* mRNA levels were compared using real time RT-PCR as described in Section 2.10.4. Data shown are fold increase in [*rr06* mRNA] in D39:p*rr06* relative to D39:pControl.

Western immunoblotting using polyclonal murine anti-CbpA. While there was no difference in CbpA expression between D39:pControl and D39:p*hk06*, both D39:p*rr06* and D39:p*rr/hk06* appeared to have substantially increased levels of CbpA (**Figure 6.9A**). In order to examine this at the level of transcription, RNA was extracted and examined by real time RT-PCR using gene specific oligonucleotides CbpAFNew & CbpAREdit (Table 2.2). While over-expression of *hk06* alone appeared to have no significant effect on *cbpA* expression, the over-expression of *rr06*, both alone and in conjunction with *hk06* led to an approximate ten-fold increase in *cbpA* mRNA (**Figure 6.9B**). Thus these data, as well as providing further evidence for the regulation of *cbpA* by RR/HK06, suggest that the system is an activator of *cbpA*.

6.2.2.3 Over-expression in TIGR4, WCH4861 and WCH4832 *S. pneumoniae*

To determine whether regulation of *cbpA* by RR/HK06 occurred across serotypes, p*hk06*, p*rr06,* and p*rr/hk06* were transformed into the serotype 4 strain TIGR4 as described in Section 2.6.2.2 (Bricker & Camilli, 1999). Due to the difference in growth of TIGR4 compared to D39, TIGR4:pControl, TIGR4:p*hk06*, TIGR4:p*rr06* and TIGR4:p*rr/hk06* were grown to A_{600} = 0.17 and induced for 30 min. Over-expression was confirmed by both real time RT-PCR for *hk06* and *rr06* and Western blotting for RR06 as described above for D39 (**Figure 6.10A, Figure 6.10B and Figure 6.11**). The level of mRNA over-expression for *rr06* and *hk06* was noticeably less than that seen in D39, although it was still approximately 30-50 fold.

Real time RT-PCR using gene specific oligonucleotides against TIGR4 *cbpA* (AS62 & AS64; Table 2.2) and Western immunoblot analysis was then undertaken to investigate any possible affects on *cbpA*. Similar results to D39 were seen, with the presence of p*rr06* and p*rr/hk06* resulting in significant increases in *cbpA* expression (approximately 10 and 5-fold respectively as measured by real time RT-PCR) (**Figure 6.12A & 6.12B**).

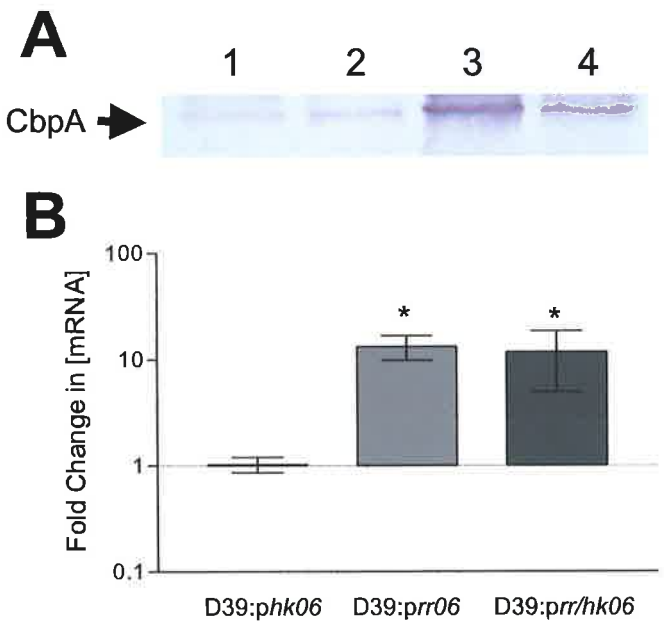

Figure 6.9. Analysis of CbpA levels in D39:pControl, D39:p*hk06*, D39:p*rr06*, and D39:p*rr/hk06*.

A) Proteins in lysates of D39:pControl (lane 1), D39:p*hk06* (lane 2), D39:p*rr06* (lane 3), and D39:p*rr/hk06* (lane 4) grown as described in Section 6.2.2.1, were separated by SDS-PAGE, transferred onto nitrocellulose and probed with polyclonal murine anti-CbpA serum.

(A) RNA was isolated from D39:pControl, D39:p*hk06*, D39:p*rr06*, and D39:p*rr/hk06* and *cbpA* mRNA levels were compared using real time RT-PCR as described in the Section 2.10.4. Data shown are fold increase (± standard error) in [*cbpA* mRNA] relative to D39:pControl. (* $P < 0.05$ as determined by One-way ANOVA with post-hoc Tuckey test).

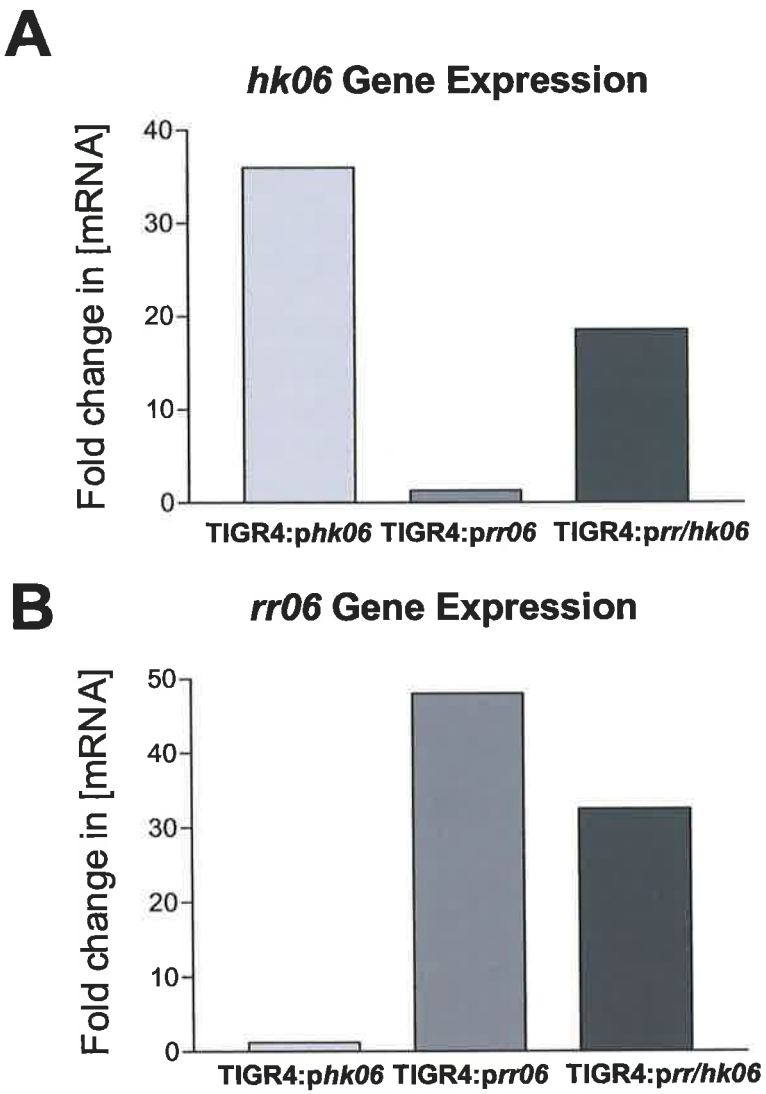

Figure 6.10. Real time RT-PCR analysis of over-expression in TIGR4.

RNA isolated from TIGR4:pControl, TIGR4:p*hk06*, TIGR4:p*rr06* and TIGR4:p*rr/hk06* and *hk06* **(A)** and *rr06* **(B)** mRNA levels were compared using real time RT-PCR as described in Section 2.10.4. Data shown are fold increase in [*cbpA* mRNA] relative to TIGR4:pControl.

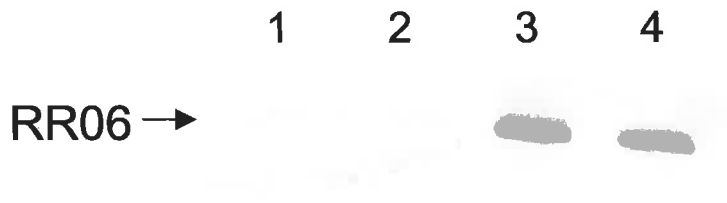

Figure 6.11 Western immunoblot analysis of RR06.

Proteins in lysates of (1) TIGR4:pControl, (2) TIGR4:p*hk06,* and (3) TIGR4:p*rr06* and (4) TIGR4:p*rr/hk06,* grown in THY as described in Section 6.2.2.3, were separated by SDS-PAGE, transferred onto nitrocellulose and probed with anti-RR06.

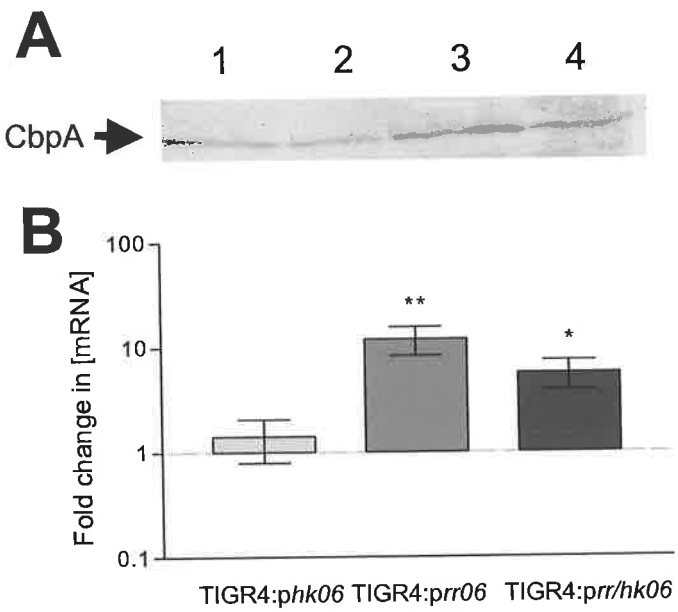

Figure 6.12. Analysis of CbpA levels in TIGR4:pControl, TIGR4:p*hk06*, TIGR4:p*rr06*, and TIGR4:p*rr/hk06*.

A) Proteins in lysates of TIGR4:pControl (lane 1), TIGR4:p*hk06* (lane 2), TIGR4:p*rr06* (lane 3), and TIGR4:p*rr/hk06* (lane 4), grown as described in Section 6.2.2.3, were separated by SDS-PAGE, transferred onto nitrocellulose and probed with polyclonal murine anti-CbpA serum.

(A) RNA was isolated from TIGR4:pControl, TIGR4:p*hk06*, TIGR4:p*rr06*, and TIGR4:p*rr/hk06,* and *cbpA* mRNA levels were compared using real time RT-PCR, as described in Section 2.10.4. Data shown are fold increase (± standard error) in [*cbpA* mRNA] relative to TIGR4:pControl. (* $P < 0.05$, ** $P < 0.01$, as determined by One-way ANOVA with post-hoc Tuckey test).

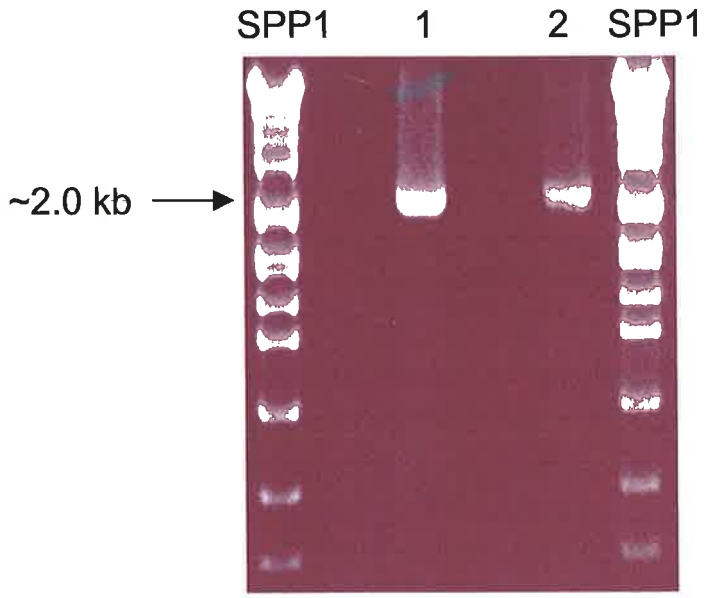

Figure 6.13. PCR analysis of WCH4832 and WCH4861

DNA from WCH4832 (lane 1) and WCH4861 (lane 2) were subjected to PCR analysis with oligonucleotides AS67 and AS70 (Table 2.2). Sizes of products were estimated based on their mobilities relative to the SPP1 markers. Products from both WCH4832 and WCH4861 were approximately 2.0 kb, the size of *rr/hk06* in D39 and TIGR4.

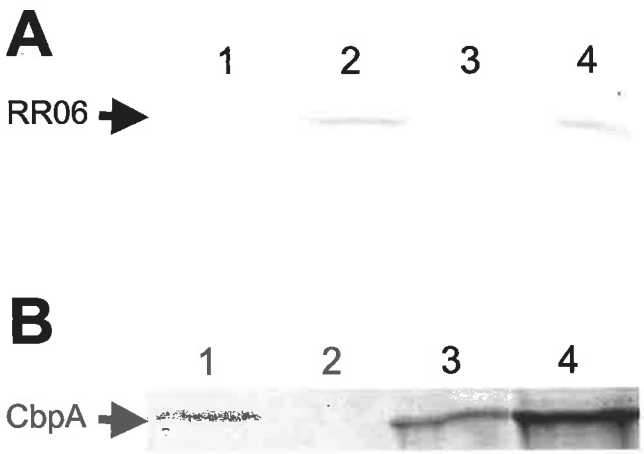

Figure 6.14. Over-expression of *rr06* in WCH4861 and WCH4832

Proteins in lysates of WCH4861 harbouring (1) pControl and (2) p*rr06* and WCH4832 harbouring (3) pControl and (4) p*rr06* were seperated by SDS-PAGE, transferred onto nitrocellulose and probed with either **(A)** anti-RR06 or **(B)** anti-CbpA.

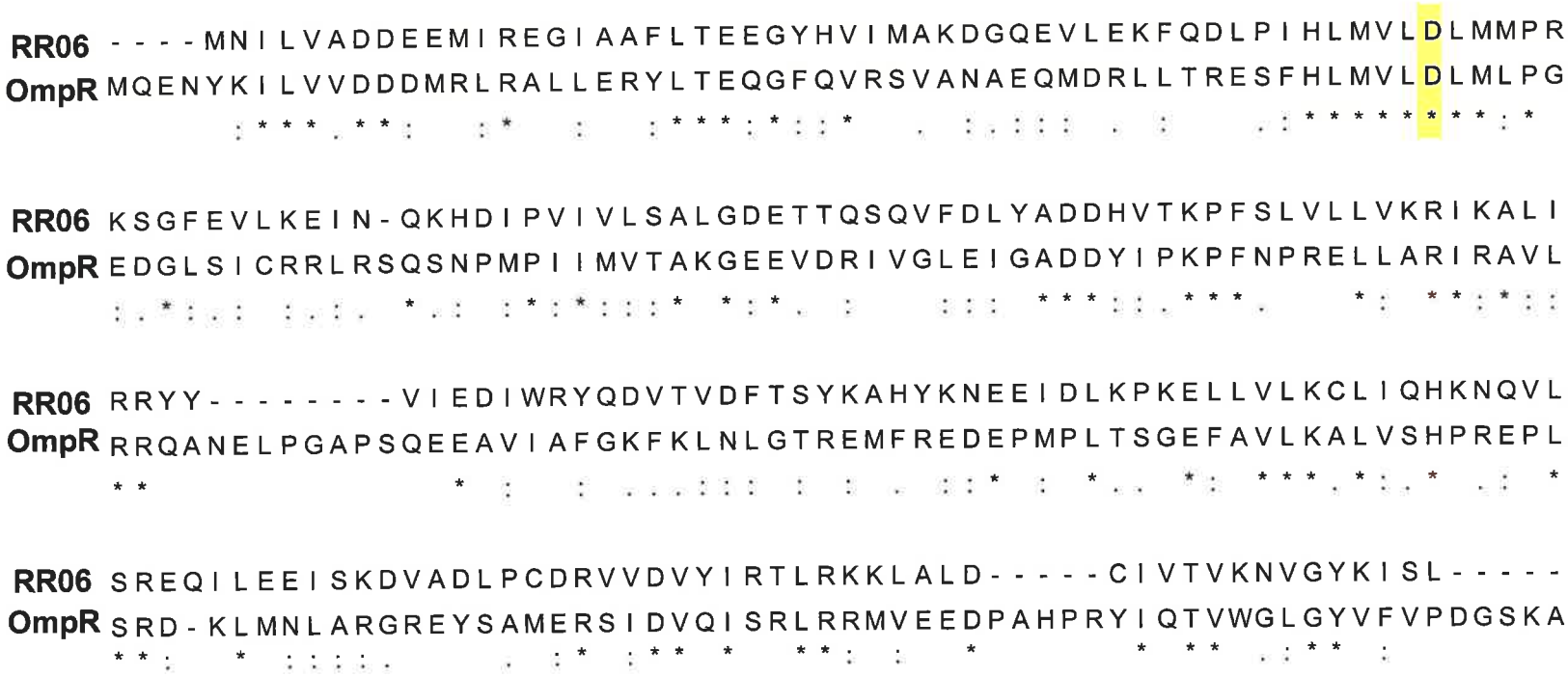

Figure 6.15. Amino Acid Alignment of RR06 and OmpR.

Amino acid sequence alignment of *S. pneumoniae* RR06 and *E. coli* OmpR. Yellow shading denotes conserved Asp_{51} that is phosphorylated in OmpR and which has been substituted with Glu and Asn in this study. * denotes exact match; : means that conserved substitutions have been observed; . means that semi-conserved substitutions are observed; - denotes absence of a residue.

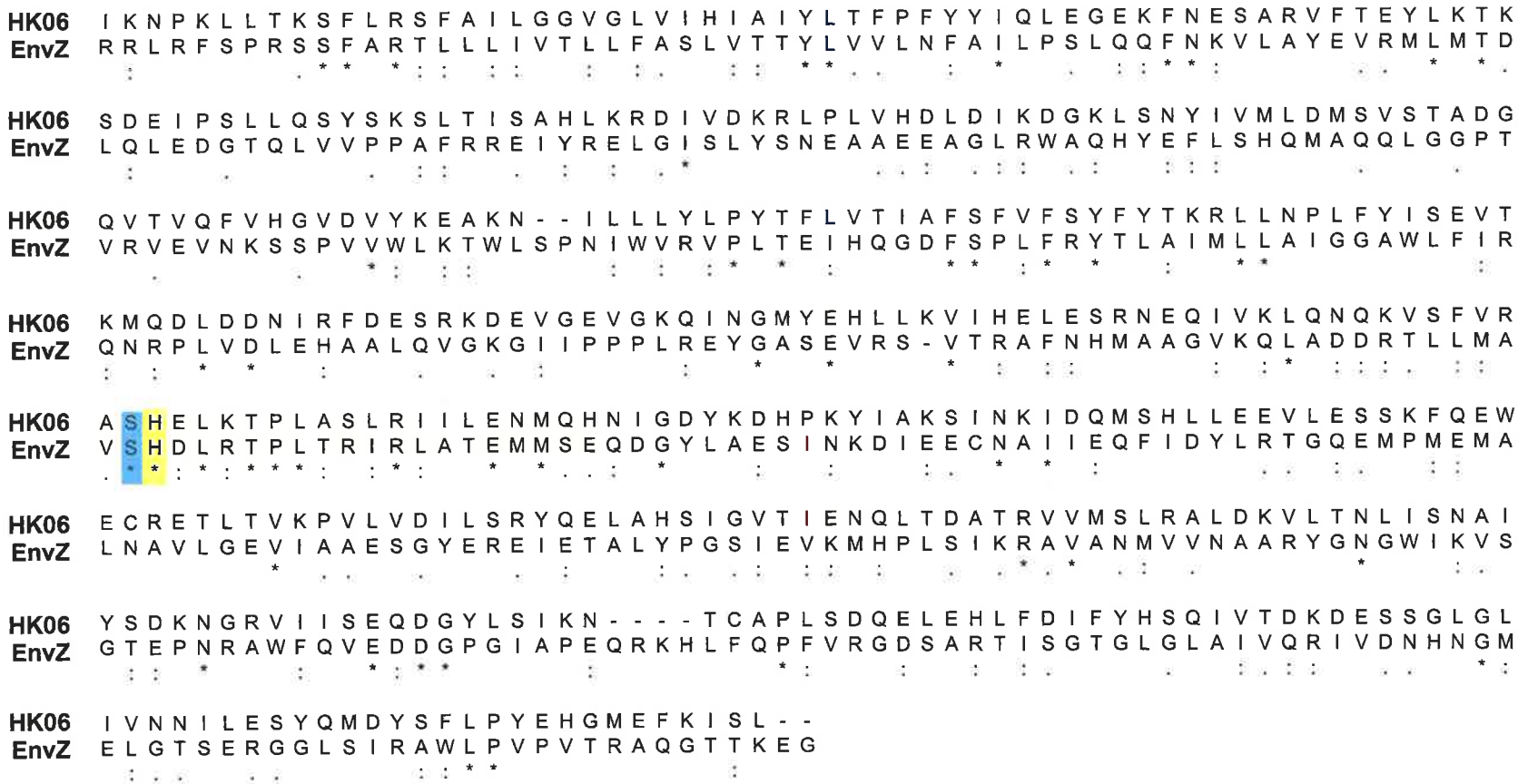

Figure 6.16. Amino Acid Alignment of HK06 and EnvZ.

Amino acid sequence alignment of *S. pneumoniae* HK06 and *E. coli* EnvZ. Yellow shading denotes conserved His_{242} that is phosphorylated in OmpR, and has been substituted with Tyr and Arg in this study. Blue shading denotes Ser_{241} which has been substituted with Asp in this study. * denotes exact match; : means that conserved substitutions have been observed; . means that semi-conserved substitutions are observed; - denotes absence of a residue.

In order to investigate whether RR/HK06 regulated CbpA in other strains, the presence of RR/HK06 in strains WCH4861 (serotype 6A) and WCH4832 (serotype 17) was confirmed by PCR analysis using oligonucleotides AS67 & AS70 (Table 2.2) (**Figure 6.13**). Subsequently, p*rr06* was transformed into these two strains as described in Section 2.6.2.2. Over-expression of RR06 in these strains was confirmed by Western blot analysis using anti-RR06 comparing WCH4861:pControl with WCH4861:p*rr06* and WCH4832:pControl with WCH4832:p*rr06* (**Figure 6.14A**). Western immunoblot analysis using anti-CbpA illustrated that CbpA was increased when *rr06* was over-expressed in WCH4832, while there was an apparent decrease in WCH4861 (**Figure 6.14B**). In order to rule out that the differences seen in regulation between these two strains is due to alterations in the RR06 binding site, the upstream region of *cbpA* in these strains was sequenced. However, there were no major differences evident, suggesting that there is unlikely to be differential binding of RR06 to this region (data not shown).

The results presented above are consistent with the data presented in Chapter Three suggesting that RR/HK06 regulates *cbpA*, with this regulation occurring across at least four pneumococcal strains. Furthermore, this data illustrates that the over-expression of a TCSTS can be used successfully to identify regulated genes.

6.2.3 Amino acid substitutions in RR/HK06

6.2.3.1 Construction of amino acid substitutions in HK06 and RR06

One hypothesis which may explain the increase in CbpA seen in D39Δ*hk06* was that RR06 binds and activates CbpA in the non-phosphorylated state. To examine this, amino acid substitutions were generated in both HK06 and RR06 in order to modulate their activity. RR/HK06 belongs to the same family of TCSTSs as the much studied EnvZ/OmpR system from *E. coli*. Sequence alignments with other family members of EnvZ/OmpR suggested that His_{242} and Asp_{51} were the conserved residues that are phosphorylated in HK06 and RR06, respectively (**Figures 6.15 and 6.16**). A large number

of HKs, including EnvZ from *E. coli*, possess both kinase and phosphatase activity in order to limit the lifetime of the activated state of the RR. Based on previous studies into EnvZ, three HK06 point mutations were constructed in D39 in an attempt to mutate kinase activity (His_{242} changed to Tyr_{242}), phosphatase activity (Ser_{241} to Asp_{241}), or both (His_{242} to Arg_{242}) (Kanamaru *et al.*, 1990; Tokishita *et al.*, 1992; Hsing & Silhavy, 1997).

A number of studies have reported that substitution of the conserved Asp_{51} of a RR, which is normally phosphorylated to provide activity, to a Glu mimics the "on" or phosphorylated state (Klose *et al.*, 1993; Lan & Igo, 1998). Alternatively, substitution with Asn or Ala for the conserved Asp_{51} blocks activation (Dahl *et al.*, 1992; Klose *et al.*, 1993; DiGiuseppe & Silhavy, 2003). Accordingly, strains were constructed with the conserved Asp_{51} of RR06 altered to Asn and Glu.

The above strains were constructed using a similar method to that described in Section 3.2.1 and is summarised in **Figure 6.17**. In order to substitute His_{242} of HK06 with Tyr, oligonucleotides AS77 & AS78 were designed such that they bound to the same but complementary region of DNA surrounding His_{242} and altered codon CAT to TAC, resulting in His_{242} being changed to Tyr_{242}. First round amplification was performed with AS5/AS77 and AS6/AS78, with AS5 and AS6 binding to flanking regions of *rr/hk06*. Overlap extension PCR using flanking oligonucleotides AS5/AS6 was then performed to combine these products resulting in a PCR product in which His_{242} of *hk06* was altered to Tyr_{242}. This PCR product was transformed into D39*rr06*$^-$, and transformants were screened for loss of Ery resistance. Substitution of His_{242} was confirmed by sequencing (data not shown), and the strain designated D39 $HK06_{H242Y}$. In order to change Ser_{241} to Asp and His_{242} to Arg in HK06, first round amplification was performed with AS5/AS87 and AS6/AS88 or AS5/AS85 and AS6/AS86, respectively. Similarly, to alter the conserved Asp_{51} of RR06 to Glu and Asn, first round amplification was undertaken with AS5/AS75 and AS6/AS76 (for Glu) or AS5/AS79 and AS6/AS80 (for Asn). The appropriate first

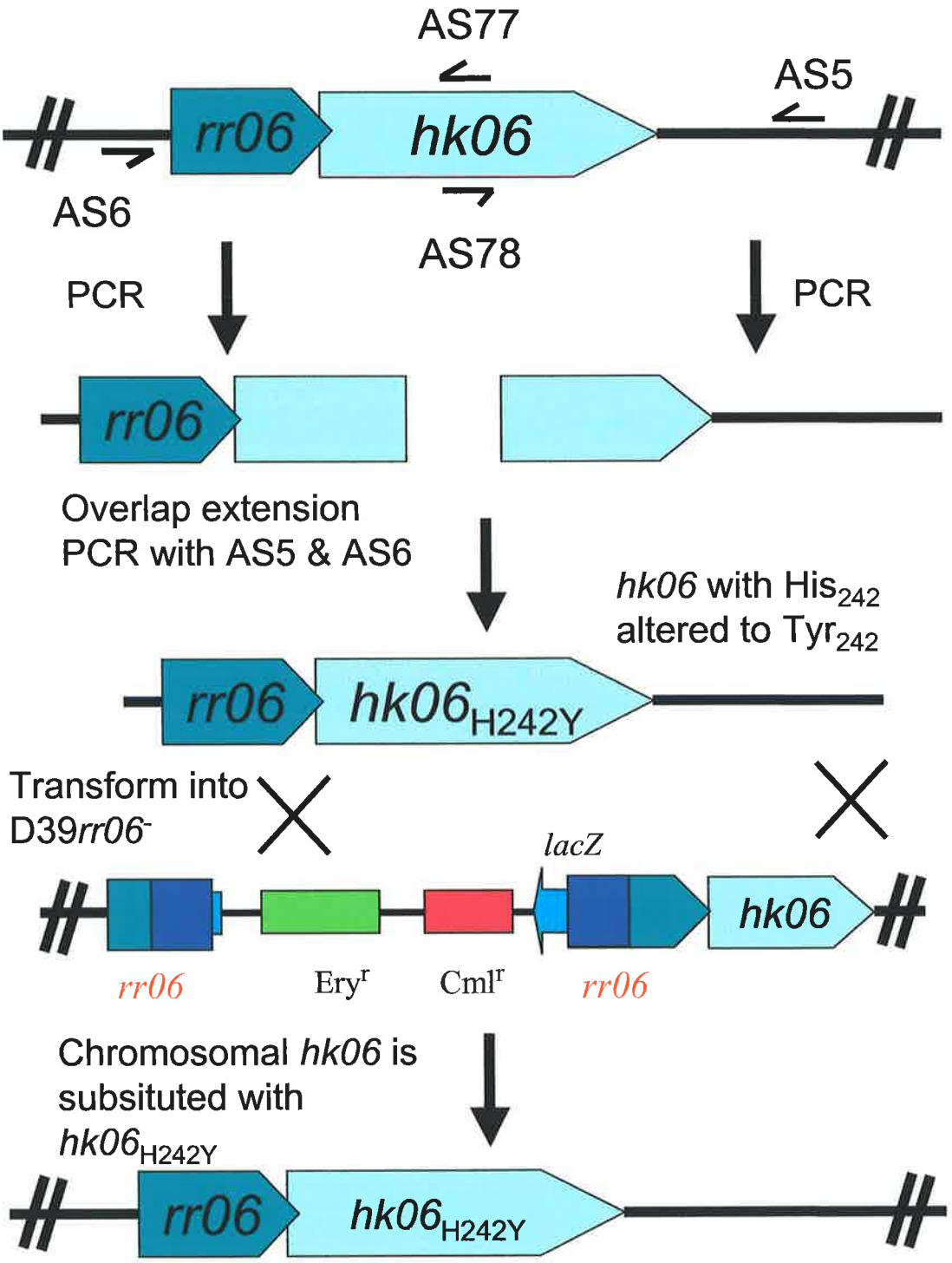

Figure 6.17. Substitution of HK06 with $HK06_{H242Y}$.

D39*rr06*⁻ *S. pneumoniae* was transformed with a PCR product created by overlap extension containing $hk06_{H242Y}$. This results in homologous recombination of the flanking regions with those on the chromosome and thus production of D39 $HK06_{H242Y}$.

round PCR products were then joined by overlap extension PCR with oligonucleotides AS5/AS6, and the subsequent PCR product was transformed into D39*rr06*⁻. Correct substitutions were confirmed by sequencing (data not shown), and the strains designated D39 $HK06_{S241D}$, D39 $HK06_{H242R}$, D39 $RR06_{D51E}$ and D39 $RR06_{D51N}$.

6.2.3.2 Effect of amino acid substitutions on *cbpA* expression

D39, D39 $HK06_{H242N}$, D39 $HK06_{S241D}$, D39 $HK06_{H242Y}$, D39 $RR06_{D51E}$, and D39 $RR06_{D51N}$ were grown to identical culture densities in THY (A_{600} of 0.35) and RNA was extracted as described in Section 2.10.1. Real time RT-PCR was used to investigate any differences in *cbpA* mRNA levels, as described previously. D39 $HK06_{H242Y}$ and D39 $HK06_{H242R}$ showed increases in *cbpA* mRNA (5-fold and 7-fold, respectively) similar to that seen in D39Δ*hk06*, which lacks *hk06* entirely (**Figure 6.18**). However, D39 $HK06_{S241D}$ showed no statistically significant difference in *cbpA* mRNA compared to the control. This suggests that it is the lack of HK06 kinase activity which results in the increase in *cbpA* expression. *cbpA* expression was decreased in both D39 $RR06_{D51E}$ and D39 $RR06_{D51N}$ to a level similar to that of D39Δ*rr06*, although this reached statistical significance only for D39 $RR06_{D51E}$ (**Figure 6.19**). Whole cell lysates of the aforementioned strains were also subjected to Western immunoblot analysis with anti-CbpA (**Figure 6.20**). Similar differences were seen, although decreases were not as marked as those seen with real time RT-PCR.

6.2.3.3 Effect of Over-expression of $RR06_{D51E}$ and $RR06_{D51N}$ on CbpA expression

$RR06_{D51E}$ and $RR06_{D51N}$ were cloned into pLS1RGFP, in order to investigate the effect of their over-expression on CbpA. Oligonucleotides AS67 and AS68 were used to amplify the *rr06* gene using *S. pneumoniae* chromosomal DNA from D39 $RR06_{D51E}$ and D39 $RR06_{D51N}$ as the template. These PCR products were digested with *Nhe*I and subsequently ligated into the unique *Xba*I site of pLS1RGFP as described in Section

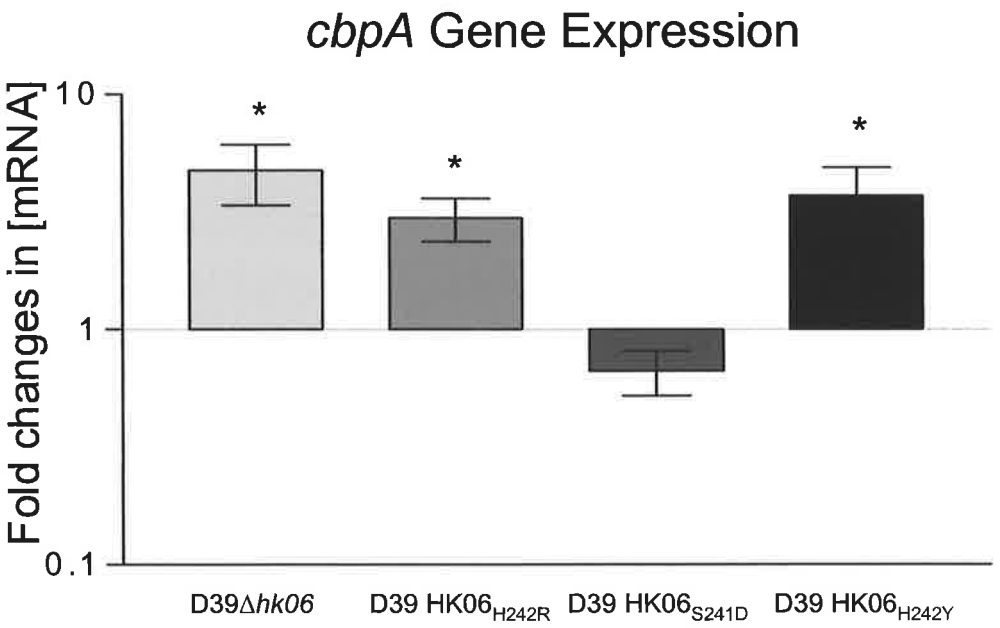

Figure 6.18 *cbpA* Gene Expression.

RNA was extracted from D39, D39Δ*hk06,* D39 $HK06_{H242Y}$, D39 $HK06_{S241D}$, D39 $HK06_{H242N}$, and [*cbpA* mRNA] was determined by real time RT-PCR. Data shown are fold increase (± standard error) in [*cbpA* mRNA] relative to D39. (* $P < 0.05$ as determined by One-way ANOVA with post-hoc Tuckey test).

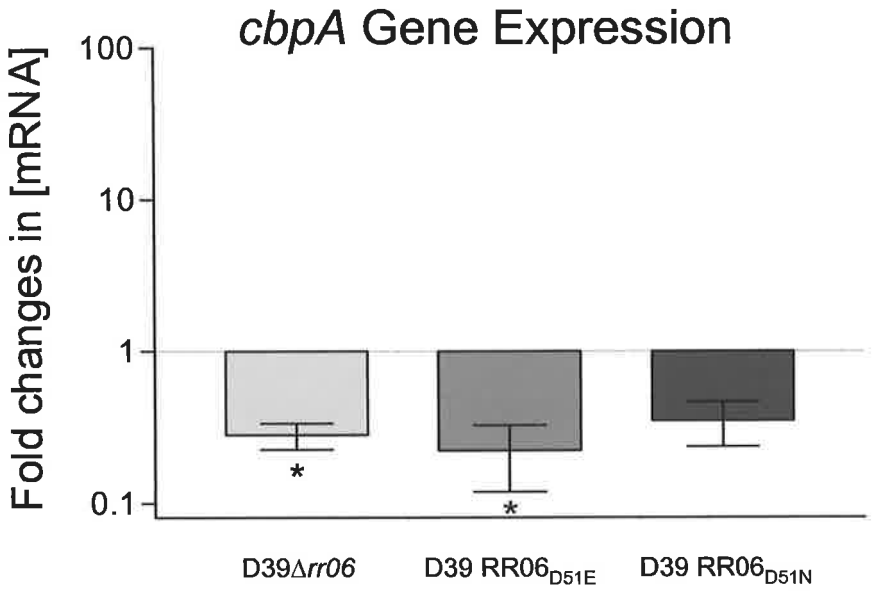

Figure 6.19 *cbpA* Gene Expression.

RNA was extracted from D39, D39Δ*rr06*, D39 $RR06_{D51E}$, D39 $RR06_{D51N}$, and [*cbpA* mRNA] was determined by real time RT-PCR. Data shown are fold increase (± standard error) in [*cbpA* mRNA] relative to D39. (* $P < 0.05$ as determined by One-way ANOVA with post-hoc Tuckey test).

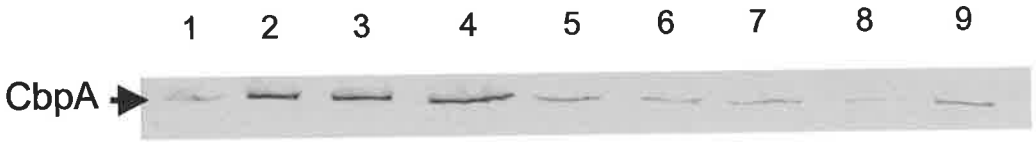

Figure 6.20. CbpA Western blot analysis.

Proteins in lysates of D39 (lane 1), D39Δ*hk06* (lane 2), D39 $HK06_{H242Y}$ (lane 3), D39 $HK06_{H242N}$ (lane 4), D39 $HK06_{S241D}$ (lane 5), D39Δ*rr06* (lane 6), D39 $RR06_{D51E}$ (lane 7), D39 $RR06_{D51N}$ (lane 8) and D39Δ*rr/hk06* (lane 9) were seperated by SDS-PAGE, transferred onto nitrocellulose and probed with anti-CbpA.

6.2.2.1. The ligation mix was transformed into *S. pneumoniae* R6, and possible transformants were selected on EryBA. Transformants were subsequently confirmed to contain the correct *rr06* by sequencing (data not shown). To ensure there would be no interference from choromosomal copies of *hk06* and *rr06*, pControl, p*rr06*, pRR06$_{D51E}$ and pRR06$_{D51N}$ were transformed into D39Δ*rr/hk06*. Strains were induced to over-express RR06 using 0.8% maltose as described previously in Section 6.2.2.1. Proteins in induced cell lysates of D39Δ*rr/hk06*:pControl, D39Δ*rr/hk06*:p*rr06*, D39Δ*rr/hk06*:pRR06$_{D51E}$ and D39Δ*rr/hk06*:pRR06$_{D51N}$ were subjected to Western blotting using anti-RR06. This confirmed the over-expression of RR06, RR06$_{D51E}$ and RR06$_{D51N}$ in D39Δ*rr/hk06* (**Figure 6.21A**).

To investigate *cbpA* mRNA levels in these strains, RNA extracted from induced cultures (Section 2.10.1) was subjected to real time RT-PCR using gene specific oligonucleotides. All constructs increased *cbpA* expression significantly, although *cbpA* expression in the strain carrying pRR06$_{D51N}$ was significantly less than in that carrying p*rr06* ($P < 0.05$) (**Figure 6.21B**). Western blot analysis of whole cell lysates of induced cultures of D39Δ*rr/hk06*:pControl, D39Δ*rr/hk06*:p*rr06*, D39Δ*rr/hk06*:pRR06$_{D51E}$ and D39Δ*rr/hk06*:pRR06$_{D51N}$ using anti-CbpA, gave similar results, and confirmed the lower production of CbpA in D39Δ*rr/hk06*:pRR06$_{D51N}$ (**Figure 6.21C**). Furthermore, D39Δ*rr/hk06*:pRR06$_{D51E}$ also appeared to produce less CbpA than D39Δ*rr/hk06*:pRR06, which was apparent by real time RT-PCR although this did not reach statistical significance.

6.2.4 Pro-Q Diamond staining

In Section 6.2.3, point mutations in HK06 and RR06 were constructed in an effort to alter the activities of these proteins. These substitutions were based on previous studies into other TCSTSs, and therefore may not have the expected effects on the activity of RR06 and HK06. Investigation of the phosphorylation state of RR06 in D39 HK06$_{H242Y}$,

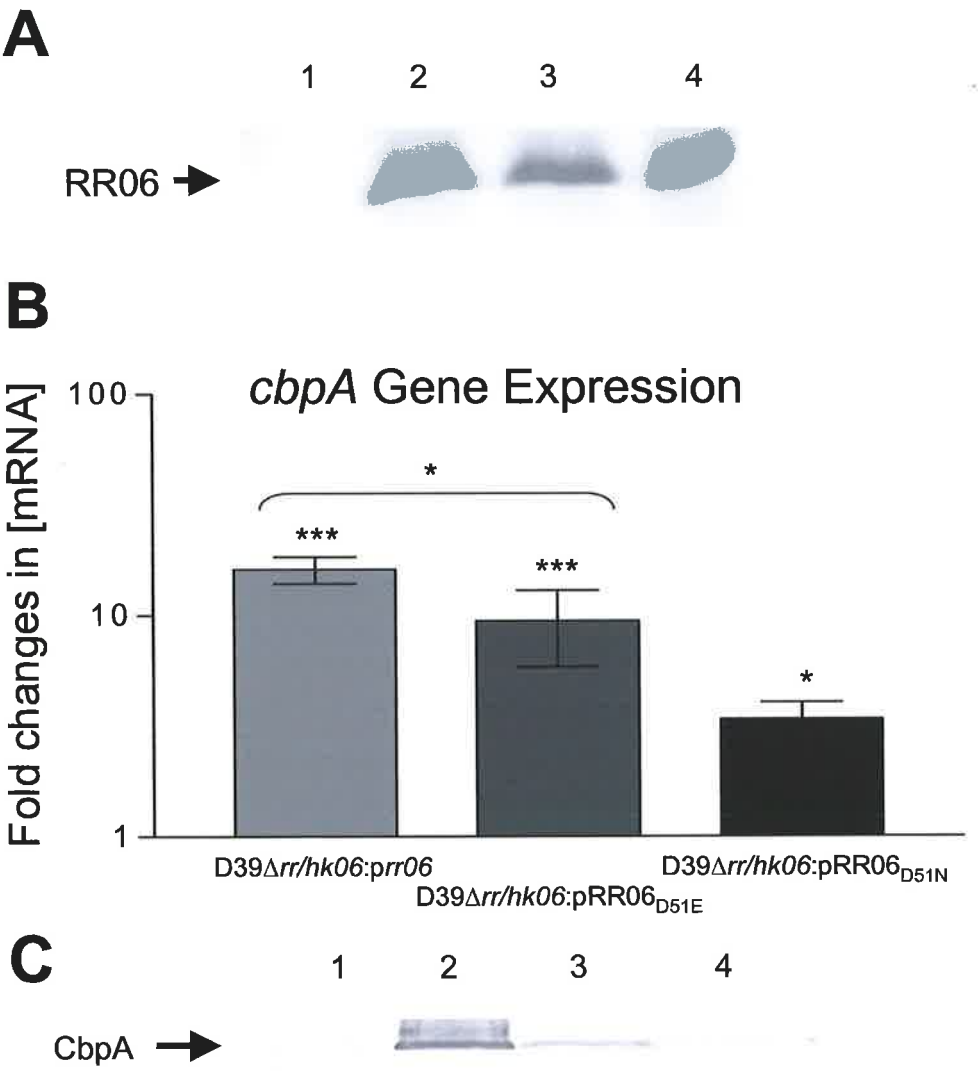

Figure 6.21. Over-expression of $RR06_{D51E}$ and $RR06_{D51N}$.

RNA and protein from lysates of D39Δ*rr/hk06*:pControl (lane 1), D39Δ*rr/hk06*:p*rr06* (lane 2), D39Δ*rr/hk06*:$pRR06_{D51E}$ (lane 3) and D39Δ*rr/hk06*: $pRR06_{D51N}$ (lane 4) were prepared as described in Section 2.10.1 and 2.8.2, respectively. Proteins were separated by SDS-PAGE, transferred to nitrocellulose and probed with anti-RR06 **(A)** or anti-CbpA **(C)**. RNA was analysed for [*cbpA* mRNA] by real time RT-PCR **(B)**. Data shown are fold increase (± standard error) in [*cbpA* mRNA] relative to D39. (* $P < 0.05$, *** $P < 0.01$ as determined by One-way ANOVA with post-hoc Tuckey test).

D39 $HK06_{H242R}$ and D39 $HK06_{S241D}$ would show whether these substitutions abrogated the expected activities of HK06. Pro-Q Diamond phosphoprotein stain binds directly to the phosphate moiety of phosphoproteins with high sensitivity, and thus it was thought this should enable the identification of the phosphorylation status of RR06. While this is specifically designed for the staining of phosphorylated Ser, Thr, and Tyr, no stain is currently available which specifically stains phosphorylated Asp residues. However, the manufacturer claimed that Pro-Q Diamond has the ability to stain phosphorylated Asp. To first investigate whether this stain would work, protein lysates from D39:pControl, D39:p*hk06*, D39:p*rr06*, and D39:p*rr/hk06* were separated by SDS-PAGE, and stained with ProQ Diamond (Section 2.8.4). It was thought that the increased level of RR06 should enable visualisation of phosphorylated RR06. However, there was no difference between the strains at the expected size of RR06 (data not shown). Asp phosphorylation is particularly labile, and thus the phosphorylation of RR06 may have been lost during electrophoresis.

6.3 Discussion

While the regulation of *cbpA* by the pneumococcal TCSTS RR/HK06 had previously been demonstrated, the mechanisms behind this were not understood. In particular, the reason why an increase in CbpA was evident in D39Δ*hk06* while a decrease was seen in D39Δ*rr06* was still unknown. In this Chapter a number of methods were used to investigate this in more detail. Mutation of the entire system in both D39 (in-frame deletion) and TIGR4 (Ery replacement) resulted in levels of CbpA similar to that seen in the wt. This result is difficult to explain. It suggests that differences seen in the *hk06* mutants of both D39 and TIGR4, along with D39Δ*rr06*, are due to the effect these mutations have on the cognate RR or HK, respectively. It is possible that another factor or TCSTS is involved in the regulation of *cbpA*, and with the deletion of *rr/hk06*, this takes

over. Recent research has shown that a pneumococcal ABC transporter is regulated by two separate TCSTSs (Knutsen *et al.*, 2004), and so such dual control is not without precedent in *S. pneumoniae*. However, further research is required to investigate this possibility.

Over-expression of TCSTS has been used successfully in the past to identify genes regulated by a number of systems (Ogura *et al.*, 2001). To over-express RR/HK06 a vector that allows maltose inducible over-expression was used. This vector, pLS1RGFP, was previously employed to investigate another pneumococcal TCSTS, YycFG (Mohedano *et al.*, 2005). When RR06 was over-expressed, both alone and in conjunction with HK06, an increase in CbpA was evident by both Western blot and real time RT-PCR. This suggested that RR/HK06 acts as an activator of CbpA, and provided further evidence for the regulation of this major virulence factor by this system. Similar results were seen when the over-expression plasmids were transformed into another pneumococcal strain, TIGR4. When p*rr06*, which directs over-expression of RR06 alone, was transformed into two further strains, differences were still evident by Western blot, although interestingly in WCH4861 over-expression of RR06 led to a decrease in CbpA. Sequencing did not identify any major differences in the upstream region of *cbpA* in this strain, suggesting that there is unlikely to be differential binding of RR06 to this region. However, it is possible that other factors present in WCH4861 may explain this. What this data does show, however, is that RR/HK06 regulates the expression of the major virulence factor CbpA across four different strains.

Most response regulators have been shown to be active in the phosphorylated form, as illustrated by the fact that mutations in the cognate histidine kinase result in a reduction in regulated gene expression. Previous research presented in Chapter Three showed that an in-frame *hk06* deletion mutant (D39Δ*hk06*) actually resulted in increased levels of *cbpA*, suggesting the possibility that this system did not follow the norm, and may activate CbpA in the non-phosphorylated form. In the current Chapter, the over-expression of *hk06* led to

no change in *cbpA* expression. Assuming HK06 has predominantly kinase activity, over-expression would not result in an increase in non-phosphorylated RR06, and thus no increase in *cbpA*. This would explain why the deletion of *hk06*, removing this kinase activity, results in an increase in *cbpA* mRNA. However, the increased *cbpA* expression seen in D39:p*rr/hk06* was unexpected. If HK06 has predominantly kinase activity, over-expression of the entire system would result in high levels of phosphorylated RR06, and thus would not be expected to increase *cbpA* expression. It is possible that the overproduction of HK06 titrates the as yet unknown signal responsible for activation of the HK, leaving phosphatase activity to predominate.

In order to further investigate the hypothesis that non-phosphorylated RR06 activates *cbpA*, point mutations in HK06 and RR06 were constructed to abrogate the activities of these molecules. These substitutions were based on previous studies into other TCSTSs, and as such it is worth noting that there is no certainty they would have the same effects on HK06 and RR06. Staining using Pro-Q Diamond was not successful in investigating the phosphorylation state of RR06, and thus further biochemical characterisation is needed to ensure that the phenotypes of these strains are as predicted.

Amino acid substitutions in HK06 predicted to mutate kinase and phosphatase or simply kinase activities of HK06 resulted in increased levels of *cbpA* similar to that seen with D39Δ*hk06*. However, D39 $HK06_{S241D}$, predicted to retain kinase but not phosphatase activity, showed a level of *cbpA* mRNA comparable to the wt D39. This suggests that the phosphatase activity or loss of kinase activity is critical to the ability of RR/HK06 to increase *cbpA* expression. In other words, this further implies that the non-phosphorylated form of RR06 activates *cbpA* expression. While activation by phosphorylation is the norm, the DegU response regulator from *B. subtilis* has been shown to function in either the phosphorylated or non-phosphorylated form depending on the target gene (Dahl *et al.*, 1992). Additionally, phosphorylation has been shown not to be essential for the activation

of a number of response regulators (Molle & Buttner, 2000; Geng *et al.*, 2004; Perron-Savard *et al.*, 2005).

Substitutions in the conserved Asp_{51} of RR06 were predicted to mimic the phosphorylated (D39 $RR06_{D51E}$) or non-phosphorylated (D39 $RR06_{D51N}$) forms. Both showed decreases in *cbpA* mRNA compared to the wt although only D39 $RR06_{D51E}$ reached statistical significance. This did not seem to correlate with substitutions in HK06 that suggested RR06 activated *cbpA* expression in the non-phosphorylated form. Therefore $RR06_{D51E}$ and $RR06_{D51N}$ were over-expressed in order to investigate their effect on *cbpA* expression. It was hypothesised that over-expression of both D39 $RR06_{D51E}$ and D39 $RR06_{D51N}$ should lead to increased CbpA due to the quantity of RR06 present. These plasmids were transformed into D39Δ*rr/hk06*, in order to eliminate any interference from the chromosomal copies of *hk06* and *rr06*. While both RR06 variants, along with the wt RR06, increased *cbpA* expression significantly, induction of *cbpA* by $RR06_{D51N}$ was significantly lower than by wt RR06. This suggests that $RR06_{D51N}$, as well as being unable to be phosphorylated, is also deficient in its ability to bind target DNA and increase expression. This would explain why D39 $RR06_{D51N}$, containing a single copy on the chromosome, does not lead to an increase in *cbpA* expression as expected if RR06 activates *cbpA* expression in the non-phosphorylated form.

This Chapter has provided further insight into the regulation of *cbpA* by RR/HK06, illustrating the cross strain regulation of *cbpA* by RR/HK06, primarily as an activator of transcription. This Chapter has also demonstrated that the over-expression of RR/HK06 can help in identifying regulated genes. Furthermore, research has suggested that RR06 may bind and activate transcription of *cbpA* in the non-phosphorylated state.

CHAPTER SEVEN – GLOBAL REGULATION BY RR/HK06

7.1 Introduction

In Chapters Three and Four, it was shown that one of the thirteen TCSTSs in *S. pneumoniae*, RR/HK06, regulates the expression of *cbpA*, a major virulence factor and protective antigen. Results from Chapter Five showed that the system plays a role in both epithelial cell adherence and pathogenesis. However, the same studies suggested that these effects were likely due to additional RR/HK06-regulated factors yet to be identified. Thus, this Chapter concentrates on identifying these factors, in order to a gain a greater understanding of the regulation mediated by this TCSTS.

7.2 Results

7.2.1 Microarray analysis

7.2.1.1 Comparison of D39:pControl and D39:p*rr06*

The over-expression of TCSTSs has been shown to be a useful tool for identifying regulated genes (Ogura *et al.*, 2001; Mohedano *et al.*, 2005). Indeed, this was shown in Chapter Six, when over-expression of RR06 and RR/HK06 led to significant increases in expression of *cbpA*, a gene we had previously shown to be regulated by the system in

Chapters Three and Four. Thus, in order to identify additional genes regulated by the system, microarray analysis was performed to investigate gene expression changes when RR06 was over-expressed in D39. D39:pControl and D39:p*rr06* were grown in THY to A_{600} = 0.25, centrifuged for 15 min at RT, and then resuspended in THY supplemented with 0.8% maltose. After 30 min, cultures were pelleted and RNA was extracted (Section 2.10.1). Microarray analysis was then undertaken as described in Section 2.10.5 using two biological replicates, along with dye swaps in order to confirm that any differences seen were not due to dye bias. Changes in expression of a given gene was deemed to be significant if the gene exhibited at least a two-fold difference in signal intensity between D39:pControl and D39:p*rr06* (with the exception of genes likely to be co-transcribed), and if this difference was found to be statistically significant ($P < 0.05$) as determined by *t*-tests (Section 2.10.5). While the majority of genes showed comparable expression in both strains, a total of 52 genes (approximately 2.5% of all pneumococcal genes) showed significant differences in expression. A complete list of genes with significantly altered transcription between D39:pControl and D39:p*rr06* is shown in **Table 7.1**. Increased transcription of both *rr06* and *cbpA* was seen in D39:p*rr06* relative to D39:pControl as expected. Transcription of the gene found immediately upstream of *cbpA*, designated *sp2191* in the TIGR4 genome sequence annotation (or *spr1996* in the R6 genome), was also increased. In order to determine whether these two genes are on the same transcript, RT-PCR on D39 mRNA was undertaken using oligonucleotides AS105 and CbpARedit (Table 2.2), which bind within *sp2191* and *cbpA*, respectively. This produced a band of 956-bp (**Figure 7.1**) indicating that these two genes are indeed co-transcribed.

Among genes with decreased expression in D39:p*rr06* relative to D39:pControl were the genes encoding a major virulence factor and protective antigen *pspA*, another pneumococcal TCSTS *vncRS*, and a putative alkaline shock protein *gls24*. The over-

Table 7.1. Genes differentially regulated with over-expression of *rr06* in D39.

Gene[a]	Mean Fold Change[b]	*P* value[c]	Annotation[d]
D39 upregulated			
Sp1826	2.29	0.0292	ABC transporter
Spr1995	7.97	0.0013	*cbpA*
Sp2191	16.68	0.0034	hypothetical protein
Sp2193	57.48	0.0014	*rr06*
D39 downregulated			
Spr0111	2.29	0.0421	hypothetical protein
Spr0112	2.04	0.1250	hypothetical protein
Spr0113	2.72	0.0294	hypothetical protein
Spr0115	2.44	0.0333	hypothetical protein
Spr0116	1.55	0.5910	hypothetical protein
Spr0117	2.60	0.1340	hypothetical protein
Spr0118	2.27	0.0120	hypothetical protein
Spr0119	1.74	0.1800	hypothetical protein
Spr0121	8.57	0.0387	*pspA*
Sp0060	70.03	0.0010	beta-galactosidase
Sp0061	62.47	0.0013	PTS system, mannose-specific IIB component
Sp0062	33.47	0.0004	PTS system, mannose-specific IIC component
Sp0063	36.50	0.0056	PTS system, mannose-specific IID component
Sp0064	7.24	0.2470	PTS system, mannose-specific IIA component
Sp0065	27.00	0.0120	sugar isomerase domain protein *AgaS*
Sp0066	42.96	0.0042	aldose 1-epimerase
Sp0368	31.89	0.0068	hypothetical protein
Sp0409	2.94	0.0084	hypothetical protein
Sp0603	3.42	0.0101	*vncR*
Sp0604	3.28	0.0162	*vncS*
Sp0648	3.41	0.0232	beta-galactosidase
Sp0703	4.89	0.0133	hypothetical protein
Sp0704	5.56	0.0428	hypothetical protein
Sp0705	5.90	0.0051	hypothetical protein
Sp0706	5.64	0.0082	hypothetical protein
Sp0707	4.17	0.0184	ABC transporter, ATP-binding protein
Sp0708	3.49	0.1370	hypothetical protein
Sp0709	5.30	0.0019	amino acid ABC transporter, ATP-binding protein
Sp0710	2.94	0.0673	amino acid ABC transporter, ATP-binding protein
Sp0711	3.02	0.1450	amino acid ABC transporter, ATP-binding protein
Sp0726	2.98	0.0378	phosphomethylpyrimidine kinase
Sp0923	2.27	0.0483	Cof family protein
Sp1470	7.41	0.0341	thiamine biosynthesis protein *ApbE*
Sp1471	6.19	0.0866	oxidoreductase
Sp1472	7.84	0.0262	oxidoreductase
Sp1708	5.70	0.0149	hypothetical protein
Sp1714	2.39	0.0291	transcriptional regulator, GntR family
Sp1715	3.77	0.0190	amino acid ABC transporter, ATP-binding protein
Sp1801	12.13	0.0043	hypothetical protein
Sp1802	7.67	0.0086	hypothetical protein
Sp1803	13.64	0.0046	hypothetical protein
Sp1804	9.42	0.0191	general stress protein 24, putative (*gls24*)
Sp1805	9.61	0.0157	hypothetical protein

Gene[a]	Mean Fold Change[b]	P value[c]	Annotation[d]
Sp1806	2.91	0.0497	hypothetical protein
Sp1852	9.92	0.0025	galactose-1-phosphate uridylyltransferase
Sp1853	4.35	0.1210	galactokinase
Sp1854	7.57	0.0015	galactose operon repressor
Sp2105	4.48	0.0371	hypothetical protein

a. Gene designation that matches microarray ORFs; Sp relates to TIGR4 sequence (GenBank Accession Number NC_003028), Spr relates to R6 sequence (GenBank Accession Number AE007317).
b. Intensity ratios of p*rr06*/pControl in microarray experiments. Genes with > two fold expression changes were selected.
c. *P* value represents the mean *P* value calculated by *t*-tests between the intensity of the individual spots.
d. Annotations as published in the TIGR4 genome.

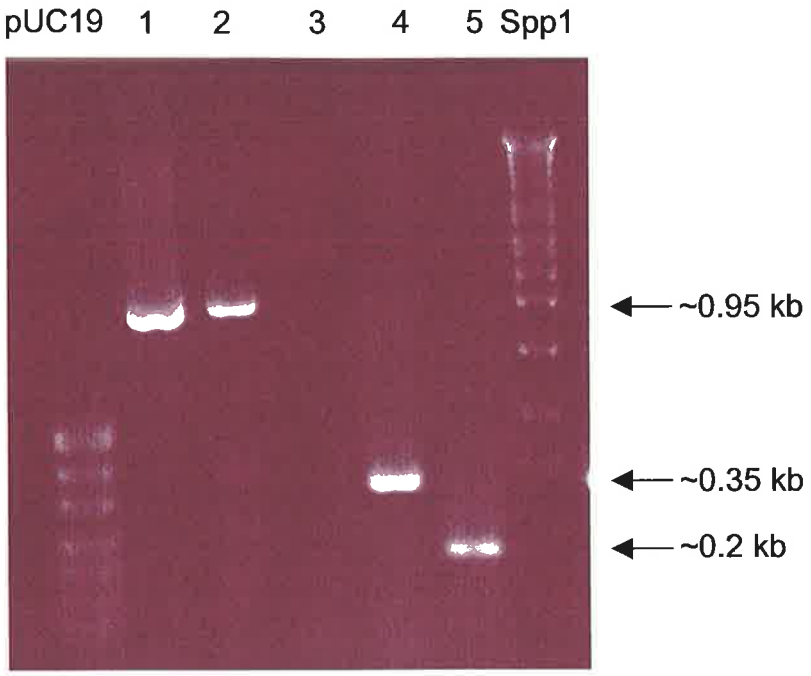

Figure 7.1. *cbpA* and *sp2191* are co-transcribed.

DNA (lane 1) and RNA (lanes 2, 3, 4 and 5) isolated from D39 *S. pneumoniae* as described in Sections 2.7.2 and 2.10.1 were subjected to RT-PCR with oligonucleotides CbpARedit & AS105 (lanes 1, 2 and 3), AS105 & AS106 (lane 4) and CbpAFnew & CbpARedit (lane 5). Oligonucleotides CbpARedit & AS105, which amplify a 956-bp region between the coding regions of *cbpA* and *sp2191*, produced products of expected size with both DNA and RNA, suggesting that these genes are co-transcribed. Lane 3 contained no reverse transcriptase and illustrated the absence of any contaminating DNA in the D39 RNA. AS105 & AS106 produce an approximately 350-bp internal fragment of *sp2191* and CbpAFnew & CbpARedit produce an approximately 200-bp internal fragment of *cbpA*.

expression of *rr06* did not appear to affect the expression of its cognate histidine kinase, *hk06*, confirming that the system does not auto-regulate its own transcription.

To validate the results from the microarray analysis, real time RT-PCR was carried out on RNA from D39:pControl and D39:p*rr06* using specific oligonucleotides against *sp0060* (AS91 & AS92), *sp0368* (AS93 & AS94), *sp0703*(AS97 & AS98), *sp1472*(AS99 & AS100), *sp1852* (AS103 & AS104), *sp1801*(AS101 & AS102), *sp2191*(AS105 & AS106) and *hk06* (HKF & HKR). Using this method, similar differences in expression of these genes between D39:pControl and D39:p*rr06* were evident (**Figure 7.2**).

7.2.1.2 Comparison of TIGR4:pControl and TIGR4:p*rr06*

In order to see if regulation by RR/HK06 is similar across serotypes, microarray analysis was also undertaken in the TIGR4 background. TIGR4:pControl and TIGR4:p*rr06* were grown to A_{600} = 0.15 in THY, pelleted at RT, and resuspended in THY supplemented with 0.8% maltose. After 30 min, the cells were pelleted and RNA was extracted as described in Section 2.10.1. The results presented are from three biological replicates, each with a dye swap to eliminate the possibility of dye bias. Microarray and subsequent analysis were undertaken as described in Section 2.10.5. As with investigation of *S. pneumoniae* TCSTS TCS04 (McCluskey *et al.*, 2004) there appeared to be a marked difference in regulation between *S. pneumoniae* D39 and TIGR4. However, while McCluskey *et al.* (2004) found no similarities in genes regulated by TCS04 between D39 and TIGR4, our studies illustrated that both *cbpA* and *sp2191* still showed a statistically significant mRNA increase when *rr06* was over-expressed (**Table 7.2**). However, no other differences in gene expression were common to these two strains. Indeed, only three additional genes were differentially expressed in TIGR4 – two hypothetical proteins and a bacteriocin formation protein (**Table 7.2**). Investigation into the upstream regions of a number of genes apparently regulated by RR/HK06 in D39, but not in TIGR4, such as *pspA*, did not show any major nucleotide differences (data not shown). This suggested that

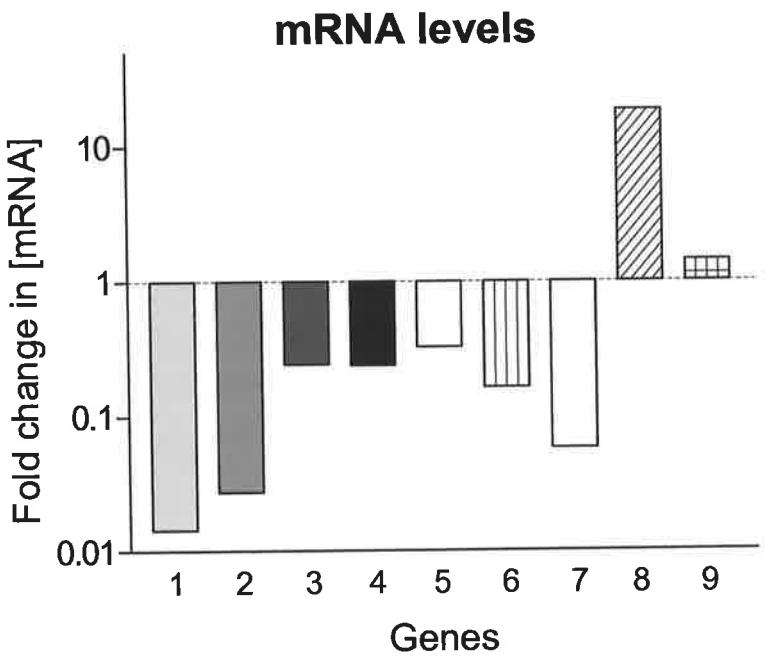

Figure 7.2. Analysis of mRNA levels by real time RT-PCR.

RNA was isolated from induced D39:pControl and D39:p*rr06* and analysed for differences in mRNA levels in D39:p*rr06* relative to D39:pControl of the following genes using real time RT-PCR: (1) *Sp0060*, (2) *Sp0368*, (3) *Sp0703*, (4) *Sp1472*, (5) *Sp1852*, (6) *Sp1801*, (7) *Sp1804*, (8) *Sp2191* and (9) *hk06*. Data show are fold change in [mRNA] relative to D39:pControl.

Table 7.2. Genes differentially regulated with over-expression of *rr06* in TIGR4.

Gene[a]	Mean Fold Change[b]	*P* value[c]	Annotation[d]
TIGR4 upregulated			
Sp2190	5.16	0.0002	*cbpA*
Sp2191	7.14	0.0019	hypothetical protein
Sp2193	19.31	0.0026	*rr06*
TIGR4 downregulated			
Sp1949	2.60	0.0285	hypothetical protein
Sp1950	2.31	0.0276	bacteriocin formation protein
Sp1954	2.05	0.0277	hypothetical protein

a. Gene designation that matches microarray ORFs; Sp relates to TIGR4 sequence (GenBank Accession Number NC_003028).
b. Intensity ratios of p*rr06*/pControl in microarray experiments. Genes with > two fold expression changes were selected.
c. *P* value represents the mean *P* value calculated by *t*-tests between the intensity of the individual spots.
d. Annotations as published in the TIGR4 genome.

the difference in gene expression profile was unlikely to be due to alterations in the DNA binding site of RR06.

7.2.2 Vancomycin resistance

Microarray analysis comparing D39:pControl and D39:p*rr06* described in Section 7.2.1.1 found that expression of another pneumococcal TCSTS, VncRS, was repressed when RR06 was over-expressed. Interestingly, RR/HK06 and VncRS have previously been shown to be closely related (Lange *et al.*, 1999). Both also share homology to VanRS from *Enterococcus faecalis*, which is responsible for the regulation of genes involved in the development of vancomycin resistance (Arthur *et al.,* 1992). Although *S. pneumoniae* possesses no homologues of the genes regulated by VanRS that are responsible for vancomycin resistance, work was undertaken to investigate whether RR/HK06 affected susceptibility to vancomycin. D39, D39Δ*hk06*, D39Δ*rr06*, D39Δ*rr/hk06* and D39:pControl, D39:p*hk06*, D39:p*rr06*, D39:p*rr/hk06* were inoculated onto BA such that sub-confluent growth resulted. Discs containing vancomycin at concentrations of 5 μg, 0.5 μg and 0.05 μg were added prior to incubation O/N at 37°C. All strains showed similar zones of inhibition around 5 and 0.5 μg, while no zones of inhibition were evident around 0.05 μg discs. Thus, this result suggested that all strains were equally susceptible to vancomycin, and that RR/HK06 is unlikely to be involved in modulating resistance or tolerance to vancomycin.

7.2.3 Comparative protein expression

7.2.3.1 D39 *S. pneumoniae*

In order to investigate changes in expression at the level of translation, proteins from cell lysates were separated by SDS-PAGE and stained with Coomassie brilliant blue as described in Section 2.8.1. Comparison of protein expression in D39, D39Δ*hk06*, D39Δ*rr06*, and D39Δ*rr/hk06* identified the presence of an approximately 32kDa band in

D39Δ*hk06* which was absent from the protein profiles of D39, D39Δ*rr06*, and D39Δ*rr/hk06* (**Figure 7.3**). When profiles of D39:pControl, D39:p*hk06*, D39:p*rr06* and D39:p*rr/hk06* were compared, a band of similar size to that seen in D39Δ*hk06* was evident in D39:pControl, but absent from the protein profiles of D39:p*hk06*, D39:p*rr06* and D39:p*rr/hk06* (**Figure 7.4**).

In order to identify this protein, the band was excised from the acrylamide gel, and sent to the Australian Proteome Analysis Facility (Sydney, Australia) for N-terminal aa sequencing. Results of the sequencing are provided in **Figure 7.5**. Blast analysis showed that the sequencing result matched only one protein, Gls24 the product of *Sp1804* from *S. pneumoniae* TIGR4 genome sequence (GenBank Accession Number NC_003028). The predicted size of this protein was 21kDa, approximately 11kDa less than the size seen on the Coomassie stained SDS-PAGE gel. Other proteins from *S. pneumoniae* are known to migrate in SDS-PAGE at anomalous molecular weights. The gene encoding this protein had been shown to be decreased approximately 10-fold in D39:p*rr06* compared to D39:pControl in microarray analysis (**Table 7.1**). Real time RT-PCR using gene specific oligonucleotides against *gls24* (AS81 & AS82; Table 2.2) was undertaken to investigate the levels of *gls24* mRNA in D39, D39Δ*hk06*, D39Δ*rr06* and D39Δ*rr/hk06*. This illustrated that transcription of *gls24* was increased substantially (50-fold) in D39Δ*hk06* (**Figure 7.6**) while there did not appear to be any apparent difference in D39Δ*rr06* and D39Δ*rr/hk06* compared to the parent strain, correlating with the results seen on the Coomassie stained SDS-PAGE.

This protein shows homology to proteins from *Enterococcus faecalis* (79%) and *Lactococcus lactis* (85%) associated with alkaline shock response (Giard *et al.*, 2000). Recent studies in *E. faecalis* illustrated that this protein is important in bile-salt resistance, and is a possible protective antigen (Teng *et al.*, 2005). Therefore, it was thought that the increased level of Gls24 in D39Δ*hk06* may lead to an increased ability for *S. pneumoniae*

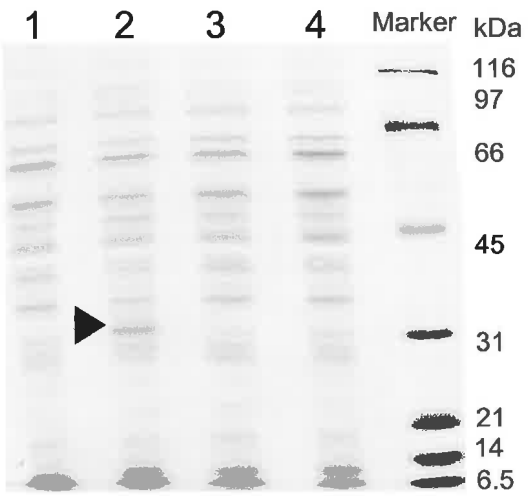

Figure 7.3. SDS-PAGE analysis of D39, D39Δ*hk06*, D39Δ*rr06* and D39Δ*rr/hk06*.

Proteins in lysates of (1) D39, (2) D39Δ*hk06*, (3) D39Δ*rr06* & (4) D39Δ*rr/hk06* grown in THY were separated by SDS-PAGE and stained with Coomassie brilliant blue (Section 2.8.1). The black arrowhead indicates the protein band shown by sequencing to be Gls24.

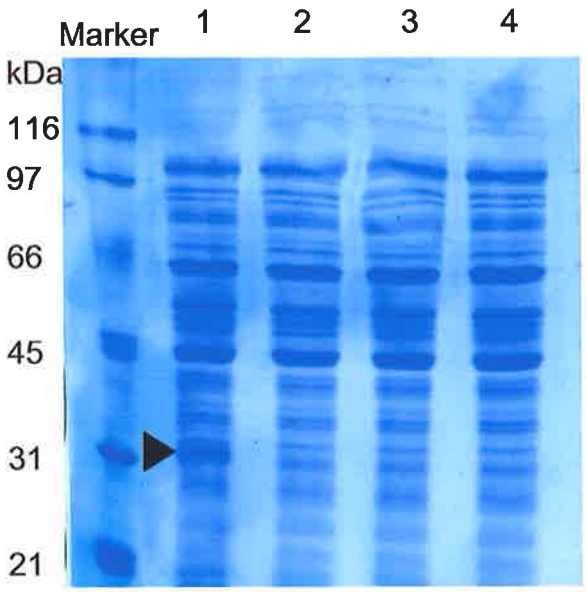

Figure 7.4. SDS-PAGE analysis of D39:pControl, D39:p*hk06*, D39:p*rr06* and D39:p*rr/hk06*.

Proteins in lysates of (1) D39:pCONTROL, (2) D39:p*hk06*, (3) D39:p*rr06* & (4) D39:p*rr/hk06* were separated by SDS-PAGE and stained with Coomassie brilliant blue (Section 2.8.1). The black arrowhead indicates the protein band shown by sequencing to be Gls24.

Cycle Number	Major Signal	Minor Signal
1	-	
2	N	
3	E	
4	K	
5	N	
6	T	F
7	N	
8	T	S, V, A
9	N	
10	V	

Figure 7.5. Protein sequencing.

The protein band from D39Δ*hk06* designated by an arrowhead in **Figure 7.3** was excised and sent to the Australian Proteome Analysis Facility for sequencing. The results for the first ten amino acids are shown.

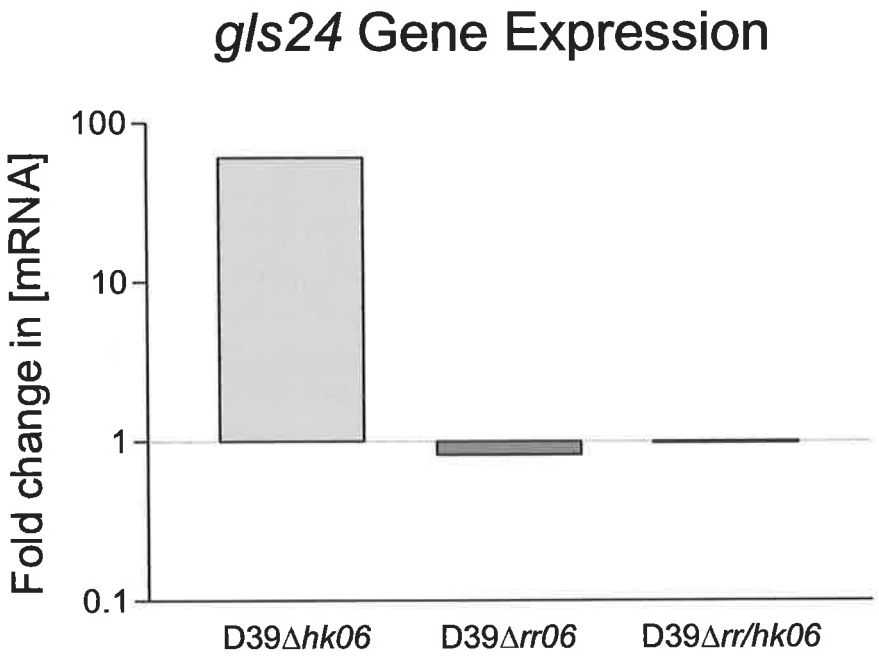

Figure 7.6. Analysis of *gls24* mRNA.

RNA was isolated from D39, D39*hk06*, D39*rr06*, and D39*rr/hk06* and real time RT-PCR (Section 2.10.4) was used to compare levels of *gls24* mRNA using specific oligonucleotides (AS81 & AS82; Table 2.2). Data shown are fold change in [*gls24* mRNA] relative to D39.

to grow in basic conditions. For this reason, the growth of D39 and D39Δ*hk06* in THY adjusted to pH 7.5, 8.0, 8.5 or 9.0 was investigated. However, there were no substantial differences in the abilities of D39 and D39Δ*hk06* to grow under any of these conditions tested, as seen in **Figure 7.7**.

As bile susceptibility is a hallmark of *S. pneumoniae*, work was undertaken to investigate whether the increased level of Gls24 altered the susceptibility of D39Δ*hk06* to the bile salt deoxycholate. D39 and D39Δ*hk06* were inoculated onto BA such that sub-confluent growth resulted. Discs containing deoxycholate at 1%, 0.5% and 0.25% were added prior to incubation O/N at 37°C. D39 and D39Δ*hk06* showed similar zones of inhibition around the 1% and 0.5% discs, while no zones of inhibition were evident around 0.25%. Thus, this suggested that the strains were equally susceptible to deoxycholate.

7.2.3.2 TIGR4, WCH4832, and WCH4861 *S. pneumoniae*

When protein profiles of TIGR4, TIGR4*hk06*, TIGR4*rr06,* and TIGR4*rr/hk06* were compared, a band corresponding to Gls24 was not increased in TIGR4*hk06* as had been previously seen in D39Δ*hk06* (**Figure 7.8**). Additionally, when proteins from lysates of TIGR4 *S. pneumoniae* harbouring pControl, p*hk06*, p*rr06*, and p*rr/hk06* were compared on Coomassie brilliant blue-stained SDS-PAGE gels, there was no apparent difference in protein profile, unlike that shown for D39 above (**Figure 7.9**). This was expected, as microarray analysis (**Table 7.2**) between TIGR4 and TIGR4:p*rr06* suggested that both expressed similar levels of *gls24* mRNA.

The protein profiles of WCH4832:pControl and WCH4832:p*rr06*, and WCH4861:pControl and WCH4861:p*rr06* were also compared. As in TIGR4, and unlike D39, there appeared to be no major differences (**Figure 7.10**). Thus, these results would suggest that Gls24 expression was only affected by RR/HK06 system in D39, and not in TIGR4, WCH4832 or WCH4861.

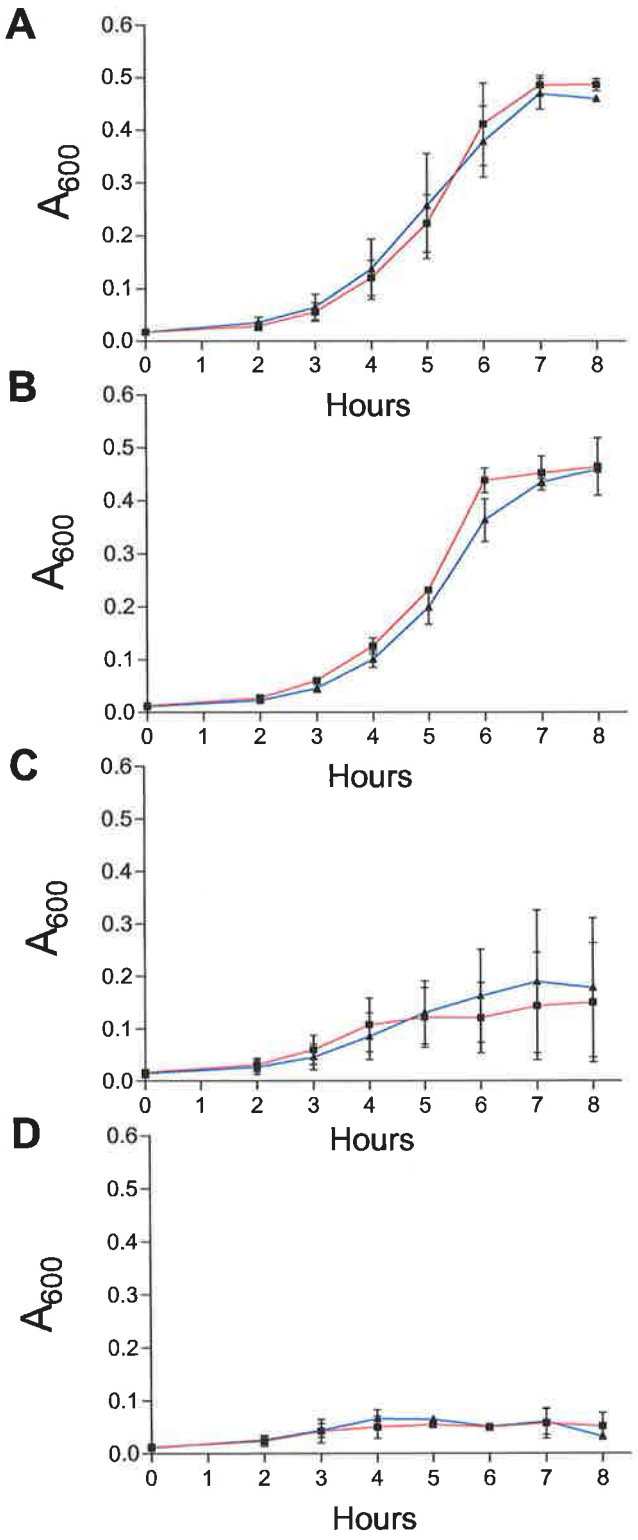

Figure 7.7. Growth of D39 and D39Δ*hk06* at basic pH.

D39 (Red) and D39Δ*hk06* (Blue) were grown O/N on BA and innoculated into 10 ml THY broth at pH (A) 7.5, (B) 8.0, (C) 8.5 and (D) 9.0. Culture density (A_{600}) was measured over the following eight h. Mean ± standard error of 2 experiments are shown.

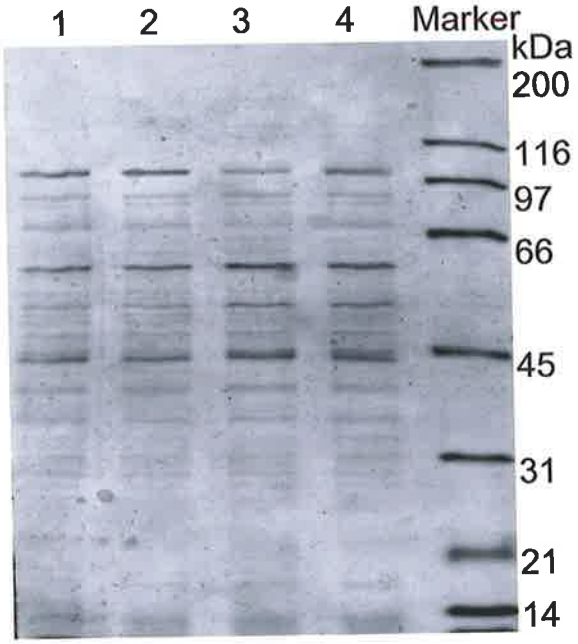

Figure 7.8. SDS-PAGE analysis of TIGR4, TIGR4*hk06*, TIGR4*rr06* and TIGR4*rr/hk06*.

Proteins in lysates of (1) TIGR4, (2) TIGR4*hk06*, (3) TIGR4*rr06* & (4) TIGR4*rr/hk06* were separated by SDS-PAGE and stained with Coomassie brilliant blue (Section 2.8.1).

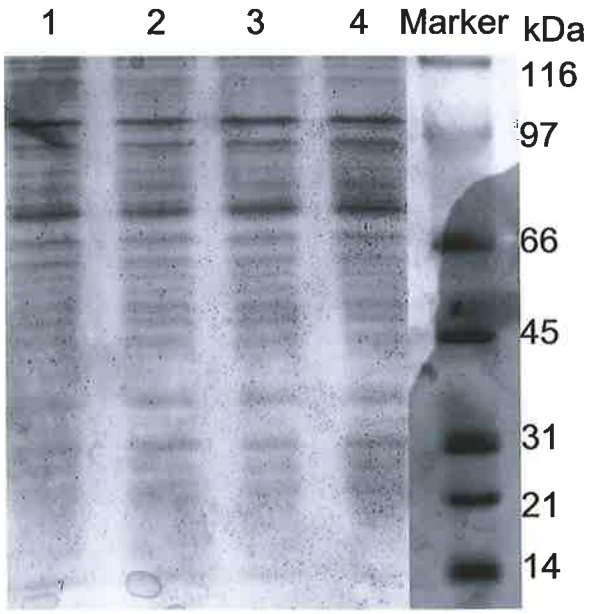

Figure 7.9. SDS-PAGE analysis of TIGR4:pControl, TIGR4:p*hk06*, TIGR4:p*rr06* and TIGR4:p*rr/hk06*.

Proteins in lysates of (1) TIGR4, (2) TIGR4*hk06*, (3) TIGR4*rr06* & (4) TIGR4*rr/hk06* were separated by SDS-PAGE and stained with Coomassie brilliant blue (Section 2.8.1).

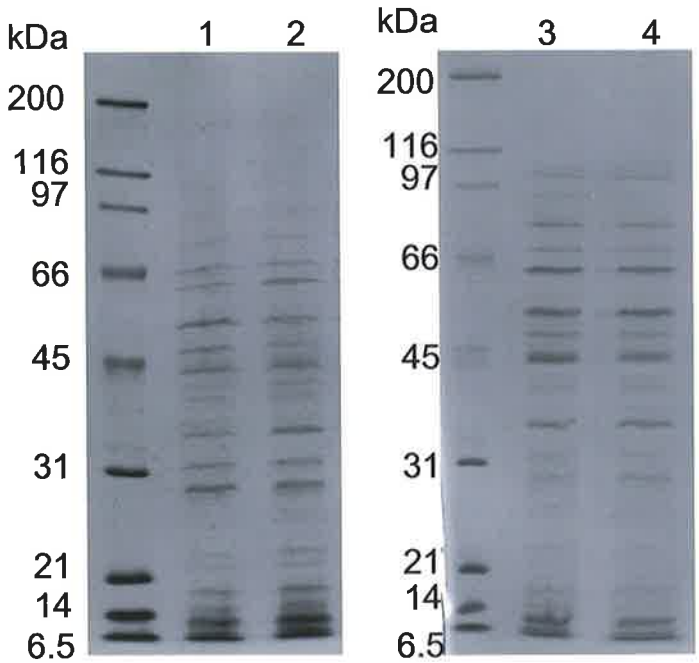

Figure 7.10. SDS-PAGE analysis of WCH4832:pControl, WCH 4832:p*rr06*, WCH4861:pControl and WCH4861:p*rr06*.

Proteins in lysates of (1) WCH4832:pControl, (2) WCH48321:p*rr06*, (3) WCH4861:pControl and (4) WCH4861:p*rr06* were separated by SDS-PAGE and stained with Coomassie brilliant blue (Section 2.8.1).

7.2.4 Effect of RR/HK06 on *pspA* Expression

Data presented above suggested that RR/HK06 regulated expression of *pspA* in D39. In order to further examine this effect, the protein and mRNA levels of PspA in D39:pControl, D39:p*hk06*, D39:p*rr06* and D39:p*rr/hk06* was investigated using Western immunoblot analysis and real time RT-PCR. Strains were grown as described previously (Section 6.2.2.1), equal numbers of cells were harvested, resuspended in lysis buffer, and subjected to SDS-PAGE and Western immunoblot analysis using anti-PspA This demonstrated that PspA was down-regulated in D39p*hk06*, D39p*rr06* and D39p*rr/hk06* (**Figure 7.11A**). Real time RT-PCR using *pspA*-specific oligonucleotides (PspAF & PspAR; Table 2.2) was then undertaken on RNA extracted from these strains, as described previously. In D39:p*hk06*, D39:p*rr06* and D39:p*rr/hk06* there was a statistically significant down-regulation of *pspA* mRNA compared to D39:pControl (**Figure 7.11B**). However, the down-regulation seen with HK06 over-expression was significantly lower than with over-expression of RR06 ($P < 0.05$). Over-expression of RR/HK06 also appeared to repress *pspA* to a greater extent than HK06, although this did not reach statistical significance. These results indicate that the RR/HK06 system acts as a repressor of *pspA*.

To examine whether the over-expression of *hk06* led to a decrease in *pspA* due to its effect on the chromosomal copy of *rr06*, pControl and p*hk06* were transformed into an in-frame *rr06* deletion mutant of D39 (D39Δ*rr06*), and selected with Ery; confirmed transformants were designated D39Δ*rr06*:pControl and D39Δ*rr06*:p*hk06*, respectively. Strains were grown in THY to A_{600} = 0.25, pelleted and then resuspended in THY supplemented with 0.8% maltose. After 30 min, cells were pelleted and RNA extracted (Section 2.10.1). Real time RT-PCR was then used to investigate the levels of *pspA* mRNA in D39Δ*rr06*:pControl and D39Δ*rr06*:p*hk06*, as described above. The level of *pspA* mRNA did not vary substantially between D39Δ*rr06*:pControl and D39Δ*rr06*:p*hk06*

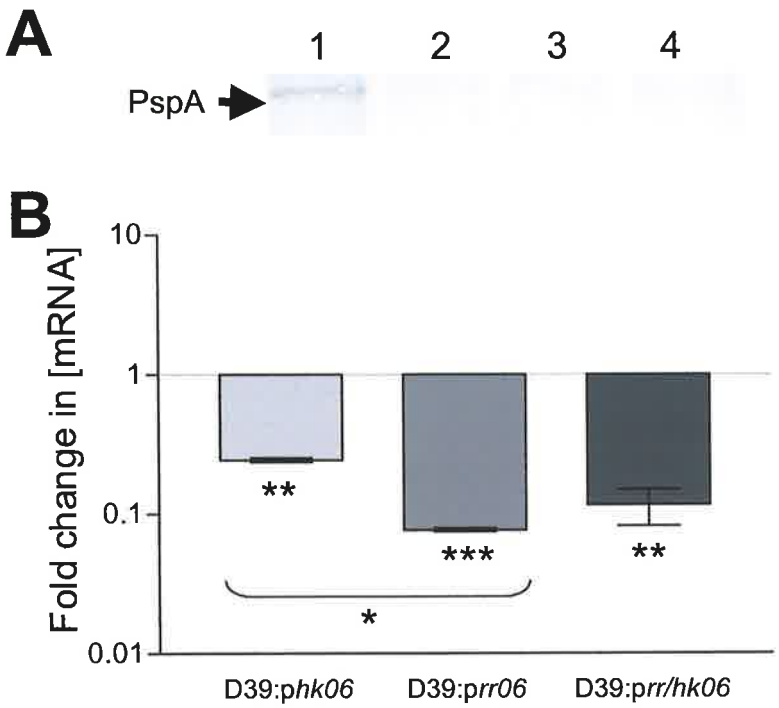

Figure 7.11. Analysis of PspA expression in D39.

(A) Proteins in lysates of (1) D39:pControl, (2) D39:p*hk06*, (3) D39:p*rr06* & (4) D39:p*rr/hk06* grown in THY, as described in Section 6.2.2.1 were separated by SDS-PAGE, transferred onto nitrocellulose and probed with polyclonal murine anti-PspA serum.

(B) RNA extracted from D39 harboring pControl, p*hk06*, p*rr06* or p*rr/hk06* were analysed for differences in *pspA* mRNA by real time RT-PCR. Data shown are fold change (± standard error) in [*pspA* mRNA] relative to D39:pControl (*** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ as determined by One-way ANOVA with post-hoc Tuckey test).

(**Figure 7.12**). This suggests that the over-expression of *hk06* was affecting *pspA* expression via the chromosomal copy of *rr06*.

Real time RT-PCR using *pspA* specific oligonucleotides, as described above, was then used to compare levels of *pspA* mRNA in D39 and the in-frame deletion mutants D39Δ*hk06*, D39Δ*rr06* and D39Δ*rr/hk06*, grown to A_{600} = 0.35 in THY. While both D39Δ*rr06* and D39Δ*rr/hk06* showed *pspA* mRNA levels similar to the wt, D39Δ*hk06* showed a significantly increased level of *pspA* mRNA (**Figure 7.13**). Thus, these results provide further evidence for the regulation of *pspA* by RR/HK06, at least in D39.

Microarray analysis failed to detect any difference in *pspA* mRNA between TIGR4:pControl and TIGR4:p*rr06* (**Table 7.2**). To confirm this result, we analysed *pspA* expression between TIGR4:pControl, TIGR4:p*hk06*, TIGR4:p*rr06* and TIGR4:p*rr/hk06* using real time RT-PCR using oligonucleotides designed against the TIGR4 *pspA* gene (PspATIGRf and PspATIGRr; Table 2.2). *pspA* expression did not vary between these strains (**Figure 7.14A**). Similarly, when we compared expression in TIGR4, TIGR4*hk06:erm*, TIGR4*rr06:erm*, TIGR4Δ*rr06,* and TIGR4*rr/hk06:erm*, we did not see any significant differences between the wt and its otherwise isogenic mutants (**Figure 7.14B**). Thus, these results would suggest that *pspA* is not regulated by RR/HK06 in TIGR4.

7.2.5 Regulation of PspA and Gls24 by RR/HK06

In Chapter Six, point mutations in RR06 and HK06 were constructed in order to investigate the mechanism by which RR/HK06 regulates CbpA. To investigate whether PspA and Gls24 were regulated by similar mechanisms to CbpA, the expression of these factors was investigated in the point mutant strains. *pspA* mRNA levels were compared using real time RT-PCR with specific oligonucleotides against D39 *pspA* (PspAF and PspAR; Table 2.2). All three substitutions in HK06 (D39 $HK06_{H242R}$, D39 $HK06_{S241D}$ and D39 $HK06_{H242Y}$) predicted to alter kinase activity, phosphatase activity or both, resulted in

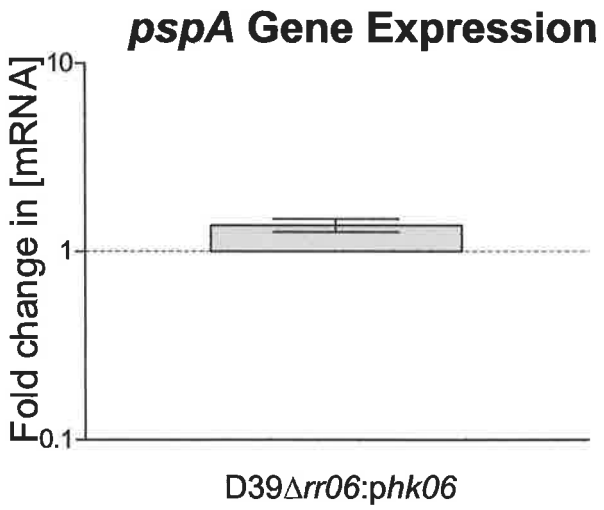

Figure 7.12. *pspA* gene expression in D39Δ*rr06*:p*hk06*.

RNA was isolated form D39Δ*rr06*:pControl and D39Δ*rr06*:p*hk06* grown as described in Section 6.2.2.1. Real time RT-PCR was subsequently used to analyse levels of *pspA* mRNA. Data shown are fold change (± standard error) in [*pspA* mRNA] relative to D39Δ*rr06*:pControl.

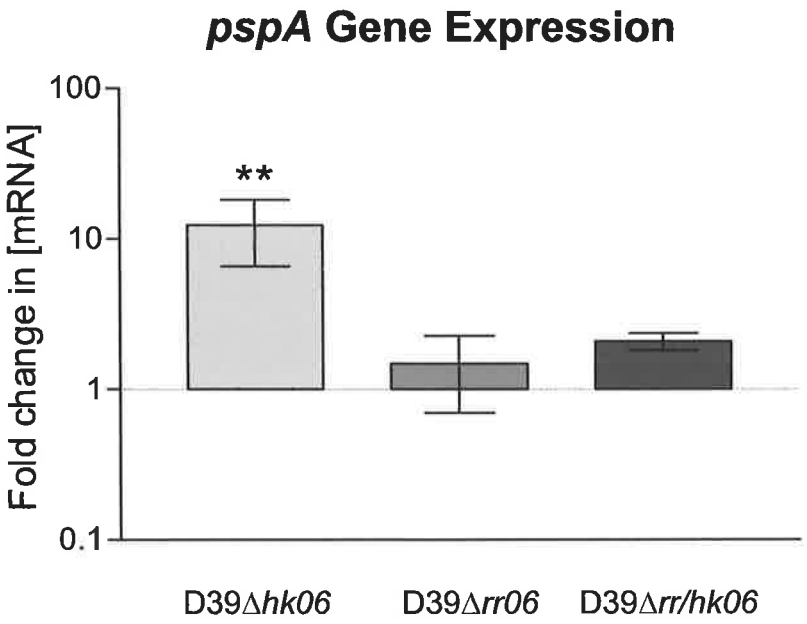

Figure 7.13. Analysis of PspA mRNA in D39.

RNA isolated from D39, D39Δ*hk06*, D39Δ*rr06* and D39Δ*rr/hk06* were analysed for differences in *pspA* mRNA by real time RT-PCR. Data shown are fold change (± standard error) in [*pspA* mRNA] relative to D39 (** $P < 0.01$ as determined by One-way ANOVA with post-hoc Tuckey test).

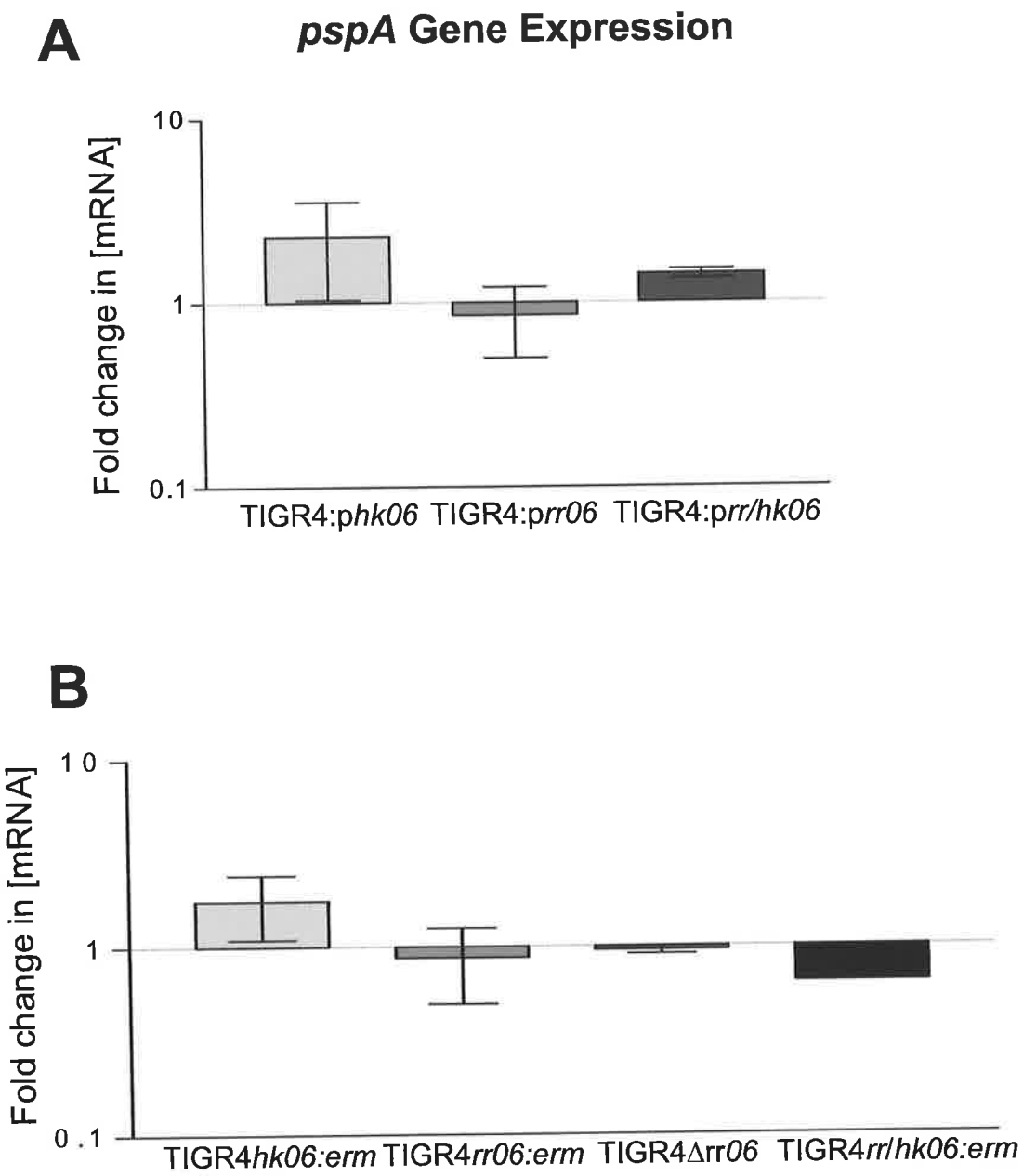

Figure 7.14. *pspA* expression in TIGR4.

RNA was isolated from (A) TIGR4:pControl, TIGR4:p*hk06*, TIGR4:p*rr06*, TIGR4:p*rr/hk06* and (B) TIGR4, TIGR4*hk06:erm*, TIGR4*rr06:erm*, TIGR4Δ*rr06*, TIGR4*rr/hk06:erm* and *pspA* mRNA levels were analysed by real time RT-PCR using oligonucleotides PspATIGRf and PspATIGRr (Table 2.2). Data shown are fold change (± standard error) in [*pspA* mRNA] relative to TIGR4:pControl and TIGR4, respectively.

increased (approximately 10-fold) *pspA* mRNA levels comparable to that seen in D39Δ*hk06* (**Figure 7.15A**). Thus, this suggests that any change in HK06 results in an increase in PspA. D39 $RR06_{D51E}$ showed no apparent difference in *pspA* expression, similar to that previously seen in D39Δ*rr06*. However, D39 $RR06_{D51N}$, predicted to mimic the non-phosphorylated form of RR06, resulted in a significantly increased (approximately 10-fold) *pspA* mRNA compared to the wt (**Figure 7.15B**). Western blot analysis of D39, D39Δ*hk06*, D39 $HK06_{H242R}$, D39 $HK06_{S241D}$, D39 $HK06_{H242Y}$, D39Δ*rr06*, D39 $RR06_{D51E}$, D39 $RR06_{D51N}$ and D39Δ*rr/hk06* using anti-PspA confirmed the results seen by real time RT-PCR (**Figure 7.16**). Although previous Western blotting (Section 3.2.3) suggested that D39Δ*hk06* produced levels of PspA similar to wt D39, real time RT-PCR described here showed that *pspA* expression is actually increased in D39Δ*hk06* (approximately 10-fold).

Real time RT-PCR analysis was then used to determine the levels of *pspA* mRNA when RR06, $RR06_{D51E}$ and $RR06_{D51N}$ were over-expressed in D39Δ*rr/hk06*. Interestingly, over-expression of RR06 in the absence of the chromosomal copies of *hk06* and *rr06* led to a significant increase in *pspA* mRNA (**Figure 7.17**). This further illustrates that the presence of functioal *hk06* is required for repression of *pspA* by this TCSTS. Over-expression of $RR06_{D51E}$, predicted to mimic the phosphorylated form, led to a decrease in *pspA* mRNA, while over-expression of $RR06_{D51N}$ resulted in levels similar to that seen in D39Δ*rr/hk06*:pControl (**Figure 7.17**).

To investigate levels of Gls24, cell lysates from strains grown to A_{600} = 0.35 in THY and resuspended in lysis byffer, were separated by SDS-PAGE and stained with Coomassie brilliant blue (Section 2.8.1). Increases in Gls24 were evident in D39Δ*hk06*, D39 $HK06_{H242R}$, D39 $HK06_{S241D}$, D39 $HK06_{H242Y}$ and D39 $RR06_{D51N}$ (**Figure 7.18**). These results are similar to those seen at the level of transcription for PspA, suggesting that these two factors are regulated in a parallel fashion, one which is clearly different to that for *cbpA*. Similarly, an increase in Gls24 was evident in D39Δ*rr/hk06*:p*rr06* (**Figure 7.19**).

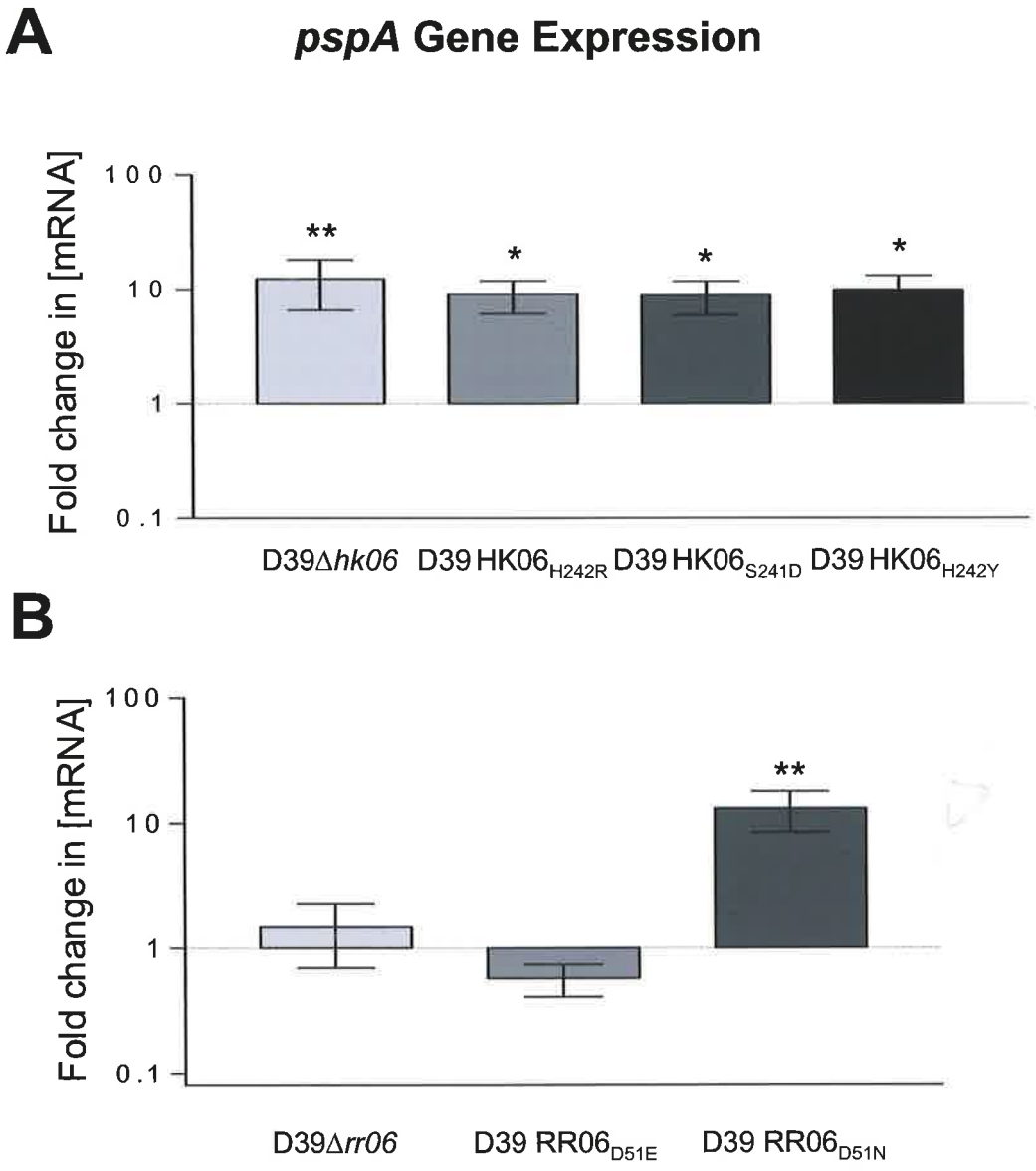

Figure 7.15. *pspA* gene expression.

RNA was isolated from **(A)** D39, D39Δ*hk06*, D39 $HK06_{H242R}$, D39 $HK06_{S241D}$, D39 $HK06_{H242Y}$ and **(B)** D39, D39Δ*rr06*, D39 $RR06_{D51E}$, D39 $RR06_{D51N}$ and *pspA* mRNA levels were analysed by real time RT-PCR using oligonucleotides PspAF and PspAR (Table 2.2). Data shown are fold change (± standard error) in [*pspA* mRNA] relative to D39. (* $P < 0.05$, ** $P < 0.01$ as determined by One way Anova with a post hoc Tuckey test).

Figure 7.16. Western blot analysis.

Proteins in cell lysates of (1) D39, (2) D39Δ*hk06*, (3) D39 $HK06_{H242R}$, (4) D39 $HK06_{S241D}$, (5) D39 $HK06_{H242Y}$, (6) D39Δ*rr06*, (7) D39 $RR06_{D51E}$, (8) D39 $RR06_{D51N}$ and (9) D39Δ*rr/hk06* were separated by SDS-PAGE, transferred onto nitrocellulose and probed with anti-PspA.

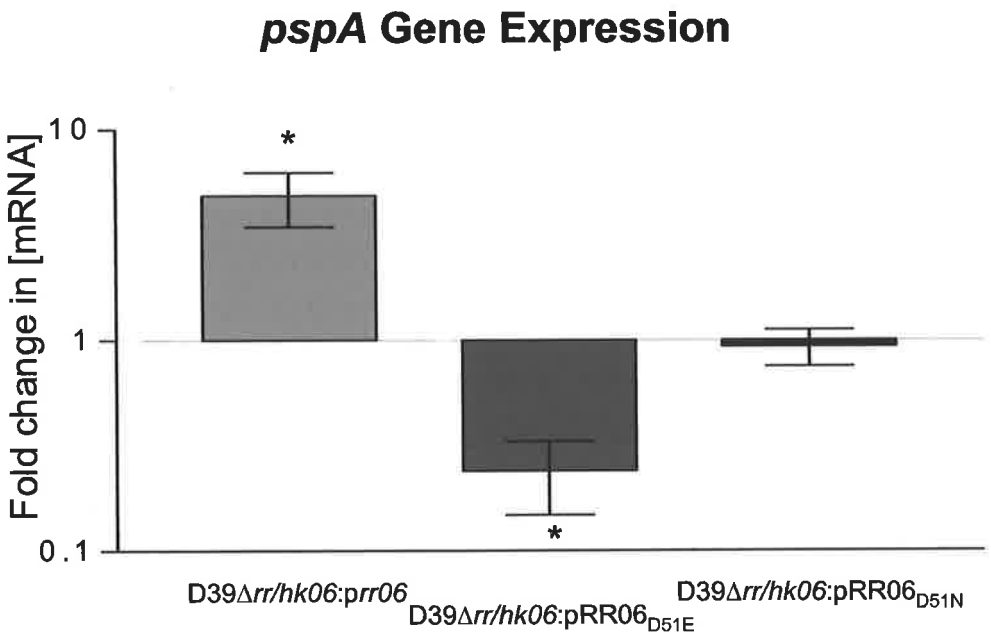

Figure 7.17. *pspA* gene expression.

RNA was isolated from D39Δ*rr/hk06*:pControl, D39Δ*rr/hk06*:p*rr06*, D39Δ*rr/hk06*:pRR06$_{D51E}$ D39Δ*rr/hk06*:pRR06$_{D51N}$ and *pspA* mRNA levels were analysed by real time RT-PCR using oligonucleotides PspATIGRf and PspATIGRr (Table 2.2). Data shown are fold change (± standard error) in [*pspA* mRNA] relative to D39Δ*rr/hk06*:pControl (* $P < 0.05$ as determined by One way Anova with a post hoc Tuckey test).

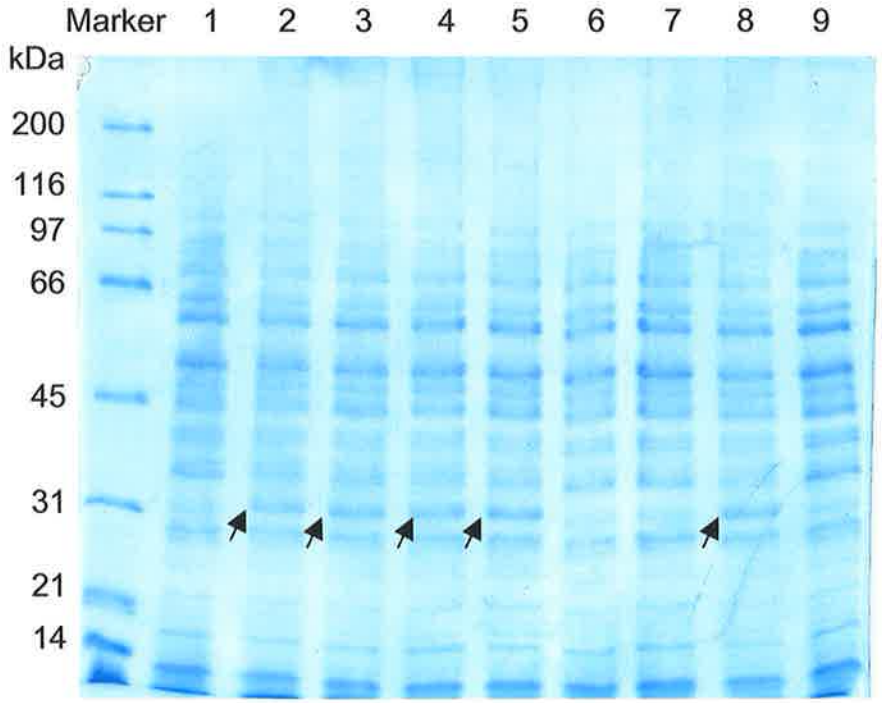

Figure 7.18. SDS PAGE analysis.

Proteins in cell lysates of (1) D39, (2) D39Δ*hk06*, (3) D39 $HK06_{H242R}$, (4) D39 $HK06_{S241D}$, (5) D39 $HK06_{H242Y}$, (6) D39Δ*rr06*, (7) D39 $RR06_{D51E}$, (8) D39$RR06_{D51N}$ and (9) D39Δ*rr/hk06* were separated by SDS-PAGE and stained with Coomassie brilliant blue (Section 2.8.1). The arrow head marks the presence of the band relating to Gls24.

Figure 7.19. SDS PAGE analysis.

Proteins in cell lysates of (1) D39Δ*rr/hk06*:pControl, (2) D39Δ*rr/hk06*:p*rr06*, (3) D39Δ*rr/hk06*:pRR06$_{D51E}$, and (4) D39Δ*rr/hk06* :pRR06$_{D51N}$, were separated by SDS-PAGE and stained with Coomassie brilliant blue (Section 2.8.1). The arrow head marks the presence of the band relating to Gls24.

Again, these results suggest that regulation of Gls24 by RR/HK06 is undertaken by a mechanism similar to that of PspA.

7.2.6 Electrophoretic mobility shift assays

The discovery that *cbpA* is co-transcribed with it upstream gene, *sp2191*, indicated that the original binding studies described in Chapter Four used a DNA target likely to be within the transcript encompassing *sp2191* and *cbpA*. It has previously been shown that *cbpA* and *hk06* are a separate transcript (Section 3.2.4), and so further RR06 binding studies used DNA upstream of *sp2191* as the target. Oligonucleotides Sp2191promf and Sp2191promr (Table 2.2) were used to amplify a 138-bp DNA region corresponding to nt -108 to +30 relative to the *sp2191* initiation codon and EMS assays were then undertaken as described in Section 2.11. Assays using 30 μg of protein could not illustrate specific binding to DNA, as cell lysates from both DH5α:pGEM-T and DH5α:RR06 (Table 2.1) resulted in a shift in the mobility of the DIG labelled DNA (data not shown). This suggested that the shift may have resulted as a consequence of the binding of an *E. coli* protein to the DNA, rather than RR06. However, it is also possible that both RR06 and the unknown *E. coli* protein interact with the same region of DNA. Thus, efforts were made to increase the binding specificity by decreasing the salt concentration and reducing the amount of protein used in the assay, in the hope of removing the binding by the *E. coli* protein. However, at a protein concentration of 5 μg and with a 10-fold reduction of KCl in TM-1 (Section 2.11), both cell lysates still caused a significant shift in the mobility off the DNA (**Figure 7.20**). Thus, specific binding of RR06 to this region of DNA could not be demonstrated.

7.3 Discussion

In Chapters Three and Four, it was shown that RR/HK06 regulates the expression of *cbpA*, which encodes a major pneumococcal virulence factor and protective antigen.

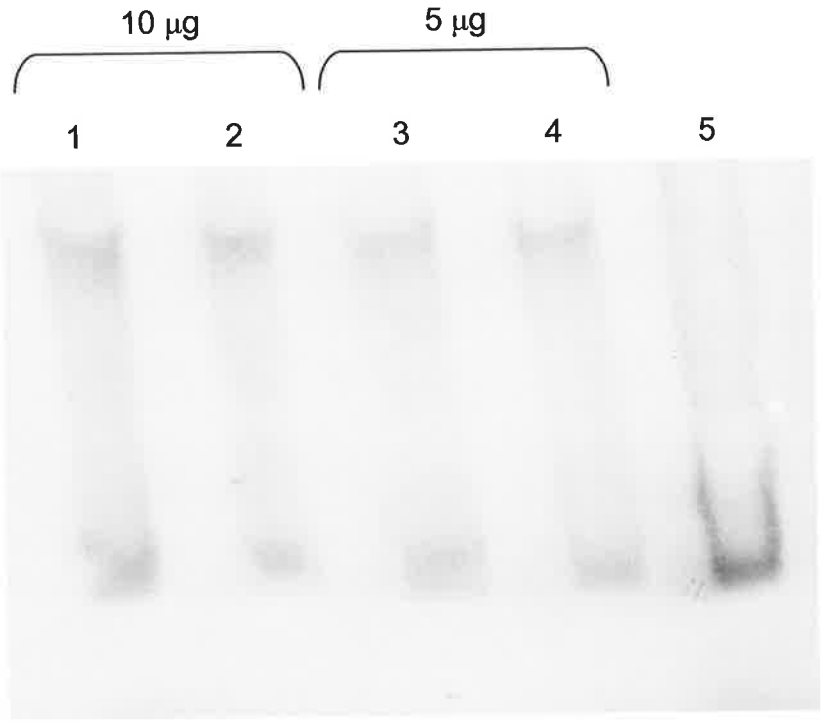

Figure 7.20. Electrophoretic mobility shift assay.

A 138-bp DNA fragment spanning the *sp2191* promoter was DIG-labeled (lane 5), and then incubated with DH5α:pGEM-T (lane 1 and 3) and DH5α:RR06 (lane 2 and 4) at the indicated protein concentrations. Protein-DNA complexes were separated by PAGE, transferred to nitrocellulose and labeled DNA was detected as described in Section 2.11.

However, regulated factors other than CbpA appeared to be at least partially responsible for the system's effects on epithelial cell adherence and virulence in mice. In this Chapter, RR/HK06 was over-expressed in order to identify additional RR/HK06-regulated genes. Using microarray analysis, 53 differentially expressed genes were identified in D39, while only 5 genes showed differences in expression in TIGR4 when grown in THY. The only commonly regulated genes were *cbpA* and its co-transcribed upstream gene, *sp2191*. Regulation of genes by another pneumococcal TCSTS, HK/RR04, has also been shown to differ markedly between these two strains (McCluskey *et al.*, 2004). Examination of the upstream regions of a number of the genes regulated in D39 but not in TIGR4, did not reveal differences in the DNA sequence which might affect the ability of RR06 to bind. Additionally, RR06 and HK06 are highly conserved between the strains. However, the genomes of these strains do vary substantially, and as such it is possible that a factor present in D39 but not in TIGR4 (or vice versa) is responsible for these differences in gene expression. While the regulated gene *pspA* shares significant homology between the strains in the 5' region, there is little homology in the remaining sequence. Replacing TIGR4 *pspA* with D39 *pspA* would indicate whether differences in the sequence of this regulated gene are responsible for the variation in regulation between these two strains.

In D39, over-expression of RR06 also led to a decrease in expression of another TCSTS, *vncRS*. Computational analysis by Lange *et al.* (1999) suggested that HK06 contains an extracellular domain closely related to that of VncS, which may suggest they respond to related stimuli. Both HKs also share homology with VanS from *E. faecalis*, which is responsible for the regulation of genes involved in the development of vancomycin resistance. However, data presented in this Chapter suggest that RR/HK06 does not affect susceptibility to vancomycin.

Another gene found to be repressed by RR/HK06 in D39 was *gls24*. This gene encodes a product with homology to proteins from *E. faecalis* and *L. lactis* associated with

alkaline shock response (Giard *et al.*, 2000; Giard *et al.*, 2002). Recent studies in *E. faecalis* illustrated that this protein is important in bile-salts resistance, and is a putative protective antigen (Teng *et al.*, 2005). This protein was also found to be substantially increased in the in-frame deletion mutant D39Δ*hk06*. Investigation of growth at alkaline pH, showed that even with increased Gls24, D39Δ*hk06* grew at the same rate as the wt. Data also showed that D39Δ*hk06* was as susceptible to deoxycholate as the wt.

pspA was markedly reduced in expression when *rr06* was over-expressed in D39. PspA, along with CbpA, is a member of the choline binding protein family of *S. pneumoniae*. PspA is an important virulence factor, enabling the pathogen to evade host defences through its ability to inhibit complement activation as well as its capacity to bind lactoferrin (Tu *et al.*, 1999; Shaper *et al.*, 2004). In contrast to CbpA, PspA appears to be repressed by RR/HK06. Differential expression of these two proteins has also been reported to occur between the transparent and opaque phase variants (Rosenow *et al.*, 1997), although as previously reported in Chapter Three, the regulation by RR/HK06 appears to be independent of colony opacity phase, at least for *cbpA*. PspA has also been reported to be regulated by another pneumococcal TCSTS, YycFG, in the strain R6, an un-encapsulated derivative of D39 (Ng *et al.*, 2005), implicating both of these TCSTS in the regulation of PspA in D39.

Further investigations into the effect of over-expression of RR/HK06 indicated that increased levels of HK06, RR06 and the cognate pair together led to significant decreases in *pspA* expression. This is in contrast to *cbpA*, where the presence of p*hk06* did not alter expression. Further research showed that the decrease was due to the effect that increased HK06 has on the chromosomally-encoded RR06. This suggests that HK06 activity is critical for the ability to repress *pspA*, while this appears not to be the case for *cbpA*.

In Chapter Six, strains were constructed in order to modulate the activity of both HK06 and RR06 in an effort to gain a more complete understanding of the regulation of

cbpA by RR/HK06. Results from these studies suggested that RR06 may activate *cbpA* expression when it is in the non-phosphorylated form. Investigation of *pspA* expression in the aforementioned strains yielded results that are not easily reconciled. Studies using D39Δ*hk06,* D39 $HK06_{H242R}$, D39 $HK06_{S241D,}$ and D39 $HK06_{H242Y}$ all produced increased levels of *pspA*. This suggests that repression is only possible in the presence of a fully functional copy of *hk06*. D39 $RR06_{D51E}$, predicted to mimic the activated state, produced wt levels of *pspA* mRNA, while D39 $RR06_{D51N}$ produced significantly increased levels. This suggests that phosphorylated RR06 represses *pspA*. This was further supported by the fact that over-expression of $RR06_{D51E}$ in D39Δ*rr/hk06* resulted in decreased levels of *pspA* mRNA.

However, there are a number of other results which do not support such a conclusion. For instance, why does D39 $HK06_{S241D}$, predicted to mutate phosphatase but not kinase activity, result in increased levels of *pspA*? In addition, when p*rr06* was transformed into D39Δ*rr/hk06,* why was an increased level of *pspA* mRNA was evident? In Chapter Six, it was hypothesised that the over-expression of $RR06_{D51E}$ led to an increase in CbpA simply due to a concentration effect. If this was the case, RR06 would be expected to repress *pspA* expression, particularly as it is common for over-expressed RRs to mimic the activity of their phosphorylated form. Additionally, if RR06 repressed *pspA* expression in the phosphorylated state, then *pspA* mRNA should have been elevated in D39Δ*rr06* and D39Δ*rr/hk06* as was the case in D39Δ*hk06*. Instead these strains produced levels of *pspA* mRNA similar to the wt. It is worth noting that regulation of PspA by a separate pneumococcal TCSTS, YycFG, may be complicating this picture. It is also possible that RR/HK06 affects *pspA* expression through another intermediate or possibly even via YycFG itself. Interestingly, Gls24 seems to be regulated by RR/HK06 in the same manner as PspA.

Differential gene regulation by a TCSTS has been seen before in *B. subtilis*, as discussed in Chapter Six. The DegU RR has been shown to function in two activated forms, with the genes affected dependent on the phosphorylation state of the RR (Msadek *et al.*, 1995). Further research may help elucidate the complex network of regulation mediated by RR/HK06.

In Chapter Four, original attempts to determine the DNA binding site of RR06 concentrated on a region of DNA upstream of *cbpA*. However, in this Chapter microarray analysis showed that *cbpA* and its upstream gene *sp2191* are actually co-regulated and co-transcribed, suggesting that the illustration of RR06 binding in Chapter Four was to a region of DNA within this transcript. Binding of RRs within the transcripts of regulated genes has been shown previously (Federle & Scott, 2002; Ng *et al.*, 2005), although this has primarily thought to result in repression rather than activation of expression, which appears to be the case here. Ng *et al.* (2005) showed that another pneumococcal RR, YycF, binds to DNA within the transcript of the regulated gene, *pcsB*, although with considerable less affinity than to the additional upstream binding sites. Investigation into the binding of RR06 to a region upstream of *sp2191* was inconclusive due to the possible binding of an *E. coli* protein to the target. If RR06 does bind upstream of *sp2191*, there is also the possibility that this occurs within the genes encoding HK06 and RR06. Thus, deletion and *erm* replacement mutants of the system might have altered *cbpA* mRNA expression due to the loss of this RR06:DNA interaction. However, two amino acid substitutions in HK06 resulted in similar changes in *cbpA* mRNA expression as D39Δ*hk06*, while over-expression of RR06 in D39Δ*rr/hk06* resulted in similar increases in *cbpA* mRNA as when RR06 was over-expressed in D39 wt. Thus, these results suggest that changes in *cbpA* expression seen in these mutants were not the result of a loss of RR06:DNA interactions, but rather the effect of the loss of these genes.

This Chapter has identified a number of additional factors regulated by RR/HK06 including another major virulence factor PspA, and the TCSTS VncRS amongst others. As with other TCSTSs in *S. pneumoniae*, regulation varied markedly between pneumococcal strains. Additionally, unlike work which suggested that CbpA was activated by the non-phosphorylated form of RR06, both PspA and Gls24 were regulated by RR/HK06 via a different mechanism.

CHAPTER EIGHT – FINAL DISCUSSION

The increasing rates of resistance of *S. pneumoniae* to antibiotics, along with the limitations of the current generation of vaccines, means that the pneumococcus remains a major cause of morbidity and mortality worldwide, particularly in the young and elderly. During disease pathogenesis, the pneumococcus encounters a variety of environmental niches in the body, including the nasopharynx, lungs, blood, brain and middle ear. In order to successfully colonise and cause disease, it is likely that the pathogen requires the co-ordinated regulation of a large number of genes. Indeed, several recent studies have shown that the gene expression profile of *S. pneumoniae* varies markedly as it encounters these different host environments (Orihuela *et al.*, 2004; LeMessurier *et al.*, 2006). However, the manner in which these genes are regulated by the pathogen remained a mystery. It is likely that the thirteen TCSTSs identified in the pneumococcus play an integral role in these global changes in gene expression (Lange *et al.*, 1999; Throup *et al.*, 2000). TCSTSs are

found throughout prokaryotes, and regulate gene expression in response to changes in environmental stimuli. In pathogenic microorganisms, a number have been shown to regulate genes essential for virulence (Beier & Gross, 2006). However, at the beginning of this study, the roles of many of the 13 TCSTSs in *S. pneumoniae* remained largely uncharacterised. Initial studies by Lange *et al.* (1999) and Throup *et al.* (2000) investigated the effects of mutations in the TCSTSs in two different virulence models, with varying findings. However, no effort was made to identify genes regulated by the systems, with only a few, such as ComDE, having been the topic of in-depth investigations. This study focused on one of the as yet uncharacterised systems, known as RR/HK06.

As the gene encoding the major virulence factor CbpA is found adjacent to *rr/hk06* on the *S. pneumoniae* genome, Chapter Three focused on the possible regulation of *cbpA* by RR/HK06. In order to do this, unmarked in-frame deletion mutants of *rr06* and *hk06* were constructed in *S. pneumoniae* D39, and the CbpA protein and mRNA levels were investigated using Western immunoblot and real time RT-PCR. D39Δ*hk06* showed increased levels of *cbpA* expression (approximately 5-fold) compared to the wt, while D39Δ*rr06* had decreased levels (approximately 3-fold) (Section 3.2.3 & 3.2.4). These changes were evident at both the mRNA and protein level, and indicated that RR/HK06 was indeed involved in the regulation of this important pneumococcal virulence factor.

Most RRs are active in the phosphorylated form. Typically, this has been demonstrated by mutating the cognate HK and showing that this resulted in reduced target gene expression due to the loss of kinase activity. However, results described above suggested this was not the case for the regulation of *cbpA* by RR/HK06, as D39Δ*hk06* exhibited increased *cbpA* expression. It was thought that RR06 may in fact be active in the non-phosphorylated form. Chapter Six used a combination of defined mutations and over-expression in order to investigate this further. An unmarked deletion mutant in both *hk06* and *rr06*, D39Δ*rr/hk06*, exhibited the same level of *cbpA* expression as the wt (Section

6.2.1). This may suggest that other factors are involved in the regulation of *cbpA*. Over-expression of RR06 and RR/HK06 led to significant increases in *cbpA* expression compared to the control, showing that *cbpA* transcription is activated by RR/HK06 (Section 6.2.2.2). However, the over-expression of HK06 alone did not alter *cbpA* expression. If HK06 has primarily kinase activity, HK06 over-expression would not lead to an increase in the non-phosphorylated form of RR06, and thus no increase in *cbpA* expression. This fits with RR06 being active in the non-phosphorylated state. To further investigate this hypothesis, amino acid substitutions were constructed in order to modulate the activities of both HK06 and RR06 (Section 6.2.3.1). Substitutions in HK06 predicted to abrogate both kinase and phosphatase activities or kinase activity alone resulted in increased levels of *cbpA* mRNA, similar to those seen in D39Δ*hk06* (Section 6.2.3.2). However, D39 expressing $HK06_{S241D}$ (predicted to be deficient in phosphatase activity) resulted in *cbpA* expression levels similar to wt. This suggested that the phosphatase activity of HK06 was responsible for the increase in *cbpA* expression, with the non-phosphorylated RR06 binding and activating *cbpA* expression. Substitutions in RR06 predicted to mimic the phosphorylated and non-phosphorylated forms of RR06 both led to decreased levels of *cbpA* mRNA (Section 6.2.3.2). However, over-expression of $RR06_{D51N}$ increased *cbpA* expression significantly less than that achieved by over-expression of wt RR06 (Section 6.2.3.3). This suggested that the D51N substitution altered the ability of RR06 to bind to its DNA target, explaining why the expected increase in *cbpA* expression was not evident in D39 $RR06_{D51N}$. Additionally, phosphorylation of amino acids other than the conserved aspartate may have occurred in D39 $RR06_{D51N}$, as has been seen in other RRs with similar substitutions (Delgado *et al.*, 1993; Moore *et al.*, 1993; Reyrat *et al.*, 1994; Appleby & Bourret, 1999). Such phosphorylation may alter the conformation of RR06, and affect interaction with its DNA target.

The effects of mutation and over-expression of RR/HK06 on *cbpA* expression in other pneumococcal strains was also investigated. While an Ery replacement mutation of *hk06* in TIGR4 *S. pneumoniae* resulted in increased *cbpA* expression, mutation of *rr06* both alone and in combination with *hk06* produced levels similar to the parent strain (Section 3.2.7 & 6.2.1). Over-expression of RR06 and RR/HK06 in TIGR4 showed increased expression levels, while HK06 had no effect (Section 6.2.2.3). RR06 over-expression in two further strains, WCH4861 and WCH4832, also resulted in an alteration of CbpA levels as seen by Western immunoblot. As in D39 and TIGR4, RR06 over-expression in WCH4861 resulted in an increase in CbpA compared to the control (Section 6.2.2.3). However, a decreased level of CbpA, was evident in WCH4832 when RR06 was over-expressed. Nevertheless, these results clearly indicated that RR/HK06 regulates CbpA (albeit not consistently) across four pneumococcal strains.

As discussed in Chapter One, TCSTSs generally act to modulate gene expression, with the RR binding to DNA and acting as a transcription factor. Previously, EMS assays illustrated the binding of RR06 to the promoter region of *cbpA* using an *E. coli* lysate over-expressing RR06 (Standish, 2002). However, in Chapter Four, His_6-RR06 purified to >95% did not result in a shift in the mobility of the same DNA fragment, possibly due to the presence of the His tag or problems associated with the activity of the protein (Section 4.2.2). However, SPB assays using an the *E. coli* lysate over-expressing RR06 illustrated the specificity of the RR06:DNA interaction, confirming that RR06 binds to this region of DNA in a specific manner, and thus providing further evidence for the regulation of CbpA by RR/HK06 (Section 4.2.4). Unfortunately, DNase I Footprinting was unable to successfully delineate the actual nucleotides required for the binding of RR06; this was perhaps due to instability of the interaction of RR06 with the DNA target during DNase I treatment (Section 4.2.5). Interestingly, later studies demonstrated that *cbpA* is in fact co-transcribed and co-regulated with an upstream gene (*sp2191*) encoding a hypothetical

protein (Section 7.2.1.1). This would suggest that the DNA used for binding studies was within the transcript encompassing *sp2191* and *cbpA*. However, further EMS assays using DNA upstream of *sp2191* did not illustrate specific binding of RR06, presumably due to the binding of an *E. coli* protein present in the cell lysate (Section 7.2.6). Thus, as an RR06 DNA binding consensus sequence was not determined, it was not possible to search the pneumococcal genome sequence for additional candidate RR/HK06-regulated genes.

In order to identify other RR/HK06-regulated genes, microarray analysis was used to investigate the effect of RR06 over-expression on global gene expression (Section 7.2.1). In D39, 53 differentially-expressed genes were identified, while only 5 genes showed differences in expression in TIGR4 when grown in THY. The only genes commonly regulated between the two strains were *cbpA* and its co-transcribed upstream gene, *sp2191*. McCluskey *et al.* (2004) saw similar variability in the genes regulated between these strains when investigating another pneumococcal TCSTS, TCS04. Although the genes encoding *hk06* and *rr06* are highly conserved, as is the promoter region upstream of potentially regulated genes, TIGR4 and D39 do vary substantially in genome sequence (Bruckner *et al.*, 2004), and hence may differ in some other regulatory element affecting RR/HK06 regulation.

In D39, only one gene other than *cbpA* and it co-transcribed upstream gene was increased in expression in D39:p*rr06* compared to D39:pControl. However, a total of 48 genes were decreased in expression. These included the genes encoding the major virulence factor and protective antigen *pspA*, another pneumococcal TCSTS *vncRS*, and a putative alkaline shock protein *gls24* (Section 7.2.1.1). Further investigation of *pspA* regulation by RR/HK06 yielded results which are not easy to reconcile (Section 7.2.4 & 7.2.5). The fact that over-expression of HK06 alone repressed *pspA* expression suggested that RR06 may repress *pspA* expression when in the phosphorylated form. This was further backed up by the fact D39 expressing $RR06_{D51E}$, predicted to mimic the phosphorylated

state, produced wt levels of *pspA* mRNA, while D39 $RR06_{D51N}$ produced significantly increased levels. However, other results did not fit with this hypothesis. For example, D39 expressing $HK06_{S241D}$, predicted to have mutated phosphatase but not kinase activity, exhibited increased levels of *pspA* mRNA. Biochemical analysis is needed to confirm the presence (or absence) of phosphatase activity in the HK06 point mutations, in order to completely understand regulation by RR/HK06. It is worth noting that regulation of *pspA* expression by a separate pneumococcal TCSTS, YycFG, may be complicating this story (Ng *et al.*, 2005), and it is possible that RR/HK06 affects *pspA* expression through another factor, possibly even YycFG.

Data presented in Chapter Five showed that RR/HK06 is important for the ability of the pneumococcus to adhere to epithelial cells *in vitro*, and to survive and proliferate in an *in vivo* model. These findings clearly implicated RR/HK06-regulated factors other than CbpA as playing a significant role in these aspects of pneumococcal pathogenesis. Both D39Δ*hk06* and D39Δ*rr06* exhibited significantly reduced adherence to epithelial cells compared to the wt, as did an insertion-duplication mutant in CbpA (Section 5.2.1.1). However, the decrease seen in D39Δ*hk06* was in spite of its increased expression of *cbpA* mRNA. Further investigation is needed to see whether other regulated factors are responsible for these deficiencies in epithelial cell adherence. Orihuela *et al.* (2004) showed that *pspA* mRNA is increased when *S. pneumoniae* are in contact with Detroit 562 cells, rather than simply in tissue culture medium. However, this study used an un-encapsulated derivative of TIGR4, a strain in which RR/HK06 does not appear to regulate *pspA* expression under the growth condition media used in the present study (Section 7.2.5). However, this does not rule out the possibility that *pspA* may be regulated in a tissue culture model.

The results from the *in vivo* mouse model did not correlate with reduced epithelial cell adherence, which was not surprising given the likelihood of differences in receptor

expression between transformed human cell lines and mouse respiratory epithelium. Indeed, while CbpA binds human pIgR, it is not able to bind the rabbit homologue (Zhang *et al.*, 2000). D39Δ*rr06* exhibited an increased ability to colonise the nasopharynx and to cause invasive disease, while D39Δ*hk06* colonised to the same level as the wt, but was deficient in its ability to invade the lungs and blood (Section 5.2.2). A D39*cbpA*⁻ insertion-duplication mutant was able to colonise and cause disease as well as the wt at all three time points. Thus, this suggested that RR/HK06-regulated factors other than CbpA play a role in pneumococcal colonisation and virulence. In order to understand this in more detail, microarray analysis of *in vivo* gene expression profiles of these strains is essential to identify factors differentially expressed in these niches. Two RR/HK06-regulated factors, *cbpA* and *pspA*, are known to be differentially regulated in different sites *in vivo*. Thus, this may suggest that regulation by RR/HK06 may differ *in vivo* (LeMessurier *et al.*, 2006). PspA is critically important for systemic pneumococcal infection through its ability to interfere with complement activation (Tu *et al.*, 1999). If D39Δ*hk06* has an increased level of *pspA* expression *in vivo* (as seen *in vitro*), this would compound the question of why this strain is unable to persist in the lungs and blood, when this increased level of PspA should reduce complement mediated opsonophagocytosis. However, the contribution of other regulated factors identified *in vitro*, such as Gls24, would need to be investigated in more detail to gain a greater understanding of the contribution this system makes to the virulence of *S. pneumoniae*.

Notwithstanding the above uncertainties, this study has provided an improved understanding of the role RR/HK06 plays in pneumococcal pathogenesis, through the identification of factors under its regulatory control, and through direct demonstration that the system contributes to epithelial cell adherence and the ability of the pneumococcus to survive and proliferate *in vivo*. An in-depth knowledge of two-component signal transduction in *S. pneumoniae* is critical to gaining a greater understanding of

pathogenesis, particularly the changes in gene expression associated with the switch from asymptomatic colonisation to invasive disease.

8.1 Future Directions

Clearly, further research is needed to better understand the results presented in this study. Binding studies using DNA from promoter regions of a number of genes differentially expressed in the microarray analysis, such as *pspA*, should enable better delineation of the actual sequence required for RR06 DNA binding. This would also enable distinction between genes directly regulated by the system, and those regulated through intermediates. Further studies are also needed to identify binding sites of RR06 involved in the regulation of *sp2191* and *cbpA*. Furthermore, biochemical characterisation of the system, such as investigation of the *in vitro* activity of both wt and mutant forms of HK06 and RR06, is needed in order to support the genetic analysis present here. The construction of *S. pneumoniae* mutants in which 2 or more TCSTS are deleted at once, may also provide evidence for possible cross-talk between pneumococcal systems RR/HK06, VncRS and YycFG. In order to understand the mechanism behind the effect mutations in RR/HK06 have on pathogenesis, microarray analysis of the gene expression profiles of bacteria from different *in vivo* sites such as the nasopharynx, lungs and blood should be undertaken. Gene expression of a number of factors, including PspA and CbpA, is known to vary between these sites, suggesting that the signal activating the system may be operating in one or other of these niches. Furthermore, mutation of individual uncharacterised regulated factors, such as Gls24, will show whether they are of importance in pneumococcal virulence, and what specific role they play. *In vivo* studies using mutants in RR/HK06 in other pneumococcal strains, such as TIGR4, would examine whether the system contributes to virulence differently across strains, as has been seen in TCS09. Collectively,

these studies would contribute to a greater understanding of pneumococcal gene regulation in general and that effected by RR/HK06 in particular.

BIBLIOGRAPHY

Abeyta, M., Hardy, G. G. and Yother, J. (2003). Genetic alteration of capsule type but not PspA type affects accessibility of surface-bound complement and surface antigens of *Streptococcus pneumoniae*. *Infect Immun.* **71**:218-25.

Agrawal, G. K. and Thelen, J. J. (2005). Development of a simplified, economical polyacrylamide gel staining protocol for phosphoproteins. *Proteomics.* **5**:4684-8.

Alex, L. A. and Simon, M. I. (1994). Protein histidine kinases and signal transduction in prokaryotes and eukaryotes. *Trends Genet.* **10**:133-8.

Alexander, J. E., Lock, R. A., Peeters, C. C., Poolman, J. T., Andrew, P. W., Mitchell, T. J., Hansman, D. and Paton, J. C. (1994). Immunization of mice with pneumolysin toxoid confers a significant degree of protection against at least nine serotypes of *Streptococcus pneumoniae*. *Infect Immun* **62**:5683-8.

AlonsoDeVelasco, E., Verheul, A. F., Verhoef, J. and Snippe, H. (1995). *Streptococcus pneumoniae*: virulence factors, pathogenesis, and vaccines. *Microbiol Rev* **59**:591-603.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**:3389-402.

Amemura, M., Makino, K., Shinagawa, H. and Nakata, A. (1990). Cross talk to the phosphate regulon of *Escherichia coli* by PhoM protein: PhoM is a histidine protein kinase and catalyzes phosphorylation of PhoB and PhoM-open reading frame 2. *J. Bacteriol.* **172**:6300-7.

Appleby, J. L. and Bourret, R. B. (1999). Activation of CheY mutant D57N by phosphorylation at an alternative site, Ser-56. *Mol Microbiol.* **34**:915-25.

Arthur, M., Molinas, C. and Courvalin, P. (1992). The VanS-VanR two-component regulatory system controls synthesis of depsipeptide peptidoglycan precursors in *Enterococcus faecium* BM4147. *J Bacteriol.* **174**:2582-91.

Austrian, R. (1981). Pneumococcus: the first one hundred years. *Rev Infect Dis* **3**:183-9.

Avery, O. T. and Dubos, R. (1931). The protective action of a specific enzyme against type 3 pneumococcus infection in mice. *Journal of Experimental Medicine* **54**:73-89.

Avery, O. T., Macleod, C. M. and McCarty, M. (1944). Studies on the chemical nature of the substance inducing tranformation of pneumococcal types: Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. *J. Exp. Med.* **79**:137-157.

Baba, H., Kawamura, I., Kohda, C., Nomura, T., Ito, Y., Kimoto, T., Watanabe, I., Ichiyama, S. and Mitsuyama, M. (2002). Induction of gamma interferon and nitric oxide by truncated pneumolysin that lacks pore-forming activity. *Infect Immun.* **70**:107-13.

Balachandran, P., Brooks-Walter, A., Virolainen-Julkunen, A., Hollingshead, S. K. and Briles, D. E. (2002). Role of pneumococcal surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of *Streptococcus pneumoniae*. *Infect. Immun.* **70**:2526-34.

Balachandran, P., Hollingshead, S. K., Paton, J. C. and Briles, D. E. (2001). The autolytic enzyme LytA of *Streptococcus pneumoniae* is not responsible for releasing pneumolysin. *J Bacteriol.* **183**:3108-16.

Barrett, J. F. and Hoch, J. A. (1998). Two-component signal transduction as a target for microbial anti-infective therapy. *Antimicrob Agents Chemother* **42**:1529-36.

Beier, D. and Gross, R. (2006). Regulation of bacterial virulence by two-component systems. *Current Opinion in Microbiology* **9**:143-152.

Bender, M. H. and Yother, J. (2001). CpsB is a modulator of capsule-associated tyrosine kinase activity in *Streptococcus pneumoniae*. *J Biol Chem.* **276**:47966-74. Epub 2001 Oct 17.

Benton, K. A., Everson, M. P. and Briles, D. E. (1995). A pneumolysin-negative mutant of *Streptococcus pneumoniae* causes chronic bacteremia rather than acute sepsis in mice. *Infect Immun.* **63**:448-55.

Berry, A. M. and Paton, J. C. (2000). Additive attenuation of virulence of *Streptococcus pneumoniae* by mutation of the genes encoding pneumolysin and other putative pneumococcal virulence proteins. *Infect. Immun.* **68**:133-40.

Berry, A. M., Yother, J., Briles, D. E., Hansman, D. and Paton, J. C. (1989). Reduced virulence of a defined pneumolysin-negative mutant of *Streptococcus pneumoniae*. *Infect. Immun.* **57**:2037-42.

Black, S., Shinefield, H., Baxter, R., Austrian, R., Bracken, L., Hansen, J., Lewis, E. and Fireman, B. (2004). Postlicensure surveillance for pneumococcal invasive disease after use of heptavalent pneumococcal conjugate vaccine in Northern California Kaiser Permanente. *Pediatr Infect Dis J.* **23**:485-9.

Blue, C. E. and Mitchell, T. J. (2003). Contribution of a response regulator to the virulence of *Streptococcus pneumoniae* is strain dependent. *Infect Immun* **71**:4405-13.

Bricker, A. L. and Camilli, A. (1999). Transformation of a type 4 encapsulated strain of *Streptococcus pneumoniae*. *FEMS Microbiol Lett* **172**:131-5.

Briles, D. E., Hollingshead, S. K., King, J., Swift, A., Braun, P. A., Park, M. K., Ferguson, L. M., Nahm, M. H. and Nabors, G. S. (2000a). Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. *J Infect Dis* **182**:1694-701.

Briles, D. E., Hollingshead, S. K., Nabors, G. S., Paton, J. C. and Brooks-Walter, A. (2000b). The potential for using protein vaccines to protect against otitis media caused by *Streptococcus pneumoniae*. *Vaccine* **19**:S87-95.

Briles, D. E., Tart, R. C., Swiatlo, E., Dillard, J. P., Smith, P., Benton, K. A., Ralph, B. A., Brooks-Walter, A., Crain, M. J., Hollingshead, S. K. and McDaniel, L. S. (1998). Pneumococcal diversity: considerations for new vaccine strategies with emphasis on pneumococcal surface protein A (PspA). *Clin Microbiol Rev* **11**:645-57.

Briles, D. E., Tart, R. C., Wu, H. Y., Ralph, B. A., Russell, M. W. and McDaniel, L. S. (1996). Systemic and mucosal protective immunity to pneumococcal surface protein A. *Ann N Y Acad Sci* **797**:118-26.

Briles, D. E., Yother, J. and McDaniel, L. S. (1988). Role of pneumococcal surface protein A in the virulence of *Streptococcus pneumoniae*. *Rev Infect Dis.* **10**:S372-4.

Brooks-Walter, A., Briles, D. E. and Hollingshead, S. K. (1999). The *pspC* gene of *Streptococcus pneumoniae* encodes a polymorphic protein, PspC, which elicits cross-reactive antibodies to PspA and provides immunity to pneumococcal bacteremia. *Infect Immun* **67**:6533-42.

Brown, S. D. (2004). PROTEKT US: results from the second year of this longitudinal surveillance study of antimicrobial resistance among respiratory tract pathogens isolated from paediatric and adult patients in the USA. Introduction. *J Antimicrob Chemother.* **54**:i5.

Bruckner, R., Nuhn, M., Reichmann, P., Weber, B. and Hakenbeck, R. (2004). Mosaic genes and mosaic chromosomes-genomic variation in *Streptococcus pneumoniae*. *Int J Med Microbiol* **294**:157-68.

Buckler, D. R., Anand, G. S. and Stock, A. M. (2000). Response-regulator phosphorylation and activation: a two-way street? *Trends Microbiol* **8**:153-6.

Burbulys, D., Trach, K. A. and Hoch, J. A. (1991). Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. *Cell.* **64**:545-52.

Cai, S. J. and Inouye, M. (2002). EnvZ-OmpR interaction and osmoregulation in *Escherichia coli*. *J Biol Chem* **277**:24155-61.

Chastanet, A., Prudhomme, M., Claverys, J. P. and Msadek, T. (2001). Regulation of *Streptococcus pneumoniae clp* genes and their role in competence development and stress survival. *J Bacteriol* **183**:7295-307.

Cheng, Q., Campbell, E. A., Naughton, A. M., Johnson, S. and Masure, H. R. (1997). The com locus controls genetic transformation in *Streptococcus pneumoniae*. *Mol. Microbiol.* **23**:683-92.

Cheng, Q., Finkel, D. and Hostetter, M. K. (2000). Novel purification scheme and functions for a C3-binding protein from *Streptococcus pneumoniae*. *Biochemistry* **39**:5450-7.

Crain, M. J., Waltman, W. D., 2nd, Turner, J. S., Yother, J., Talkington, D. F., McDaniel, L. S., Gray, B. M. and Briles, D. E. (1990). Pneumococcal surface protein A (PspA) is serologically highly variable and is expressed by all clinically important capsular serotypes of *Streptococcus pneumoniae*. *Infect Immun.* **58**:3293-9.

Crook, D., Brrueggemann, A., Sleeman, K. and Peto, T. (2004). Pneumococcal carriage. *The Pneumococcus*. E. Tuomanen. Washington D.C., ASM Press: 136-147.

Cundell, D. R., Gerard, N. P., Gerard, C., Idanpaan-Heikkila, I. and Tuomanen, E. I. (1995). *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor. *Nature* **377**:435-8.

Cundell, D. R. and Tuomanen, E. I. (1994). Receptor specificity of adherence of *Streptococcus pneumoniae* to human type-II pneumocytes and vascular endothelial cells in vitro. *Microb Pathog.* **17**:361-74.

Dahl, M. K., Msadek, T., Kunst, F. and Rapoport, G. (1992). The phosphorylation state of the DegU response regulator acts as a molecular switch allowing either degradative enzyme synthesis or expression of genetic competence in *Bacillus subtilis*. *J Biol Chem.* **267**:14509-14.

Daines, D. A. and Silver, R. P. (2000). Evidence for multimerization of neu proteins involved in polysialic acid synthesis in *Escherichia coli* K1 using improved LexA-based vectors. *J Bacteriol.* **182**:5267-70.

de Saizieu, A., Gardes, C., Flint, N., Wagner, C., Kamber, M., Mitchell, T. J., Keck, W., Amrein, K. E. and Lange, R. (2000). Microarray-based identification of a novel *Streptococcus pneumoniae* regulon controlled by an autoinduced peptide. *J. Bacteriol.* **182**:4696-703.

Delgado, J., Forst, S., Harlocker, S. and Inouye, M. (1993). Identification of a phosphorylation site and functional analysis of conserved aspartic acid residues of OmpR, a transcriptional activator for ompF and ompC in *Escherichia coli*. *Mol Microbiol* **10**:1037-47.

DiGiuseppe, P. A. and Silhavy, T. J. (2003). Signal detection and target gene induction by the CpxRA two-component system. *J Bacteriol.* **185**:2432-40.

Dmitrova, M., Younes-Cauet, G., Oertel-Buchheit, P., Porte, D., Schnarr, M. and Granger-Schnarr, M. (1998). A new LexA-based genetic system for monitoring and analyzing protein heterodimerization in *Escherichia coli*. *Mol Gen Genet* **257**:205-12.

Douglas, R. M., Paton, J. C., Duncan, S. J. and Hansman, D. J. (1983). Antibody response to pneumococcal vaccination in children younger than five years of age. *J Infect Dis.* **148**:131-7.

Duthy, T. G., Ormsby, R. J., Giannakis, E., Ogunniyi, A. D., Stroeher, U. H., Paton, J. C. and Gordon, D. L. (2002). The human complement regulator factor H binds pneumococcal surface protein PspC via short consensus repeats 13 to 15. *Infect. Immun.* **70**:5604-11.

Dziejman, M. and Mekalanos, J. J. (1995). Two-component signal transduction and its role in the expression of bacterial virulence factors. *Two-Component Signal Transduction*. J. A. Hoch, Silhavy, T.J. Washington DC, American Society for Microbiology: 305-317.

Echenique, J. R., Chapuy-Regaud, S. and Trombe, M. C. (2000). Competence regulation by oxygen in *Streptococcus pneumoniae*: involvement of *ciaRH* and *comCDE*. *Mol Microbiol* **36**:688-96.

Echenique, J. R. and Trombe, M. C. (2001). Competence repression under oxygen limitation through the two-component MicAB signal-transducing system in *Streptococcus pneumoniae* and involvement of the PAS domain of MicB. *J Bacteriol.* **183**:4599-608.

Elm, C., Braathen, R., Bergmann, S., Frank, R., Vaerman, J. P., Kaetzel, C. S., Chhatwal, G. S., Johansen, F. E. and Hammerschmidt, S. (2004). Ectodomains 3 and 4 of human polymeric Immunoglobulin receptor (hpIgR) mediate invasion of *Streptococcus pneumoniae* into the epithelium. *J Biol Chem.* **279**:6296-304. Epub 2003 Dec 3.

Eskola, J., Kilpi, T., Palmu, A., Jokinen, J., Haapakoski, J., Herva, E., Takala, A., Kayhty, H., Karma, P., Kohberger, R., Siber, G. and Makela, P. H. (2001). Efficacy of a pneumococcal conjugate vaccine against acute otitis media. *N Engl J Med.* **344**:403-9.

Fabret, C. and Hoch, J. A. (1998). A two-component signal transduction system essential for growth of *Bacillus subtilis*: implications for anti-infective therapy. *J Bacteriol* **180**:6375-83.

Federle, M. J. and Scott, J. R. (2002). Identification of binding sites for the group A streptococcal global regulator CovR. *Mol Microbiol.* **43**:1161-72.

Feldman, C., Mitchell, T. J., Andrew, P. W., Boulnois, G. J., Read, R. C., Todd, H. C., Cole, P. J. and Wilson, R. (1990). The effect of *Streptococcus pneumoniae* pneumolysin on human respiratory epithelium *in vitro*. *Microb Pathog.* **9**:275-84.

Feng, J., Atkinson, M. R., McCleary, W., Stock, J. B., Wanner, B. L. and Ninfa, A. J. (1992). Role of phosphorylated metabolic intermediates in the regulation of glutamine synthetase synthesis in *Escherichia coli*. *J. Bacteriol.* **174**:6061-70.

Ferrante, A., Rowan-Kelly, B. and Paton, J. C. (1984). Inhibition of in vitro human lymphocyte response by the pneumococcal toxin pneumolysin. *Infect Immun.* **46**:585-9.

Fisher, S. L., Jiang, W., Wanner, B. L. and Walsh, C. T. (1995). Cross-talk between the histidine protein kinase VanS and the response regulator PhoB. Characterization and identification of a VanS domain that inhibits activation of PhoB. *J Biol Chem.* **270**:23143-9.

Forst, S., Comeau, D., Norioka, S. and Inouye, M. (1987). Localization and membrane topology of EnvZ, a protein involved in osmoregulation of OmpF and OmpC in *Escherichia coli*. *J Biol Chem* **262**:16433-8.

Fukuchi, K., Kasahara, Y., Asai, K., Kobayashi, K., Moriya, S. and Ogasawara, N. (2000). The essential two-component regulatory system encoded by *yycF* and *yycG* modulates expression of the *ftsAZ* operon in *Bacillus subtilis*. *Microbiology.* **146**:1573-83.

Gabrielsen, O. S. and Huet, J. (1993). Magnetic DNA affinity purification of yeast transcription factor. *Methods Enzymol.* **218**:508-25.

Gasson, M. J. (1983). Genetic transfer systems in lactic acid bacteria. *Antonie Van Leeuwenhoek.* **49**:275-82.

Geng, H., Nakano, S. and Nakano, M. M. (2004). Transcriptional activation by *Bacillus subtilis* ResD: tandem binding to target elements and phosphorylation-dependent and -independent transcriptional activation. *J Bacteriol.* **186**:2028-37.

Giammarinaro, P. and Paton, J. C. (2002). Role of RegM, a homologue of the catabolite repressor protein CcpA, in the virulence of *Streptococcus pneumoniae*. *Infect. Immun.* **70**:5454-61.

Giard, J. C., Rince, A., Capiaux, H., Auffray, Y. and Hartke, A. (2000). Inactivation of the stress- and starvation-inducible *gls24* operon has a pleiotrophic effect on cell morphology, stress sensitivity, and gene expression in *Enterococcus faecalis*. *J Bacteriol* **182**:4512-20.

Giard, J. C., Verneuil, N., Auffray, Y. and Hartke, A. (2002). Characterization of genes homologous to the general stress-inducible gene *gls24* in *Enterococcus faecalis* and *Lactococcus lactis*. *FEMS Microbiol Lett* **206**:235-9.

Graham, R. M. A. (2005). CXC Chemokine Responses of Respiratory Epithelial Cells to *Streptococcus pneumoniae*. School of Molecular and Biomedical Sciences, University of Adelaide.

Gray, B. M., Converse, G. M., 3rd and Dillon, H. C., Jr. (1980). Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life. *J Infect Dis.* **142**:923-33.

Gray, B. M., Dillon, H. C., Jr. and Briles, D. E. (1983). Epidemiological studies of *Streptococcus pneumoniae* in infants: development of antibody to phosphocholine. *J Clin Microbiol.* **18**:1102-7.

Guenzi, E., Gasc, A. M., Sicard, M. A. and Hakenbeck, R. (1994). A two-component signal-transducing system is involved in competence and penicillin susceptibility in laboratory mutants of *Streptococcus pneumoniae*. *Mol. Microbiol.* **12**:505-15.

Haas, W., Sublett, J., Kaushal, D. and Tuomanen, E. I. (2004). Revising the role of the pneumococcal *vex-vncRS* locus in vancomycin tolerance. *J Bacteriol.* **186**:8463-71.

Hakansson, A., Roche, H., Mirza, S., McDaniel, L. S., Brooks-Walter, A. and Briles, D. E. (2001). Characterization of binding of human lactoferrin to pneumococcal surface protein A. *Infect Immun.* **69**:3372-81.

Hammerschmidt, S., Bethe, G., Remane, P. H. and Chhatwal, G. S. (1999). Identification of pneumococcal surface protein A as a lactoferrin-binding protein of *Streptococcus pneumoniae*. *Infect. Immun.* **67**:1683-7.

Hammerschmidt, S., Talay, S. R., Brandtzaeg, P. and Chhatwal, G. S. (1997). SpsA, a novel pneumococcal surface protein with specific binding to secretory immunoglobulin A and secretory component. *Mol. Microbiol.* **25**:1113-24.

Hardy, G. G., Magee, A. D., Ventura, C. L., Caimano, M. J. and Yother, J. (2001). Essential role for cellular phosphoglucomutase in virulence of type 3 *Streptococcus pneumoniae*. *Infect Immun.* **69**:2309-17.

Harlocker, S. L., Bergstrom, L. and Inouye, M. (1995). Tandem binding of six OmpR proteins to the *ompF* upstream regulatory sequence of *Escherichia coli*. *J. Biol. Chem.* **270**:26849-56.

Hava, D. L. and Camilli, A. (2002). Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors. *Mol. Microbiol.* **45**:1389-406.

Havarstein, L. S., Coomaraswamy, G. and Morrison, D. A. (1995). An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. *Proc. Natl. Acad. Sci. U S A* **92**:11140-4.

Henrichsen, J. (1995). Six newly recognized types of *Streptococcus pneumoniae*. *J Clin Microbiol.* **33**:2759-62.

Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R. (1989). Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* **77**:51-9.

Hoch, J. A. (1993). Regulation of the phosphorelay and the initiation of sporulation in *Bacillus subtilis*. *Annu Rev Microbiol.* **47**:441-65.

Hoch, J. A. (2000). Two-component and phosphorelay signal transduction. *Curr. Opin. Microbiol.* **3**:165-70.

Horton, R. M. (1993). *In vitro* recombination and mutangenesis of DNA. *Methods Mol. Biol.* **15**:251-261.

Hoskins, J., Alborn, W. E., Jr., Arnold, J., Blaszczak, L. C., Burgett, S., DeHoff, B. S., Estrem, S. T., Fritz, L., Fu, D. J., Fuller, W., Geringer, C., Gilmour, R., Glass, J. S., Khoja, H., Kraft, A. R., Lagace, R. E., LeBlanc, D. J., Lee, L. N., Lefkowitz, E. J., Lu, J., Matsushima, P., McAhren, S. M., McHenney, M., McLeaster, K., Mundy, C. W., Nicas, T. I., Norris, F. H., O'Gara, M., Peery, R. B., Robertson, G. T., Rockey, P.,

Sun, P. M., Winkler, M. E., Yang, Y., Young-Bellido, M., Zhao, G., Zook, C. A., Baltz, R. H., Jaskunas, S. R., Rosteck, P. R., Jr., Skatrud, P. L. and Glass, J. I. (2001). Genome of the bacterium *Streptococcus pneumoniae* strain R6. *J. Bacteriol.* **183**:5709-17.

Houldsworth, S., Andrew, P. W. and Mitchell, T. J. (1994). Pneumolysin stimulates production of tumor necrosis factor alpha and interleukin-1 beta by human mononuclear phagocytes. *Infect Immun.* **62**:1501-3.

Hsing, W. and Silhavy, T. J. (1997). Function of conserved histidine-243 in phosphatase activity of EnvZ, the sensor for porin osmoregulation in *Escherichia coli*. *J Bacteriol* **179**:3729-35.

Huang, S. S., Platt, R., Rifas-Shiman, S. L., Pelton, S. I., Goldmann, D. and Finkelstein, J. A. (2005). Post-PCV7 changes in colonizing pneumococcal serotypes in 16 Massachusetts communities, 2001 and 2004. *Pediatrics.* **116**:e408-13.

Hung, D. T., Shakhnovich, E. A., Pierson, E. and Mekalanos, J. J. (2005). Small-molecule inhibitor of *Vibrio cholerae* virulence and intestinal colonization. *Science* **310**:670-4.

Iannelli, F., Chiavolini, D., Ricci, S., Oggioni, M. R. and Pozzi, G. (2004). Pneumococcal surface protein C contributes to sepsis caused by *Streptococcus pneumoniae* in mice. *Infect Immun.* **72**:3077-80.

Iannelli, F., Oggioni, M. R. and Pozzi, G. (2002). Allelic variation in the highly polymorphic locus *pspC* of *Streptococcus pneumoniae*. *Gene* **284**:63-71.

Ibrahim, Y. M., Kerr, A. R., McCluskey, J. and Mitchell, T. J. (2004a). Role of HtrA in the virulence and competence of *Streptococcus pneumoniae*. *Infect. Immun.* **72**:3584-91.

Ibrahim, Y. M., Kerr, A. R., McCluskey, J., Mitchell, T. J., Mascher, T., Zahner, D., Merai, M., Balmelle, N., de Saizieu, A. B. and Hakenbeck, R. (2004b). Control of virulence by the two-component system CiaR/H is mediated via HtrA, a major virulence factor of *Streptococcus pneumoniae*. *J. Bacteriol.* **186**:5258-66.

Janoff, E. N., O'Brien, J., Thompson, P., Ehret, J., Meiklejohn, G., Duvall, G. and Douglas, J. M., Jr. (1993). *Streptococcus pneumoniae* colonization, bacteremia, and immune response among persons with human immunodeficiency virus infection. *J. Infect. Dis.* **167**:49-56.

Jedrzejas, M. J. (2001). Pneumococcal virulence factors: structure and function. *Microbiol. Mol. Biol. Rev.* **65**:187-207.

Jiang, M., Shao, W., Perego, M. and Hoch, J. A. (2000). Multiple histidine kinases regulate entry into stationary phase and sporulation in *Bacillus subtilis*. *Mol Microbiol.* **38**:535-42.

Johnston, J. W., Briles, D. E., Myers, L. E. and Hollingshead, S. K. (2006). Mn2+-dependent regulation of multiple genes in *Streptococcus pneumoniae* through PsaR and the resultant impact on virulence. *Infect Immun.* **74**:1171-80.

Jones, R. N., Biedenbach, D. J. and Beach, M. L. (2003). Influence of patient age on the susceptibility patterns of *Streptococcus pneumoniae* isolates in North America (2000-2001): report from the SENTRY Antimicrobial Surveillance Program. *Diagn Microbiol Infect Dis.* **46**:77-80.

Kadioglu, A., Echenique, J., Manco, S., Trombe, M. C. and Andrew, P. W. (2003). The MicAB two-component signaling system is involved in virulence of *Streptococcus pneumoniae*. *Infect Immun.* **71**:6676-9.

Kadioglu, A., Taylor, S., Iannelli, F., Pozzi, G., Mitchell, T. J. and Andrew, P. W. (2002). Upper and lower respiratory tract infection by *Streptococcus pneumoniae* is affected by pneumolysin deficiency and differences in capsule type. *Infect Immun.* **70**:2886-90.

Kanamaru, K., Aiba, H. and Mizuno, T. (1990). Transmembrane signal transduction and osmoregulation in *Escherichia coli*: I. Analysis by site-directed mutagenesis of the amino acid residues involved in phosphotransfer between the two regulatory components, EnvZ and OmpR. *J Biochem (Tokyo).* **108**:483-7.

Khorchid, A. and Ikura, M. (2005). Bacterial histidine kinase as signal sensor and transducer. *Int J Biochem Cell Biol* **26**:26.

Kim, J. O., Romero-Steiner, S., Sorensen, U. B., Blom, J., Carvalho, M., Barnard, S., Carlone, G. and Weiser, J. N. (1999). Relationship between cell surface carbohydrates and intrastrain variation on opsonophagocytosis of *Streptococcus pneumoniae*. *Infect Immun* **67**:2327-33.

Kim, J. O. and Weiser, J. N. (1998). Association of intrastrain phase variation in quantity of capsular polysaccharide and teichoic acid with the virulence of *Streptococcus pneumoniae*. *J Infect Dis* **177**:368-77.

Klein, D. L. (2000). Pneumococcal disease and the role of conjugate vaccines. *Streptococcus pneumoniae Molecular Biology and Mechanisms of Disease*. A. Tamasz. New York, Mary Ann Liebert Inc: 467-477.

Klose, K. E., Weiss, D. S. and Kustu, S. (1993). Glutamate at the site of phosphorylation of nitrogen-regulatory protein NTRC mimics aspartyl-phosphate and activates the protein. *J Mol Biol.* **232**:67-78.

Klugman, K. P. (1990). Pneumococcal resistance to antibiotics. *Clin Microbiol Rev.* **3**:171-96.

Knutsen, E., Ween, O. and Havarstein, L. S. (2004). Two separate quorum-sensing systems upregulate transcription of the same ABC transporter in *Streptococcus pneumoniae*. *J Bacteriol* **186**:3078-85.

Kustu, S., Santero, E., Keener, J., Popham, D. and Weiss, D. (1989). Expression of sigma 54 (*ntrA*)-dependent genes is probably united by a common mechanism. *Microbiol Rev.* **53**:367-76.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680-5.

Lan, C. Y. and Igo, M. M. (1998). Differential expression of the OmpF and OmpC porin proteins in *Escherichia coli* K-12 depends upon the level of active OmpR. *J Bacteriol.* **180**:171-4.

Lange, R., Wagner, C., de Saizieu, A., Flint, N., Molnos, J., Stieger, M., Caspers, P., Kamber, M., Keck, W. and Amrein, K. E. (1999). Domain organization and molecular characterization of 13 two-component systems identified by genome sequencing of *Streptococcus pneumoniae*. *Gene* **237**:223-34.

Lau, G. W., Haataja, S., Lonetto, M., Kensit, S. E., Marra, A., Bryant, A. P., McDevitt, D., Morrison, D. A. and Holden, D. W. (2001). A functional genomic analysis of type 3 *Streptococcus pneumoniae* virulence. *Mol Microbiol.* **40**:555-71.

Lee, C. J., Banks, S. D. and Li, J. P. (1991). Virulence, immunity, and vaccine related to *Streptococcus pneumoniae*. *Crit Rev Microbiol.* **18**:89-114.

LeMessurier, K. S. (2002). Characterisation of a nasopharyngeal carriage model of *Streptococcus pneumoniae* D39 in outbred CD1 mice. Department of Molecular Biosciences, University of Adelaide.

LeMessurier, K. S., Ogunniyi, A. D. and Paton, J. C. (2006). Differential expression of key pneumococcal virulence genes in vivo. *Microbiology.* **152**:305-11.

Lexau, C. A., Lynfield, R., Danila, R., Pilishvili, T., Facklam, R., Farley, M. M., Harrison, L. H., Schaffner, W., Reingold, A., Bennett, N. M., Hadler, J., Cieslak, P. R. and Whitney, C. G. (2005). Changing epidemiology of invasive pneumococcal disease among older adults in the era of pediatric pneumococcal conjugate vaccine. *Jama.* **294**:2043-51.

Lopez de Felipe, F., Magni, C., de Mendoza, D. and Lopez, P. (1995). Citrate utilization gene cluster of the *Lactococcus lactis* biovar diacetylactis: organization and regulation of expression. *Mol Gen Genet.* **246**:590-9.

Lu, L., Lamm, M. E., Li, H., Corthesy, B. and Zhang, J. R. (2003). The human polymeric immunoglobulin receptor binds to *Streptococcus pneumoniae* via domains 3 and 4. *J Biol Chem.* **278**:48178-87. Epub 2003 Sep 17.

Lukat, G. S., McCleary, W. R., Stock, A. M. and Stock, J. B. (1992). Phosphorylation of bacterial response regulator proteins by low molecular weight phospho-donors. *Proc. Natl. Acad. Sci. U S A* **89**:718-22.

Macrina, F. L., Evans, R. P., Tobian, J. A., Hartley, D. L., Clewell, D. B. and Jones, K. R. (1983). Novel shuttle plasmid vehicles for *Escherichia-Streptococcus* transgeneric cloning. *Gene.* **25**:145-50.

Magee, A. D. and Yother, J. (2001). Requirement for capsule in colonization by *Streptococcus pneumoniae*. *Infect Immun.* **69**:3755-61.

Marra, A., Asundi, J., Bartilson, M., Lawson, S., Fang, F., Christine, J., Wiesner, C., Brigham, D., Schneider, W. P. and Hromockyj, A. E. (2002). Differential fluorescence induction analysis of *Streptococcus pneumoniae* identifies genes involved in pathogenesis. *Infect. Immun.* **70**:1422-33.

Martin, M., Turco, J. H., Zegans, M. E., Facklam, R. R., Sodha, S., Elliott, J. A., Pryor, J. H., Beall, B., Erdman, D. D., Baumgartner, Y. Y., Sanchez, P. A., Schwartzman, J. D., Montero, J., Schuchat, A. and Whitney, C. G. (2003). An outbreak of conjunctivitis due to atypical *Streptococcus pneumoniae*. *N Engl J Med.* **348**:1112-21.

Martinez-Hackert, E. and Stock, A. M. (1997). The DNA-binding domain of OmpR: crystal structures of a winged helix transcription factor. *Structure.* **5**:109-24.

Marton, A., Gulyas, M., Munoz, R. and Tomasz, A. (1991). Extremely high incidence of antibiotic resistance in clinical isolates of *Streptococcus pneumoniae* in Hungary. *J Infect Dis.* **163**:542-8.

Maus, U. A., Srivastava, M., Paton, J. C., Mack, M., Everhart, M. B., Blackwell, T. S., Christman, J. W., Schlondorff, D., Seeger, W. and Lohmeyer, J. (2004). Pneumolysin-induced lung injury is independent of leukocyte trafficking into the alveolar space. *J Immunol.* **173**:1307-12.

Mbelle, N., Huebner, R. E., Wasas, A. D., Kimura, A., Chang, I. and Klugman, K. P. (1999). Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. *J Infect Dis.* **180**:1171-6.

McCluskey, J., Hinds, J., Husain, S., Witney, A. and Mitchell, T. J. (2004). A two-component system that controls the expression of pneumococcal surface antigen A (PsaA) and regulates virulence and resistance to oxidative stress in *Streptococcus pneumoniae*. *Mol. Microbiol.* **51**:1661-75.

McDaniel, L. S., Yother, J., Vijayakumar, M., McGarry, L., Guild, W. R. and Briles, D. E. (1987). Use of insertional inactivation to facilitate studies of biological properties of pneumococcal surface protein A (PspA). *J Exp Med.* **165**:381-94.

McGee, L. (2000). Serotypes and clones of antibiotic-resistant pneumococci. *Streptococcus pneumoniae; Molecular Biology and Mechanisms of Disease.* A. Tomasz. New York, Mary Ann Liebert Inc.

Mera, R. M., Miller, L. A., Daniels, J. J., Weil, J. G. and White, A. R. (2005). Increasing prevalence of multidrug-resistant *Streptococcus pneumoniae* in the United States over a 10-year period: Alexander Project. *Diagn Microbiol Infect Dis.* **51**:195-200.

Miller, J. H. (1972). Experiments in molecular genetics. Cold Spring Harbor, New York, N.Y., Cold Spring Harbor Laboratory Press.

Mohedano, M. L., Overweg, K., de la Fuente, A., Reuter, M., Altabe, S., Mulholland, F., de Mendoza, D., Lopez, P. and Wells, J. M. (2005). Evidence that the essential response regulator YycF in *Streptococcus pneumoniae* modulates expression of fatty acid biosynthesis genes and alters membrane composition. *J Bacteriol* **187**:2357-67.

Molle, V. and Buttner, M. J. (2000). Different alleles of the response regulator gene *bldM* arrest *Streptomyces coelicolor* development at distinct stages. *Mol Microbiol.* **36**:1265-78.

Moore, J. B., Shiau, S. P. and Reitzer, L. J. (1993). Alterations of highly conserved residues in the regulatory domain of nitrogen regulator I (NtrC) of *Escherichia coli. J Bacteriol.* **175**:2692-701.

Morona, J. K., Miller, D. C., Morona, R. and Paton, J. C. (2004). The effect that mutations in the conserved capsular polysaccharide biosynthesis genes *cpsA*, *cpsB*, and *cpsD* have on virulence of *Streptococcus pneumoniae. J Infect Dis.* **189**:1905-13. Epub 2004 Apr 27.

Mortier-Barriere, I., de Saizieu, A., Claverys, J. P. and Martin, B. (1998). Competence-specific induction of *recA* is required for full recombination proficiency during transformation in *Streptococcus pneumoniae. Mol Microbiol.* **27**:159-70.

Msadek, T. (1999). When the going gets tough: survival strategies and environmental signaling networks in *Bacillus subtilis*. *Trends Microbiol.* **7**:201-7.

Msadek, T., Kunst, F. and Rapoport, G. (1995). A Signal Transduction Network in *Bacillus subtilis* Includes the DegS/DegU and ComP/ComA Two-Component Systems. *Two-Component Signal Transduction*. J. A. Hoch and T. J. Silhavy. Washington D.C., ASM Press: 447-471.

Musher, D. M. (1992). Infections caused by *Streptococcus pneumoniae*: clinical spectrum, pathogenesis, immunity, and treatment. *Clin Infect Dis.* **14**:801-7.

Musher, D. M., Groover, J. E., Reichler, M. R., Riedo, F. X., Schwartz, B., Watson, D. A., Baughn, R. E. and Breiman, R. F. (1997). Emergence of antibody to capsular polysaccharides of *Streptococcus pneumoniae* during outbreaks of pneumonia: association with nasopharyngeal colonization. *Clin Infect Dis.* **24**:441-6.

Ng, W. L., Robertson, G. T., Kazmierczak, K. M., Zhao, J., Gilmour, R. and Winkler, M. E. (2003). Constitutive expression of PcsB suppresses the requirement for the essential VicR (YycF) response regulator in *Streptococcus pneumoniae* R6. *Mol Microbiol* **50**:1647-63.

Ng, W. L., Tsui, H. C. and Winkler, M. E. (2005). Regulation of the *pspA* Virulence Factor and Essential *pcsB* Murein Biosynthetic Genes by the Phosphorylated VicR (YycF) Response Regulator in *Streptococcus pneumoniae*. *J Bacteriol* **187**:7444-59.

Nieto, C., Fernandez de Palencia, P., Lopez, P. and Espinosa, M. (2000). Construction of a tightly regulated plasmid vector for *Streptococcus pneumoniae*: controlled expression of the green fluorescent protein. *Plasmid.* **43**:205-13.

Ninfa, A. J., Ninfa, E. G., Lupas, A. N., Stock, A., Magasanik, B. and Stock, J. (1988). Crosstalk between bacterial chemotaxis signal transduction proteins and regulators of transcription of the Ntr regulon: evidence that nitrogen assimilation and chemotaxis are controlled by a common phosphotransfer mechanism. *Proc Natl Acad Sci U S A.* **85**:5492-6.

Nourti, J. P., Butler, J. C., Farley, M. M., Harrison, L. H., McGeer, A., Kolczak, M. S. and Breiman, R. F. (2000). Cigarette smoking and invasive pneumococcal disease. *N Engl J Med* **342**:681-689.

Novak, R., Henriques, B., Charpentier, E., Normark, S. and Tuomanen, E. (1999). Emergence of vancomycin tolerance in *Streptococcus pneumoniae*. *Nature* **399**:590-3.

Obaro, S. K., Adegbola, R. A., Banya, W. A. and Greenwood, B. M. (1996). Carriage of pneumococci after pneumococcal vaccination. *Lancet.* **348**:271-2.

Ogunniyi, A. D., Folland, R. L., Briles, D. E., Hollingshead, S. K. and Paton, J. C. (2000). Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*. *Infect. Immun.* **68**:3028-33.

Ogunniyi, A. D., Giammarinaro, P. and Paton, J. C. (2002). The genes encoding virulence-associated proteins and the capsule of *Streptococcus pneumoniae* are upregulated and differentially expressed *in vivo*. *Microbiology* **148**:2045-53.

Ogura, M., Yamaguchi, H., Yoshida, K., Fujita, Y. and Tanaka, T. (2001). DNA microarray analysis of *Bacillus subtilis* DegU, ComA and PhoP regulons: an approach to comprehensive analysis of *B.subtilis* two-component regulatory systems. *Nucleic Acids Res.* **29**:3804-13.

Okeke, I. N., Klugman, K. P., Bhutta, Z. A., Duse, A. G., Jenkins, P., O'Brien, T. F., Pablos-Mendez, A. and Laxminarayan, R. (2005). Antimicrobial resistance in developing countries. Part II: strategies for containment. *Lancet Infect Dis.* **5**:568-80.

Orihuela, C. J., Gao, G., Francis, K. P., Yu, J. and Tuomanen, E. I. (2004a). Tissue-specific contributions of pneumococcal virulence factors to pathogenesis. *J Infect Dis* **190**:1661-9. Epub 2004 Sep 21.

Orihuela, C. J., Radin, J. N., Sublett, J. E., Gao, G., Kaushal, D. and Tuomanen, E. I. (2004b). Microarray analysis of pneumococcal gene expression during invasive disease. *Infect Immun.* **72**:5582-96.

Park, H. and Inouye, M. (1997). Mutational analysis of the linker region of EnvZ, an osmosensor in *Escherichia coli*. *J Bacteriol.* **179**:4382-90.

Parkinson, J. S. and Kofoid, E. C. (1992). Communication modules in bacterial signaling proteins. *Annu Rev Genet.* **26**:71-112.

Paton, J. C. (2004). New pneumococcal vaccines: Basic science and developments. *The Pneumococcus*. E. Tuomanen. Washington D.C., ASM Press**:** 383-402.

Paton, J. C., Lock, R. A. and Hansman, D. J. (1983). Effect of immunization with pneumolysin on survival time of mice challenged with *Streptococcus pneumoniae*. *Infect Immun.* **40**:548-52.

Paton, J. C., Rowan-Kelly, B. and Ferrante, A. (1984). Activation of human complement by the pneumococcal toxin pneumolysin. *Infect Immun.* **43**:1085-7.

Perraud, A. L., Weiss, V. and Gross, R. (1999). Signalling pathways in two-component phosphorelay systems. *Trends Microbiol.* **7**:115-20.

Perron-Savard, P., De Crescenzo, G. and Le Moual, H. (2005). Dimerization and DNA binding of the *Salmonella enterica* PhoP response regulator are phosphorylation independent. *Microbiology.* **151**:3979-87.

Pestova, E. V., Havarstein, L. S. and Morrison, D. A. (1996). Regulation of competence for genetic transformation in *Streptococcus pneumoniae* by an auto-induced peptide pheromone and a two-component regulatory system. *Mol. Microbiol.* **21**:853-62.

Peterson, S. N., Sung, C. K., Cline, R., Desai, B. V., Snesrud, E. C., Luo, P., Walling, J., Li, H., Mintz, M., Tsegaye, G., Burr, P. C., Do, Y., Ahn, S., Gilbert, J., Fleischmann, R. D. and Morrison, D. A. (2004). Identification of competence pheromone responsive genes in *Streptococcus pneumoniae* by use of DNA microarrays. *Mol Microbiol* **51**:1051-1070.

Polissi, A., Pontiggia, A., Feger, G., Altieri, M., Mottl, H., Ferrari, L. and Simon, D. (1998). Large-scale identification of virulence genes from *Streptococcus pneumoniae*. *Infect Immun.* **66**:5620-9.

Reichmann, P. and Hakenbeck, R. (2000). Allelic variation in a peptide-inducible two-component system of *Streptococcus pneumoniae*. *FEMS Microbiol Lett* **190**:231-6.

Reichmann, P., Konig, A., Linares, J., Alcaide, F., Tenover, F. C., McDougal, L., Swidsinski, S. and Hakenbeck, R. (1997). A global gene pool for high-level cephalosporin resistance in commensal *Streptococcus* species and *Streptococcus pneumoniae*. *J Infect Dis.* **176**:1001-12.

Ren, B., McCrory, M. A., Pass, C., Bullard, D. C., Ballantyne, C. M., Xu, Y., Briles, D. E. and Szalai, A. J. (2004a). The virulence function of *Streptococcus pneumoniae* surface protein A involves inhibition of complement activation and impairment of complement receptor-mediated protection. *J Immunol.* **173**:7506-12.

Ren, B., Szalai, A. J., Hollingshead, S. K. and Briles, D. E. (2004b). Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface. *Infect Immun.* **72**:114-22.

Reyrat, J. M., David, M., Batut, J. and Boistard, P. (1994). FixL of *Rhizobium meliloti* enhances the transcriptional activity of a mutant FixJD54N protein by phosphorylation of an alternate residue. *J Bacteriol.* **176**:1969-76.

Riley, I. D. and Douglas, R. M. (1981). An epidemiologic approach to pneumococcal disease. *Rev Infect Dis* **3**:233-45.

Robertson, G. T., Zhao, J., Desai, B. V., Coleman, W. H., Nicas, T. I., Gilmour, R., Grinius, L., Morrison, D. A. and Winkler, M. E. (2002). Vancomycin tolerance induced by erythromycin but not by loss of *vncRS*, *vex3*, or *pep27* function in *Streptococcus pneumoniae*. *J Bacteriol* **184**:6987-7000.

Robinson, V. L., Buckler, D. R. and Stock, A. M. (2000). A tale of two components: a novel kinase and a regulatory switch. *Nat Struct Biol* **7**:626-33.

Rosenow, C., Ryan, P., Weiser, J. N., Johnson, S., Fontan, P., Ortqvist, A. and Masure, H. R. (1997). Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*. *Mol. Microbiol.* **25**:819-29.

Roychoudhury, S., Zielinski, N. A., Ninfa, A. J., Allen, N. E., Jungheim, L. N., Nicas, T. I. and Chakrabarty, A. M. (1993). Inhibitors of two-component signal transduction systems: inhibition of alginate gene activation in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **90**:965-9.

Rubins, J. B., Charboneau, D., Paton, J. C., Mitchell, T. J., Andrew, P. W. and Janoff, E. N. (1995). Dual function of pneumolysin in the early pathogenesis of murine pneumococcal pneumonia. *J Clin Invest.* **95**:142-50.

Schnarr M and M, G.-S. (1993). LexA, the self-cleaving transcriptional repressor of the SOS system. *Nucleic acids and molecular biology, vol. 7.* Eckstein F and L. DMJ. Berlin, Springer-Verlag. **7:** 170-189.

Sebert, M. E., Palmer, L. M., Rosenberg, M. and Weiser, J. N. (2002). Microarray-based identification of *htrA*, a *Streptococcus pneumoniae* gene that is regulated by the CiaRH two-component system and contributes to nasopharyngeal colonization. *Infect. Immun.* **70**:4059-67.

Shaper, M., Hollingshead, S. K., Benjamin, W. H., Jr. and Briles, D. E. (2004). PspA protects *Streptococcus pneumoniae* from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin [corrected]. *Infect Immun.* **72**:5031-40.

Shoemaker, N. B., Smith, M. D. and Guild, W. R. (1979). Organization and transfer of heterologous chloramphenicol and tetracycline resistance genes in pneumococcus. *J Bacteriol.* **139**:432-41.

Skerker, J. M., Prasol, M. S., Perchuk, B. S., Biondi, E. G. and Laub, M. T. (2005). Two-component signal transduction pathways regulating growth and cell cycle progression in a bacterium: a system-level analysis. *PLoS Biol* **3**:e334. Epub 2005 Sep 27.

Smith, B. L. and Hostetter, M. K. (2000). C3 as substrate for adhesion of *Streptococcus pneumoniae*. *J. Infect. Dis.* **182**:497-508. Epub 2000 Jul 19.

Standish, A. J. (2002). Two component signal transduction in *Streptococcus pneumoniae*. Microbiology, University of Adelaide.

Standish, A. J., Stroeher, U. H. and Paton, J. C. (2005). The two-component signal transduction system RR06/HK06 regulates expression of *cbpA* in *Streptococcus pneumoniae*. *Proc Natl Acad Sci U S A* **102**:7701-6.

Stock, A. M., Robinson, V. L. and Goudreau, P. N. (2000). Two-component signal transduction. *Annu Rev Biochem* **69**:183-215.

Stock, J. B., Ninfa, A. J. and Stock, A. M. (1989). Protein phosphorylation and regulation of adaptive responses in bacteria. *Microbiol Rev.* **53**:450-90.

Talbot, U. M., Paton, A. W. and Paton, J. C. (1996). Uptake of *Streptococcus pneumoniae* by respiratory epithelial cells. *Infect. Immun.* **64**:3772-7.

Teng, F., Nannini, E. C. and Murray, B. E. (2005). Importance of *gls24* in virulence and stress response of *Enterococcus faecalis* and use of the Gls24 protein as a possible immunotherapy target. *J Infect Dis* **191**:472-80. Epub 2004 Dec 28.

Thore, M., Lofgren, S., Tarnvik, A., Monsen, T., Selstam, E. and Burman, L. G. (1985). Anaerobic phagocytosis, killing, and degradation of *Streptococcus pneumoniae* by human peripheral blood leukocytes. *Infect Immun.* **47**:277-81.

Throup, J. P., Koretke, K. K., Bryant, A. P., Ingraham, K. A., Chalker, A. F., Ge, Y., Marra, A., Wallis, N. G., Brown, J. R., Holmes, D. J., Rosenberg, M. and Burnham, M. K. (2000). A genomic analysis of two-component signal transduction in *Streptococcus pneumoniae*. *Mol. Microbiol.* **35**:566-76.

Tokishita, S., Kojima, A. and Mizuno, T. (1992). Transmembrane signal transduction and osmoregulation in *Escherichia coli*: functional importance of the transmembrane regions of membrane-located protein kinase, EnvZ. *J Biochem (Tokyo).* **111**:707-13.

Towbin, H., Staehelin, T. and Gordon, J. (1992). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. 1979. *Biotechnology* **24**:145-9.

Tsuzuki, M., Ishige, K. and Mizuno, T. (1995). Phosphotransfer circuitry of the putative multi-signal transducer, ArcB, of *Escherichia coli*: *in vitro* studies with mutants. *Mol Microbiol.* **18**:953-62.

Tu, A. H., Fulgham, R. L., McCrory, M. A., Briles, D. E. and Szalai, A. J. (1999). Pneumococcal surface protein A inhibits complement activation by *Streptococcus pneumoniae*. *Infect Immun.* **67**:4720-4.

Tuomanen, E., Rich, R. and Zak, O. (1987). Induction of pulmonary inflammation by components of the pneumococcal cell surface. *Am Rev Respir Dis.* **135**:869-74.

Ulijasz, A. T., Andes, D. R., Glasner, J. D. and Weisblum, B. (2004). Regulation of Iron Transport in *Streptococcus pneumoniae* by RitR, an Orphan Response Regulator. *J Bacteriol* **186**:8123-8136.

van Dam, J. E., Fleer, A. and Snippe, H. (1990). Immunogenicity and immunochemistry of *Streptococcus pneumoniae* capsular polysaccharides. *Antonie Van Leeuwenhoek.* **58**:1-47.

van Rossum, A. M., Lysenko, E. S. and Weiser, J. N. (2005). Host and Bacterial Factors Contributing to the Clearance of Colonization by *Streptococcus pneumoniae* in a Murine Model. *Infect Immun* **73**:7718-26.

Ween, O., Gaustad, P. and Havarstein, L. S. (1999). Identification of DNA binding sites for ComE, a key regulator of natural competence in *Streptococcus pneumoniae*. *Mol Microbiol* **33**:817-27.

Weiser, J. N., Austrian, R., Sreenivasan, P. K. and Masure, H. R. (1994). Phase variation in pneumococcal opacity: relationship between colonial morphology and nasopharyngeal colonization. *Infect Immun* **62**:2582-9.

Welch, M., Oosawa, K., Aizawa, S. and Eisenbach, M. (1993). Phosphorylation-dependent binding of a signal molecule to the flagellar switch of bacteria. *Proc Natl Acad Sci U S A.* **90**:8787-91.

Wellmer, A., Zysk, G., Gerber, J., Kunst, T., Von Mering, M., Bunkowski, S., Eiffert, H. and Nau, R. (2002). Decreased virulence of a pneumolysin-deficient strain of *Streptococcus pneumoniae* in murine meningitis. *Infect Immun.* **70**:6504-8.

Winkelstein, J. A. (1981). The role of complement in the host's defense against *Streptococcus pneumoniae*. *Rev Infect Dis.* **3**:289-98.

Wu, H. Y., Virolainen, A., Mathews, B., King, J., Russell, M. W. and Briles, D. E. (1997). Establishment of a *Streptococcus pneumoniae* nasopharyngeal colonization model in adult mice. *Microb Pathog.* **23**:127-37.

Wyman, C., Rombel, I., North, A. K., Bustamante, C. and Kustu, S. (1997). Unusual oligomerization required for activity of NtrC, a bacterial enhancer-binding protein. *Science.* **275**:1658-61.

Yindeeyoungyeon, W. and Schell, M. A. (2000). Footprinting with an automated capillary DNA sequencer. *Biotechniques* **29**:1034-6, 1038, 1040-1.

Yuste, J., Botto, M., Paton, J. C., Holden, D. W. and Brown, J. S. (2005). Additive inhibition of complement deposition by pneumolysin and PspA facilitates *Streptococcus pneumoniae* septicemia. *J Immunol.* **175**:1813-9.

Zhang, J. R., Mostov, K. E., Lamm, M. E., Nanno, M., Shimida, S., Ohwaki, M. and Tuomanen, E. (2000). The polymeric immunoglobulin receptor translocates pneumococci across human nasopharyngeal epithelial cells. *Cell.* **102**:827-37.

Zimmerman, R. K. (2001). Pneumococcal conjugate vaccine for young children. *Am Fam Physician* **63**:1991-8.

APPENDIX

Publications

Standish, AJ, Stroeher, UH and Paton JC. (2005) The two-component regulatory transduction system regulates expression of *cbpA* in Streptococcus pneumoniae. *Proc Natl Acad Sci U S A* **102,** 7701-6.

Standish, AJ, Stroeher, UH and Paton JC. CbpA Expression and the two component regulatory system RR06 and HK06. Abstract PATH-45, p 177. 4th International Symposium on Pneumococci and Pneumococcal Diseases, 9-13 May 2004, Helsinki, Finland.

Standish, AJ, Stroeher, UH and Paton JC. The two-component regulatory transduction system regulates expression of *cbpA* in Streptococcus pneumoniae. 7th European Meeting on the Molecular Biology of the Pneumococcus, 8-11 May 2005, Braunschweig, Germany.

Standish, AJ, Stroeher, UH and Paton JC. The two-component signal transduction system RR/HK06. Abstract P05.04, p 197. 5th International Symposium on Pneumococci and Pneumococcal Diseases, 2-6 April 2006, Alice Springs, Australia.

Standish, AJ, Stroeher, UH and Paton JC (2006). Pneumococcal two-component signal transduction system RR/HK06 regulates CbpA and PspA by two distinct mechanisms. Manuscript in preparation.

The two-component signal transduction system RR06/HK06 regulates expression of *cbpA* in *Streptococcus pneumoniae*

Alistair J. Standish, Uwe H. Stroeher, and James C. Paton*

Australian Bacterial Pathogenesis Program, School of Molecular and Biomedical Science, University of Adelaide, Adelaide SA 5005, Australia

Edited by Stanley Falkow, Stanford University, Stanford, CA, and approved April 14, 2005 (received for review December 16, 2004)

Streptococcus pneumoniae **encounters a number of environmental niches in the body, including the nasopharynx, lungs, blood, middle ear, and brain. Recent studies have identified 13 putative two-component signal-transduction systems in *S. pneumoniae*, which are likely to be important for gene regulation in response to external stimuli. Here, we present conclusive evidence for the regulation of choline binding protein A (CbpA), a major pneumococcal virulence factor and protective antigen, by one of these two-component signal-transduction systems. We have demonstrated divergent expression of *cbpA* in unmarked *hk06* and *rr06* deletion mutants relative to wild-type *S. pneumoniae* D39 by using Western immunoblotting and real-time RT-PCR. Electrophoretic mobility-shift and solid-phase binding assays have demonstrated the binding of RR06 to the promoter region of *cbpA*, suggesting that RR06/HK06 directly regulates *cbpA* transcription. We have also shown that this system is important for the ability of the pneumococcus to adhere to epithelial cells *in vitro* and to survive and proliferate in an *in vivo* mouse model. Thus, the RR06/HK06 system has a significant role in pathogenesis, both in colonization and invasive disease.**

adherence | two-component system | pneumococcus | colonization

The major human pathogen *Streptococcus pneumoniae* is responsible for diseases, including pneumonia, sepsis, meningitis, and otitis media. During the course of an infection, the pneumococcus encounters a number of different environmental niches, including the nasopharynx, lungs, blood, and potentially the brain. Thus, the regulation of virulence factors in response to external stimuli may be a principal determinant of the success or failure of the infection. Recently, several virulence-associated genes of *S. pneumoniae* have been shown to be differentially expressed in varying *in vitro* and *in vivo* conditions, but the underlying mechanism is unknown (1, 2).

Two-component signal-transduction systems (TCSTSs) control the expression of virulence factors in a wide range of bacterial species in response to external stimuli (reviewed in ref. 3). The typical TCSTS consists of a His kinase (HK) and a response regulator (RR). The HK, usually present in the cell membrane, responds to external stimuli by autophosphorylating a conserved His residue, with subsequent transfer of this phosphate group to the RR. The resultant conformational change enables the RR to bind to the promoter region of target gene(s) and act as a transcription factor.

The *S. pneumoniae* genome sequence contains 13 putative TCSTSs, along with one orphan RR (4, 5). The best characterized of these is ComDE, which activates the genetic competence system in response to a competence-stimulating peptide (6, 7). Another system, CiaRH, also affects competence and has been shown to control a novel virulence factor, HtrA (8, 9). More recently, HK/RR04 has been linked to the control of the Mn transporter PsaA, although this regulation was apparently strain-specific (10). In this study, we used unmarked deletion mutants to examine the role of the RR06/HK06 TCSTS (4) (also referred to as 478; ref. 5) in pathogenesis and identify a crucial target gene, *cbpA*. These findings provide conclusive evidence for regulation of a major pneumococcal virulence factor and protective antigen by a TCSTS, and they demonstrate binding of RR06 to the promoter region of *cbpA*.

Materials and Methods

Bacterial Strains, Plasmids, Growth Conditions, and Transformation. Bacterial strains and plasmids are given in Table 1, which is published as supporting information on the PNAS web site. *S. pneumoniae* were grown in Todd Hewitt broth with 1% yeast extract (THY), serum broth (SB), or on blood agar, as described (2). *Escherichia coli* strains were grown in LB broth or LB agar (11). *S. pneumoniae* D39 and TIGR4 were transformed as described, with competence-stimulating peptide-2 used to transform TIGR4 (12–14). Transformation of *E. coli* K-12 with plasmid DNA was carried out with $CaCl_2$-treated cells (15).

Oligonucleotide Primers, DNA Isolation, and Manipulation. The primers that were used are given in Table 2, which is published as supporting information on the PNAS web site. *S. pneumoniae* chromosomal DNA was isolated, purified, and analyzed as described (16). DNA amplification was performed by high-fidelity PCR using the Expand Long Template PCR system (Roche Diagnostics). Overlap-extension PCR products were generated from the initial products as described (17, 18). Amplification products were purified by using the UltraClean PCR CleanUp DNA purification kit (Qiagen, Valencia, CA) and sequenced by using the Big Dye system (Applied Biosystems) on a model 3700 automated sequencer.

Construction of *S. pneumoniae* with Deletions in *rr06* and *hk06*. Initially, an insertion-duplication mutant in *rr06* was constructed essentially as described (19) by using oligonucleotides Sp7c and Sp7d. Because insertion duplication can result in the production of truncated gene products, as well as possible polar effects on downstream genes, unmarked deletion mutations in the *hk06* and *rr06* genes were constructed by using overlap-extension PCR mutagenesis (17, 18). For *hk06*, separate 5′- and 3′-flanking fragments were generated by using PCR with D39 DNA template and primer pairs AS5/AS14 and AS6/AS13. The two PCR products were then combined and subjected to a second round of amplification by using the primer pair AS5/AS6. This PCR generated a 5,720-bp linear DNA fragment comprising the *hk06* gene with flanking sequences but with nucleotides 73–1,308 of the *hk06* ORF deleted. Nucleotides 23–639 of the *rr06* gene were similarly deleted by using primers AS6/AS10 and AS5/AS11 in

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: TCSTS, two-component signal-transduction system; RR, response regulator; HK, His kinase; DIG, digoxigenin; CFU, colony-forming unit; SB, serum broth; THY, Todd Hewitt broth with 1% yeast extract; SPB, solid-phase binding; CbpA, choline binding protein A.

*To whom correspondence should be addressed. E-mail: james.paton@adelaide.edu.au.

round one, followed by a second round of amplification using the primer pair AS5/AS6. These constructs were then used to transform the insertion-duplication mutant D39*rr06*$^-$. Putative mutants were checked by PCR using flanking oligonucleotides, and in-frame deletion was confirmed by sequence analysis. These mutants were designated D39Δ*hk06* and D39Δ*rr06*, respectively.

Deletion mutations in *hk06* and *rr06* could not be constructed in TIGR4; however, mutants in which most of the gene was deleted and replaced with an erythromycin resistance cassette were created by using the method described in ref. 5. Oligonucleotide combinations AS53/AS5 and AS54/AS6, along with AS60/AS5 and AS61/AS6, were used for production of flanking DNA of *rr06* and *hk06*, respectively. The erythromycin cassette was amplified by using J214 and J215, and products were joined by using overlap-extension PCR and subsequently transformed into TIGR4. The mutants designated TIGR4*hk06* and TIGR4*rr06* were confirmed by PCR and sequencing.

SDS/PAGE and Western Blotting. Bacteria were subjected to SDS/PAGE as described by Laemmli (20). Separated samples were then electroblotted onto nitrocellulose (Pall) as described (21). After transfer, the membrane was probed with polyclonal murine antisera at a dilution of 1:3,000 and then reacted with blotting grade goat anti-mouse IgG alkaline phosphatase conjugate (BioRad). Labeled bands were visualized by using a nitroblue tetrazolium/X-phosphate substrate system (Roche Diagnostics).

Expression and Purification of RR06. The RR06 ORF from *S. pneumoniae* D39 was amplified by using AS1 and AS2. The PCR product was digested with BamHI and HindIII, cloned into pQE30, and transformed into *E. coli* M15 (Qiagen) to generate a His-6 fusion. His-6–RR06 expression was induced by the addition of 2 mM IPTG. The recombinant protein was purified by nickel–nitrilotriacetic acid (Ni-NTA) affinity chromatography (Qiagen).

Immunization of Mice. His-6–RR06 antigen was formulated in aluminum hydroxide adjuvant (Alum) to a final ratio of 100 μg of antigen to 1 mg of Alum adjuvant. CD-1 mice were then immunized i.p. with three doses of 10 μg at 14-day intervals. Polyclonal mouse serum was collected by cardiac puncture 7 days after the final immunization and stored at 4°C until use. Mouse antisera against choline binding protein A (CbpA), PspA, Ply, and PsaA were prepared previously in the laboratory by using methods similar to those described for RR06.

RNA Extraction. RNA was extracted from *S. pneumoniae* strains by using the RiboPure bacteria kit (Ambion, Austin, TX) according to the manufacturer's instructions. RNA was resuspended in nuclease-free water in the presence of RNasin RNase inhibitor (Promega). Contaminating DNA was digested with DNase I (Roche Diagnostics), and the purity of all RNA isolations was confirmed by subjecting them to one-step RT-PCR analysis with or without reverse transcriptase by using 16s rRNA-specific primers (see Table 2).

Real-Time RT-PCR. Real-time RT-PCR was performed on a Rotorgene RG-2000 (Corbett Research, Mortlake NSW, Australia) by using the Access RT-PCR system (Promega) according to the manufacturer's instructions and essentially as described (22).

Electrophoretic Mobility-Shift Assays. We labeled 10 pmol of PCR-amplified promoter DNA by using digoxigenin (DIG)-11-dideoxy-UTP and terminal transferase according to the manufacturer's instructions (Roche Diagnostics). Cell extracts were prepared by lysing overnight cultures of *E. coli* K-12 expressing the appropriate protein by using a French pressure cell (Aminco–SLM, Urbana, IL). The protein concentrations in the cell extracts were determined by using a Bio-Rad protein-assay kit. Lysates containing specified protein concentrations were preincubated on ice with 100 μg of herring sperm DNA in TM-1 buffer (25 mM Tris·HCl, pH 7.6/100 mM KCl/0.5 mM DTT/5 mM $MgCl_2$/0.5 mM EDTA/10% glycerol) for 20 min. We then incubated ≈1 pmol of labeled DNA target with the mixture on ice for an additional 20 min. Binding reactions were loaded onto a preelectrophoresed 4% nondenaturing PAGE containing 1× TBE and electrophoresed at 100 V for ≈2–3 h at 4°C. The DNA–protein complexes were then transferred onto Hybond-N$^+$ membrane (Amersham Pharmacia) at 400 mA for 30 min by using 1× TBE, and the DNA was then cross-linked and reacted with goat anti-DIG alkaline phosphatase conjugate (Roche Diagnostics). Labeled bands were visualized by using nitroblue tetrazolium/X-phosphate substrate.

Solid-Phase Binding (SPB) Assay. The specific binding of RR06 to the *cbpA* promoter region was also investigated by using an SPB assay essentially as described (23). We biotin end-labeled ≈40 pmol of DNA by using biotin-dideoxy-UTP and terminal transferase, according to the manufacturer's instructions (Roche Diagnostics). Strepdavidin MagneSphere Paramagnetic Particles (Promega) were washed three times in 1× CB buffer (1 M NaCl/5 mM Tris, pH 8.0/0.5 mM EDTA). Labeled DNA was then bound to the beads in 1× CB buffer with gentle agitation for 60 min at room temperature, followed by three washes in 1× CB and three washes in TM-1. The protein–DNA binding reaction was performed in a total volume of 100 μl, containing 50 μg of specified lysate, 100 μg of herring sperm DNA, and 0.6 mg of coated beads for 20 min on ice. After washing three times vigorously in binding buffer, bound proteins were eluted by boiling the beads for 5 min in lysis buffer and then analyzed by SDS/PAGE and Western blotting using anti-RR06.

Adherence Assay. Adherence of pneumococci to A549 cells (human type II pneumocytes) and Detroit 562 cells (human nasopharyngeal cells) was determined as described (24).

Intranasal Colonization Model. The ability of pneumococci to colonize the nasopharynx and translocate to the lungs and blood was investigated as described (25).

Results

Effect of *hk06* and *rr06* Deletion on Pneumococcal Virulence Protein Expression. We have demonstrated that several well characterized virulence genes of *S. pneumoniae*, including *ply*, *pspA*, *psaA*, and *cbpA* are differentially expressed under different environmental conditions (2). Of these genes, *cbpA* (also known as *pspC*) was of particular interest, because it is located only 750 nt downstream of *rr06/hk06*, and a number of prokaryotic TCSTSs regulate adjacent genes on the chromosome, including the *blpH/R* system from the pneumococcus (26). CbpA is an important pneumococcal virulence factor associated with nasopharyngeal colonization, adherence of pneumococci to epithelial cells, and the ability of the pathogen to cause lung infection (27, 28). It binds the secretory component of sIgA, and also inhibits complement activation by binding C3 and factor H (29–31). CbpA is also a protective immunogen in mice and has been proposed as a component of protein-based pneumococcal vaccines for use in humans (32).

Accordingly, we used Western immunoblot analysis with polyclonal murine antisera to compare the levels of CbpA, as well as those of Ply, PspA, and PsaA, in lysates of D39, D39Δ*hk06*, and D39Δ*rr06*. Because a number of proteins are reported to be differentially expressed in opaque and transparent phase pneumococci (28), all strains were confirmed to be in the transparent phase before Western immunoblot analysis.

Fig. 1. Western blot analysis. Proteins in lysates of D39 (lanes 1 and 4), D39Δ*hk06* (lanes 2 and 5), and D39Δ*rr06* (lanes 3 and 6) grown in THY or SB, to an A_{600} of 0.35 and 0.09 respectively, were separated by SDS/PAGE, transferred onto nitrocellulose, and probed with polyclonal murine anti-CbpA serum.

Strains were grown to identical culture densities ($A_{600} = 0.35$ and 0.09 in THY and SB, respectively); equal numbers of cells were harvested, resuspended in lysis buffer, and then subjected to SDS/PAGE and Western blotting. No differences were observed in the levels of Ply, PspA, and PsaA in D39, D39Δ*hk06*, and D39Δ*rr06* lysates (data not shown). However, as shown in Fig. 1, CbpA expression in both THY and SB appeared to be up-regulated in D39Δ*hk06* and down-regulated in D39Δ*rr06* compared with the parent strain. The culture supernatants from each strain were also tested for the presence of CbpA, and showed a similar result (data not shown), indicating that the differences seen in CbpA expression are not due to effects on anchorage of CbpA to the cell surface. The same changes in CbpA expression were also detected when the strains were in the opaque phase (data not shown).

Effect of Δ*hk06* and Δ*rr06* Mutations on Transcription of *cbpA*. To determine whether *cbpA* is transcriptionally coupled with *hk/rr06*, RT-PCR was performed by using D39 RNA template and primers from within the *cbpA* and *hk06* genes (RTHis-Rev and RtcbpA-For; Table 2). No product was obtained (data not shown) indicating that *cbpA* and *hk06* are transcribed separately. We then used real time RT-PCR with *cbpA*-specific primers (CbpAFNew and CbpAREdit; Table 2) to determine the relative levels of *cbpA* mRNA in cultures of D39, D39Δ*hk06*, and D39Δ*rr06* grown in THY to an A_{600} of 0.35. The 16S rRNA levels (determined in parallel using primers 16sF and 16sR) were used as an internal control. Real time RT-PCR was performed in quadruplicate on three independently isolated mRNA samples for each strain. In D39Δ*hk06*, the *cbpA* mRNA level was up-regulated ≈5-fold relative to that in D39 ($P < 0.01$), whereas in D39Δ*rr06*, *cbpA* mRNA was down-regulated 3-fold compared with the wild-type parent ($P < 0.01$) (Fig. 2). This result correlates with the Western immunoblot analysis data, and it indicates that the regulation occurs at the level of transcription.

Real time RT-PCR was also used to study possible autoregulatory effects by examining the levels of *rr06* and *hk06* mRNA in D39Δ*hk06* and D39Δ*rr06*, respectively, in comparison with the wild type, by using gene-specific oligonucleotides (RRf/RRr and HKf/HKr). No significant differences in level of *rr06* and *hk06* gene expression were observed (data not shown).

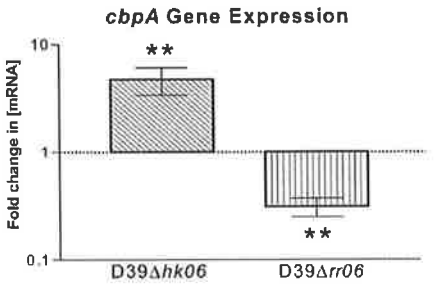

Fig. 2. Real time RT-PCR analysis of *cbpA* mRNA. RNA was isolated from D39, D39Δ*hk06*, D39Δ*rr06*, and *cbpA* mRNA levels were compared by using real time RT-PCR as described in *Materials and Methods*. Data shown are fold increase (±SE) in [*cbpA* mRNA] relative to D39. **, $P < 0.01$, compared with *cbpA* mRNA levels in D39, as determined by Tukey test.

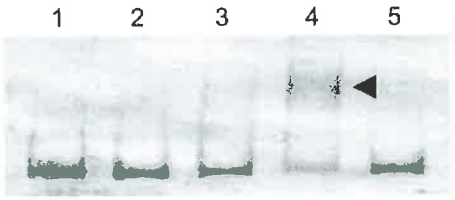

Fig. 3. Electrophoretic mobility-shift assay. A 377-bp DNA fragment spanning the *cbpA* promoter was DIG-labeled (lane 1) and then incubated with lysates of DH5α:pGEMT (lane 2), DH5α:RR13 (lane 3), DH5α:RR06 (lane 4), and DH5α:RR06 with 100-fold excess unlabeled competitor DNA (lane 5). Protein–DNA complexes were separated by PAGE and transferred to nitrocellulose, and labeled DNA was detected as described in *Materials and Methods*. Protein concentration was 10 μg per binding reaction. Arrowhead indicates the mobility of the retarded fragment when incubated with DH5α:RR06.

Binding of RR06 to the *cbpA* Promoter Region. The finding that deletion of either *hk06* or *rr06* results in altered transcription of *cbpA* compared with the wild type, suggests that transcription of *cbpA* might be directly regulated by binding of RR06 to DNA in the vicinity of the *cbpA* promoter. A 377-bp DNA region corresponding to nucleotides −299 to 78 relative to the *cbpA* initiation codon (nucleotides 1,989,574–1,989,939 of the *S. pneumoniae* R6 genome; GenBank accession no. AE007317) was PCR-amplified and labeled with DIG-11-dideoxy-UTP, as described in *Materials and Methods*. To overexpress RR06, the *rr06* gene was cloned into pGEM-T Easy and transformed into *E. coli* DH5α. As a control, the *rr13* gene, which encodes another pneumococcal RR, was also cloned into pGEM-T Easy and transformed into DH5α. Cell lysates of DH5α, carrying either pGEM-T Easy (DH5α:pGEMT), or its derivatives, containing *rr06* (DH5α:RR06) and *rr13* (DH5α:RR13), were prepared. Aliquots of each lysate containing 10 μg of protein were incubated with the labeled *cbpA* promoter fragment. Reaction mixtures were then electrophoresed, transferred to a nylon filter, and developed. Incubation of the DH5α:RR06 lysate with the labeled *cbpA* promoter DNA caused a marked decrease in the electrophoretic mobility of the DNA fragment, which was not seen with the control lysate (DH5α:pGEMT) or the lysate from DH5α:RR13 (Fig. 3). The mobility shift could be completely inhibited by coincubation of the DIG-labeled DNA fragment with DH5α:RR06 and a 100-fold excess of unlabeled *cbpA* promoter DNA (Fig. 3). These results suggest that this shift is specific for RR06, providing strong evidence for the binding of RR06 to the *cbpA* promoter region.

To investigate the specificity of RR06-*cbpA* promoter interaction further, a SPB assay was undertaken. The *cbpA* promoter region, or an internal fragment of the 16S rRNA gene, was biotin labeled and attached to Strepdavidin MagneSphere Paramagnetic Particles. The beads were subsequently incubated with cell lysates of DH5α:pGEMT or DH5α:RR06. After washing in the presence of herring sperm competitor DNA, bound protein was eluted by boiling the beads in lysis buffer. These samples along with crude *E. coli* lysates were then probed for presence of RR06 by Western blot using anti-RR06 serum. The antiserum reacted strongly with a 25-kDa (the expected size of RR06) species in the DH5α:RR06 crude lysate, but it did not label any protein species in the crude lysate of DH5α:pGEMT (data not shown). The antiserum labeled a 25-kDa species in the eluate from the *cbpA*

Fig. 4. SPB assay. A 377-bp DNA fragment spanning the *cbpA* promoter (lanes 1 and 2) or the 16S rRNA gene (lanes 3 and 4) was biotin labeled, attached to streptavidin magnetic beads, and then incubated with DH5α:pGEM-T (lanes 1 and 3) or DH5α:RR06 (lanes 2 and 4) lysates. Samples were subsequently separated by SDS/PAGE, transferred onto nitrocellulose, and probed with polyclonal murine anti-RR06 serum. Arrow indicates the 25-kDa immunoreactive species in lane 2.

promoter-coated beads that had been incubated with the DH5α:RR06 lysate, but no such species was labeled in the eluate of beads incubated with DH5α:pGEMT (Fig. 4). As a further control, an internal fragment of the 16S rRNA gene was coated onto the beads and incubated with either lysate, but no RR06 binding was detected (Fig. 4). These data confirm the specificity of binding of RR06 to the *cbpA* promoter region.

Adherence to A549 and Detroit 562 Cells. Ref. 28 demonstrated that a mutant deficient in CbpA production has reduced adherence to A549 cells (type II pneumocytes). Given the apparent role of RR06/HK06 in regulation of *cbpA* expression, the *in vitro* adherence of D39, D39Δ*hk06*, and D39Δ*rr06* to human lung (A549) and nasopharyngeal (Detroit 562) cell lines was investigated. For comparative purposes, a D39 *cbpA*$^-$ insertion-duplication mutant that was previously constructed in this laboratory was tested also (33).

We incubated ≈2 × 10^6 pneumococci with confluent cell monolayers in 24-well plates, and the number of adherent bacteria was determined as described in *Materials and Methods*. Total adherence of wild-type D39 to the A549 cells was ≈4.4 × 10^4 colony-forming units (CFU) per well, but that of either D39Δ*hk06* or D39Δ*rr06* was ≈75% lower ($P < 0.001$; Fig. 5*A*). The D39 CbpA$^-$ mutant exhibited an additional significant decrease in adherence compared with the TCSTS mutants (D39Δ*hk06* vs. CbpA$^-$, $P < 0.001$; D39Δ*rr06* vs. CbpA$^-$, $P < 0.05$). Adherence of D39 to Detroit 562 cells was ≈3.1 × 10^4 CFU per well, and again, both the D39Δ*hk06* and D39Δ*rr06* mutants showed a statistically significant reduction in adherence ($P < 0.001$). D39Δ*rr06* also exhibited significantly lower adherence than D39Δ*hk06* ($P < 0.05$). The adherence of the D39CbpA$^-$ mutant to Detroit 562 cells was ≈75% lower than that of D39 ($P < 0.001$) but was not significantly different from that of either D39Δ*hk06* or D39Δ*rr06* (Fig. 5*B*). Also, Western immunoblot analysis was undertaken to investigate the level of CbpA expression during the adherence assay. In accordance with data presented above for bacteria grown in THY or SB, D39Δ*hk06* exhibited up-regulation of CbpA relative to the wild type, whereas D39Δ*rr06* exhibited down-regulation (data not shown).

***In Vivo* Studies.** To investigate the role of RR06/HK06 in nasopharyngeal colonization and virulence, groups of 18 CD-1 mice were inoculated intranasally with D39, D39Δ*hk06*, D39Δ*rr06*, or D39*CbpA*$^-$ at a dose of ≈2 × 10^7 CFU. At least five mice from each group were killed after 24, 48, and 96 h, and the numbers of pneumococci in the nasopharynx, lungs, and blood were determined (Fig. 6). The results presented are pooled from two independent experiments. Although neither D39Δ*hk06* nor D39*CbpA*$^-$ appeared to show any significant difference in nasopharyngeal colonization compared with the parent strain, D39Δ*rr06* showed a statistically significant increase in number of pneumococci colonizing the nasopharynx at both 48 and 96 h ($P < 0.05$ and $P < 0.01$, respectively; unpaired two-tailed

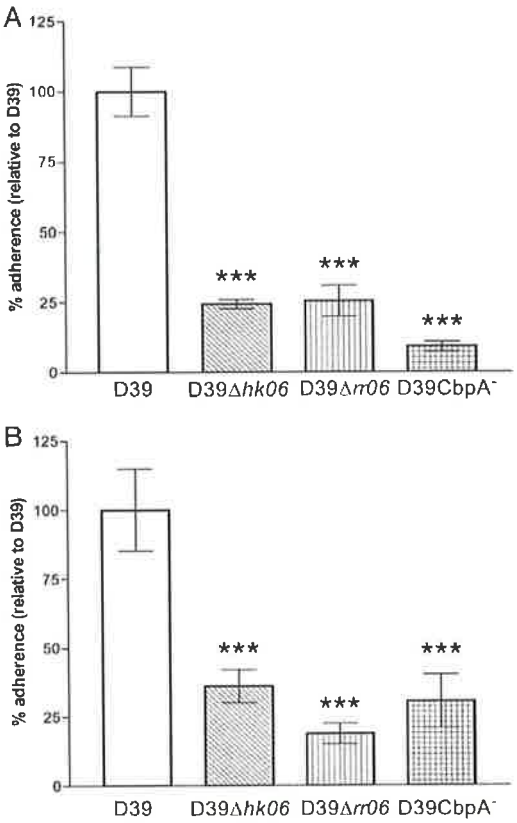

Fig. 5. Adherence of D39, D39Δ*hk06*, D39Δ*rr06*, and D39CbpA$^-$ to A549 (human lung; *A*) and Detroit 562 (human nasopharyngeal; *B*) cells. D39 and its otherwise isogenic mutants were incubated with confluent cell monolayers for 2 h, and adherent bacteria were then enumerated. Data are presented as percentage of adherence relative to that of wild-type D39. Mean adherence of D39 to A549 and Detroit 562 cells (100%) was 4.4 × 10^4 CFU per well and 3.1 × 10^4 CFU per well, respectively. ***, $P < 0.001$, compared with wild type; unpaired two-tailed Student's *t* test).

Student's *t* test). Translocation to the lungs and blood at 96 h appeared to be severely affected in D39Δ*hk06*, with only 1 of 16 mice showing evidence of bacteria in the lungs or blood in the two experiments. In contrast, 13 and 9 of the 16 mice infected with D39 had pneumococci in the lungs and blood, respectively ($P < 0.001$ and $P < 0.025$; Fisher's exact test). However, at the same time point, D39Δ*rr06* showed a significant increase in its ability to cause infection in the lung ($P < 0.05$). D39*CbpA*$^-$ showed no differences relative to the wild type in either its ability to colonize the nasopharynx or translocate to the lungs and blood.

Mutagenesis of *rr06*/*hk06* in Strain TIGR4. To examine whether the effects of RR06/HK06 on *cbpA* expression were influenced by host strain, insertion mutations were constructed in *rr06* and *hk06* in the type 4 strain TIGR4 (see *Materials and Methods*). The TIGR4*hk06* mutant exhibited increased *cbpA* expression relative to the wild-type parent, as judged by both Western immunoblotting and real time RT-PCR (using oligonucleotides AS62 and AS64 specifically designed for the TIGR4 *cbpA* gene). However, *cbpA* expression in TIGR4*rr06* was similar to that of the parent strain (data not shown).

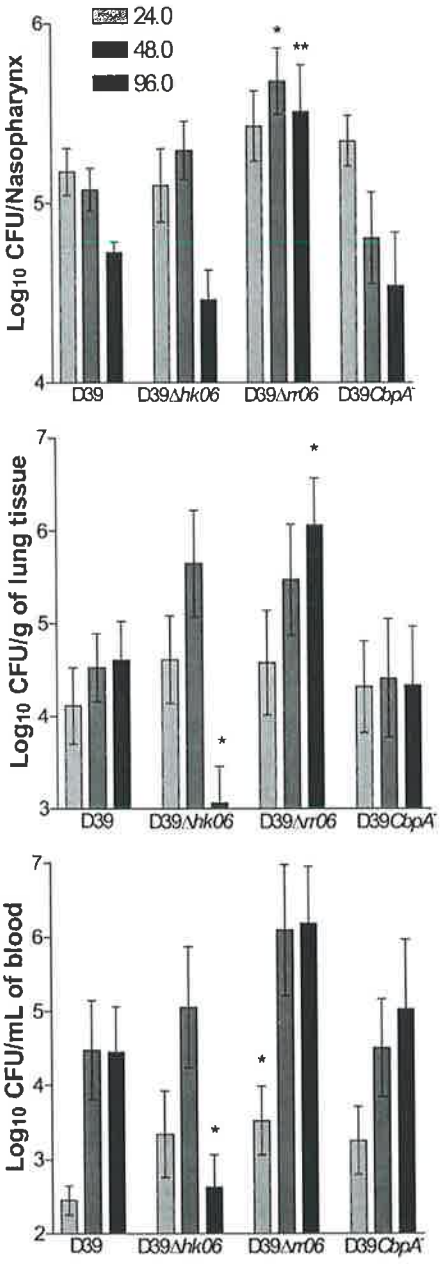

Fig. 6. Numbers of D39, D39Δ*hk06*, D39Δ*rr06*, and D39*CbpA*⁻ in the nasopharynx (*Top*), lungs (*Middle*), and blood (*Bottom*) after intranasal challenge. Mice were challenged with $\approx 2 \times 10^7$ CFU and killed at 24, 48, and 96 h, and the numbers of bacteria in the various compartments were determined. Data are mean $\log_{10}$ CFU ± standard error. Significant difference from the wild type was determined (**, $P < 0.01$; *, $P < 0.05$; two-tailed unpaired Student's *t* test).

Discussion

Coordinated regulation of virulence genes is likely to be fundamental to the capacity of *S. pneumoniae* to asymptomatically colonize the nasopharynx and cause invasive disease in humans. In this study, we investigated the role of the TCSTS RR06/HK06 in regulation of the expression of CbpA, an important pneumococcal virulence factor. By using Western immunoblot analysis, we have demonstrated differential expression of *cbpA* in the TCSTS mutants D39Δ*hk06* and D39Δ*rr06* relative to the wild-type parent. RT-PCR was also used to exclude the possibility that *cbpA* and *rr06/hk06* are cotranscribed with *cbpA*. Moreover, real time RT-PCR analysis indicated that the regulation of *cbpA* occurred at the level of transcription and not through a post-transcriptional effect. These data suggested that, in D39, RR06/HK06 exerts regulatory control over *cbpA*.

Confirmation of the above findings in the unrelated TIGR4 strain was complicated by inability to construct unmarked in-frame deletion mutants in this background. However, an insertion mutant, TIGR4*hk06*, also exhibited up-regulation of *cbpA* at both the protein and mRNA level compared with the wild type. However, differences in *cbpA* expression were not observed for TIGR4*rr06*. A difference in findings between the same two *S. pneumoniae* strains (D39 and TIGR4) has recently been reported with respect to the effects of mutations in a different TCSTS (TCS04) on expression of the Mn transporter PsaA (10). Although the genes encoding *hk06* and *rr06* are highly conserved, as is the promoter region upstream of *cbpA*, TIGR4 and D39 vary substantially in genome sequence (34) and, hence, may differ in some other regulatory element affecting *cbpA* expression.

The up-regulation of *cbpA* detected in D39Δ*hk06* suggests that RR06 can be activated independently of HK06, perhaps through small-molecular-weight phosphodonors such as acetyl phosphate or other noncognate HKs. Systems such as CheY, PhoB/PhoR, and NtrB/NtrC from *E. coli* are examples of systems that are able to be activated in this manner (35–37). The higher level of CbpA is likely to occur because of HK06 possessing phosphatase as well as kinase activity, a trait commonly found among HK proteins. Thus, in noninducing conditions, a higher level of the regulated gene would be produced in the absence of HK phosphatase activity. More RR06 would be phosphorylated through other intermediaries, leading to an increased level of *cbpA*. This other phosphate intermediary remains to be identified, and it remains to be determined whether it is a noncognate HK or other phosphodonor.

In most prokaryotic TCSTSs, regulation occurs by means of the RR binding to the promoter region of the regulated gene. In this study, electrophoretic mobility shift and SPB assays provided strong evidence for the specific binding of RR06 to the promoter region of *cbpA*. The electrophoretic mobility-shift assay showed a marked, albeit incomplete, retardation in mobility, which was only observed when the target DNA fragment was incubated with lysates of *E. coli* K-12 expressing the RR06 protein. This shift in mobility could be competitively inhibited by excess unlabeled target DNA (Fig. 3), indicating that the interaction was specific for the *cbpA* promoter region. SPB assays rely on the ability of target DNA coupled to a substrate to bind the RR (in this case, RR06). Our results indicate that a protein of ≈25 kDa in the DH5α:RR06 lysate bound to immobilized *cbpA* DNA target and that this protein reacted with anti-RR06. We conclude that the *cbpA* promoter region acts as one possible binding site for RR06. However, BLAST searches have not identified regions with significant DNA homology elsewhere in the *S. pneumoniae* genome. This fact may not be surprising because OmpR, the founder member of the family to which RR06 belongs, binds to a basic consensus sequence that varies markedly (5, 38).

The ability of the pneumococcus to adhere to host epithelial cells is particularly important to the pathogenesis of disease, especially in the nasopharynx where the pathogen can reside for extended periods without causing disease. Such asymptomatic colonization is a precursor to invasive disease, but carriers are also the principal reservoir for *S. pneumoniae*, which is a

human-specific pathogen. Work by Rosenow and coworkers (28) has shown that CbpA mediates the adherence of pneumococci to A549 cells (human type II pneumocytes). In this study, both D39Δ*hk06* and D39Δ*rr06* showed a reduction in adherence to A549 as well as Detroit 562 (human nasopharyngeal) cells. The findings for D39Δ*hk06* were unexpected, given the increase in *cbpA* expression in this mutant. Additional Western immunoblot analysis confirmed that the relative expression of CbpA was unaffected by either tissue culture medium or contact with the eukaryotic cells. These findings clearly implicate additional, as yet uncharacterized, RR06/HK06-regulated factors in adherence to epithelial cells of human origin.

The results from the *in vivo* experiments are not easy to reconcile with the *in vitro* adherence data. This discrepancy is not surprising given the likelihood of differences in receptor expression between transformed human cell lines and mouse respiratory epithelium. Although CbpA is clearly important for *in vitro* adherence, mutation of *cbpA* had no obvious effect on either colonization of the murine nasopharynx or progression to pulmonary or systemic disease. In contrast, D39Δ*rr06* exhibited increased colonization capacity, whereas D39Δ*hk06* was deficient in its capacity to invade the lungs and blood relative to D39. These data clearly indicate that other uncharacterized RR06/HK06-regulated factors have a significant role in both colonization and invasive disease, at least in the mouse model. Identification of such factors may possibly yield important new targets for vaccines and novel drugs, capable of interfering with the capacity of *S. pneumoniae* to establish infection and progress to invasive disease in humans.

This work was supported by National Health and Medical Research Council of Australia Program Grant 284214 and the Meningitis Research Foundation (U.K.).

1. Marra, A., Asundi, J., Bartilson, M., Lawson, S., Fang, F., Christine, J., Wiesner, C., Brigham, D., Schneider, W. P. & Hromockyj, A. E. (2002) *Infect. Immun.* **70,** 1422–1433.
2. Ogunniyi, A. D., Giammarinaro, P. & Paton, J. C. (2002) *Microbiology* **148,** 2045–2053.
3. Dziejman, M. & Mekalanos, J. J. (1995) in *Two-Component Signal Transduction*, eds. Hoch, J. A. & Silhavy, T. J. (Am. Soc. Microbiol., Washington, DC), pp. 305–317.
4. Lange, R., Wagner, C., de Saizieu, A., Flint, N., Molnos, J., Stieger, M., Caspers, P., Kamber, M., Keck, W. & Amrein, K. E. (1999) *Gene* **237,** 223–234.
5. Throup, J. P., Koretke, K. K., Bryant, A. P., Ingraham, K. A., Chalker, A. F., Ge, Y., Marra, A., Wallis, N. G., Brown, J. R., Holmes, D. J., *et al.* (2000) *Mol. Microbiol.* **35,** 566–576.
6. Cheng, Q., Campbell, E. A., Naughton, A. M., Johnson, S. & Masure, H. R. (1997) *Mol. Microbiol.* **23,** 683–692.
7. Pestova, E. V., Havarstein, L. S. & Morrison, D. A. (1996) *Mol. Microbiol.* **21,** 853–862.
8. Guenzi, E., Gasc, A. M., Sicard, M. A. & Hakenbeck, R. (1994) *Mol. Microbiol.* **12,** 505–515.
9. Sebert, M. E., Palmer, L. M., Rosenberg, M. & Weiser, J. N. (2002) *Infect. Immun.* **70,** 4059–4067.
10. McCluskey, J., Hinds, J., Husain, S., Witney, A. & Mitchell, T. J. (2004) *Mol. Microbiol.* **51,** 1661–1675.
11. Maniatis, T. E., Fritsh, E. & Sambrrook, J. (1989) *Molecular Cloning: A Laboratory Manual.* (Cold Spring Harbor Lab. Press, Woodbury, NY).
12. Berry, A. M., Yother, J., Briles, D. E., Hansman, D. & Paton, J. C. (1989) *Infect. Immun.* **57,** 2037–2042.
13. Yother, J., McDaniel, L. S. & Briles, D. E. (1986) *J. Bacteriol.* **168,** 1463–1465.
14. Berry, A. M., Ogunniyi, A. D., Miller, D. C. & Paton, J. C. (1999) *Infect. Immun.* **67,** 981–985.
15. Brown, M. C. M., Weston, A., Saunders, J. R. & Humphreys, G. O. (1979) *FEMS Microbiol. Lett.* **5,** 219–222.
16. Morona, J. K., Morona, R. & Paton, J. C. (1999) *J. Bacteriol.* **181,** 5355–5364.
17. Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. & Pease, L. R. (1989) *Gene* **77,** 51–59.
18. Horton, R. M. (1993) *Methods Mol. Biol.* **15,** 251–261.
19. Giammarinaro, P. & Paton, J. C. (2002) *Infect. Immun.* **70,** 5454–5461.
20. Laemmli, U. K. (1970) *Nature* **227,** 680–685.
21. Towbin, H., Staehelin, T. & Gordon, J. (1992) *Biotechnology* **24,** 145–149.
22. Rogers, T. J., Paton, A. W., McColl, S. R. & Paton, J. C. (2003) *Infect. Immun.* **71,** 5623–5632.
23. Gabrielsen, O. S. & Huet, J. (1993) *Methods Enzymol.* **218,** 508–525.
24. Talbot, U. M., Paton, A. W. & Paton, J. C. (1996) *Infect. Immun.* **64,** 3772–3777.
25. Strocher, U. H., Paton, A. W., Ogunniyi, A. D. & Paton, J. C. (2003) *Infect. Immun.* **71,** 3206–3212.
26. de Saizieu, A., Gardes, C., Flint, N., Wagner, C., Kamber, M., Mitchell, T. J., Keck, W., Amrein, K. E. & Lange, R. (2000) *J. Bacteriol.* **182,** 4696–4703.
27. Balachandran, P., Brooks-Walter, A., Virolainen-Julkunen, A., Hollingshead, S. K. & Briles, D. E. (2002) *Infect. Immun.* **70,** 2526–2534.
28. Rosenow, C., Ryan, P., Weiser, J. N., Johnson, S., Fontan, P., Ortqvist, A. & Masure, H. R. (1997) *Mol. Microbiol.* **25,** 819–829.
29. Hammerschmidt, S., Talay, S. R., Brandtzaeg, P. & Chhatwal, G. S. (1997) *Mol. Microbiol.* **25,** 1113–1124.
30. Smith, B. L. & Hostetter, M. K. (2000) *J. Infect. Dis.* **182,** 497–508.
31. Duthy, T. G., Ormsby, R. J., Giannakis, E., Ogunniyi, A. D., Strocher, U. H., Paton, J. C. & Gordon, D. L. (2002) *Infect. Immun.* **70,** 5604–5611.
32. Ogunniyi, A. D., Folland, R. L., Briles, D. E., Hollingshead, S. K. & Paton, J. C. (2000) *Infect. Immun.* **68,** 3028–3033.
33. Berry, A. M. & Paton, J. C. (2000) *Infect. Immun.* **68,** 133–140.
34. Bruckner, R., Nuhn, M., Reichmann, P., Weber, B. & Hakenbeck, R. (2004) *Int. J. Med. Microbiol.* **294,** 157–168.
35. Lukat, G. S., McCleary, W. R., Stock, A. M. & Stock, J. B. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 718–722.
36. Amemura, M., Makino, K., Shinagawa, H. & Nakata, A. (1990) *J. Bacteriol.* **172,** 6300–6307.
37. Feng, J., Atkinson, M. R., McCleary, W., Stock, J. B., Wanner, B. L. & Ninfa, A. J. (1992) *J. Bacteriol.* **174,** 6061–6070.
38. Harlocker, S. L., Bergstrom, L. & Inouye, M. (1995) *J. Biol. Chem.* **270,** 26849–26856.