Investigation of a Low Molecular Weight Protein Tyrosine Phosphatase in Streptococcus pneumoniae

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B. Sc (Biomedical Science), B. Health Sciences (Hons)

THE UNIVERSITY of ADELAIDE

Submitted for the degree of Doctor of Philosophy

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This thesis is dedicated to my sister, Zurinah, who always chooses to love
Science, I had come to learn, is as political, competitive, and fierce a career as you can find, full of the temptations to find easy paths.

One could count on V to always choose the honest (and, often, self-effacing) way forward. While most scientists connived to publish in the most prestigious journals and get their names out there, V maintained that our only obligation was to be authentic to the scientific story and to tell it uncompromisingly.

— Paul Kalanithi, When Breath Becomes Air
Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, 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. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Adelaide, Australia, April 2018

Zuleeza Ahmad
Abstract

Tyrosine phosphorylation is a critical regulator of bacterial virulence, with the associated protein tyrosine phosphatases (PTPs) and bacterial tyrosine kinases (BY-kinases) recognised as major virulence factors in a range of bacterial pathogens including *Streptococcus pneumoniae* (the pneumococcus). The pneumococcus has a phosphoregulatory system comprising of CpsB (a PTP) and CpsC and CpsD, which together form an active BY-kinase. This system plays a crucial role in the regulation of the pathogen’s major virulence factor, the capsular polysaccharide (CPS). One open reading frame in the pneumococcal chromosome (designated *spd1837*) shows homology to the low molecular weight protein tyrosine phosphatases (LMWPTPs). LMWPTPs mediate CPS regulation in many other bacteria. Thus, investigating what role this protein plays in pneumococcal biology is the overreaching goal of this study. Purification of the phosphatase expressed in *E. coli* showed that Spd1837 was indeed a LMWPTP, with specificity against phosphotyrosine. *spd1837* mutation was constructed on the chromosome of the pneumococcus and it was found that Spd1837 does not play a role in the regulation of CPS. The use of substrate-trapping assays, demonstrated that the phosphatase may interact with a variety of metabolic enzymes such as ATP-dependent-6-phosphofructokinase and Hpr kinase/phosphorylase, suggesting that the phosphatase may have roles in pneumococcal metabolism.

In the chromosome of approximately 90% of pneumococcal strains with available genome sequence, *spd1837* is co-transcribed together in the *OM001* operon with the upstream translocase subunit YajC (Spd1838), and a downstream hypothetical protein (Spd1836). The *OM001* operon was previously implicated to be important for pneumococcal virulence in a number of *in vivo* models. Here, we found that Spd1836 was essential for the bacterial ability to cause invasive disease in an established mouse model. Additionally, a previous genome-wide screen identified the *OM001* operon to be important for pneumococcal growth and survival in human saliva. The data collected from this study suggest that human saliva can support the survival of the wildtype pneumococcal strain but not the mutant strain that carries a chromosomal deletions in *spd1836* and *spd1838*. 
The pneumococcus is known to produce large quantities of hydrogen peroxide (H$_2$O$_2$) predominantly via the pyruvate oxidase, SpxB. It was found that the phosphatase activity of Spd1837 could be inhibited by H$_2$O$_2$ in vitro and Spd1837 itself confers protection against killing by H$_2$O$_2$. Whether SpxB played a role in regulating the activity of Spd1837 was then further investigated. Interestingly, in SpxB-deficient backgrounds and under aerobic conditions, Spd1837 modulated CPS biosynthesis, with Δ$spd1837ΔspxB$ and Spd1837$_{C8S}$Δ$spxB$ showing significantly reduced CPS relative to both the wildtype and the Δ$spxB$ strains. Therefore, the phosphatase Spd1837 does play a role in the pneumococcal CPS biosynthesis in an SpxB-dependent manner.

The outcomes of this thesis highlight the importance of a number of previously unknown and uncharacterised bacterial factors during different stages of pneumococcal pathogenesis. Such research is critical to identify novel targets for anti-microbials against pneumococcal infection.
Acknowledgements

First and foremost, Praise Da Lord! I went in to do a PhD as another thing in my bucket list (as if I can’t find something more practical to add to my bucket list) and came out being (I hope) a better servant to You and a better person.

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This Thesis is submitted in the style of a ‘Thesis by Publication’. As such, the results chapters are replaced by three Research Articles. Author contributions for each publication are stated in preceding ‘Statements of Authorship’. Article chapters retain the section order layout style, reference style and formatting (US versus Australian spelling) of the journal publisher. Article supplementary data is included in this thesis (and (S) after the figure or table number). Please note that formatting may be slightly different between articles (i.e. US versus Australian spelling).
Publications

Peer-reviewed research articles;


Results chapter written in a publication style;

Ahmad Z, Morona R,* & Standish, AJ.* *Streptococcus pneumoniae* protein tyrosine phosphatase Spd1837 confers resistance to hydrogen peroxide and modulates capsular polysaccharide production in an SpxB-dependent manner. Intended for submission to Journal of Bacteriology, – Chapter 5

*equal authorship
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>~</td>
<td>approximately</td>
</tr>
<tr>
<td>AGRF</td>
<td>Australian Genome Research Facility</td>
</tr>
<tr>
<td>amp</td>
<td>ampicillin</td>
</tr>
<tr>
<td>ANOVA</td>
<td>one-way analysis of variance</td>
</tr>
<tr>
<td>anti-PY</td>
<td>anti-phosphotyrosine</td>
</tr>
<tr>
<td>BA</td>
<td>blood agar</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>BY-kinase</td>
<td>bacterial tyrosine kinase</td>
</tr>
<tr>
<td>C8S</td>
<td>cysteine 8 to serine</td>
</tr>
<tr>
<td>CAN</td>
<td>acetonitrile</td>
</tr>
<tr>
<td>CAP</td>
<td>community-acquired pneumonia</td>
</tr>
<tr>
<td>CbpA</td>
<td>choline-binding protein A</td>
</tr>
<tr>
<td>CFU</td>
<td>colony-forming unit</td>
</tr>
<tr>
<td>ChoP</td>
<td>phosphoryl choline</td>
</tr>
<tr>
<td>CID</td>
<td>collision-induced dissociation</td>
</tr>
<tr>
<td>cml</td>
<td>chloramphenicol</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
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<tr>
<td>CPS</td>
<td>capsular polysaccharide</td>
</tr>
<tr>
<td>CSP</td>
<td>competence-stimulating peptide</td>
</tr>
<tr>
<td>CW-CPS</td>
<td>cell wall-associated CPS</td>
</tr>
<tr>
<td>DDM</td>
<td>n-dodecyl-β-D-maltoside</td>
</tr>
<tr>
<td>DFI</td>
<td>differential fluorescence induction</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>dNTPs</td>
<td>deoxynucleic triphosphates</td>
</tr>
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<td>DOC</td>
<td>sodium deoxycholate</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<tr>
<td>DUSP</td>
<td>eukaryotic-like and dual-specificity phosphatases</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>Eno</td>
<td>α-enolase</td>
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<td>EPS</td>
<td>exopolysaccharide</td>
</tr>
<tr>
<td>FA</td>
<td>formic acid</td>
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<tr>
<td>FBP</td>
<td>fructose 1,6-bisphosphate</td>
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<tr>
<td>G6P</td>
<td>glucose 6-phosphate</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>gm</td>
<td>gentamicin</td>
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<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
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<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
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<td>~</td>
<td>mass to charge ratio</td>
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<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>MORN</td>
<td>membrane occupation and recognition nexus</td>
</tr>
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<td>MQ</td>
<td>MilliQ water</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>Na₂B₄O₄</td>
<td>di-sodium tetraborate</td>
</tr>
<tr>
<td>Na₂VO₃</td>
<td>sodium orthovanadate</td>
</tr>
<tr>
<td>NaF</td>
<td>sodium fluoride</td>
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<tr>
<td>NanA</td>
<td>neuraminidase A</td>
</tr>
<tr>
<td>NEB</td>
<td>New England Biolab</td>
</tr>
<tr>
<td>NH₄HCO₃</td>
<td>ammonium bicarbonate</td>
</tr>
<tr>
<td>NHMRC</td>
<td>National Health and Medical Research Council</td>
</tr>
<tr>
<td>O₂</td>
<td>oxygen</td>
</tr>
<tr>
<td>OD₆₀₀</td>
<td>optical absorbance at 600 nm</td>
</tr>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PavA</td>
<td>pneumococcal adherence and virulence factor A</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PCV</td>
<td>pneumococcal conjugate vaccine</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PHP</td>
<td>the polymerase and histidinol family of phosphoesterases</td>
</tr>
<tr>
<td>P-Loop</td>
<td>phosphate-binding loop</td>
</tr>
<tr>
<td>pNP</td>
<td>p-nitrophenol</td>
</tr>
<tr>
<td>pNPP</td>
<td>p-nitrophenyl phosphate</td>
</tr>
<tr>
<td>PsaA</td>
<td>pneumococcal surface polysaccharide vaccine</td>
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<tr>
<td>pH</td>
<td>phosphorylated serine</td>
</tr>
<tr>
<td>pSer</td>
<td>phosphorylated serine</td>
</tr>
<tr>
<td>PspA</td>
<td>pneumococcal surface protein A</td>
</tr>
<tr>
<td>pH</td>
<td>phosphorylated threonine</td>
</tr>
<tr>
<td>PTK</td>
<td>protein tyrosine kinase</td>
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<td>PTM</td>
<td>post-translational modification</td>
</tr>
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<td>PTP</td>
<td>protein tyrosine phosphatase</td>
</tr>
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<td>pTYr</td>
<td>phosphorylated tyrosine</td>
</tr>
<tr>
<td>RO</td>
<td>reverse osmosis</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>revolution per minute</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>sm</td>
<td>streptomycin</td>
</tr>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>i.n.</td>
<td>intranasal</td>
</tr>
<tr>
<td>i.p.</td>
<td>intraperitoneal</td>
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<tr>
<td>IAA</td>
<td>iodoacetic acid</td>
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<tr>
<td>IC₅₀</td>
<td>the half maximal inhibitory concentration</td>
</tr>
<tr>
<td>IDT</td>
<td>Integrated DNA Technologies</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl β-β-thiogalactopyranoside</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobases</td>
</tr>
<tr>
<td>km</td>
<td>Kanamycin</td>
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<tr>
<td>LB</td>
<td>Lysogeny Broth</td>
</tr>
<tr>
<td>LMWM</td>
<td>low molecular weight markers</td>
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<tr>
<td>LMWPTP</td>
<td>low molecular weight protein tyrosine phosphatase</td>
</tr>
<tr>
<td>LTA</td>
<td>lipoteichoic acids</td>
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**Abbreviations:**
- **kb**: kilobases
- **km**: kanamycin
- **LB**: Lysogeny Broth
- **LMWM**: low molecular weight markers
- **LMWPTP**: low molecular weight protein tyrosine phosphatase
- **LTA**: lipoteichoic acids
- **v/v**: volume per volume
- **w/v**: weight per volume
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Chapter One

INTRODUCTION
1.1 *Streptococcus pneumoniae*: disease burden, vaccines and challenges

*Streptococcus pneumoniae* or the pneumococcus is a Gram positive, human-specific bacterial pathogen. The bacteria are normally observed as lancet-shaped diplococci or in short chains (Ramirez, 2015). Despite contributing to significant morbidity and mortality worldwide, the pneumococcus is a frequent commensal of the upper respiratory tract (Lipsitch *et al*., 2000). The pneumococcus can be the aetiological agent of mucosal diseases such as acute otitis media (Syrjanen *et al*., 2006) and sinusitis (Petraitienė *et al*., 2015). However, more concerning is the ability of the bacteria to invade deeper, normally sterile tissues causing pneumonia, bacteraemia and meningitis (Simell *et al*., 2012). In 2015, pneumococcal pneumonia was responsible for 921,000 deaths of children under the age of five (Wang *et al*., 2017). Indeed, the pneumococcus is the leading cause of community-acquired pneumonia (CAP) in both adults and children including in developed countries (McIntosh, 2002, O'Brien *et al*., 2009, Said *et al*., 2013, Cilloniz *et al*., 2016). The elderly and patients with chronic respiratory diseases and immunosuppression are more likely to succumb to CAP with the survivors having a higher chance of being readmitted after recovery (Blasi *et al*., 2012, Prescott *et al*., 2014). Additionally, not only does pneumococcal meningitis result in 34% mortality (van de Beek *et al*., 2006), 30 - 50% of survivors end up with persistent neurological sequelae (van de Beek *et al*., 2002). Despite implementation of the World Health Organisation recommendations for treatment, pneumococcal bacteraemia and meningitis can still rapidly lead to fatalities due to delay in 24 to 48 hours window required for causative agent identification (Berkley *et al*., 2005).

To date, there are two classes of vaccines that have been developed against pneumococcal disease; these are the polysaccharide vaccines and the conjugate vaccines. A 23-valent pneumococcal polysaccharide vaccine (PPV23)\(^1\) was introduced in 1983. PPV23 is generally effective against invasive pneumococcal disease in the elderly (Falkenhorst *et al*., 2017). However, to overcome PPV23’s poor immunogenicity in children (Douglas *et al*., 1983, Huss *et al*., 2009, Postma *et al*., 2012, Moberley *et al*., 2013), the capsular

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\(^1\) PNEUMOVAX\(^®\) 23, (pneumococcal vaccine polyvalent). Full Prescribing Information, Merck & Co., Inc., Whitehouse Station, NJ 08889, USA 2013
polysaccharide was conjugated to a carrier protein giving rise to pneumococcal conjugate vaccines (PCVs) containing 7 (PCV7), 10 (PCV10), or 13 (PCV13) serotypes. The conjugation to a carrier protein has the ability to induce a T-cell-dependent antibody response leading to the much needed immunological memory in children (O'Brien et al., 1996). As of June 2017, 141 countries have included the PCVs in their infant National Immunisation Program as reported by Johns Hopkins Bloomberg School of Public Health International Vaccine Access Center (2017).

The widespread administration of the vaccines has resulted in some success by limiting the carriage of serotypes included in the vaccine formulation (Whitney et al., 2003, Bonten et al., 2015). Unfortunately, due to the sheer number of pneumococcal serotypes (almost reaching 100 to date), based on its capsular polysaccharide, the problem of serotype replacement has arisen (Nigrovic et al., 2008, Aguiar et al., 2010, Miller et al., 2011, Weinberger et al., 2011). Serotype replacement describes the phenomena whereby the non-vaccine serotypes have replaced the niches vacated by the serotypes included in the vaccine formulation (Hicks et al., 2007, Singleton et al., 2007, van der Linden et al., 2015). There are also increasing problems with geographical and temporal vaccine coverage with the advent of vaccine escape strains (Lynch & Zhanel, 2010, Davis et al., 2013). Another apparent problem is the observed declining levels of antibody against pneumococcus below the protective threshold just two years after immunisation (De Schutter et al., 2014).

Furthermore, pneumococcal strains resistant to antibiotics including cephalosporins, macrolides and fluoroquinolones continue to emerge (Lau et al., 2001, Song & Chung, 2010). This resistance against almost all classes of available antibiotics means that treatment is becoming more and more difficult (Mendes et al., 2014). Not only does overprescribing and overuse of antibiotics exacerbate this problem (Keenan et al., 2015), the vaccines can act as a double-edge sword, putting a selective pressure for current

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2 Prevnar/Prevenar®, (Wyeth Lederle Vaccines) [Prevnar® (pneumococcal 7-valent conjugate vaccine [diphtheria CRM197 protein]). Full Prescribing Information, Wyeth Pharmaceuticals, Inc., Philadelphia, PA, 2008]
3 Synflorix®, (GlaxoSmithKline Biologicals S.A.) [SYNFLORIX Product Monograph (pneumococcal conjugate vaccine [non-typeable *Haemophilus influenzae* (NTHi) protein D, diphtheria or tetanus toxoid conjugates]). Full Prescribing Information, GlaxoSmithKline, Mississauga, ON, 2015]
4 Prevnar 13/Prevenar 13®, (Wyeth/Pfizer Vaccines) [Prevnar 13® (pneumococcal 13-valent conjugate vaccine [diphtheria CRM197 protein]). Full Prescribing Information, Pfizer Inc, Collegeville, PA, 2016]
strains to undergo clonal expansion (Song et al., 2012). Additionally, the pneumococcus is naturally competent – it readily acquires new antibiotic resistance genes, pathogenicity islands and also undergoes capsule switching, rendering vaccine administration ineffective (Ferrandiz et al., 2000, Johnston et al., 2014). Based on high-throughput genome comparisons of 240 S. pneumoniae isolates of one pneumococcal lineage, PMEN1 (Spain^{23F-1}), more than 700 recombination events were detected and non-essential antigens were shown to be quickly removed from the chromosome. The study therefore showed that this single pneumococcal lineage has acquired drug resistance and the ability to evade vaccine pressure on a number of occasions in just over a few decades (Croucher et al., 2011).

1.2 Pathogenesis of S. pneumoniae

The first step in pneumococcal pathogenesis is asymptomatic colonisation of the nasopharynx. Most children are transiently colonised by the pneumococcus at some point of their life right after birth up to six years of age, with the peak being at three years (Bogaert et al., 2004, Regev-Yochay et al., 2004, Mackenzie et al., 2010, Tan, 2012, Le Polain de Waroux et al., 2014). Generally less than 10% of adults are colonised by the pneumococcus and they typically acquire the bacteria from children, although the risk of severe disease increases in the elderly (Henriques-Normark & Tuomanen, 2013, Mosser et al., 2014). The colonisation of the nasopharynx always precedes the pneumococcal disease state (Simell et al., 2012). Interestingly, while successful pneumococcal colonisation is associated with increased viral carriage (Glennie et al., 2016), pneumococcal carriage does not increased the likelihood of co-colonisation with other common nasopharyngeal flora such as Staphylococcus aureus, Moraxella catarrhalis or Haemophilus influenzae (Shak et al., 2014).

From an evolutionary point of view, it is important for the pneumococcus to be able to exit the current host and successfully transmit to the next host. Although transmission is the important first step that precedes carriage and disease (in fact none of the pneumococcal disease states facilitate contagion (Musher, 2003)), pneumococcal factors that foster transmission are not well characterised due to a lack of tractable models to study this process until recently (Zafar et al., 2017). Indeed, pneumococcal disease occurrence is directly linked to the strains circulating in carriage (Simell et al., 2012).
Transmission is thought to require close contact, such as between individuals within the same households or day care centre (Pessoa et al., 2013, Mosser et al., 2014). While it is generally accepted that the pneumococcus is a human-obligate pathogen with no known environmental or animal reservoir, evidence accumulating is that the bacteria can survive outside of the human host. For instance, rehydrated pneumococci were able to infect mice after being left desiccated for four weeks (Walsh & Camilli, 2011).

It is believed that pneumococci exist in very low numbers in the nasopharynx (LeMessurier et al., 2006, Oggioni et al., 2006, Mahdi et al., 2008). This possibly explains why although adherence of the bacteria onto bronchio-epithelial cells triggers cytokine-induced cell activation and inflammation (Bergeron et al., 1998, Catterall, 1999), the overall carriage process remains relatively asymptomatic. The appropriate expression of capsular polysaccharide was also shown to be essential for prolonged colonisation in mice (Bender & Yother, 2001, Magee & Yother, 2001, Morona et al., 2004).

The precise mechanisms underlying the pneumococcal transition from a harmless commensal to an invasive pathogen remain elusive. It is understood that S. pneumoniae can utilise both clathrin- and caveolae-mediated endocytosis to enter the endothelial host cell. The majority of the endocytosed bacteria do get neutralised by lysosomes, however, a small proportion of the bacteria was shown to have the capacity to evade lysosomal degradation. These surviving bacteria can later be translocated out of the cell, further disseminating the bacteria throughout the host (Gradstedt et al., 2013). The bacteria in the bloodstream can then cross the blood–brain barrier via receptor-mediated transcytosis across endothelial and epithelial cell layers (Ring et al., 1998, Zhang et al., 2000, Mook-Kanamori et al., 2011) to enter the brain meninges and cause infection (Iovino et al., 2016).

1.2.1 S. pneumoniae major virulence factors

S. pneumoniae possess the exquisite ability to alter the expression of complex sets of genes according to the new microenvironment (Ogunniyi et al., 2002, Orihuela et al., 2004, LeMessurier et al., 2006). Some of the main pneumococcal virulence factors and their roles are outlined below;
1.2.1.1 Teichoic acids

Lipoteichoic acids (LTA) and wall teichoic acids (WTA) are mainly masked by the capsular polysaccharide layer (Figure 1.1) (Skov Sorensen et al., 1988). The distinction between WTA and LTA is that WTA are covalently linked to the cell wall peptidoglycan while LTA is tethered on the cytoplasmic membrane by a lipid anchor (Fischer, 2000). Otherwise, they both are decorated with phosphoryl choline (ChoP) and share the same structural repeating unit (Fischer, 2000, Gisch et al., 2013). Although the mechanism is not fully understood, ChoP was shown to facilitate bacterial adherence and the subsequent invasion of eukaryotic cells and transmigration of the bacteria to the basolateral surface during invasive pneumococcal disease (Cundell et al., 1995b, Swords et al., 2001).

![Figure 1.1: S. pneumoniae major virulence factors.](image)

Important pneumococcal virulence factors include the capsular polysaccharide, teichoic acids, pneumolysin, pneumococcal surface antigen A (PsaA), choline-binding protein A (CbpA), pneumococcal surface protein A (PspA), neuraminidase A (NanA), pneumococcal adherence and virulence factor A (PavA), α-enolase (Eno) and the autolysin, LytA.

1.2.1.2 Pneumolysin

Pneumolysin is a cholesterol-binding toxin containing a choline-binding domain that oligomerises to form pores in eukaryotic cell membranes (Rossjohn et al., 1998). The cytolysin pneumolysin, being one of the more widely-studied pneumococcal virulence factors is known to contribute to the invasive nature of pneumococcal infections (Canvin

1.2.1.3 Pneumococcal surface antigen A (PsaA)

PsaA is the lipoprotein component of an iron uptake ABC transporter that functions to transport Mn$^{2+}$ and Zn$^{2+}$ into the bacterial cytoplasm (Dintilhac et al., 1997). A psaA deletion mutant displayed growth perturbation, reduced competence, adherence and virulence and was also more sensitive to oxidative stress (Dintilhac et al., 1997, Briles et al., 2000, Tseng et al., 2002, Johnston et al., 2004, McAllister et al., 2004).

1.2.1.4 Choline-binding protein A (CbpA)

CbpA (also known as PspC) is the most abundant choline-binding protein in the pneumococcus (Jedrzejas, 2001). CbpA is anchored to the surface of the pneumococcus by its binding to the terminal choline residues of wall teichoic acid and lipoteichoic acids. CbpA itself is an adhesin and it mediates the binding of pneumococci to human respiratory epithelial cells and later, pneumococcal invasion and translocation across human nasopharyngeal epithelial layer (Rosenow et al., 1997, Zhang et al., 2000).

1.2.1.5 Pneumococcal surface protein A (PspA)

PspA is another choline-binding protein, highly variable and expressed by all important clinical pneumococcal serotypes. PspA consists of five domains including an α-helical domain and a proline-rich region (Yother & White, 1994). PspA protects pneumococcus from host immune response during colonisation and invasion by neutralising the antimicrobial activity of apolactoferrin (Shaper et al., 2004) and inhibiting complement-mediated opsonisation (Mukerji et al., 2012).

1.2.1.6 Neuraminidase A (NanA)

S. pneumoniae expresses at least three types of neuraminidases, the three characterised ones so far are NanA, NanB and NanC. However, only NanA contains an LPxTG anchoring motif and is expressed by all pneumococcal strains. NanA cleaves terminal sialic acid residues from glycolipids, glycoproteins and oligosaccharides on host cell surfaces, promoting pneumococcal adherence to lung epithelial cells (Brittan et al., 2012).
Additionally, NanA was also shown to be important for resistance to opsonophagocytic killing in ex vivo killing assays using human neutrophils (Dalia et al., 2010).

1.2.1.7 LytA

LytA is an amidase responsible for autolysis during the stationary phase of the pneumococcal growth (Goebel & Avery, 1929) and implicated to be important for the release of pneumolysin (Martner et al., 2008) and bacterial fratricide (Eldholm et al., 2009). Fratricide promotes the release of virulence factors in a small portion of non-competent cells (Claverys et al. 2007), releasing cell components including pneumolysin, which damage host cells directly and provides benefits for nearby pneumococcus cells. Autolysis by LytA also mediates gene transfer as the competence regulon is activated within local pneumococcal populations (Claverys & Havarstein, 2007).

1.2.1.8 Moonlighting proteins

Moonlighting proteins are mainly housekeeping cytosolic enzymes that are secreted and attached to the bacterial cell wall (Bittaye & Cash, 2015). The mechanism that results in the surface exposure of these proteins is still unclear – it has been proposed that these proteins are either actively transported to the pneumococcal surface or they are derived from lysed cells in the vicinity. The best characterised moonlighting proteins in S. pneumoniae are pneumococcal adherence and virulence factor A (PavA), α-enolase (Eno) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). PavA was shown to modulate the immune response by conferring resistance from phagocytosis by dendritic cells (Noske et al., 2009). On the other hand, Eno and GAPDH mediate pneumococcal attachment to plasminogen, subsequently contributing to bacterial migration through the basement membrane (Bergmann et al., 2004, Bergmann et al., 2005). Eno also interferes with complement activation by interacting with the complement inhibitor C4b-binding protein (Agarwal et al., 2012).

1.3 Capsular polysaccharide (CPS)

As mentioned previously, capsular polysaccharide (CPS) which makes up the outermost layer of the pneumococcus is the antigen of which the current pneumococcal vaccines is targeted. Although unencapsulated or rough pneumococci have been
implicated in outbreaks of conjunctivitis (Ramirez, 2015), CPS remains the single most important virulence factor of the pneumococcus especially during invasive disease (Avery & Dubos, 1931, Winkelstein, 1984, Brown, 1985, Hardy et al., 2001, Magee & Yother, 2001). The role of CPS is multi-faceted (Figure 1.2) – perhaps the most important one is to shield the bacteria from phagocytosis and complement-mediated killing mounted by the host.

Specifically, CPS limits the deposition of complement and recognition of cell wall antigens (Winkelstein, 1981, Hardy et al., 2001, Abeyta et al., 2003, Hyams et al., 2010). Also, for IgG or C3b/iC3b that is successfully bound to the bacterial cell surface, CPS may prevent the interaction of its Fc region to the phagocytic cells (Avery & Dubos, 1931, Mac & Kraus, 1950, Musher, 1992, Hardy et al., 2001, Magee & Yother, 2001, Ogunniyi et al., 2002, Kjos et al., 2015) such as neutrophils that are critical for the bacterial clearance (Standish & Weiser, 2009). CPS also limits mucus-mediated clearance during colonisation (Nelson et al., 2007). Additionally, most serotypes possess highly-charged CPS at physiological pH and this may interfere with cell-to-cell interactions with phagocytes (Kozel et al., 1980, Lee et al., 1991, Weinberger et al., 2009). Furthermore, there is evidence that released CPS, particularly anionic CPS, can act as a decoy to neutralise cationic antimicrobial peptides (Llobet et al., 2008). Recently, CPS is found to have a possible role in transmission as bacterial shedding was shown to require CPS expression in the mouse infant model (Zafar et al., 2016).

**Figure 1.2: Roles of *S. pneumoniae* capsular polysaccharide (CPS).**
Characterised roles of *S. pneumoniae* CPS include (A) limits the deposition of complement and recognition of cell wall antigens, (B) prevent the interaction of complement and antibodies to the phagocytic cells, (C) limits mucus-mediated clearance during colonisation.
and (D) potentially mediates transmission as bacterial shedding was shown to require CPS expression in mouse infant model.

1.3.1 Phase variation

During pathogenesis, the pneumococci are able to switch from highly encapsulated to a less encapsulated variant depending on the host environment that the bacteria is currently reside in. Indeed, *S. pneumoniae* clinical isolates derived from different host environments displayed these phenotypic differences. Termed phase variation, this reversible process is still ill-defined, with the mechanisms and environmental conditions which govern the switching between the two phenotypes, opaque and transparent not well understood (Weiser *et al.*, 1994, Cundell *et al.*, 1995a, Weiser *et al.*, 1996, Kim & Weiser, 1998, Morona *et al.*, 2000). Phase variation has been linked to changes in DNA methylation (Manso *et al.*, 2014, Li *et al.*, 2016). Additionally, a recent proteomic analysis of the opaque and transparent variants of three pneumococcal strains with different pathogenicity patterns suggest that a combination of metabolic activities and overall protein expression patterns contribute to the phase variations and these are likely to be strain-dependent (Chai *et al.*, 2017). Phase variation could still be observed in unencapsulated pneumococcal mutants (Weiser *et al.*, 1994) suggesting that factors other than CPS are contributing to this bidirectional change.

The proposed basis for phase variation is that minimal CPS expression is important during colonisation as CPS thickness determines how exposed bacterial surface factors are such as adhesins which are important for the process. In contrast, maximal expression of CPS is regarded to be advantageous during systemic infections, providing resistance to opsonophagocytosis and also masking a potent activator of complement pathway, namely the cell wall teichoic acid (Winkelstein & Tomasz, 1978). Pneumococcal phase variants were shown to also differ in the amount of teichoic acids, particularly cell wall teichoic acid, with teichoic acids in transparent variants being more abundant than in opaque variants (Weiser *et al.*, 1994, Cundell *et al.*, 1995a), contrary to what was found for CPS.
1.3.2 CPS biosynthesis

To date, there are 98 pneumococcal serotypes that have been identified, each differing in sugar composition and linkage (Bentley et al., 2006, Geno et al., 2017). CPS biosynthesis is mainly controlled by cps locus. The overall locus is conserved across all serotypes except in serotype 3 and 37 (Figure 1.3). At the 5’ end of the locus, there are four common regulatory genes, cpsA, cpsB, cpsC and cpsD followed by serotype-specific genes. These serotype-specific regions encode the enzymes responsible for the synthesis of NDP-sugars unique to the CPS structure, polymerisation (Wzy polymerase), transport (Wzx flippase), glycosidic linkages (glycosyltransferases), and sugar modification (O-acetylases) (Garcia & Lopez, 1997, Morona et al., 1999a, Morona et al., 1999b). CPS is synthesised via lipid-linked repeat unit intermediates termed Wzy-dependent mechanism in a manner similar to O-antigen biosynthesis in Gram negative bacteria (Whitfield, 1995, Morona et al., 1999b, Morona et al., 1999a), except in serotypes 3 and 37 in which CPS is synthesised via synthase-dependent mechanism by a processive transferase in the same manner as hyaluronic acid synthesis in Group A Streptococci (DeAngelis et al., 1994, Arrecubieta et al., 1995, Llull et al., 1999).

Figure 1.3: Schematic organisation of the pneumococcal cps locus for serotype 2 and 3.

The cps locus for serotype 2 D39 strain is represented as (A) and for serotype 3 WU2 (B). The genes that encode for CPS assembly machinery are denoted in blue, glycosyl transferases in pink, phosphotyrosineregulatory system in green and UDP-sugar synthases in yellow. Only genes that do not possess mutations and/or are functional are shown for serotype 3.
Figure 1.4: The model of Wzy-dependent mechanism of CPS assembly in S. pneumoniae. CPS assembly starts in the cytoplasm with the synthesis of CPS subunits. The repeat unit is assembled onto the undecaprenyl-diphosphate lipid carrier by CpsE. Then, these subunits are flipped across by CpsJ. CpsH polymerises the polysaccharide repeat units. The polymer is eventually transferred onto peptidoglycan by the phosphotransferase CpsA. CpsC is required for the localisation of CpsD and likely acts as a scaffold, organising the others. CpsC triggers CpsD kinase activity, allowing autophosphorylation of its C-terminal cluster. Phosphorylated CpsD can be dephosphorylated by CpsB.

1.3.2.1 Serotype 2

To delve further into Wzy-dependent synthesis of CPS, we are utilising serotype 2 as an example. The cps locus that encodes the enzymes required to produce the serotype 2 CPS is approximately 18 kb in length and is predicted to comprise a single operon (Iannelli et al., 1999) (Figure 1.3A). Similar to other Gram positive bacteria, in this serotype, CPS is linked either to the cytoplasmic membrane or the cell wall.

It is understood that the first step in CPS biosynthesis in serotype 2 is the transfer of a sugar-phosphate to a lipid acceptor on the cytoplasmic side by the UDP-glycosyl transferase CpsE (Cartee et al., 2005). Glucose-1-phosphate is the most common initiating sugar but other sugars can be used (Bentley et al., 2006). Following that is the addition of monosaccharide to the repeat unit by serotype-specific glycosyltransferases (James &
Yother, 2012, James et al., 2013). The completed repeat subunit is then translocated across the cytoplasmic membrane by a Wzx flippase, CpsJ. Wzy polymerase, CpsH then links the repeat units into long-chain polymers at the reducing end of the polysaccharide (Robbins et al., 1967). In serotype 2, the repeat unit contains a backbone of Glc-Rha-Rha-Rha and a side chain of Glc-GlcUA (Xayarath & Yother, 2007). Once synthesised, some or all of the polymers are attached to the peptidoglycan by CpsA (Eberhardt et al., 2012, Chan et al., 2014) with the remainder being membrane-associated (Sorensen et al., 1990). The attachment occurs via the reducing end glucose of CPS and the β-D-N-acetylglucosamine (GlcNAc) residues of peptidoglycan via 1,6 glycosidic bond (Larson & Yother, 2017). CPS-peptidoglycan linkage in serotype 2, 8 and 31 which all utilise different initiating sugars is similar, suggesting a common linking mechanism across serotypes utilising the Wzy-dependent mechanism (Larson & Yother, 2017).

The full assembly and transfer of CPS to peptidoglycan appears to be essential as any mutations that inhibit this process results in lethality. Toxic accumulation of lipid intermediate and/or reduced turnover of undecaprenyl phosphate (Und-P) for other pathways such as peptidoglycan and teichoic acids synthesis has been proposed to be responsible for the lethality phenotype (Xayarath & Yother, 2007, James et al., 2013). Experimental data also suggest there are a limited number of available CPS attachment sites on the surface of the pneumococcus and this overrides any observable increase in the efficiency of the ligation machinery (Byrne et al., 2011).

1.3.2.2 Serotype 3

In serotype 3, most of the genes in the cps locus are truncated or otherwise mutated (Figure 1.3B), and the functions they encode are irrelevant to CPS synthesis (Dillard & Yother, 1994, Arrecubieta et al., 1995, Caimano et al., 1998, Cartee et al., 2000). Synthesis of serotype 3 CPS requires a UDP-glucose dehydrogenase, Cps3D which converts UDP-glucose to UDP-glucuronic acid and the polysaccharide synthase, Cps3S. Both enzymes are encoded in the serotype 3 capsule locus, which is transcribed as a single operon (cps3DSUM-tnpApIpaA) (Figure 1.3B) (Dillard & Yother, 1994, Arrecubieta et al., 1995, Dillard et al., 1995, Caimano et al., 1998, Magee & Yother, 2001). Serotype 3 CPS is synthesised by a processive mechanism in which repeat units are not formed (Cartee et al., 2000), and polysaccharide that is released from the membrane into the surrounding cell.
wall and environment is not covalently attached to the peptidoglycan (Sorensen et al., 1990, Forsee et al., 2000, Hardy et al., 2000). Spontaneous sequence duplications tend to occur within the \textit{cps3D} (\textit{cap3A}) gene, causing high-frequency CPS phase variations (Waite et al., 2001).

1.4 Post-translational modifications

Post-translational modifications (PTM) are changes in proteins that are not genetically encoded. PTMs have the ability to alter protein folding, stability, structure, cellular localisation and interaction with other macromolecules (Mijakovic et al., 2016). PTM may take the form of covalently modified amino acids within a protein structure resulting in, for example, phosphorylation or oxidation which are the two PTMs which will be the main focus of this thesis. Phosphorylated proteins are abundant - about 50% of all eukaryotic proteins are phosphorylated once in their lifetime and about 30% of all human proteins are phosphorylated at a given time (Olsen et al., 2006).

1.4.1 Tyrosine phosphorylation

The first evidence that protein phosphorylation on hydroxyl amino acids serine, threonine and tyrosine was not phylogenetically confined to eukaryotes but could occur in bacteria as well, was provided by two independent works conducted in the late 1970s in \textit{Escherichia coli} and \textit{Salmonella} (Wang & Koshland, 1978, Garnak & Reeves, 1979, Manai & Cozzone, 1979). Phosphorylated serine (pSer) and phosphorylated threonine (pThr) are chemically distinct from phosphorylated tyrosine (pTyr) such that the distance between the phosphoester group to the peptide chain in pTyr is longer due to the para position of the hydroxyl in the benzene group of tyrosine. This more exposed position of the phosphate is speculated to facilitate a better interaction with phosphotyrosine-binding protein domains and the phosphoester bond is also thermodynamically stable (Mijakovic et al., 2016). It is now recognised that tyrosine phosphorylation is critical for bacterial virulence (Whitmore & Lamont, 2012). Protein phosphorylation on tyrosine in bacteria has been reported to be implicated in the control of heat shock response (Klein et al., 2003), adaptation to cold (Ray et al., 1994), adaptation to light (Warner & Bullerjahn, 1994), flagellin export (South et al., 1994), cell aggregation and sporulation (Frasch & Dworkin, 1996), and cell division and
differentiation (Wu et al., 1999) as reviewed by Cozzone (2005) and Chao et al. (2014). More prominently, tyrosine phosphorylation is closely linked to CPS and exopolysaccharide (EPS) regulation as reviewed by Standish & Morona (2014). CPSs are high-molecular weight polysaccharides that are covalently/non-covalently attached to cell as discussed previously while EPS is loosely in association with the cell surface, and are usually secreted to the extracellular environment to facilitate biofilm formation (Schmid et al., 2015).

The two protein classes that modulate tyrosine phosphorylation are protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). PTKs function to transfer the γ-phosphate from ATP to the side chains of specific tyrosine residues and PTPs reverse this process (Hanks & Hunter, 1995). Advances in phosphoproteomics revealed that tyrosine phosphorylation in bacteria is more important than originally thought. This was highlighted by two recent studies; 512 unique phosphotyrosine sites were discovered on 384 E. coli proteins, corresponding to up to 6% of the E. coli proteome (Hansen et al., 2013) and 905 unique phosphotyrosine sites was discovered on at least 573 Shigella flexneri proteins, corresponding to approximately 15% of all S. flexneri proteins (Standish et al., 2016). These identified tyrosine-phosphorylated proteins are involved in important cellular processes including cell division, virulence, transport, transcription, translation, and are central to numerous metabolic pathways (Hansen et al., 2013, Standish et al., 2016). Another study detected a total of 272 phosphorylation events in Bacillus subtilis with the ratio of pSer:pThr:pTyr sites in humans is 86:12:2 compared to 70:20:10 in B. subtilis (Ravikumar et al., 2014). Overall, these studies suggest that bacteria rely on phosphotyrosine signalling more heavily than eukaryotes.

1.4.2 Tyrosine phosphorylation in S. pneumoniae

The predominant research into tyrosine phosphorylation in the pneumococcus has focused on the phosphoregulatory system and its role in the regulation of CPS which will be explained in greater details below. Tyrosine phosphorylation also plays a significant role in regulating the autolysin LytA (Standish et al., 2014). Additionally, Nourikyan et al. (2015) demonstrated that, in order for the CPS assembly machinery to localise at the division site to synthesise CPS for the daughter cells, CpsD localisation and autophosphorylation is required. These coordinated actions ensure the concealment of the daughter cell by CPS.
1.4.2.1 The phosphoregulatory circuit in *S. pneumoniae* and its link to CPS biosynthesis and cell division

This section also refers to Figure 1.4. As mentioned previously, the first four genes in the *cps* locus are highly conserved across all pneumococcal serotypes except in serotype 3 and 37. Homologs of *cpsB*, *cpsC*, and *cpsD* are also found in capsule loci from other Gram positive genera (Morona *et al.*, 2002) and they are also arranged in the genome in a similar manner (Standish & Morona, 2014).

1.4.2.1.1 CpsA

Technically not a part of the phosphoregulatory system, CpsA was shown to interact with the pyrophosphoryl-lipid carrier of the polysaccharide precursor and is proposed to attach CPS to cell wall peptidoglycan as mentioned previously (Kawai *et al.*, 2011, Eberhardt *et al.*, 2012). *cpsA* mutant colonies appear smaller and duller. However the smooth, partially encapsulated strain was as virulent as the wildtype strain in mice (Morona *et al.*, 2004).

1.4.2.1.2 CpsB

The only verified PTP in the pneumococcus before the start of this study is CpsB – a manganese-dependent PTP from the polymerase and histidinol phosphatase family (Morona *et al.*, 2002). *cpsB* mutants were attenuated in virulence following intravenous inoculation of mice and were unable to colonise the nasopharynx (Bender *et al.*, 2003). While *cpsB* mutants produce significantly lower levels of CPS compared to the wildtype, they attach significantly more CPS to the cell wall (Morona *et al.*, 2006).

1.4.2.1.3 CpsC

CpsC is a membrane protein that contains two short cytoplasmic regions at the amino and carboxy terminals, two transmembrane helices and a series of alternating α-helices and β-strands within a large extracellular loop region of the protein (Byrne *et al.*, 2011). Deletion of *cpsC* induces the Wzy polymerase, CpsH delocalisation (Nourikyan *et al.*, 2015). For clarity, the function of CpsC will be discussed together with CpsD as below.
CpsD is an autophosphorylating bacterial tyrosine kinase (BY-kinase) which requires interaction with CpsC for its function. CpsC and CpsD belong to polysaccharide copolymerase 2b protein family (Morona et al., 2000). Although CpsC is required for the initial autophosphorylation of CpsD, it is not needed for subsequent transphosphorylation (Bender & Yother, 2001). Deletion of cpsD resulted in the loss of most of the CPS while the relative amounts of CPS attached to the cell wall remained similar to the wildtype (Morona et al., 2000, Bender et al., 2003, Morona et al., 2006, Geno et al., 2014). Additionally, a cpsC deletion mutant essentially had no detectable level of CpsD despite having similar level of cpsD transcript compared to the wildtype and as the result, the mutant failed to achieve full encapsulation (Morona et al., 2000, Bender et al., 2003). Interestingly, mucoid strains containing mutations in the [YGX]3-repeat domain of CpsD were unable to cause bacteraemia after intranasal challenge of CD1 mice, even though such strains were capable of killing BALB/c mice after intraperitoneal challenge. This suggests that the ability of S. pneumoniae to regulate CPS production, via CpsD phosphorylation, appears to be required for its transition from the lung to the bloodstream (Kadioglu et al., 2001, Morona et al., 2004).

While the mechanism of how the phosphoregulatory system regulates CPS biosynthesis is still not completely understood, the cycling between phosphorylated and non-phosphorylated form of the BY-kinase, CpsD is thought to be essential as the phosphorylated form of BY-kinase in a number of bacteria has been shown to either promote (Wugeditsch et al., 2001, Bender et al., 2003) or block CPS synthesis (Morona et al., 2003, Nakar & Gutnick, 2003, Obadia et al., 2007). BY-kinase can adopt distinct configurations depending on whether it is phosphorylated or not. For instance, the non-phosphorylated form of BY-kinase domain of Wzc (the E. coli homolog of CpsC and CpsD as CpsCD homologs in Gram negative bacteria are encoded as a single protein) forms an octomer and this configuration is disrupted when Wzc becomes phosphorylated (Wugeditsch et al., 2001). Similarly, homologs from the Gram positive bacteria, S. aureus, CapB (equivalent to S. pneumoniae CpsD) and C-terminal of CapA (equivalent to S. pneumoniae CpsC) forms a ring-shaped octomer and the oligomerisation is disrupted upon phosphorylation and CapAB then becomes a monomer (Paiment et al., 2002, Olivares-Illana et al., 2008, Bechet et al., 2010). CpsC is proposed to work in concert to ensure that the
conformational change in CpsD is relayed to the CPS assembly machinery. The dissociation of CpsD into monomers would be transmitted to CpsC which then modifies its interaction with the other members of CPS assembly (Grangeasse, 2016).

The role of tyrosine phosphoregulatory system in pneumococcal cell division has become more apparent in the recent years. This was initiated by the observations that CpsC and CpsD are both localised to the division site in the serotype 14 strain (Henriques et al., 2011). It was later demonstrated that CpsC is not only required for CpsD autophosphorylation but also for CpsD localisation at mid-cell. Following that, the CpsC-CpsD complex further contributes to proper cell division by recruiting the Wzy polymerase, CpsH. As a homolog of ParA-like ATPases, CpsD also interact with the Noc-like, chromosome partitioning protein, ParB (Nourikyan et al., 2015).

1.4.2.2 The association between tyrosine phosphorylation, CPS and oxygen levels in the pneumococcus

*S. pneumoniae* is an aerotolerant anaerobe that encounters a range of oxygen pressures in the host. In sites such as the middle ear or pleural fluid, oxygen pressure may be 20 mmHg or lower (Treacher & Leach, 1998). *In vitro*, low oxygen levels have been shown to increase CPS levels in clinical isolates of various serotypes compared to the same isolates grown in atmospheric oxygen (159 mmHg) (Treacher & Leach, 1998, Weiser et al., 2001). In a highly aerobic microenvironment such as the mucosal airways, the production of CPS is suppressed (Weiser et al., 2001). This reduced level of CPS was correlated with decreased tyrosine phosphorylation of CpsD (Magee & Yother, 2001, Weiser et al., 2001). Intriguingly, at lower oxygen levels, CpsB protein but not its phosphatase activity is needed for parental CPS levels (Geno et al., 2014). Therefore, *S. pneumoniae* may alter CPS production in response to environmental conditions by sensing and responding to environmental oxygen via the tyrosine phosphoregulatory system.

1.5 Protein tyrosine phosphatases in bacteria

PTPs can function as important effector proteins during active infections. For instance, *Yersinia* YopH and *Salmonella* SptP are secreted out of the bacterial cell via type III secretion systems to prevent bacterial internalisation in macrophages and to promote
bacterial intracellular replication respectively (Dean, 2011). More recently, a plant pathogen, *Pseudomonas syringae* was shown to possess a PTP HopAOI with a unique property; it targets the pattern recognition receptors (most are kinases), a component of innate immunity in the plant, *Arabidopsis* (Macho *et al.*, 2014).

In the context of bacteria, PTPs can be categorised into three families; i. eukaryotic-like and dual-specificity phosphatases (DUSPs); ii. low molecular weight protein tyrosine phosphatases (LMWPTPs); and iii. the polymerase and histidinol family of phosphoesterases (PHPs), one example we encountered earlier being CpsB (Section 1.4.2). PHPs have a completely different, conserved 30-kDa active site with a series of coordinated histidine and aspartic acid residues while DUSPs and LMWPTPs harbour the same active site, the C(X)$_5$R motif (Aravind & Koonin, 1998, Mijakovic *et al.*, 2003, Madhurantakam *et al.*, 2005, Hagelueken *et al.*, 2009, Kim *et al.*, 2011). A few important distinctions between the active site of these two families include the consensus amino acid sequence, the number of flanking residues between the C(X)$_5$R motif and the downstream aspartic acid, and the relative location of the phosphate binding site along the polypeptide chain (Cozzone *et al.*, 2004). The DUSPs are also capable of dephosphorylating pSer and pThr in addition to pTyr.

1.5.1 Low molecular weight protein tyrosine phosphatases (LMWPTPs)

1.5.1.1 Role in CPS and EPS biosynthesis

Based on Table 1.1, the control of CPS and EPS by a BY-kinase-LMWPTP pair encoded in the *cps* or *cps*-like operon has been found to be a conserved feature among Gram negative bacteria (Vincent *et al.*, 2000). In fact, there are significant differences in chromosomal and operon structures between LMWPTPs in Gram negative bacteria and the Gram positive bacteria, *B. subtilis* and *S. aureus* (Soulat *et al.*, 2002, Musumeci *et al.*, 2005) and also in the pneumococcus (our observations). One exception is the Gram negative bacteria, *Porphyromonas gingivalis* whereby the LMWPTP, Ltp1 and its cognate BY-kinase which contribute to EPS and biofilm formation, are present at distant sites on the chromosome (Maeda *et al.*, 2008).
Table 1.1: Bacterial LMWPTPs involved in capsular polysaccharide (CPS)/exopolysaccharide (EPS) biosynthesis

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>LMWPTP</th>
<th>BY-kinase</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli K-30</td>
<td>Wzb</td>
<td>Wzc</td>
<td>Group 1 CPS assembly</td>
<td>(Wugeditsch et al., 2001)</td>
</tr>
<tr>
<td>E. coli K-12</td>
<td>Wzb</td>
<td>Wzc</td>
<td>Colanic acid production</td>
<td>(Vincent et al., 2000)</td>
</tr>
<tr>
<td>Enteropathogenic E. coli</td>
<td>Etp</td>
<td>Etk</td>
<td>Secretion and assembly of the group 4 CPS</td>
<td>(Ilan et al., 1999, Peleg et al., 2005)</td>
</tr>
<tr>
<td>Acinetobacter iwoffii</td>
<td>Wzb</td>
<td>Wzc</td>
<td>Emulsan production</td>
<td>(Nakar &amp; Gutnick, 2003)</td>
</tr>
<tr>
<td>Acinetobacter johnsonii</td>
<td>Ptp</td>
<td>Ptk</td>
<td>Colanic acid/EPS synthesis</td>
<td>(Grangeasse et al., 1998)</td>
</tr>
<tr>
<td>Erwinia amylovora</td>
<td>AsmI</td>
<td>AsmH</td>
<td>Amylovoran production</td>
<td>(Bugert &amp; Geider, 1997)</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>Yor5/Wzb</td>
<td>Yco6/Wzc</td>
<td>CPS production</td>
<td>(Preneta et al., 2002)</td>
</tr>
<tr>
<td>Pseudomonas solanacearum</td>
<td>EpsP</td>
<td>EpsK</td>
<td>EPS I production</td>
<td>(Huang &amp; Schell, 1995)</td>
</tr>
</tbody>
</table>

### 1.5.1.2 Role in processes other than CPS and EPS biosynthesis

*P. gingivalis* Ltp1 does have a second function which is to regulate transcriptional activity of the global regulator LuxS (Maeda et al., 2008). In addition, while *Burkholderia contaminans* LMWPTP BceD does not affect the production of EPS (cepacian), the bceD mutant forms biofilms at a much lower level than the wildtype (Ferreira et al., 2007, Ferreira et al., 2015). In another study, *E. coli* Etp was shown to regulate heat shock resistance by dephosphorylating the sigma factor RpoH and the anti-sigma factor RseA (Klein et al., 2003).

Similar to a number of bacterial high molecular weight PTPs such as *Yersinia* YopH, a couple of LMWPTPs can also be secreted into host cells and subvert the regular host signalling process. *Mycobacterium tuberculosis* PtpA is secreted into the host macrophage during infection. In the host, PtpA binds to subunit H of the human vacuolar-H⁺-ATPase pump and dephosphorylates human vacuolar protein sorting 33B to inhibit phagosome acidification and block fusion with lysosomes (Bach et al., 2008, Wong et al., 2002).
2011, Poirier et al., 2014). Ultimately, PtpA is also required for successful long-term M. tuberculosis infection (Bach et al., 2008). More recently, Burkholderia cenocepacia, an opportunistic pathogen associated with cystic fibrosis and chronic granulomatous disease was also shown to possess a secreted LMWPTP, Dpm. Dpm facilitates B. cenocepacia survival in membrane-bound vacuoles of macrophages by directly contributing to the phagosome maturation arrest, independent of its phosphatase activity (Dpm is an inactive phosphatase) (Andrade & Valvano, 2014).

1.5.2 Eukaryotic LMWPTPs

Biochemical and structural studies have demonstrated that the eukaryotic LMWPTPs share a similar catalytic mechanism to their bacterial counterparts, and that common steps are involved in dephosphorylation process (Su et al., 1994, Zhang et al., 1998, Wang et al., 2000). While in general, eukaryotic and prokaryotic PTPs are quite different, LMWPTPs are found abundantly in both eukaryotes and bacteria and also Archaea (Mustelin, 2007). The conservation of LMWPTPs or Class II PTPs through evolution to humans indicates that LMWPTPs are likely involved in fundamental processes in cell physiology. As S. pneumoniae is a human-adapted pathogen and as any potential drug that is developed against the putative pneumococcal LMWPTP (more details in Section 1.6) runs a chance of cross-reacting with human LMWPTP, it is of interest to look further into the human LMWPTP.

All mammals including humans possess a single gene encoding LMWPTP which would then be spliced into two active isoforms, HCPTP-A and HCPTP-B (Wo et al., 1992, Dissing et al., 1993, Modesti et al., 1998). This results in the change of the surface charges near the active site in otherwise minor shape variation between these two isoforms (Zabell et al., 2006). Human LMWPTP is known to downregulate the platelet-derived growth factor (PDGF)-stimulated cell proliferation by dephosphorylating the PDGF receptor (Berti et al., 1994, Chiarugi et al., 1995). This event has the overall effects of modulating cytoskeleton rearrangement, cell motility, cell proliferation and cell adhesion (Chiarugi et al., 2000a, Chiarugi et al., 2000b, Raugei et al., 2002). Mammalian LMWPTPs have been observed to be overexpressed in certain tumours, and thus are considered oncogenes (Kikawa et al., 2002, Malentacchi et al., 2005). Human LMWPTP has also identified as a key promoter of obesity-induced diabetes and a recent study has characterised the first orally bioavailable
human LMWPTP inhibitor to combat obesity-associated insulin resistance (Stanford et al., 2017), a testament to the potential of LMWPTP as a potential drug target.

1.5.3 LMWPTP structural topology and specificity

In general, LMWPTPs exhibit low sequence identities, although they form similar folds and overall 3D structure. LMWPTP consists of a central four-stranded parallel β-sheet flanked by five α-helices; α1, α2, α5 on one side and α3, α4 on the other side with three loops connecting β2-α2, α2-α3 and β4-α5. Sequence identities do exist in two domains, P-loop and D-loop (Figure 1.5) which harbour the two signature motif, the C(X)₅R motif and the DPY motif respectively.

Figure 1.5: 3D representation of LMWPTP structure modelled from solved crystal structures of *E. coli* Wzb.

The structure of *E. coli* Wzb as modelled by The NGL Viewer (http://proteinformatics.charite.de/ngl) according to its solved crystal structures. The image generated is colour-coded according to its secondary structure; magenta represents α-helix, yellow represents β-strand and purple represent 3₁₀ helix. (A) shows a clearer representation of Wzb’s secondary structure with its N- and C-terminal while (B) shows the domains, P-loop and D-loop with greater clarity.
1.5.3.1 The C(X)₅R motif

The C(X)₅R motif resides within the phosphate-binding loop or P-loop (Tabernero et al., 2008). The catalytic cysteine, absolutely critical for the phosphatase activity, is positioned within this motif (Vega et al., 2011). Structural studies of LMWPTPs revealed that the phosphate ion is cradled and stabilised between the catalytic cysteine and the downstream arginine, giving rise to a cysteinyl-phosphate intermediate (Pannifer et al., 1998). In the first step of the dephosphorylation process, the catalytic cysteine functions as the nucleophile and its thiolate form attacks and binds the phosphate ion of the substrate (Su et al., 1994). Also, the backbone nitrogens of the P-loop form hydrogen bonds with the phosphate group of the substrate (Madhurantakam et al., 2005). In the second step, the cysteinyl-phosphate intermediate is then hydrolysed by a water molecule, generating free phosphate and regenerating the thiol (Hagelueken et al., 2009, Stanford et al., 2014). This step is rate-limiting for most substrates (Zhang & VanEtten, 1991).

1.5.3.2 The DPY motif

The DPY motif in the D-loop between the last two helices is also conserved in LMWPTPs. The hydrophobic nature and the orientation of the aromatic residue in this motif are important for the affinity of the enzyme towards different substrates (Xu et al., 2006). This loop becomes displaced by substrate binding and closes around the side chain of the pTyr residue. This conformational change places the distant aspartate residue in a position where it can function as a general acid for the first step of catalysis and a general base in the second (Stanford et al., 2014). In addition, LMWPTPs commonly have two adjacent tyrosine residues in the D-loop, whose phosphorylation status appear to regulate its functional activity (Tailor et al., 1997, Bucciantini et al., 1999). For human LMWPTP HCPTP-A, phosphorylation of Tyr131 increases the enzyme activity 25-fold while phosphorylation of Tyr132 does not affect the enzyme activity but leads to the recruitment of an adaptor protein, important for downstream signal transduction (Tailor et al., 1997, Bucciantini et al., 1999, Raugei et al., 2002). In NIH3T3 cells, human LMWPTP is constitutively localised in both cytoplasmic and cytoskeleton-associated fractions, however, only the cytoskeleton-associated LMWPTP fraction is specifically phosphorylated by c-Src after PDGF stimulation (Cirri et al., 1998). In *E.coli* Etp and *B. cenocepacia* BCAL2200, the consecutive tyrosine residues are also tyrosine phosphorylated (Nadler et al., 2012, Andrade et al., 2015). The
effect of this phosphorylation on BCAL2200 is still unknown although the overall deletion of BCAL2200 led to growth defects in minimal media (Andrade, 2015). In the case of Etp, it was found that the non-phosphorylated form of Etp inhibits CPS biosynthesis regardless of its phosphatase activity while the phosphorylated form alleviates this inhibition (Nadler, 2012).

1.5.4 LMWPTP substrate specificity

Despite the conserved fold, LMWPTPs have highly specific substrate preferences. The active sites in LMWPTPs are relatively deep (≈9 Å) compared to that of DUSPs and this is predicted to exclude pSer and pThr from being recognised (Su et al., 1994, Jia et al., 1995, Moorhead et al., 2009). Both the catalytic domain and non-catalytic domain of the LMWPTPs contribute to substrate specificity in vivo. Distinct charge distribution around the catalytic site of different LMWPTPs is expected to recognize amino acids with different charges (Zhang, 2003a). Likewise, the non-catalytic segments of LMWPTPs can facilitate substrate specificity by targeting LMWPTPs to specific intracellular compartments whereby the effective local concentration of substrate is high (Andersen et al. 2001; Fischer 1999; Forman-Kay & Pawson 1999). In summary, the three known regulation mechanisms for LMWPTPs are: i. phosphate binding in the C(X)₅R active site motif, ii. phosphorylation of the adjacent tyrosines in the DPY motif and iii. oxidation of catalytic cysteine residue which will be discussed next.

1.5.5 Redox regulation of LMWPTPs

Oxidation of the protein backbone could lead to direct protein fragmentation or to irreversibly oxidised, non-functional proteins (Berlett, 1997). On the other hand, regulated oxidation of amino acid side chains and in particular of cysteine residues is a functional regulation of proteins because the oxidation can be reversed by the redox cellular systems (thioredoxin and GSH/glutaredoxin) (Chiarugi, 2001). Redox regulation is now recognised as a critical mechanism in regulating the activity of PTPs with the C(X)₅R active site as reviewed by Tanner et al. (2011). In fact, PTPs are emerging as important redox sensors in cells.
Experimental and computational studies of several eukaryotic PTPs, including DUSPs and LMWPTPs, have demonstrated that the cysteine within the C(X)₅R motif exhibits a perturbed pKa and exists as a thiolate anion at physiological conditions (Denu & Dixon, 1995, Evans et al., 1996, Czyryca & Hengge, 2001). The low pKa (pKa of around 5 instead of 8) of the conserved cysteine ensures that it remains deprotonated which is essential for its function as a nucleophile and has the overall effect of enhancing the rate of reaction (Jackson & Denu, 2001, Jensen et al., 2009). The highly positively-charged environment of the active site however, results in the catalytic cysteine being very sensitive to oxidation (Zhang & Dixon, 1993, Peters et al., 1998). As the result, the oxidised cysteine is unable to act as a nucleophile, rendering the phosphatase inactive as cysteinyl-phosphate intermediate cannot be formed during the first step of the catalysis (Böhmer et al., 2013). Oxidation is an attractive regulatory mechanism for PTPs because reactive oxygen species (ROS) are readily formed in response to various stimuli, and as mentioned previously, it is also reversible (Denu & Tanner, 1998, den Hertog et al., 2005, Groen et al., 2005).

Intramolecular disulfide bond formation of both cysteines in the active site of human LMWPTP, Cys12 and Cys17 has been observed, given their proximity (Caselli et al., 1998). By forming the disulfide bond, the catalytic cysteine is protected against further, irreversible oxidation to sulfinic acid or sulfonic acid (Caselli et al., 1998, Chiarugi et al., 2001, Lee et al., 2002, Savitsky & Finkel, 2002, Jensen et al., 2009). ROS-mediated inhibition of PTPs is essential for PDGF signalling (Meng et al., 2002) because strict subcellular localisation of PTP oxidation provides specificity to the PDGF response in that not all PTPs throughout the stimulated cell are inactivated, but only the ones very close to the PDGF receptor (den Hertog et al., 2005).

Caselli and colleagues have demonstrated that hydrogen peroxide (H₂O₂) and nitric oxide can lead to the specific oxidation of Cys12 and Cys17 in the catalytic pocket of human LMWPTP. The oxidation/inactivation of LMWPTP both endogenously and when overexpressed is transient, and the enzyme is reduced/reactivated after the removal of the oxidants (Caselli et al., 1995, Caselli et al., 1998). H₂O₂, being produced in cells downstream of many surface receptors (Rhee et al., 2000, Veal et al., 2007), is a physiologically relevant PTP oxidant. PTP oxidation has therefore been suggested to be highly compartmentalised to areas of decreased H₂O₂ clearance (Ostman et al., 2011).
1.6 Spd1837 is a *S. pneumoniae* putative LMWPTP

In the annotated genome of all sequenced pneumococcal strains to date, lies as yet uncharacterised PTP, designated Spd1837. The PTP shows homology to the members of LMWPTPs (Figure 1.6). As expected for Gram positive bacteria, further analysis of *S. pneumoniae* genome sequence revealed no potential neighbouring BY-kinase within *spd1837* gene vicinity.

![Figure 1.6: A sequence alignment for selected bacterial LMWPTPs.](image)

The alignment was generated using Clustal Omega program. Identical amino acids are indicated by (*), conserved amino acids are depicted by (:), whereas semi-conserved amino acids are depicted by (.). The cysteine residue critical for enzymatic activity is framed. GenBank accession numbers for the LMWPTPs are as follows; *Streptococcus pneumoniae* Spd1837, WP_000737448; *Erwinia amylovora* Amsl, CBA21355; *Acinetobacter johnsonii* Ptp, O52787; *Escherichia coli* Wzb, NP_416565; *Escherichia coli* Etp, NP_415502; *Klebsiella pneumoniae* Wzb, BAF47013; *Bacillus subtilis* YfKJ, NP_388669; and *Mycobacterium tuberculosis* PtpA, NP_216750. Shaded areas indicate the location of the P-loop and D-loop. The numbers indicate amino acid position.
1.6.1 Spd1837 is encoded in the *OM001* operon

On the serotype 2 D39 *S. pneumoniae* chromosome, *spd1837* is arranged in *OM001* operon together with an upstream translocase, YajC subunit, Spd1838 (99 a.a) and a downstream hypothetical protein, Spd1836 (136 a.a) (Figure 1.7). In *E. coli*, a YajC subunit participates in the Sec-dependent secretion by forming a complex with SecDF and YidC which may associate with the SecYEG and SecA ATPase to improve protein translocation efficiency (Schulze et al., 2014). Although the Sec-dependent pathway has been extensively studied, the precise role of SecDF-YidC-YajC complex is largely unknown. On the other hand, Spd1836 contains a multiple membrane occupation and recognition nexus (MORN)-repeat motif. According to studies in eukaryotes especially in the parasite, *Toxoplasma gondii* and the plant, *Arabidopsis*, the MORN-repeat motif may function to localise and tether specific proteins to the membrane (Lorestani et al., 2010, Mikami et al., 2010). Using differential fluorescence induction (DFI) technique, Marra et al. (2002) showed that the *OM001* operon might be important for pneumococcal virulence in a number of animal models.

*Figure 1.7: Schematic representation of the OM001 operon.*

In the chromosome, the operon consists of *spd1838* which encodes for a translocase, YajC (99 amino acids); *spd1837* which encodes for a low molecular weight protein tyrosine phosphatase (142 amino acids); and *spd1836* which encodes for a Membrane Occupation and Recognition Nexus (MORN) repeats-containing protein (136 amino acids). The arrow indicates the direction of the transcription.

The DFI technique employed by Marra et al. (2002) used a library of random small fragments of *S. pneumoniae* chromosomal DNA fused upstream of a promoterless *gfp* gene on an *E. coli* shuttle plasmid (Bartilson et al., 2001). The resulting library was transformed into *S. pneumoniae* and grown under *in vitro* conditions that mimic infections (high osmolarity, temperature shift, change in carbon dioxide (CO₂) concentration, change to blood agar medium and iron limitation). If a promoter fragment controls genes that are important for survival under the said condition, *gfp* would be expressed and fluorescent cells could be isolated and analysed by flow cytometry (Marra et al., 2002). Subsequently,
the sequence of the promoter containing the specific fragment was determined and compared to the \textit{S. pneumoniae} genome sequence, facilitating identification of genes downstream of the promoter; one of such was the \textit{OM001} operon. The contribution of genes in this operon to infection was then assessed by directed mutagenesis and virulence studies of the mutants (Marra \textit{et al.}, 2002, Schneider \textit{et al.}, 2002).

In a model for more localised infection, the respiratory tract infection mice models, deleting this entire operon was found to severely attenuate the pneumococcus abilities to cause lung infection and the bacteria could not disseminate through the blood. Similar levels of attenuation were also found when using two other infection models, gerbil model of otitis media and the intraperitoneal chamber implant model (Marra \textit{et al.}, 2002). This study therefore provide some evidence for the importance of \textit{spd1837} and the other two genes in the operon, \textit{spd1838} and \textit{spd1836} in some aspect of bacterial growth, survival or/and virulence. Other LMWPTPs that had prominent effects on virulence as shown by animal studies include \textit{Streptococcus pyogenes} SP-PTP (Kant \textit{et al.}, 2015) and \textit{M. tuberculosis} PtpA (Singh \textit{et al.}, 2003).

\section{1.7 The pyruvate oxidase, SpxB}

\textit{S. pneumoniae} is known to produce large quantities of H$_2$O$_2$ (Pericone \textit{et al.}, 2003). However, it lacks the typical peroxide-detoxifying enzymes and regulators such as catalase, OxyR, PerR and NADH peroxidase (Tettelin \textit{et al.}, 2002, Hua \textit{et al.}, 2014). The pyruvate oxidase, SpxB, is the main enzyme responsible for H$_2$O$_2$ production in the pneumococcus (Spellerberg \textit{et al.}, 1996). SpxB catalyses the conversion of pyruvate, inorganic phosphate (Pi), and molecular oxygen (O$_2$) to hydrogen peroxide (H$_2$O$_2$), carbon dioxide (CO$_2$) and acetyl phosphate (Blanchette-Cain \textit{et al.}, 2013). Interestingly, aside from the pneumococcus, the \textit{spxB} gene is only present in some streptococcal species that colonise the oropharynx, such as \textit{Streptococcus gordonii}, \textit{Streptococcus oralis}, and \textit{Streptococcus sanguinis} (Okahashi \textit{et al.}, 2013).

\subsection{1.7.1 SpxB is required for H$_2$O$_2$ resistance}

A peculiar phenotype displayed by the pneumococcus when expressing SpxB is its inherent ability to resist death by SpxB’s own toxic byproduct, H$_2$O$_2$. \textit{spxB} mutant was
previously shown to have 10²- to 10³-fold lower survival after exposure to 20 mM of exogenously added H₂O₂ compared to the SpxB-expressing wildtype strain (Pericone et al., 2003). De novo protein synthesis does not appear to be required for pneumococcal H₂O₂ resistance (Pericone et al., 2003). Additionally, prior exposure to sub-inhibitory concentrations of H₂O₂ for several bacterial generations did not improve pneumococcal H₂O₂ resistance (Syk et al., 2014). H₂O₂ is known to cause damage via the Fenton reaction which involves the generation of hydroxyl radicals by interacting with Fe²⁺ ions. However, treatment with the iron chelators dipyridyl or desferrioxamine did not alter the survival of both spxB mutant and wildtype strain (Pericone et al., 2003). The pneumococcus also expresses Dpr which has the capacity to protect the bacteria against death by Fenton reaction (Hua et al., 2014). Echlin et al. (2016) and Carvalho et al. (2013) showed that spxB mutation resulted in only 20% of H₂O₂ being produced relative to the wildtype. However, it is worth noting that during infection of human alveolar epithelial cells, the ability of S. pneumoniae to produce H₂O₂ appears to be strain-dependent, at least in the three serotypes tested, serotype 19F, 3 and 4 (Rai et al., 2015).

1.7.2 The effects of SpxB on pneumococcal pathogenesis

The contribution of SpxB to pneumococcal pathogenesis is still unclear. The H₂O₂ produced was shown to be able to kill or inhibit the growth of other co-colonisers of the nasopharynx such as H. influenzae and Neisseria meningitidis (Pericone et al., 2000, Regev-Yochay et al., 2006). The lack of spxB was shown to reduce virulence in a number of in vivo murine models (Spellerberg et al., 1996, Regev-Yochay et al., 2007, Ramos-Montanez et al., 2008). However, another recent study has shown that the lack of spxB can actually contribute to pneumococcal hypervirulence during invasive disease in mice and spontaneous spxB mutants could be recovered from patients with invasive disease. The authors suggested that the expression of SpxB is detrimental for survival in the bloodstream given the mutants were cleared later by splenic macrophages (but not neutrophils) from the bloodstream despite having similar growth rate (Syk et al., 2014).

The inconsistency in the in vivo data is not surprising given the intricate interaction between SpxB and other pneumococcal factors such as pneumolysin (Bryant et al., 2016), the overall colonisation process (Orihuela et al., 2004, Regev-Yochay et al., 2007) and metabolism (Echlin et al., 2016). For instance, a study found that spxB mutant strains
are less efficient in colonising (Syk et al., 2014) while SpxB was shown to contribute to the initial ability to colonise a naïve animal and prolonged nasopharyngeal colonisation in another study (Orihuela et al., 2004). Additionally, based on real-time PCR data, various pneumococcal serotypes including serotype 2 D39 were implicated to require SpxB for colonisation and the spxB transcripts were dramatically downregulated in the lung and bloodstream (Orihuela et al., 2004, LeMessurier et al., 2006, Mahdi et al., 2008). One known regulator of SpxB is SpxR, which regulates other genes during colonisation. SpxR positively regulates SpxB and this was proposed to be in response to differences in metabolic state as SpxR can bind adenosyl and CoA-containing products (Ramos-Montanez et al., 2008). Furthermore, SpxB was also shown to be required for competence (Battig & Muhlemann, 2008) and resistance to fluoroquinolone antibiotics (Ferrandiz et al., 2015).

1.7.3 The effects of SpxB on CPS and metabolism

The reported effects of spxB mutations on CPS production have varied from either increased CPS levels (Carvalho et al., 2013), decreased CPS levels (Echlin et al., 2016) to no change in CPS levels (Echlin et al., 2016) and this seems to be dependent on the serotype tested and the detection method used. SpxB also appears to play a role in phase variation as the opaque variant was found to produce less SpxB (Overweg et al., 2000). It should be noted that the R6x and Rx1 (unencapsulated derivatives of D39) had a lower resistance to H2O2 than D39, the strain from which they were derived. Although these strains do possess null mutations in the hexA locus, which confer a DNA mismatch repair defect and increased sensitivity to DNA damage (Tiraby & Fox, 1973), this cannot explain the more severe defect in Rx1 resistance to H2O2, which had lower expression of SpxB, compared to R6x strain (Pericone et al., 2003).

Additionally, SpxB has recently been recognised as a link between CPS biosynthesis and metabolism as reduced acetyl-CoA availability resulted from spxB deletion led to CPS defects in pneumococcal serotypes possessing CPS with acetylated sugars (Echlin et al., 2016). spxB deletion was also shown to alter sugar utilisation pattern in the pneumococcus such that, the carbon sources are likely being redirected away from glycolysis to produce more CPS. The authors observed a reduction in the levels of upper glycolytic metabolites glucose 6-phosphate (G6P) and fructose 1,6-bisphosphate (FBP) in late-exponential phase. G6P is a key metabolite at the hub of glycolysis and several
biosynthetic pathways, and its conversion to α-glucose 1-phosphate (α-G1P) is the first step that commits it to the synthesis of many structural polysaccharides, including serotype 2 CPS, the strain of which the study was conducted on (Carvalho et al., 2013). It should be noted that the intracellular concentration of α-G1P and the UDP-activated CPS precursors, UDP-Glc and UDP-GlcUA were similar in the wildtype and its spxB mutant.

1.7.4 The effects of H$_2$O$_2$ produced by the pneumococcus

For the pneumococcus, the H$_2$O$_2$ produced can cause changes in membrane fatty acid composition (Pesakhov et al., 2007) and fatty acid saturation and chain length (Benisty et al., 2010). Other studies showed that the frequency of spontaneous mutations in pneumococcal genes is influenced by endogenous H$_2$O$_2$ production (Pericone et al., 2000, Pericone et al., 2002). From the host perspective, H$_2$O$_2$ produced by S. pneumoniae was shown to induce toxic DNA double-strand breaks in human alveolar epithelial cells and this occurs in a bacterial contact-independent manner. The damage to alveolar epithelium was also demonstrated to disintegrate pulmonary architecture and weaken the alveolar-blood barrier which was speculated to assist bacterial entry into the blood and therefore facilitate systemic bacterial dissemination (Rai et al., 2015). In addition, the presence of H$_2$O$_2$ slows ciliary beating, thereby promoting pneumococcal progression to the lungs (Hirst et al., 2000).

1.7.5 The possible interaction between SpxB-produced H$_2$O$_2$ and Spd1837

As with other protein tyrosine phosphatases which harbour the CX$_5$R motif in their active site, Spd1837’s catalytic cysteine is predicted to be deprotonated at physiological pH. As alluded to in Section 1.5.5, this highly positive environment of the cysteine’s thiol group is required for the phosphatase enzymatic activity (Tanner et al., 2011). However, this also potentially renders the phosphatase susceptible to oxidation leading to its transient inactivation (Chiarugi & Buricchi, 2007). This observation has been documented for many eukaryotic phosphatases with the CX$_5$R active site including PTP-IB, the dual-specificity phosphatase PTEN, and PRL-1 (phosphatase of regenerating liver-1) (van Montfort et al., 2003, den Hertog et al., 2005, Sun et al., 2005). The H$_2$O$_2$ produced by the pneumococcus is therefore predicted to have the ability to oxidise and inactivate PTP with CX$_5$R active site such as Spd1837 (Figure 1.8).
Figure 1.8: Schematic representation of the model of Spd1837 regulation by SpxB-produced H$_2$O$_2$.

ROS appear to oxidise selected PTPs, leading to inactivation by modification of the catalytic cysteine (shown in the thiolate state) to different possible oxidation products (denoted SO$_x$).

1.8  Research Questions and Aims

As shown in Figure 1.6, Spd1837 is putative PTP in the pneumococcus which shows homology to the members of LMWPTPs in bacteria. Members of the LMWPTP family in bacteria most commonly play a role in CPS and EPS biosynthesis, although they can also facilitate a range of other processes which emphasises the versatility of LMWPTPs. Cumulatively, this suggests that LMWPTPs are pivotal for the physiology and pathogenicity of many bacteria and this may also apply to Spd1837 in the pneumococcus. This study also set out to investigate the role of the $OM001$ operon, the operon which encodes Spd1837, in CPS biosynthesis, virulence and survival in human saliva. The possibility that the SpxB-produced H$_2$O$_2$ may regulate Spd1837 activity also drove us to investigate if Spd1837 may have a role in CPS production in the pneumococcus in certain conditions.

Hypothesis 1: Spd1837 is an active LMWPTP in vitro which modulates important metabolic enzymes in the pneumococcus.

Aim 1.1: To enzymatically and biochemically characterise Spd1837 as a LMWPTP in vitro.
Aim 1.2: To identify potential substrates of Spd1837 using a combination of pull-down assays and mass spectrometry.

Hypothesis 2: The operon encoding spd1837, the OM001 operon has a role in CPS biosynthesis, in vivo virulence and survival in human saliva in S. pneumoniae.

Aim 2.1: To generate non-polar, markerless mutations in the genes of the OM001 operon in the chromosome of S. pneumoniae.

Aim 2.2: To assess the effects of the mutations in OM001 operon on pneumococcal CPS biosynthesis.

Aim 2.3: To determine the contributions of the genes in the OM001 operon to pneumococcal in vivo virulence.

Aim 2.4: To determine the contributions of the genes in the OM001 operon to pneumococcal survival in human saliva which could potentially further our understanding of the bacterial transmission process.

Hypothesis 3: Spd1837 modulates pneumococcal CPS biosynthesis in a SpxB-dependent manner.

Aim 3.1: To examine the sensitivity of Spd1837 to H2O2 as a C(X)5R active site-containing PTP.

Aim 3.2: To discover the link between SpxB, Spd1837 and the regulation of CPS, the major virulence factor of the pneumococcus.
Chapter Two

MATERIALS AND METHODS
Chapter 2: Materials and Methods

2.1 Growth Media

2.1.1 Liquid growth media, solid growth media and selection

All *E. coli* strains were routinely grown at 37 °C in Lysogeny Broth (LB) (10 g l⁻¹ tryptone (Becton, Dickinson and Co.; BD), 5 g l⁻¹ yeast extract (BD), 5 g l⁻¹ NaCl) with aeration at 180 rpm. For spread and streak plating, all *E. coli* strains were grown on LB agar (LB, 15 g l⁻¹ agar (BD)) for 16 hr at 37 °C. To store *E. coli* strains, bacteria were grown on solid media as a lawn, harvested by a sterile loop, and stored at -80 °C in 30 % (v/v) glycerol, 1 % (w/v) peptone in glass vials (Wheaton).

All *S. pneumoniae* strains were routinely grown at 37 °C with 5% CO₂ in Todd-Hewitt broth (Oxoid) with 1% yeast extract (Oxoid) (THY) without aeration. For spread, streak, and patch plating, all *S. pneumoniae* strains were grown on Columbia blood agar (39 g l⁻¹ Columbia base agar (Oxoid), 1% (w/v) agar (Bacto), 5% (v/v) defibrinated horse blood (Australian Ethical Biologicals)) for 16 hr at 37 °C with 5% CO₂. On blood agar (BA), *S. pneumoniae* forms alpha haemolytic colonies, 1-2 mm in diameter (Ramirez et al., 2015). For mouse challenge, *S. pneumoniae* strains were grown in serum broth (10% (v/v) heat-inactivated horse serum in nutrient broth (25 g l⁻¹ of nutrient broth no. 2 (Oxoid) in MilliQ water (MQ) (Millipore) (18.2 MQ cm⁻¹)). To store *S. pneumoniae* strains, bacteria were grown in THY until high optical density, and stored at -80 °C in 30 % (v/v) glycerol.

Antibiotics were added as required to the media at the following final concentrations: for *E. coli*, ampicillin (Amp) at 100 μg ml⁻¹ (Roche) and for *S. pneumoniae*, streptomycin (Sm) at 150 μg ml⁻¹ (Sigma), kanamycin (Km) at 200 μg ml⁻¹ (A.G. Scientific Inc), chloramphenicol (Cml) at 6 μg ml⁻¹ (Sigma) and gentamicin (Gm) at 10 μg ml⁻¹ (Sigma).

Bacterial concentration in liquid growth media was measured by optical density at 600 nm (OD₆₀₀) where an OD₆₀₀ reading of 1.0 was equivalent to 5 x 10⁸ CFU ml⁻¹.
2.2 Bacterial strains and plasmids

All *E. coli* and *S. pneumoniae* strains and plasmids utilised or constructed in this work are listed in Appendix A.

2.3 Antibodies and antisera

THE™ His Tag Antibody (mouse monoclonal, GenScript) was used at 1 in 10,000 dilution. Monoclonal mouse anti-phosphotyrosine (anti-PY) 4G10 antibodies (Bio X Cell) were used at 1 in 5,000 dilution. Polyclonal affinity-purified rabbit anti-CpsB and rabbit anti-CpsD antibodies were produced and validated as described previously (Whittall *et al.*, 2015) and both were used at 1 in 500 dilution. Polyclonal affinity-purified mouse anti-SpxB was gifted by Prof James Paton and validated as described previously (Chai *et al.*, 2017). Polyclonal affinity-purified anti-CbpA was validated as described previously (Standish *et al.*, 2005). Both anti-SpxB and anti-CbpA were used at 1 in 5,000 dilution. Horseradish peroxidase-conjugated goat ant-rabbit secondary antibodies (KPL) (1 mg ml⁻¹) were used at 1 in 30,000 dilution. Horseradish peroxidase-conjugated goat ant-mouse secondary antibodies (Cell Signalling) were used at 1 in 5,000 dilution.

Antibodies were raised against Spd1837 (purified protein > 95% pure as determined by Coomassie-stained SDS-PAGE) in rabbits (Institute of Medical and Veterinary Science, Veterinary Services (Gilles Plain, SA, Australia). The antiserum was produced under the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and was approved by the University of Adelaide Animal Ethics Committee. The crude antibodies were enriched and affinity-purified as described previously (Van den Bosch *et al.*, 1997) before being stored at -20 °C in 50% (v/v) glycerol. Anti-Spd1837 antibodies were used at 1 in 500 dilution.
2.4 DNA techniques

2.4.1 Oligonucleotides

All oligonucleotides as listed in Appendix B were ordered from Integrated DNA Technologies (IDT), resuspended in MQ to a storage stock concentration of 100 µM, and stored at -20 °C. Oligonucleotides were diluted to 10 µM before use in polymerase chain reactions (PCR) at 0.2 – 0.5 µM.

2.4.2 Polymerase chain reaction (PCR)

PCR reactions were conducted according to the supplied protocols either in a 25 or 50 µl volume. In general, Taq DNA polymerase with 1 X ThermoPol Reaction Buffer (New England Biolab; NEB) was only used for screening purposes. For cloning and maintenance of sequence fidelity, Phusion Pfu High-Fidelity DNA Polymerase (NEB) was used. For difficult amplification, Q5® Hot Start High-Fidelity DNA Polymerase (NEB) was used. Deoxynucleic triphosphates (dNTPs) (Sigma) was used at a final reaction concentration of 200 µM. An Eppendorf Mastercycler Gradient thermocycler was used for all reactions. Standard cycles for Taq reaction were 30 × denaturation (98 °C, 30 sec), annealing (55 °C, 30 sec), and extension (68 °C, 1 min kb⁻¹). Standard cycles for Phusion reaction were 25 × denaturation (95 °C, 10 sec), annealing (55 °C, 3 min), and extension (68 °C, 30 sec kb⁻¹). Standard cycles for Q5 reaction were 35 × denaturation (98 °C, 30 sec), annealing (55 °C, 30 sec), and extension (72 °C, 30 sec kb⁻¹).

2.4.3 Agarose gel electrophoresis

Prior to loading DNA samples, 5 µl of loading buffer (1 mg ml⁻¹ bromophenol blue, 20 % (v/v) glycerol, 0.1 mg ml⁻¹ RNase) for every 5 µl of sample was added. Samples were separated through horizontal 1% (w/v) agarose TBE (70 mM Tris, 20 mM boric acid, 1 mM EDTA) gels supplemented with the required volume of 20,000 x RedSafe nucleic acid staining solution (iNtRON Biotechnology). Size markers used were SPP1 phage DNA EcoRI fragments made in-house (sizes (kb): 8.51, 7.35, 6.11, 4.84, 3.59, 2.81, 1.95, 1.95, 1.86, 1.51 1.39, 1.16, 0.98, 0.72, 0.48 0.36 and 0.09). The EcoRI digested SPP1 molecular weight standards
were prepared as described previously (Ratcliff et al., 1979). Gels were run at 120 V for 30 min and visualised using a GelDoc XR system (BioRad).

2.4.4 PCR product purification

A QIAquick PCR purification kit (Qiagen) or illustra™ GFX™ PCR DNA Purification Kit (GE Healthcare) was used according to the supplied protocols for purifying PCR products. DNA was eluted in 20 - 50 μl of MQ and stored at -20 °C.

2.4.5 DNA quantification

NanoDrop 2000c Spectrophotometer (Thermo Scientific) was used for measurements of DNA sample concentration by absorption at 260 nm.

2.4.6 DNA sequencing

The samples (purified DNA in the form of double-stranded plasmid or PCR product) were sequenced by the Australian Genome Research Facility (AGRF) sequencing service. In the case of PCR product, sequencing primer was designed such that it binds more internally than the oligonucleotides used to amplify the PCR product. The sequencing primer was added at a final concentration of 0.8 μM to purified DNA (at a concentration following the facility’s recommendation depending on the type and/or size of the DNA) and adjusted to a volume of 12 μl using MQ in a 1.5 ml Eppendorf tube. DNA sequencing data obtained from AGRF was checked for quality using Chromas version 2.6.4 and aligned with the native DNA sequence using DNAMAN version 4.22.

2.4.7 Cloning of spd1837 into pET-15b

2.4.7.1 The vector pET-15b

The vector pET-15b (Novagen) carries an N-terminal His-tag sequence followed by a thrombin site and three cloning sites. The cloning/expression region of the coding strand is transcribed by T7 RNA polymerase. The spd1837 gene from S. pneumoniae serotype 2 D39 strain (NCBI protein ID ABJ55438) was cloned between the NdeI and BamHI sites and the transformants were selected by plating on ampicillin plate.
2.4.7.2 The plasmid pET-15b isolation

pET-15b was isolated from overnight bacterial culture of RMA2302 (LB, 10 ml) according to the QIAprep Spin Miniprep kit (Qiagen) protocol, eluted in MQ and was stored at -4 °C.

2.4.7.3 Restriction endonuclease digests

In a total volume of 20 µl, 2 µl of CutSmart buffer (NEB), 1 µl of each BamHI and NdeI-HF (NEB), 10 µl of isolated pET-15b or PCR-amplified spd1837 and 16 µl MQ were added. Digestion was incubated for 1.5 hr at 37 °C. Following digestion, the sample was PCR purified as BamHI cannot be heat-inactivated.

2.4.7.4 Ligation into pET-15b

Ligation reaction was performed following the manufacturer’s (NEB) protocol where PCR product and plasmid for ligations were mixed in a molar ratio of 3:1 (insert:vector) in a total volume of 10 µl containing 2 U of T4 DNA ligase (NEB) and 1 X T4 DNA ligase buffer (NEB) for 1 hr at 25 °C.

2.4.7.5 Preparation of chemically competent E. coli DH5α

Overnight DH5α culture was diluted 1:20 in 10 ml LB. Mid-exponential phase (OD600 ~ 0.5) DH5α was harvested by centrifugation (2,200 × g, 10 min, 4 °C), washed in 5 ml of ice-cold 100 mM MgCl2, resuspended in 1 ml of ice-cold 100 mM CaCl2, and incubated on ice for 1 hr. Bacteria were then centrifuged (16,000 × g, 1 min, 4 °C), resuspended in 500 µl of 100 mM CaCl2 containing 15% (v/v) glycerol, and split into 100 µl aliquots. Aliquots were stored at -80 °C.

2.4.7.6 Heat-shock transformation of chemically competent E. coli

The entire volume of the ligation reaction was added to the thawed, chemically competent E. coli DH5α aliquot and incubated on ice for 30 min. Bacteria were then heat-shocked at 37 °C for 3 min and then incubated on ice for 5 min. 1 ml of LB was added and the mixture was incubated at 37 °C for 30 min to allow the expression of the ampicillin antibiotic resistance genes in the plasmids before spread-plating. The resultant strain is ZA1 (Appendix A).
2.4.7.7 Site-directed mutagenesis

Single amino acid substitution in spd1837 was constructed using the QuikChange Lightning Site-directed Mutagenesis kit (Agilent Technologies) according to the supplied protocols with some modifications. For the synthesis of the mutant strand, Q5® Hot Start High-Fidelity DNA Polymerase was used with primers ZA11 and ZA12 primers (Appendix B) instead of the supplied QuikChange Lightning Enzyme. Plasmid DNA was added at a final concentration of approximately 40 ng µl⁻¹. Then, during the transformation of XL-10 Gold Ultracompetent cells, the recommended NZY+ broth was substituted to regular LB. The resultant strain is ZA11 (Appendix A).

2.4.7.8 Strains construction for Spd1837 and Spd1837_C8S protein over-expression

The constructed pET-15b-Spd1837 and pET-15b-Spd1837_C8S were isolated from ZA1 and ZA11 strain respectively and transformed into Lemo21 (DE3) strain as per section 2.4.7.5 and 2.4.7.6, giving rise to ZA2 and ZA12 strains (Appendix A).

2.4.8 Construction of chromosomal mutations in S. pneumoniae

2.4.8.1 S. pneumoniae chromosomal DNA isolation

Pneumococcal chromosomal DNA was isolated using the Wizard Genomic DNA purification kit (Promega) according to the manufacturer’s instructions. Sodium deoxycholate (DOC) (Sigma) was used at 0.1% to lyse the cells. DNA isolated was rehydrated in 20 – 50 µl MQ.

2.4.8.2 Overlap-extension PCR

All the deletion and point mutation in S. pneumoniae chromosome (except spxB deletion) were constructed using the Janus cassette system (Sung et al., 2001). This involved a two-step transformation process which resulted in non-polar, markerless mutation of the target gene. For example, to generate D39Δspd1837 strain, firstly, PCR products which encodes for the 2kb upstream region of spd1837 gene and the 2kb downstream region of spd1837 gene were amplified using overlap-extension PCR with oligonucleotides which have homologous region to the Janus cassette (Horton, 1993). Next, these three separate PCR products were PCR-purified and combined, serving as the
template DNA for the second round of PCR using only the external oligonucleotides/primers (Figure 2.1A). Then, a streptomycin-resistant D39 was transformed with PCR product generated earlier. The Janus cassette carries a kanamycin-resistant cassette and the dominant rpsl’ gene that encodes for streptomycin-sensitivity. Hence, for this first transformation, D39spd1837::janus strain was generated by selecting for colonies which were streptomycin-sensitive and kanamycin-resistant (Figure 2.1B). In the second round of overlap-extension PCR, similar process was repeated except that this time, the primers utilised were designed to delete spd1837 in place of the Janus cassette (Figure 2.1C). Thus, for this second transformation, D39Δspd1837 mutants were generated by selecting for colonies which were streptomycin-resistant and kanamycin-sensitive due to the loss of the Janus cassette (Figure 2.1D). It is also critical that before the next amplification process, the PCR products which would serve as the template DNA in the second step was PCR-purified twice, one in separate tubes then again in the same tube (Section 2.4.4)
Figure 2.1: Construction of spd1837 chromosomal deletion in *S. pneumoniae* D39.

All the deletion and point mutation in *S. pneumoniae* chromosome (except spxB deletion) were constructed using the Janus cassette system and overlap-extension PCR (A) and (C), in combination with two-step transformation process (B) and (D), which resulted in non-polar, markerless mutation of the target gene. Transformation of *S. pneumoniae*

The strain was grown to mid-log phase (OD$_{600}$ ~ 0.5) and 100 µl of the culture was added to 10 ml THY, 10 µl 0.1 M CaCl$_2$, 25 µl 8% (w/v) Bovine Serum Albumin (BSA) and 10 µl of the PCR product. The PCR product containing the desired mutation or deletion was transformed directly into *S. pneumoniae* without being PCR-purified first. For transformation into D39, the culture was also supplemented with 10 ng ml$^{-1}$ competence-stimulating peptide-1 (CSP-1) (amino acid sequence: MRLSKFFDFILQRKK (Chirontech (Victoria, Australia)) while for transformation into WU2, both CSP-1 and competence-stimulating peptide-2 (CSP-2) (amino acid sequence: EMRISRIILDFLFLRKK (Mimotopes (Victoria, Australia)) were added (Havarstein *et al.*, 1995).

2.4.8.3 Complementation

To create the OM001 complemented strain, firstly, the 2 kb region upstream of the deleted OM001 operon was amplified using the primers ZA3 and ZA16 and the 2 kb
region downstream of the deleted OM001 operon was amplified using the primers ZA4 and ZA19. These two PCR products and the amplified Janus cassette were combined and amplified again using just the primers ZA3 and ZA4. The approximately 2.4 kb PCR product was then used to transform D39ΔOM001. The transformants were selected on kanamycin plates, resulted in the intermediate strain, D39ΔOM001::janus. Next, the OM001 operon region including 1 kb of flanking genomic DNA from D39 was amplified using the primers ZA36 and ZA37. This product was then used to transform D39ΔOM001::janus to replace the Janus cassette with the wild type copy of the OM001 operon. The successful transformants were selected on streptomycin plate and one of them was sequenced and verified (Section 2.4.6) to have acquired the OM001 operon back and this strain is called D39ΔOM001::OM001⁺.

2.4.8.4 Deletion of spxB

To construct spxB deletion mutation, a chloramphenicol resistance cassette with 2 kb homology to upstream and downstream region of spxB gene in S. pneumoniae serotype 2 D39 genome was amplified using the primers AS253 and AS254 (Appendix B). The PCR products were then transformed into the D39 WT, Δspd1837 and Spd1837CBS strains as per Section 0 to delete and replace the open reading frame encoding SpxB with the chloramphenicol resistance cassette.

2.5 Protein techniques

2.5.1 Whole bacterial lysate samples

A total of 2.5 x 10⁸ bacteria from a mid-log phase (OD₆₀₀ ~ 0.5) culture were harvested (16,000 x g, 1 min, 4 °C) and resuspended in 50 μl of sample buffer (4 % (w/v) SDS, 20 % (v/v) glycerol, 10 % (v/v) β-mercaptoethanol, 1 % (w/v) bromophenol blue, 0.25 M Tris pH 6.8). Samples were heated at 100 °C for 5 min and either stored at -20 °C or used immediately for SDS-PAGE.
2.5.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was conducted using BioRad self-cast Mini-Protean System III and a Tris-Glycine buffer system. Acrylamide and ammonium persulfate were purchased from BioRad while N,N,N',N'-Tetramethyl-ethylenediamine (TEMED) was purchased from Sigma. Samples were electrophoresed on 12% or 15% (w/v) acrylamide gels depending on the size of the protein(s) of interest. The running buffer (pH 8.3) was consisted of 25 mM Tris-HCl, 200 mM glycine, and 0.1 % (w/v) SDS. Gels were generally electrophoresed between 100 – 200 V for 1 – 2 hr. Low molecular weight markers (LMWM) (Invitrogen) (sizes (kDa): 97.0, 66.0, 45.0, 30.0, 20.1, 14.4) were used as guides to estimate the protein molecular mass if the gel was intended for Coomassie staining (Section 2.5.3). BenchMark Prestained Protein standard (Invitrogen) (sizes (kDa): 190, 120, 85, 60, 50, 40, 25, 20, 15, 10) or SeeBlue™ Plus2 Pre-stained Protein Standard (Thermo Scientific) (sizes (kDa): 155, 100, 65, 41, 33, 23, 12) were used as guides to estimate the protein molecular mass if the gel was intended for Western immunoblotting (Section 2.5.4). For mass spectrometry analysis (Section 2.5.7), the samples were electrophoresed on a 4-12% Bolt™ Bis-Tris Plus Gel (Thermo Scientific).

2.5.3 Coomassie blue staining

SDS-PAGE-separated proteins were stained by incubating the gel in Coomassie blue stain solution (0.3 % (w/v) Coomassie Brilliant Blue R-250 (Thermo Scientific), 10 % (v/v) acetic acid, 45 % (v/v) methanol) at room temperature with shaking at least 1 hr to overnight. Gels were destained with repeated washes of Destain solution (10 % (v/v) acetic acid, 50 % (v/v) methanol).

2.5.4 Western immunoblotting and detection

SDS-PAGE-separated proteins were transferred to nitrocellulose membranes (NitroBind, pure nitrocellulose, 0.45 μm (BioRad)) using BioRad Trans-Blot® Turbo™ Transfer System. Transfer was conducted under turbo mode according to manufacturer’s recommendations (7 min at 1.3 A, 25 V for one mini gel or 7 min at 2.5 A, 25V for two mini gels in 1 × transfer buffer (200 ml 5 × transfer buffer, 600 ml reverse osmosis (RO) water and 200 ml ethanol). Ponceau S stain (0.1% (w/v) Ponceau S (Sigma), 5% acetic acid) was used to visualise if the transfer was successful. For the detection using all primary
antibodies except anti-PY 4G10, the blots were blocked with 5 % (w/v) skim milk in TTBS buffer (16 mM TrisHCl, 120 mM NaCl, 0.05 % (v/v) Tween-20 (Sigma)) for 1 hr before overnight incubation with primary antibody diluted in 5 % (w/v) skim milk in TTBS. Blots were then washed three times with TTBS for 10 min and incubated with HRP-conjugated secondary antibody diluted in TTBS for 2 hr. For anti-PY blots, following transfer, the blots were blocked with 5 % (w/v) BSA in TTBS overnight. The blots were then incubated in primary antibody diluted in 5% (w/v) BSA in TTBS for 1 hr. Following washes as described previously, the blots were incubated with HRP-conjugated secondary antibody diluted in 5 % (w/v) skim milk in TTBS for 2 hr. The following steps applied for all blots including anti-PY blots; blots were then washed three times with TTBS and three times with TBS (TTBS without Tween-20) for 5 min. Blots were incubated with Chemiluminescent substrate (Sigma) and then developed digitally using a ChemiDoc MP System (BioRad). Where appropriate, the blots were re-probed using another primary antibodies after incubation with Restore™ Western Blot Stripping Buffer (Thermo Scientific) following the manufacturer’s instructions.

2.5.5 Over-expression and purification of Spd1837 and Spd1837_C8S

The Spd1837 from *S. pneumoniae* D39 (serotype 2) and the protein with a point mutation in its active site, Spd1837_C8S were expressed as His6-recombinant proteins using the vector pET-15b as described in Section 2.4.7. Spd1837 and Spd1837_C8S proteins were expressed in *E. coli* Lemo21 (DE3), grown at 37 °C for 16 hr LB, sub-cultured 1:20 in 1 l LB at 37 °C for 2 hr with the expression of recombinant protein induced with 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Biovectra) and incubation was proceeded for another 3 hr at 37 °C.

Spd1837 and Spd1837_C8S were purified essentially as described by Romero et al. (2007). Briefly, to harvest the cells, the cultures of the indicated strains were centrifuged at 8,000 × g for 20 min at 4 °C. The cell pellet was resuspended in 10 ml Buffer A (100 mM Tris, 200 mM NaCl, 20% (v/v) glycerol, 20 mM imidazole (Sigma), pH 7.4) containing 100 µg ml⁻¹ deoxyribonuclease I (Sigma) and 1 x protease inhibitor (BioSciences). The cells were disrupted by French press at >1000 p.s.i. (~6.9 MPa). The crude lysate was ultracentrifuged at 288,000 × g for 1 hr at 4 °C to remove insoluble material. The isolated soluble fraction was loaded onto a 5 ml HisTrap FF column (GE Healthcare) previously equilibrated with
Buffer A. The protein was then eluted with Buffer B (100 mM Tris, 200 mM NaCl, 20% (v/v) glycerol, 500 mM imidazole, pH 7.4). The homogeneity of the purified proteins was determined using 15% (v/v) SDS-PAGE. Fractions containing the proteins were pooled and desalted using a PD-10 column (GE Healthcare) pre-equilibrated with Buffer A. The 6×His-tag was then cleaved with Thrombin (Sigma) (10 µl per 1.5 ml of sample). The final purification of the protein from contaminating proteins was achieved by buffer exchange, size-exclusion filtration using HiLoad 16/600 and 26/600 Superdex 200 prep grade column (GE Healthcare). The concentrations of both wild type and mutant proteins were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific).

2.5.6 **In vitro substrate-trapping assay**

The method involved substituting the critical cysteine residue to a serine residue which was successfully constructed as per Section 2.4.7.8. Replacement of the catalytic site cysteine by a serine has been shown to completely abolish phosphatase activity (Castandet *et al.*, 2005, Maeda *et al.*, 2008, Linford *et al.*, 2014, Nath *et al.*, 2014). Such mutation has been shown previously to result in a mutant form of the enzyme that is able to bind substrates to the same affinity as the wildtype enzyme i.e. the substrate is ‘trapped’ in the catalytic pocket but can no longer be dephosphorylated, resulting in a stable non-covalent PTP-Ser-PO₃ complex (Davis *et al.*, 1994, Buist *et al.*, 2000, Zhang, 2003a, Zhang, 2003b, Blanchetot *et al.*, 2005, Trentini *et al.*, 2014).

The assay was performed essentially as described by Blanchetot *et al.* (2005) with some modifications. 500 ml THY media was inoculated with D39Δspd1837 strain and grown for 6 hr (OD₆₀₀ ~ 0.2). 1 mM of freshly-prepared pervanadate (1 mM H₂O₂, 1 mM sodium orthovanadate (Na₂VO₃)) was added to the culture and the incubation was continued for another 30 min. Pervanadate is a strong oxidant of the PTPs active site cysteine (Chiarugi, 2001). Once added to the cells, pervanadate disrupts the balance between tyrosine phosphatases and tyrosine kinases in favour of the tyrosine kinases which results in increased tyrosine phosphorylation. To harvest the cells, the culture was centrifuged at 8,000 × g for 20 min at 4 °C, the supernatant removed and the pellet frozen at -80 °C. 100 µl of Ni-charged MagBeads slurry (GenScript) per sample was equilibrated with cold lysis buffer (20 mM Tris, pH 7.5, 300 mM NaCl, 0.1 mM EDTA, 1% (v/v) Triton X-100 (Sigma), 10% (v/v) glycerol, 1 mM imidazole) twice. 125 µg of His₆-Spd1837, His₆-
Spd1837CBS, or BSA in conjugation buffer (1 × TBS, 10% (v/v) glycerol, 1 mM dithiothreitol (DTT)) were incubated with the equilibrated magnetic beads at 4 °C overnight, and beads only sample was incubated with conjugation buffer only.

The next day, the frozen pellet was resuspended in cold lysis buffer freshly supplemented with 5 mM iodoacetic acid (IAA) (Sigma), 1 mM Na3VO4 and 1 × proteases inhibitor (BioSciences). IAA irreversibly inactivates the endogenous tyrosine phosphatase and EDTA chelates and inactivates the vanadate(s). The resuspended cells were then disrupted by sonication on ice (Branson B15). Cleared cell lysate was incubated with 5 mM DTT on ice for 15 min. The crude lysate was ultracentrifuged at 450,000 × g for 1 hr at 4 °C to separate the soluble and insoluble fractions. After the supernatant (the soluble fraction) was collected, the pellet (the insoluble fraction) was solubilised with 1% (w/v) n-dodecyl-β-ᴅ-maltoside (DDM) (Anatrace). The conjugation buffer from the beads was removed and the soluble fraction and insoluble fraction were incubated separately with the beads overnight at 4 °C. Step-wise elution with imidazole during the washes was implemented with 250 mM imidazole as the final elution step. The eluted samples were collected and subjected to SDS-PAGE on a 4-12% Bolt™ Bis-Tris Plus Gel (Thermo Scientific) and subsequently Coomassie-stained.

2.5.7 Liquid chromatography – electrospray ionisation tandem mass spectrometry

2.5.7.1 Sample preparation

The substrate-trapping assay were repeated three times with consistent results and one of the Coomassie-stained whole gels was submitted to the Adelaide Proteomics Centre for trypsin digestion and mass spectrometry analysis of tryptic peptides of the selected bands. Firstly, the gel bands were destained with 100 mM ammonium bicarbonate in 30% acetonitrile (ACN), washed with 50 mM ammonium bicarbonate (NH₄HCO₃) and digested with 100 ng of sequencing grade modified trypsin (Promega) in 5 mM ammonium bicarbonate in 10% ACN. Resulting peptides were extracted using 3 washes of 1% formic acid (FA) in water, 1% FA in 50% ACN and 100% ACN respectively. The volumes of the resulting peptide extracts were reduced by vacuum centrifugation to approximately 1 µl then resuspended with 0.1% FA in 2% ACN to a total volume of ~10 µl.
2.5.7.2 Data acquisition

Data acquisition was also performed by Adelaide Proteomics Centre. Nano-liquid chromatography-electrospray ionisation tandem mass spectrometry was performed on an Ultimate 3000 RSLC system coupled to a LTQ Orbitrap XL ETD MS instrument (both Thermo Scientific). Peptide samples (5 µl) were pre-concentrated onto a C18 trapping column (Acclaim PepMap100 C18 75 µm × 20 mm, Thermo Scientific) at a flow rate of 5 µl min\(^{-1}\) in 2% ACN 0.1% FA for 5 min. Peptide separation was performed using a 75 µm ID C18 column (Acclaim PepMap100 C18 75 µm × 15 cm, Thermo Scientific) at a flow rate of 0.3 µl min\(^{-1}\) using a linear gradient from 5 to 45% B (A: 5% ACN 0.1% FA, B: 80% ACN 0.1% FA) over 30 minutes, followed by a 10 min wash with 90% B, and an 15 min equilibration with 5% B. Mass spectrometry (MS) scans were acquired in the mass range of 300 to 2,000 m/z at a resolution of 60,000. The six most intense precursor ions selected for isolation and were subjected to collision-induced dissociation (CID) fragmentation using a dynamic exclusion of 5 sec. Dynamic exclusion criteria included a minimum relative signal intensity of 1,000, and ≥ 2+ charge state. An isolation width of 3.0 was used with a normalised collision energy of 35.

RAW files were submitted directly to Mascot via Proteome Daemon (1.3, Thermo Scientific). Acquired data was searched against the Swiss-Prot database in MASCOT (V2.3.02). Search parameters were set as *Streptococcus pneumoniae* strain D39 (Taxonomy), trypsin digestion with 2 missed cleavages, fixed modification of carbamidomethyl of cysteine, variable modification of oxidation of methionine, precursor ion mass tolerance of 10 ppm, and product ion mass tolerance of 0.8 Da. Further analysis of the data was carried out in Proteome Discoverer (V1.1, Thermo Scientific). Data was searched against decoy database for false discovery rate calculations (approximately 1%). Peptides with \(p < 0.05\) are reported. Identifications can be made if at least two unique peptides were sequenced from a protein and had individual ion scores above the homology threshold. Multiple charge states were not considered as unique.
2.6 Tissue culture techniques

2.6.1 Tissue culture and maintenance

The cell line used was A549 (human type II pneumocytes) ATCC CCL-185. Cells were grown and maintained in Falcon 75 cm² vented tissue culture flasks (BD Corning). Cells were grown in 20 ml Dulbecco’s Modified Eagle’s medium (DMEM) with HEPES minus sodium pyruvate (Life Technologies). The medium was supplemented with 5 % (v/v) foetal calf serum, 4 mM L-glutamine (replaced every 7 days), 100 U ml⁻¹ penicillin and 100 U ml⁻¹ streptomycin. Cells were maintained at 37 °C in a humidified incubator with a constant 5 % CO₂. Upon reaching confluence, A549 cells were washed three times in 1 x PBS, detached with 1 ml 0.25% (w/v) trypsin and 0.02% (w/v) EDTA and counted. Flasks were re-seeded at a ratio of 1:4 (cell suspension:media). Cells were stored in the growth medium supplemented with 10% (v/v) demethylsulfoxide (DMSO) (Sigma) at -80 °C for long-term storage.

2.6.2 Mycoplasma detection by PCR-based method

As the A549 was a new cell line to enter the laboratory, it was subjected to identification of mycoplasma contamination before any assays were conducted using the cell line. Firstly, 300 µl of media supernatant was heated at 100 °C for 3 min. Following that, 300 µl of phenol/chloroform mix were added and the mixture was centrifuged at 13,000 x g for 10 min. 25 µl of the supernatant was removed and added to 75 µl MQ. 1 µl of this mixture was used as the template for PCR reaction using Taq polymerase as described in Section 2.4.2 and MycoF and MycoR oligonucleotides (Appendix B). With the appropriate positive (supernatant of Caco-2 cells that were previously shown to be mycoplasma positive) and negative (miliQ) controls, the A549 cell line was determined to be mycoplasma negative.

2.6.3 Adherence assays

The method was adapted from Talbot et al. (1996). To achieve approximately 90% confluency so that the adherence sites remained fully exposed, 4.5 x 10⁴ cells/well were seeded in 24-well plates and incubated overnight at 37 °C in 5% CO₂. The strains were
grown in THY until the OD_{600} of 0.5, washed and resuspended in culture media before being
added to four wells per strain at a density of 5 \times 10^6 CFU ml^{-1}. Infected A549 cells were
incubated for 1.30 hr at 37 °C in 5% CO_2 followed by three washes in Dulbecco’s PBS (0.1% (v/v) CaCl_2, 0.1% (v/v) MgCl_2 in 1 \times PBS). To detach the adherent bacteria, 100 µl of 0.25% (v/v) trypsin with 0.02% (w/v) EDTA and 400 µl of 0.25% (v/v) Triton X-100 were added to the wells. 100 µl lysate from each well and serial dilutions (up to 10^{-3}) thereof were plated onto BA. Adherent pneumococci were then quantified and expressed as percentage of adherent cells relative to the wildtype. Results were analysed using Student’s unpaired t-test (2-tailed).

2.7 Phosphatase assays

Phosphatase activity was monitored at 37 °C by using a continuous method based on the detection of \( p \)-nitrophenol (pNP) formed from \( p \)-nitrophenyl phosphate (pNPP). Assay linearity over at least 10 min was established. The amount of pNP released was estimated by using a molar extinction coefficient of 18,000 M^{-1} cm^{-1} (Cirri et al., 1993). The assay was optimised with respect to protein concentration, time, and pH. Absorbance readings at 410 nm were carried out on a PowerWaveX340 microplate spectrophotometer (Bio-Tek Instruments, Inc.). Kinetic reaction mixtures were thermally equilibrated for 30 min at 37°C prior to reaction initiation. Kinetic parameters were determined by fitting the data to the Michaelis-Menten equation, using non-linear regression (GraphPad Prism 6 Software). Phosphatase activities at different temperatures were compared at temperatures ranging from 25 to 50 °C using 100 mM Tris pH 7.0 as buffer. Phosphatase activities at different pH values were compared with the following buffers: 100 mM sodium citrate (pH 4.0-6.5), and 100 mM Tris (pH 7.0-9.5) at 37 °C. In varied pH, temperature and inhibitors concentration assays, 400 ng of Spd1837 and 8.0 mM pNPP was used. Na_2VO_3 (Sigma) was added from 200 mM stocks according to manufacturer’s instructions. Phosphotyrosine phosphatase activity was also analysed using the Tyrosine Phosphatase Assay System (Promega), according to the manufacturer’s instructions.

For the Na_2VO_3 and the sodium fluoride (NaF) (Sigma) inhibition assay, both Na_2VO_3 and NaF were added in the range of 0-100 mM. For the \( H_2O_2 \) inhibition assay, \( H_2O_2 \) was added in the range of 0-500 µM. To rescue the phosphatase activity, catalase from
Bovine liver (Sigma) in the range of 2-175 ng ml⁻¹ was added to the reaction containing 100 μM of H₂O₂ in a separate assay. In all inhibition assays, 400 ng of Spd1837 and 8.0 mM pNPP was used in 100 mM Tris pH 7.0 as buffer at 37 °C.

2.8 Quantification of pneumococcal CPS

2.8.1 Preparation of pneumococcal CPS

CPS was prepared from the indicated strains grown either aerobically (BA at 37 °C with 5% CO₂) or anaerobically (BA at 37 °C with 5% CO₂ in a BD GasPak™ Anaerobic Jar (BD)). The cells were resuspended in 10 ml PBS and adjusted to an OD₆₀₀ of 0.5. The resuspended bacteria were then separated into two 5 ml aliquots and centrifuged (4,500 × g, 30 min). The aliquots were then resuspended differently depending on the preparation as described below;

2.8.1.1 Total CPS (T-CPS) samples

The cell pellet was resuspended in 150 mM Tris (pH 7.0), 1 mM MgSO₄ to a final volume of 250 µl. 5 µl of 10 % (w/v) DOC was added and incubated for 30 min at 37 °C to lyse the cells.

2.8.1.2 Cell wall-associated (CW-CPS) samples

The cell pellet was resuspended in 5 ml of 2% SDS in 1 × PBS and heated at 100 °C for 30 min. The cells were cooled to room temperature and then centrifuged at 3,500 x g and washed in 1 × PBS three times. The pellet was resuspended in 150 mM Tris (pH 7.0), 1 mM MgSO₄ to a final volume of 250 µl.

Following that, 100 U of mutanolysin and 0.5 mg of both DNasel and RNase (all from Sigma) were added and the solution was incubated at 37 °C overnight. Then, 50 μg of proteinase K (Thermo Scientific) was added before a further incubation at 56 °C for 4 hr. The T-CPS and CW-CPS samples were stored at -20 °C.
2.8.2 Uronic acid assays

The uronic acid assay was performed as described previously (Morona et al., 2006, Standish et al., 2012). 600 µl of 0.0125 M di-sodium tetraborate (Na₂B₄O₄) in concentrated H₂SO₄ was added to 35 µl of sample plus 65 µl MQ water while on ice. Samples were vortexed, heated at 100 °C for 5 min and then immediately cooled on ice. To one tube of the aliquot, 10 µl of 0.15% (w/v) 3-phenylphenol (Sigma) dissolved in 0.5% (v/v) NaOH was added, while the second tube (the internal negative control) had 10 µl of 0.5% NaOH, and the tubes were immediately inverted several times to mix the samples. The A₅₂₀nm of a 200 µl aliquot was measured on a PowerWaveX340 microplate spectrophotometer (Bio-Tek Instruments, Inc.). The data were processed by first subtracting the value of the NaOH control from 3-phenylphenol/NaOH value. Then, the amount of CPS present in each sample was expressed as a percentage of the T-CPS present in D39 or WU2. Levels were related back to a standard curve of D-glucuronic acid (Sigma). Differences in CPS levels were analysed by one-way analysis of variance (ANOVA) with Dunnett’s post-hoc test.

2.9 Hydrogen peroxide sensitivity assay

Hydrogen peroxide sensitivity assays were conducted essentially as described previously (Pericone et al., 2003). Briefly, bacteria were grown until early-log phase (OD₆₀₀ 0.3-0.4), and each culture was added to 100 µl of THY medium or 100 µl of THY medium containing either 15 mM or 5 mM H₂O₂, followed by incubation at 37 °C for 30 min. Serial dilutions from each tube were then prepared in ice-cold 1 × PBS to minimise Fenton reaction (Pesakhov et al., 2007), and duplicate aliquots were spotted onto BA plates with half of the plate spotted with the strain treated with H₂O₂ and the other half without H₂O₂ treatment. The percent survival was calculated by dividing the CFU of cultures after exposure to H₂O₂ by the CFU of cultures without H₂O₂. Results were analysed using Student’s unpaired t-test (2-tailed).
2.10 Animal studies

2.10.1 Ethics statement

This study was carried out in strict accordance with the recommendations in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition (2004) and 8th Edition (2013)) and the South Australian Animal Welfare Act 1985. The protocol was approved by the Animal Ethics Committee at The University of Adelaide (approval number S/2013/053).

2.10.2 Intranasal challenge of Swiss mice

Outbred 5-to-6-week-old female CD1 (Swiss) mice were used in all animal experiments. For intranasal (i.n.) challenge, mice were anesthetised by intraperitoneal (i.p.) injection of pentobarbital sodium (Nembutal; Rhone-Merieux) at a dose of 66 μg per g of body weight, followed by i.n. challenge with 50 μl of bacterial suspension containing approximately $1 \times 10^7$ CFU ml$^{-1}$ bacteria in serum broth. The challenge dose was confirmed retrospectively by serial dilution and plating on BA. Mice were euthanised by CO$_2$ asphyxiation at the 48 hr post-challenge. Blood was collected by syringe from the posterior vena cava. The pleural cavity was lavaged with 1 ml sterile PBS containing 2 mM EDTA introduced through the diaphragm. Pulmonary vasculature was perfused by infusion of sterile PBS through the heart. Lungs were subsequently excised into 2-ml vials containing 1 ml sterile PBS and 2.8-mm-diameter ceramic beads (GeneWorks) for CFU counts. To obtain unattached pneumococci, the nasopharynx was subjected to lavage by insertion of a 26-gauge needle sheathed in tubing into the tracheal end of the upper respiratory tract and injection of 1 ml 0.5% trypsin in 1× PBS through the nasopharynx. Additionally, the upper palate and nasopharynx were excised and placed into 2-ml vials containing 1 ml sterile PBS and 2.8-mm-diameter ceramic beads to obtain attached pneumococci. Lung and nasopharyngeal tissues were homogenised using a Precellys 24 tissue homogenizer (Bertin Technologies) at 3 cycles of 30 sec and 5,000 rpm. 40 μl aliquots of lung homogenate, nasopharyngeal tissues homogenate and pleural lavage, and 20 μl aliquots of blood were serially diluted and plated on BA supplemented with gentamicin to determine the number of CFU in these niches. CFU counts for both the nasal wash and nasal tissue samples were combined to determine the total number of bacteria in the nasopharynx. Data were
analysed using non-parametric Mann-Whitney test. The incidence of pneumococcal invasion into the lungs and blood of mice were compared using two-tailed Fisher’s exact test.

2.11 Evaluation of the survival of *S. pneumoniae* strains in human saliva

The University of Adelaide Human Research Ethics Committee approved the study protocol and the written informed consent form with approval number of H-2016-224. Saliva collection and *S. pneumoniae* survival tests were conducted essentially as described by Verhagen *et al.* (2014) with a few modifications. The additional criteria for recruiting participants include ‘currently a non-smoker’ and ‘no respiratory or periodontal disease or infection’ as smokers and individuals with such disease or infection were shown previously to have human leukocyte elastase in their saliva and therefore is not representative of general, healthy population (Nedzi-Gora *et al.*, 2014, Patel *et al.*, 2015). Briefly, fasting saliva of the donors was pooled and centrifuged at 16,000 × g at 4 °C for 15 minutes. The supernatant was sterilised by ultrafiltration with 0.45 µm Minisart filters (Sartorius Stedim Biotech). Before inoculation in saliva, the strains were grown in THY for 2 hr, diluted to a starting concentration of 10^6 CFU ml⁻¹ and washed twice in sterile PBS. The bacteria was incubated with at least 500 µl saliva at two conditions: 37°C with 5% CO₂ (representing in-host carriage) and 25°C without CO₂ (representing transmission). At t = 0, t = 3, t = 22, and t = 24 hr, samples were taken for CFU count. The number of bacteria at specific time point was enumerated by plating serial dilutions on BA plates. Experiments were performed in duplicates and repeated three times independently. Statistical differences between survival of *S. pneumoniae* in multiple dilutions of saliva were assessed by a one-way ANOVA and Dunnett’s post hoc tests.
Chapter Three

**Research Article One:**

*In vitro* characterization and identification of potential substrates of a low molecular weight protein tyrosine phosphatase in *Streptococcus pneumoniae*
**In vitro** characterization and identification of potential substrates of a low molecular weight protein tyrosine phosphatase in *Streptococcus pneumoniae*

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Keywords: tyrosine phosphorylation; low molecular weight phosphatase; pneumococcus; *Streptococcus pneumoniae*; phosphatase substrates identification

Subject category: Regulation

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Abbreviations: BA, Colombia blood agar; BY-kinase, bacterial tyrosine kinase; C8S, cysteine 8 to serine; CPS, capsular polysaccharide; DDM, n-dodecyl-β-d-maltoside; EPS, exopolysaccharide; IAA, iodoacetic acid; LB, Lysogeny Broth; LMWPTP, low molecular weight protein tyrosine phosphatase; NaF, sodium fluoride; Na₃VO₄, sodium orthovanadate; pNP, p-nitrophenol; pNPP, p-nitrophenyl phosphate; PTP, protein tyrosine phosphatase; THY, Todd-Hewitt broth with 1% Bacto yeast extract.
# Statement of Authorship

<table>
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<th>In vitro characterization and identification of potential substrates of a low molecular weight protein tyrosine phosphatase in <em>Streptococcus pneumoniae</em></th>
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- [ ] Submitted for Publication
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## Principal Author

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<th>Name of Principal Author (Candidate)</th>
<th>Zuleez Ahmad</th>
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| Contribution to the Paper | Performed all experiments, performed analysis on all samples, interpreted data, constructed all figures, tables, and supplementary, wrote manuscript and acted as corresponding author for the submission. |

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| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. |

| Signature | Date | 14/12/17 |

## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate’s stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate’s stated contribution.

<table>
<thead>
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| Contribution to the Paper | Supervised development of work, helped in data interpretation, helped to evaluate and edit the manuscript and provision of laboratory and materials. |

<p>| Signature | Date | 14/12/17 |</p>
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<td>Supervised development of work, helped in data interpretation, helped to evaluate and edit the manuscript. Act as the final corresponding author.</td>
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Chapter 3: Research Article 1: *In vitro* characterization and identification of potential substrates of a low molecular weight protein tyrosine phosphatase in *Streptococcus pneumoniae*

3.1 Abstract

*Streptococcus pneumoniae* is a major human pathogen responsible for significant mortality and morbidity worldwide. Within the annotated genome of the pneumococcus lies a previously uncharacterized protein tyrosine phosphatase which shows homology to Low Molecular Weight Protein Tyrosine Phosphatases (LMWPTPs). LMWPTPs modulate many processes critical for the pathogenicity of a number of bacteria including capsular polysaccharide biosynthesis, stress response and persistence in host macrophages. Here, we demonstrate that Spd1837 is indeed a LMWPTP, by purifying the protein, and characterizing its phosphatase activity. Spd1837 showed specific tyrosine phosphatase activity, and it did not form higher order oligomers in contrast to many other LMWPTPs. Substrate-trapping assays using the wild-type and the phosphatase-deficient Spd1837 identified potential substrates/interacting proteins including major metabolic enzymes such as ATP-dependent-6-phosphofructokinase and Hpr kinase/phosphorylase. Given the tight association between the bacterial basic physiology and virulence, this study hopes to prompt further investigation of how the pneumococcus controls its metabolic flux via the LMWPTP Spd1837.

3.2 Introduction

*Streptococcus pneumoniae* is a human specific bacterial pathogen responsible for a range of diseases such as pneumonia, bacteremia and meningitis. Research into tyrosine phosphorylation in the pneumococcus has focused on the role of protein tyrosine phosphatase (PTP) CpsB and the bacterial tyrosine kinase (BY-kinase) CpsD, on capsular polysaccharide (CPS) biosynthesis [1-3]. Further, tyrosine phosphorylation can alter the activity of the pneumococcal amidase LytA [4] and the Noc-like protein ParB during cell division [5] suggesting tyrosine phosphorylation plays a diversity of roles in the pneumococcus.
Our analysis of the *S. pneumoniae* genome sequence identified another putative PTP besides CpsB, designated as Spd1837. Spd1837 shows homology to the low molecular weight protein tyrosine phosphatase (LMWPTP) family (Figure 3.4S). Members of the LMWPTP family in bacteria most commonly play a role in CPS and exopolysaccharide biosynthesis [6]. Spd1837 is not present in an operon with a BY-kinase [7] which has been shown to reliably predict a LMWPTP’s role in CPS and exopolysaccharide regulation. LMWPTPs encoded independently of a BY-kinase often play species-specific functions such as stress response and heat shock resistance which emphasizes the versatility of LMWPTPs [8, 9]. Thus, identification of potential substrates for Spd1837 would greatly assist in determining this putative phosphatase’s role in the pneumococcus.

Here, we present evidence that Spd1837 of *S. pneumoniae* is indeed a PTP *in vitro* with kinetic parameters and characteristics typical of a LMWPTP. A substrate-trapping approach and subsequent identification via mass spectrometry revealed possible substrates that may also act as binding partners. The identification of possible interacting proteins sheds light on the potential role of Spd1837 in the physiology of the pneumococcus, especially in central carbon metabolism.

### 3.3 Materials and Methods

#### 3.3.1 Growth media, growth conditions, DNA manipulation, *E. coli* and *S. pneumoniae* transformation

*S. pneumoniae* and *E. coli* strains (listed in Table 3.2S) were routinely grown as described previously [4]. DNA manipulation, PCR and transformation into *E. coli* were performed as previously described [10]. Oligonucleotides (Integrated DNA Technologies) are listed in Table 3.3S. The gene encoding Spd1837 was amplified from *S. pneumoniae* D39 genomic DNA with primers ZA1 and ZA2. The PCR product was digested with BamHI and NdeI, the restriction sites for which were included in ZA1 and ZA2. The PCR product was then ligated into similarly digested pET-15b and transformed into strain DH5α and screened by PCR, with the correct plasmid confirmed by DNA sequencing (pET-15b-Spd1837) (Australian Genome Research Facility Ltd). Site-directed mutagenesis of cysteine 8 to serine (C8S) was conducted according to the manufacturer’s instructions using
oligonucleotides ZA11 and ZA12 (Quikchange® Lightning Site-Directed Mutagenesis - Agilent Technologies). The mutation was confirmed by DNA sequencing (pET-15b-Spd1837CBS). Markerless, non-polar, in-frame deletion in spd1837 was constructed in a Serotype 2 D39 streptomycin resistant strain essentially as previously described [4].

### 3.3.2 Purification of Spd1837 and Spd1837CBS

Spd1837 and Spd1837CBS proteins were expressed in Lemo21 (DE3), grown at 37 °C for 16 hr in Lysogeny Broth (LB), sub-cultured 1/20 in 1 L LB at 37 °C for 2 hr with expression of recombinant protein then induced with 0.1 mM IPTG for 3 hr. Spd1837 and Spd1837CBS were purified essentially as described [11]. The 6×His-tag was cleaved with Thrombin (Sigma Aldrich) and the final purification from contaminating proteins was achieved by buffer exchange, size-exclusion filtration using HiLoad 16/600 and 26/600 Superdex 200 prep grade columns (GE Healthcare). The concentrations of both wild-type and mutant proteins were determined using Pierce BCA Protein Assay Kit (Thermo Scientific). Approximately 44 mg of both purified Spd1837 and Spd1837CBS was obtained from 1 l of bacterial culture.

### 3.3.3 Phosphatase assays

Phosphatase activity was monitored at 37 °C by using a continuous method based on the detection of p-nitrophenol (pNP) formed from p-nitrophenyl phosphate (pNPP) as described previously [12]. Kinetic parameters were determined by fitting the data to the Michaelis-Menten equation, using non-linear regression (GraphPad Prism 6 Software). Phosphatase activities at different temperatures were compared at temperatures ranging from 25 to 50 °C using 100 mM Tris pH 7.0 as the buffer. Phosphatase activities at different pH values were compared with the following buffers: 100 mM sodium citrate (pH 4.0-6.5), and 100 mM Tris (pH 7.0-9.5) at 37 °C. In varied pH, temperature and inhibitors concentration assays, 400 ng of Spd1837 and 8.0 mM pNPP was used. Phosphotyrosine phosphatase activity was also analysed using the Tyrosine Phosphatase Assay System (Promega), according to the manufacturer’s instructions. All experiments were conducted in duplicates and repeated three times independently and values reported represent the means and the standard errors.
3.3.4  **In vitro substrate-trapping assay**

The assay was performed essentially as described by Blanchetot *et. al.* [13] with some modifications. 500 ml THY (Todd-Hewitt broth with 1% Bacto yeast extract) media was inoculated with D39Δspd1837 strain and grown for 6 hr until the OD$_{600\text{nm}}$ was approximately 0.2. 1 mM pervanadate was added to the culture and the incubation was continued for another 30 min. To harvest the cells, the culture was centrifuged at 8000 g for 20 min at 4 °C, the supernatant removed and the pellet frozen at -80 °C. Then, 100 µL of Ni-charged MagBeads slurry (GenScript) per sample was equilibrated with cold lysis buffer (20 mM Tris, pH 7.5, 300 mM NaCl, 0.1 mM EDTA, 1% (v/v) Triton X-100, 10% (v/v) glycerol, 1 mM imidazole) twice. In total, 125 µg of His$_6$-Spd1837, His$_6$-Spd1837$_{\text{C8S}}$, or Bovine Serum Albumin (BSA) in conjugation buffer (1 × TBS, 10% (v/v) glycerol, 1 mM dithiothreitol (DTT)) were incubated with the equilibrated magnetic beads at 4 °C overnight, and the bead’s only sample was incubated with conjugation buffer only.

The next day, the frozen pellet was resuspended in cold lysis buffer freshly supplemented with 5 mM iodoacetic acid (IAA), 1 mM sodium orthovanadate (Na$_3$VO$_4$) and 1 × proteases inhibitor (BioSciences). The resuspended cells were then disrupted by sonication on ice (Branson B15). Cleared cell lysate was incubated with 5 mM DTT on ice for 15 min. The crude lysate was ultracentrifuged at 450 000 g for 1 hr at 4 °C to separate the soluble and insoluble fractions. After the supernatant (the soluble fraction) was collected, the pellet (the insoluble fraction) was solubilized with 1% (w/v) n-dodecyl-β-d-maltoside (DDM) (Anatrace). The conjugation buffer from the beads was removed and the soluble fraction and insoluble fraction were incubated separately with the beads overnight at 4 °C. Step-wise elution with imidazole during the washes was implemented with 250 mM imidazole as the final elution step. The eluted samples were collected and subjected to SDS-PAGE on a 4-12% Bolt™ Bis-Tris Plus Gel (Thermo-Fisher Scientific) and subsequently Coomassie-stained.

3.3.5  **Liquid chromatography – electrospray ionisation tandem mass spectrometry**

The substrate-trapping assays were repeated three times with consistent results and one of the Coomassie-stained whole gels was submitted to the Adelaide Proteomics Centre for trypsin digestion and mass spectrometry analysis of tryptic peptides.
of the selected bands. A total of six bands were excised from the gel. Liquid chromatography – electrospray ionisation tandem mass spectrometry was performed on an Ultimate 3000 RSLC system coupled to a LTQ Orbitrap XL ETD MS instrument (both Thermo-Fisher Scientific) as previously described [14]. MS scans were acquired in the mass range of 300 to 2000 m/z at a resolution of 60 000. The six most intense precursor ions selected for isolation and were subjected to CID fragmentation using a dynamic exclusion of 5 s. Dynamic exclusion criteria included a minimum relative signal intensity of 1000 and ≥ 2+ charge state. An isolation width of 3.0 was used with a normalized collision energy of 35. RAW files were submitted directly to Mascot via Proteome Daemon (1.3, Thermo-Fisher Scientific). Acquired data was searched against the Swiss-Prot database in MASCOT (V2.3.02). Search parameters were set as S. pneumoniae strain D39 (Taxonomy), trypsin digestion with two missed cleavages, fixed modification of carbamidomethyl of cysteine, variable modification of oxidation of methionine, precursor ion mass tolerance of 10 ppm, and product ion mass tolerance of 0.8 Da. Further analysis of the data was carried out in Proteome Discoverer (V1.1, Thermo Scientific). Data was searched against decoy database for false discovery rate calculations (approximately 1%). Peptides with p < 0.05 are reported.

### 3.3.6 SDS-PAGE and Western immunoblotting

Samples from substrate-trapping assay were also subjected to 12% (v/v) SDS-PAGE and Western immunoblotting using mouse anti-phosphotyrosine 4G10 antibodies (Bio X Cell) or rabbit anti-CpsD antibodies as described previously [4]. The experiment was repeated three times independently with similar results and the representative blots are presented.
3.4 Results

3.4.1 Spd1837 possesses tyrosine phosphatase activity

In order to investigate whether Spd1837 was indeed a tyrosine phosphatase, Spd1837 along with a protein with a mutation in the putative active site cysteine (Spd1837$_{C8S}$) were purified from *E. coli* as described in Materials and Methods (Figure 3.1A). Using size exclusion chromatography as the final purification step, the apparent molecular mass of Spd1837 was approximately 15.8 kDa (Figure 3.5S), suggesting that the native protein exists as a monomer. The phosphatase activity of Spd1837 was determined *in vitro* by using the cleavage of pNPP as a substrate. Spd1837 could dephosphorylate pNPP in a concentration-dependent manner while Spd1837$_{C8S}$ did not have activity against pNPP (Figure 3.1B).

In order to verify that Spd1837 possessed specific tyrosine phosphatase activity, we utilized the Tyrosine Phosphatase Assay System (Promega) which measures the release of inorganic phosphate from two different phosphotyrosine-containing peptides. Our results indicated that 1687 ± 76 pmol and 1515 ± 223 pmol of inorganic phosphate was released from the phosphotyrosine-containing peptide 1 and 2 respectively per 300 pmol of Spd1837. This assay also showed the importance of cysteine-8 in the activity of Spd1837 as the mutant protein Spd1837$_{C8S}$ lacked any activity against these two phosphotyrosine-containing phosphopeptides (Figure 3.1C).
Figure 3.1: *In vitro* activity of Spd1837.

(A) Proteins were purified by affinity chromatography from the soluble fraction of *E. coli* Lemo21 (DE3) cells grown at 37°C in the presence of 0.1 mM IPTG followed by separation by SDS-PAGE and staining by Coomassie. Lane 1, Spd1837 protein purified from Lemo21[pET15b-Spd1837]. Lane 2, Spd1837<sub>C8S</sub> protein purified from Lemo21[pET15b-Spd1837<sub>C8S</sub>]. Approximately 6.5 µg of protein was loaded into each lane. (B) Spd1837 steadily dephosphorylated the synthetic phosphatase substrate, pNPP, releasing pNP, a yellow product that can be detected by absorbance at 410 nm. (C) Spd1837 had activity against two phosphotyrosine-containing peptides, phosphopeptide-1 (END(pY)INASL) and phosphopeptide-2 (DADE(pY)LIPQQG) as the mutant protein Spd1837<sub>C8S</sub> lacked activity against these two phosphotyrosine-containing phosphopeptides. For (B) and (C), data were from three independent experiments represented as mean and SEM.

We also demonstrated that Spd1837 dephosphorylated pNPP according to Michaelis-Menten kinetics (Figure 3.2A), with a $K_m$ of 8.0 mM and a $V_{max}$ of 1.34 µmol min<sup>-1</sup> mg<sup>-1</sup>. These $K_m$ values are within the range of $K_m$ reported for other LMWPTPs (Table 3.4S). Spd1837 showed optimum activity at 37 °C (Figure 3.2B) and pH 7.0 (Figure 3.2C), both of which are similar to the optimum conditions for most LMWPTPs (Table 3.5S). The

---

$K_m$ and $V_{max}$ inherently have large variances or standard errors, therefore these are not routinely reported (Ritchie RJ, Prvan T. Current statistical methods for estimating the $K_m$ and $V_{max}$ of Michaelis-Menten kinetics. Biochemical Education. 1996;24(4):196-206. doi: https://doi.org/10.1016/S0307-4412(96)00089-1).
strict specificity of Spd1837 for phosphotyrosine residues was confirmed by analysing the effect of sodium orthovanadate (Na₃VO₄), which specifically inhibits tyrosine phosphatases. Na₃VO₄ strongly inhibited Spd1837 phosphatase activity (IC₅₀ ≈ 0.1 µM) (Figure 3.2D). No reduction of Spd1837 phosphatase activity was observed when a serine and threonine phosphatase inhibitor, sodium fluoride was added up to 100 mM of concentration in a separate inhibition assay (Figure 3.2D).

Figure 3.2: Phosphatase activity of Spd1837.
(A) Spd1837 enzymatic activity in the presence of increasing concentrations of the substrate pNPP. The graph represents mean activities and non-linear fits of the experimental data to the Michaelis-Menten equation. The Kₘ value of Spd1837 for pNPP in 100 mM Tris buffer (pH 7.0) was measured when 400 ng Spd1837 was used for the assay. (B) Relative Spd1837 phosphatase activity with various temperatures. The absolute value of enzyme activity corresponds to 100% is 0.409 µmol min⁻¹. (C) Relative Spd1837 phosphatase activity with various pHs. The assays contained 8.0 mM pNPP as the substrate. Buffers used were sodium citrate (pHs 4.5-6.5), and Tris-HCl (pHs 7.0-9.5). The absolute value of enzyme activity corresponds to 100% is 0.487 µmol min⁻¹. (D) Effects of sodium orthovanadate (Na₃VO₄) and sodium fluoride (NaF) inhibitors on the phosphatase activity of Spd1837. Results were expressed as a percentage of the phosphatase activity measured in the absence of inhibitor, taken as 100%. The absolute value of enzyme activity corresponds to 100% is 0.502 µmol min⁻¹. Error bars in all graphs represent the standard errors.
3.4.2 Spd1837 potential substrates include major metabolic enzymes

Having confirmed that Spd1837 was a PTP, we then investigated whether the purified form of the mutant enzyme could pull-down potential substrates from a *S. pneumoniae* lysate. A strain deficient in Spd1837 was used to prevent any competition for substrate binding from endogenous Spd1837. Three unique bands of ~37 (numbered 1 and 4 on Figure 3.3), 35 (numbered 2 and 5 on Figure 3.3) and 25 (numbered 3 and 6 on Figure 3.3) kDa were present in Coomassie-stained SDS-PAGE gels when Spd1837 and Spd1837\textsubscript{CBS} were incubated with lysate samples (both in the soluble and insoluble fractions) that were not present when the fractions were incubated with BSA or beads only (Figure 3.3A). Putative substrates identified by mass spectrometry analysis are listed in Table 3.1 while specific proteins identified from each band and their tryptic peptides are listed in Table 3.6S.

![Figure 3.3: Substrate-trapping assay using wildtype and mutant Spd1837.](image)

(A) A representative Coomassie-stained gel of pulldowns was submitted for tryptic digest and mass spectrometry analysis. Wild-type Spd1837, Spd1837\textsubscript{CBS} or BSA were coupled to the beads, and beads alone were incubated with buffer only. Beads were incubated with lysate from *S. pneumoniae* D39\textdelta spd1837 pre-treated with pervanadate. Samples were subjected to SDS–PAGE and Coomassie-stained. Bolded numbers 1-6 indicate band excised for mass spectrometry analysis (B) Western immunoblot of *S. pneumoniae* D39\textdelta spd1837 pull-down samples probed for tyrosine-phosphorylated proteins using an anti-PY (anti-4G10) antibody (upper panel) and anti-CpsD antibodies (lower panel). Arrow indicates bands of CpsD at ~25 kDa. Red-coloured numbers represent common bands in lanes 1-2.
Table 3.1: Putative substrate proteins identified by mass spectrometry. Candidates from the ~25 kDa (bands 1 & 2 on Fig. 3A), ~35 kDa (bands 3 & 4 on Fig. 3A) and ~37 kDa (bands 5 & 6 on Fig. 3A) bands were included if they were approximately the correct size (± 2 kDa).

<table>
<thead>
<tr>
<th>#</th>
<th>Identified protein</th>
<th>Gene</th>
<th>GenBank accession number</th>
<th>Spd no</th>
<th>Number of unique peptides</th>
<th>Peptide coverage of protein</th>
<th>Gene Ontology (GO) category</th>
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</thead>
<tbody>
<tr>
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<td>2</td>
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<td>WP_000115140.1</td>
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<td>30s ribosomal protein S4</td>
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<td>Translation</td>
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<tr>
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<td>Glycerol-3-phosphate dehydrogenase</td>
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<td>Redox-sensing transcriptional repressor Rex</td>
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<td>7</td>
<td>Nucleotide binding protein SPD1396</td>
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<td>Aspartate carbamoyltransferase</td>
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<td>ABJ55115.1</td>
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<td>9</td>
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<td>ABJ54561.1</td>
<td>spd1107</td>
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<td>ABJ53898.1</td>
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<td>11</td>
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<td>spd1468</td>
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<tr>
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<td>aroD</td>
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<td>spd1211</td>
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<td>26.2%</td>
<td>Amino acid biosynthesis</td>
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We observed qualitatively that the intensity of bands in the wild-type His6-Spd1837 were greater than when His6- Spd1837C85 incubated with lysate (compare lanes 1 and 2, and lanes 5 and 6 on Figure 3.3A) when the same amount of total protein was loaded for each sample. To further investigate if the identified proteins are actual phosphatase substrates, pulled-down proteins were probed with a specific anti-phosphotyrosine antibody (Figure 3.3B, top panel). Bands with apparent molecular weight of ~15 kDa, ~25 kDa, ~40 kDa and ~50 kDa (labelled as 1-4 on Figure 3.3B, top panel) were detected in lanes 1 and 2. The ~25 kDa band is particularly strong and was also present in the beads only control (lane 3). A separate blot (Figure 3.3B, bottom panel), revealed the ~25 kDa band to most likely correspond to strongly tyrosine-phosphorylated CpsD [15], which bound non-specifically to the beads. Ultimately, by comparing lanes 1 and 2 in Fig. 3B, top panel, the proteins pulled-down with His6-Spd1837C85 did not appear to be more tyrosine-phosphorylated than those pulled-down with His6-Spd1837.

3.5 Discussion

Protein tyrosine phosphorylation in bacteria is now recognized as a critical post-translational regulatory system for bacterial survival and virulence, modulating the pathogenic ability of many human pathogens [6]. For this reason, we set out to confirm if a gene encoding a protein of high homology to the family of LMWPTPs, Spd1837, did indeed exhibit PTP activity, and to discover putative substrates or interacting proteins in the major human pathogen, S. pneumoniae.

Purification and enzymatic activity assays showed that the spd1837 gene did encode an active PTP, with specific activity against synthetic tyrosine phosphatase substrates, but not serine and threonine. While the specific phosphatase activity of Spd1837 ($K_m$, $V_{max}$ and optimum pH and temperature) was similar to other LMWPTPs, a difference was that Spd1837 existed as a monomer in solution similar to Erwinia amylovora AmsI [16]. Many other LMWPTPs such as Bovine LMWPTP and Bacillus subtilis YwlE form dimers [17-19], with data suggesting dimers are inactive enzymes. The only known LMWPTP that forms active dimer instead of a monomer is Vibrio cholerae VcLMWPTP-1 [20]. It is interesting to speculate that the lack of oligomerization may suggest alternative
methods of regulation for Spd1837, although further studies are required to investigate what occurs in vivo.

Substrate-trapping studies were subsequently undertaken in order to help determine Spd1837 function in the pneumococcus. The presence of proteins of similar sizes interacting with both Spd1837 and Spd1837_{C8S} suggest these may be interacting proteins rather than phosphatase substrates, with phosphotyrosine Western immunoblots providing further evidence for this. This is not unprecedented as several PTP substrates do interact with other domains of the phosphatase (away from the active site) before dephosphorylation takes place [13]. One also cannot exclude the possibility that Spd1837 exerts functions independent of its phosphatase activity in the pneumococcus. A number of LMWPTPs have phosphatase-independent functions, including Burkholderia cenocepacia Dpm and Mycobacterium tuberculosis PtpA [21, 22]. In the pneumococcus, the other verified PTP, CpsB also has a phosphatase-independent role, modulating CPS levels under reduced-oxygen conditions [23]. Interestingly, while purified Spd1837 was an active phosphatase in vitro, we could not detect any in vivo phosphatase activity from Spd1837 unlike for CpsB (data not shown, [2]), suggesting that its phosphatase activity may not be critical for its function in the pneumococcus.

Regardless of whether these proteins are phosphatase substrates or not, their identity may prove invaluable in order to determine Spd1837’s function in the pneumococcus. Two out of the thirteen proteins identified are ribosomal proteins, while amongst the others, many participate in precursors biosynthesis and metabolic processes (Table 3.1). These findings are perhaps unsurprising given many ribosomal proteins are tyrosine phosphorylated [24, 25], while enzymes involved in central carbon metabolism makes up the single largest subset of phosphorylated proteins in E. coli, Bacillus subtilis and Lactobacillus lactis [24, 25]. Currently only 14 proteins are known to be tyrosine phosphorylated in the pneumococcus [26], none of which were identified from our pull-down studies. Therefore, an updated tyrosine phosphoproteome analysis as performed in other bacteria may help with the interpretation of our finding. Although we do not know if these are substrates or interacting proteins, their identification suggests Spd1837 may play a role in growth in different carbon sources, and subsequently in the ability of the pneumococcus to survive in the different niches it encounters during human infection.
Our current work is focused on verifying if the identified proteins are biological interactants of Spd1837, and using these findings to uncover the role that Spd1837 plays in the physiology and virulence of the pneumococcus. Investigating the role of such factors is critical if we are to uncover novel methods to combat pneumococcal disease in the age of ever-increasing antimicrobial resistance.

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Conflicts of interest: All authors declared no conflict of interest.

3.6 References


3.7 Supplementary Material Files

<table>
<thead>
<tr>
<th>Bacterial Species</th>
<th>Sequence Alignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. pneumoniae Ptp</td>
<td>MKKLGFNYGICRSPMAEFVMSMTDNY---IQRSATSWEHGNPIHKG</td>
</tr>
<tr>
<td>E. amylovora Amsl</td>
<td>MINSILVNCIGCRSPTGLRLAALPER---KIASAGKAM-VGSADETA</td>
</tr>
<tr>
<td>A. johnsonii Ptp</td>
<td>MQIKHILVNCIGCRSPMAEYLLCAQPEL---HIESAGIAAM-VGHGADDAK</td>
</tr>
<tr>
<td>E. coli Wzb</td>
<td>MFNIIILVNCIGCRSPAERLQLQRYPHEL---KVESAGIAL-VKKBAPTD</td>
</tr>
<tr>
<td>E. coli Etp</td>
<td>MQAKLFNSILVNCIGCRSPIERLLRLRPGV---KVKASGVHGL-VKHAPDATA</td>
</tr>
<tr>
<td>K. pneumoniae Wzb</td>
<td>MAQLMFDSILVNCIGCRSPIERLLRLRNLW---KIDSAGVGL-IDHAAADA</td>
</tr>
<tr>
<td>B. subtilis YfkJ</td>
<td>MINSVLFVNCIGCRSPMAAIFRDLAKKGKLEKAPSAGIGGGHGINPHEGT</td>
</tr>
<tr>
<td>M. tuberculosis PtpA</td>
<td>MSPLHNTFVNCIGCRSPMAEKMFAQQLRLRGLDAVRVSATSAIGNWHVGSCADA</td>
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</tbody>
</table>

Figure 3.4S: A sequence alignment for selected bacterial LMWPTPs.

The alignment was generated using Clustal Omega program. Identical amino acids are indicated by (*), conserved amino acids are depicted by (:), whereas semi-conserved amino acids are depicted by (.). The cysteine residue critical for enzymatic activity is framed. GenBank accession numbers for the LMWPTPs are as follows; *Streptococcus pneumoniae* Spd1837, WP_000734748; *Erwinia amylovora* Amsl, CBA21355; *Acinetobacter johnsonii* Ptp, OS2787; *Escherichia coli* Wzb, NP_416565; *Escherichia coli* Etp, NP_415502; *Klebsiella pneumoniae* Wzb, BAF47013; *Bacillus subtilis* YfkJ, NP_388669; and *Mycobacterium tuberculosis* PtpA, NP_216750. Shaded areas indicate the location of the P-loop and D-loop. The numbers indicate amino acid position.
<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Description/antibiotic resistance</th>
<th>Source/reference</th>
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<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td><em>E. coli</em> transformation strain</td>
<td>Gibco-BRL</td>
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<tr>
<td>Lemo21 (DE3)</td>
<td><em>E. coli</em> expression strain</td>
<td>New England BioLabs</td>
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<tr>
<td><strong>Plasmid</strong></td>
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</tr>
<tr>
<td>pET-15b</td>
<td>Amp</td>
<td>Novagen</td>
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<td>Amp</td>
<td>This work</td>
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<td>This work</td>
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<tr>
<td>D39</td>
<td>Sm</td>
<td>[1]</td>
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<td>D39&lt;sub&gt;spd1837::janus&lt;/sub&gt;</td>
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<td>D39&lt;sub&gt;Δspd1837&lt;/sub&gt;</td>
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<td>This work</td>
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</table>

<sup>#</sup> Amp, Ampicillin; Sm, Streptomycin; Km, Kanamycin
Table 3.3S: List of oligonucleotides used. All primers were designed according to *S. pneumoniae* serotype 2 D39 sequence.

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<th>Sequence</th>
<th>Purpose</th>
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<tbody>
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<td>ZA1</td>
<td>5’-GCCCATATGATGAAAAATTAGCTTTTGCTGTCTG-3’</td>
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</tr>
<tr>
<td>ZA2</td>
<td>5’-CCGGGATCTTTAATACCTCTCTTTTCTAAACGTTCTAAC-3’</td>
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</tr>
<tr>
<td>ZA3</td>
<td>5’-ATACCTAGGTATCTGTGGG-3’</td>
<td>F, amplify spd1837 upstream region</td>
</tr>
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<td>ZA4</td>
<td>5’-AAGGAGGCTTGGAAACGTCCCGG-3’</td>
<td>R, amplify spd1837 downstream region</td>
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<td>ZA5</td>
<td>5’-GGAAAGGCCCAGGTCCTGAAAGAGAAGTGGGAAATC-3’</td>
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<td>ZA6</td>
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<td>ZA7</td>
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<td>R, amplify rpsl</td>
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Forward and reverse primers are represented by plus (F) or minus (R), respectively.
Figure 3.5S: Elution profile of the size-exclusion chromatography.

This was carried out as the final step of the purification using a HiLoad 16/600 and 26/600 Superdex 200 prep grade column (GE Healthcare). The injection volume was 500 µl protein solution (the blue line represents the UV absorption at 280 nm). This step was used as a desalting step as well (the red line represents the conductivity of the solution). The column was calibrated with protein mixtures of known molecular weight (cytochrome, MW 12.4 kDa; carbonic anhydrate, MW 29.0 kDa and Bovine serum albumin, MW 66.0 kDa). Using the standard curve drawn based on the elution profile of these protein mixtures, we found that Spd1837 exist in monomeric form in solution (MW 15.8 kDa).
Table 3.4S: Known $K_m$ and $V_{\text{max}}$ for pNPP of LMWPTPs.

<table>
<thead>
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<th>Organism</th>
<th>LMWPTP</th>
<th>$K_m$ (mM)</th>
<th>$V_{\text{max}}$ (µmol min$^{-1}$ mg$^{-1}$)</th>
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<td>BceD</td>
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<td></td>
<td>PtpB</td>
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### Table 3.5S: Known optimum temperature and optimum pH of LMWPTPs.

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<th>LMWPTP</th>
<th>Optimum temperature (°C)</th>
<th>Optimum pH (pH)</th>
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Table 3.6S: Putative substrate proteins identified by mass spectrometry. The Mascot protein identities (maximum of top 10 for each gel band) are listed. Identifications can be made if at least two unique peptides were sequenced from a protein and had individual ion scores above the homology threshold. Multiple charge states were not considered as unique. The individual unique peptide sequences are shown with the number in parentheses denotes the number of spectra correspond to each peptide.

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<th>Excised region</th>
<th>Identified protein</th>
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<th>Observed mass (kDa)</th>
<th>Unique peptides identified</th>
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<td>(insoluble fraction)</td>
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<td>37</td>
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NAGDIALWAGIATGAEIIEPEAGFK (5)
NAGDIALWAGIATGAEIIEPEAGFKMEDIVASIK (3)
QAISEGMEVFGIYDGYAGMVAGEIHPLDASVGDIIWR (5)
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HPr kinase/phosphorylase

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Chapter Four

RESEARCH ARTICLE TWO:
Role of *Streptococcus pneumoniae OM001* operon in capsular polysaccharide production, virulence and survival in human saliva
Role of *Streptococcus pneumoniae OM001* operon in capsular polysaccharide production, virulence and survival in human saliva

Short title: *S. pneumoniae OM001* operon

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<td>James C. Paton</td>
<td>Supervised development of work, helped to evaluate and edit the manuscript and provision of laboratory and materials.</td>
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<td>Renato Morona</td>
<td>Supervised development of work, helped in data interpretation, helped to evaluate and edit the manuscript and provision of laboratory and materials.</td>
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<td>Alistair J. Standish</td>
<td>Carried out mouse experiments and assisted in data analysis and interpretation, supervised development of work, helped in data interpretation, helped to evaluate and edit the manuscript. Will act as the final corresponding author.</td>
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**Chapter 4: Research Article 2: Role of *Streptococcus pneumoniae OM001* operon in capsular polysaccharide production, virulence and survival in human saliva**

### 4.1 Abstract

*Streptococcus pneumoniae* is the leading cause of community-acquired pneumonia in all ages worldwide, and with ever-increasing antibiotic resistance, the understanding of its pathogenesis and spread is as important as ever. Recently, we reported the presence of a Low Molecular Weight Tyrosine Phosphatase (LMWPTP) Spd1837 in the pneumococcus. This protein is encoded in an operon, *OM001* with two other genes, with previous work implicating this operon as important for pneumococcal virulence. Thus, we set out to investigate the role of the individual genes in the operon during pneumococcal pathogenesis. As LMWPTPs play a major role in capsular polysaccharide (CPS) biosynthesis in many bacteria, we tested the effect of mutating *spd1837* and its adjacent genes, *spd1836* and *spd1838* on CPS levels. Our results suggest that individual deletion of the genes, including the LMWPTP, did not modulate CPS levels, in multiple conditions, and in different strain backgrounds. Following *in vivo* studies, Spd1836 was identified as a novel virulence factor during pneumococcal invasive disease, in both the lungs and blood, with this protein alone responsible for the effects of operon’s role in virulence. We also showed that a deletion in *spd1836, spd1838* or the overall *OM001* operon reduced survival in human saliva during the conditions that mimic transmission compared to the wildtype strain. With studies suggesting that survival in human saliva may be important for transmission, this study identifies Spd1836 and Spd1838 as transmission factors, potentially facilitating the spread of the pneumococcus from person to person. Overall, this study hopes to further our understanding of the bacterial transmission that precedes disease and outbreaks.

### 4.2 Introduction

*Streptococcus pneumoniae* (the pneumococcus) predominantly colonizes the nasopharynx as a commensal in healthy individuals [1]. However, the bacteria can transition to be an opportunistic pathogen, leading to diseases with significant morbidity.
and mortality such as pneumonia, bacteremia and meningitis. By blocking the colonization or carrier state with widespread immunization, rates of transmission within the community for the strains that are included in the vaccine formulations have declined and this in turns provides herd immunity for the unvaccinated populations [2, 3]. These epidemiological studies have also shown that older populations mainly acquire the pneumococcus from colonized children [4]. Therefore, this suggests that the vaccine exerts its efficacy by limiting the spread between immunized individuals and it is possible to target a specific step in pneumococcal pathogenesis which is the colonization stage.

Much of our work has previously focused on determining the role that tyrosine phosphorylation plays in the virulence of *S. pneumoniae* [5-8]. We have recently characterized Spd1837 as a protein tyrosine phosphatase (PTP) of the Low Molecular Weight Protein Tyrosine Phosphatase family (LMWPTP) that may interact with proteins associated with pneumococcal metabolism (Chapter 3). LMWPTPs are also widely established to play a role in regulating capsular polysaccharide (CPS) and exopolysaccharide (EPS) biosynthesis [9].

In the chromosome of the serotype 2 *S. pneumoniae* strain D39, *spd1837* is co-transcribed together in the *OM001* operon with the upstream translocase subunit YajC (Spd1838), and a downstream hypothetical protein (Spd1836) (Figure 1.7S). While the operon is conserved across approximately 90% of pneumococcal strains with available genome sequence, little is known concerning the function of Spd1836 and Spd1838. Spd1836 consists of one conserved motif, the Membrane Occupation and Recognition Nexus (MORN) repeats. Despite being found in all domains of life and some viruses, very little is known about the MORN motif function. Based on limited studies conducted in apicomplexan parasites and *Arabidopsis*, the MORN motifs confer the ability of lipid binding, however, the subsequent role differs between species from regulating cell size to cell budding [10]. On the other hand, Spd1838 homologs have only been studied in Gram negative bacteria. In *E. coli*, YajC participates in Sec-dependent secretion by forming a complex with SecDF and YidC which may associate with the SecYEG and SecA ATPase to improve protein translocation efficiency [11]. Although the Sec-dependent pathway has been extensively studied, the precise role of SecDF-YidC-YajC complex is largely unknown.
Using a differential fluorescence induction (DFI) technique, the OM001 operon was previously identified to be significantly upregulated in several in vitro conditions that mimic infection. Subsequent deletion of this operon severely attenuated the ability of the pneumococcus to cause infection in multiple in vivo infection models [12], however, the role of the individual genes of the operon remained unknown.

In a recent study, Verhagen et. al. [13] conducted a genome-wide negative selection screening using Tn-seq and found 147 genes potentially required for the pneumococcal survival and growth in human saliva. Of these, two out of the three genes from the OM001 operon (spd1836 and spd1837) were identified. Indirect evidence from studies in humans suggests saliva is a possible medium for person-to-person spread [14]. Not only could live pneumococci be isolated and cultured from human saliva [15], saliva culture was also found to be a more robust and sensitive method for detecting the bacteria compared to conventional and the more invasive methods of trans-nasal and trans-oral swabs [16]. Verhagen et. al [13] have shown that the pneumococcus could survive and even grow in pure human saliva in 24 hours period. This highlights the extreme ability of the pneumococcus to adapt to different environments, namely the nasopharynx and potentially the oropharynx during the colonization step of pneumococcal pathogenesis.

Although transmission is the important first step that precedes carriage and disease (in fact none of pneumococcal disease states facilitate contagion [17]), pneumococcal factors that foster transmission are not well characterized due to a lack of tractable models to study this process until recently [18]. Indeed, pneumococcal disease occurrence is directly linked to the strains circulating in carriage [19]. Transmission is thought to require close contact, such as between individuals within the same households or day care centre [20, 21]. While it is generally accepted that the pneumococcus is a human-obligate pathogen with no known environmental or animal reservoir, evidence accumulating is that the bacteria can survive outside of human host. For instance, rehydrated pneumococci were able to infect mice after being left desiccated for four weeks [22].

This study set out to investigate the role of the OM001 operon in CPS biosynthesis, virulence and survival in human saliva. While there was a minimal role for the operon in CPS production, we have shown that the operon is important for the ability of S.
*Pneumoniae* to cause invasive disease and the ability to survive in human saliva. Specifically, we have identified Spd1836 as a previously uncharacterized virulence factor, while Spd1836 and Spd1838 are essential for the pneumococcal survival in human saliva at 25 °C, a condition to mimic how the bacteria would survive outside of the human body during transmission. With ever-increasing antibiotic resistance, the continued identification of factors important for the virulence and transmission of the pneumococcus is critical to identify new targets for the development of antimicrobials.

### 4.3 Materials and Methods

#### 4.3.1 Growth media and growth conditions

*S. pneumoniae* strains (listed in Table 4.1) were routinely grown either in Todd-Hewitt broth with 1% Bacto yeast extract (THY) at 37 °C as indicated or on Columbia blood agar (BA) plates supplemented with 5% (v/v) horse blood and grown at 37 °C in 5% CO₂ or, for mouse challenge, in serum broth (10% heat-inactivated horse serum in nutrient broth). Where appropriate, antibiotics were supplemented at the following concentrations: streptomycin at 150 µg mL⁻¹, kanamycin at 200 µg mL⁻¹ and gentamycin at 10 µg mL⁻¹.

**Table 4.1: List of strains used.**

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<tr>
<th>Strain</th>
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<td>Sm</td>
<td>[8]</td>
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<td>D39<em>spd1837</em>::<em>janus</em></td>
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<td>Sm</td>
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*a Sm, Streptomycin; Km, Kanamycin

#### 4.3.2 Construction of chromosomal mutation in *S. pneumoniae* D39

Markerless, non-polar mutant strains were constructed in a serotype 2 D39 streptomycin resistant strain and serotype 3 WU2 streptomycin resistant strain essentially
as previously described [8]. First, the Janus cassette was used to target and replace the \textit{spd1837} operon region in D39 and WU2 background strains [23]. Then the D39\textit{spd1837::janus} strain was transformed with PCR products containing the in-frame deletion or point mutation in \textit{spd1837}, or deletion in \textit{spd1836}, \textit{spd1838} or \textit{OM001}. Additionally, PCR products containing the in-frame deletion of \textit{spd1837} was also transformed into WU2\textit{spd1837::janus} strain. All oligonucleotides used are listed in Table 4.2S. Transformations were carried out as described previously [24]. To create the \textit{OM001} complemented strain, firstly, the 2 kb region upstream of the deleted \textit{OM001} operon was amplified using the primers ZA3 and ZA16 and the 2 kb region downstream of the deleted \textit{OM001} operon was amplified using the primers ZA4 and ZA19. These two PCR products and the amplified Janus cassette were combined and amplified again using just the primers ZA3 and ZA4. The approximately 2.4 kb PCR product was then used to transform D39\textit{ΔOM001}. The transformants were selected on kanamycin plates, resulted in the intermediate strain, D39\textit{ΔOM001::janus}. Next, the \textit{OM001} operon region including 1 kb of flanking genomic DNA from D39 was amplified using the primers ZA36 and ZA37. This product was then used to transform D39\textit{ΔOM001::janus} to replace the Janus cassette with the wild type copy of the \textit{OM001} operon. The successful transformants were selected on streptomycin plate and one of them was sequenced and verified to have acquired the \textit{OM001} operon back and this strain is called D39\textit{ΔOM001::OM001+}.

### 4.3.3 The production of polyclonal antibodies against Spd1837

Antibodies were raised against Spd1837 (purified protein > 95% pure as determined by Coomassie-stained SDS-PAGE (Figure 3.1)) (Institute of Medical and Veterinary Science, Veterinary Services (Gilles Plain, SA, Australia)) in rabbits. The antiserum was produced under the National Health and Medical Research Council (NHMRC) Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and was approved by the University of Adelaide Animal Ethics Committee. The crude antibodies were enriched and affinity-purified using purified Spd1837 before being stored at -20 °C in 50% (v/v) glycerol [25].
4.3.4 SDS-PAGE and Western immunoblotting

The whole cell bacterial lysates were prepared from cultures grown in THY to an OD<sub>600nm</sub> of approximately 0.3 and then subjected to SDS-PAGE and Western immunoblotting as described previously [26]. The concentrations of primary antibodies used were as follows; mouse anti-phosphotyrosine 4G10 antibodies (Bio X Cell) and mouse anti-CbpA at 1/5000 dilution, and rabbit anti-CpsD, rabbit anti-CpsB and rabbit anti-Spd1837 at 1/500 [27].

4.3.5 Uronic acid assay

CPS was prepared from the indicated strains grown either aerobically (BA at 37 °C with 5% CO<sub>2</sub>) or anaerobically (BA at 37 °C with 5% CO<sub>2</sub> in a BD GasPak™ Anaerobic Jar (Becton, Dickinson and Company)). The uronic acid assay was performed as described previously [7, 26]. Levels were related back to a standard curve of D-glucuronic acid (Sigma Aldrich). Differences in CPS levels were analyzed by one-way analysis of variance (ANOVA) with Dunnett’s post-hoc test.

4.3.6 Mouse infection model

This study was carried out in strict accordance with the recommendations in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition (2004) and 8th Edition (2013)) and the South Australian Animal Welfare Act 1985. The protocol was approved by the Animal Ethics Committee at The University of Adelaide (approval number S/2013/053). Outbred 5-to-6-week-old female CD1 (Swiss) mice were used in all animal experiments. For intranasal (i.n.) challenge, mice were anesthetized by intraperitoneal (i.p.) injection of pentobarbital sodium (Nembutal; Rhone-Merieux) at a dose of 66 μg per g of body weight, followed by i.n. challenge with 50 μL of bacterial suspension containing approximately 1×10<sup>7</sup> CFU mL<sup>−1</sup> bacteria in serum broth. The challenge dose was confirmed retrospectively by serial dilution and plating on BA. Mice were euthanized by CO<sub>2</sub> asphyxiation at the 48 hr post-challenge. Blood was collected by syringe from the posterior vena cava. The pleural cavity was lavaged with 1 mL sterile PBS containing 2 mM EDTA introduced through the diaphragm. Pulmonary vasculature was perfused by infusion of sterile PBS through the heart. Lungs were subsequently excised into
2-mL vials containing 1 mL sterile PBS and 2.8-mm-diameter ceramic beads for CFU counts. To obtain unattached pneumococci, the nasopharynx was subjected to lavage by insertion of a 26-gauge needle sheathed in tubing into the tracheal end of the upper respiratory tract and injection of 1 mL 0.5% trypsin–1×PBS through the nasopharynx. Additionally, the upper palate and nasopharynx were excised and placed into 2-mL vials containing 1 mL sterile PBS and 2.8-mm-diameter ceramic beads to obtain attached pneumococci. CFU counts for both the nasal wash and nasal tissue samples were combined to determine the total number of bacteria in the nasopharynx. Lung and nasopharyngeal tissues were homogenized using a Precellys 24 tissue homogenizer (Bertin Technologies) at 3 cycles of 30 s and 5,000 rpm. 40 μL aliquots of lung homogenate, nasopharyngeal tissues homogenate and pleural lavage, and 20 μL aliquots of blood were serially diluted and plated on BA supplemented with gentamycin to determine the number of CFU in these niches. Data were analyzed using non-parametric Mann-Whitney test. The incidence of pneumococcal invasion into the lungs and blood of mice were compared using two-tailed Fisher’s exact test.

4.3.7 Evaluation of the survival of *S. pneumoniae* strains in human saliva

The University of Adelaide Human Research Ethics Committee approved the study protocol and the written informed consent form with approval number of H-2016-224. Saliva collection and *S. pneumoniae* survival tests were conducted essentially as described by Verhagen et. al. [13] with a few modifications. The additional criteria for recruiting participants include ‘currently a non-smoker’ and ‘no respiratory or periodontal disease or infection’ as smokers and individuals with such disease or infection were shown previously to have human leukocyte elastase in their saliva and therefore is not representative of general, healthy population [28, 29]. Briefly, fasting saliva of the donors was pooled and centrifuged at 16,000 g at 4 °C for 15 minutes. The supernatant was sterilized by ultrafiltration with 0.45 μm Minisart filters (Sartorius Stedim Biotech). Before inoculation in saliva, the strains were grown in THY for 2 hr, diluted to a starting concentration of $1 \times 10^6$ CFU mL$^{-1}$ and washed twice in sterile PBS. The bacteria was incubated with at least 500 μL saliva at two conditions: 37°C with 5% CO$_2$ (representing in-host carriage) and 25°C without CO$_2$ (representing transmission). At t = 0, t = 3, t = 22, and t = 24 hr, samples were taken for CFU count. The number of bacteria at specific time point was enumerated by plating serial dilutions on BA plates. Experiments were performed in
duplicates and repeated three times independently. Statistical differences between survival of *S. pneumoniae* in multiple dilutions of saliva were assessed by a one-way ANOVA and Dunnett’s post hoc tests.

## 4.4 Results

### 4.4.1 The proteins encoded in OM001 operon do not play a role in CPS regulation

In another study, we showed that the Spd1837 was a PTP from the LMWPTP family (Chapter 3). As a number of LMWPTPs modulate CPS and EPS biosynthesis, we investigated if Spd1837 and the co-transcribed genes encoding Spd1836 and Spd1838 played a role in the regulation of CPS in *S. pneumoniae*. Separate non-polar markerless deletion mutations in *spd1836*, *spd1837*, *spd1838* and of all three genes of the OM001 operon were constructed in the chromosome of D39. We also constructed an in-frame unmarked point mutant (D39Spd1837<sub>C8S</sub>) which would not have any phosphatase activity. The strains (D39Δspd1837, D39Spd1837<sub>C8S</sub>, D39Δspd1838, D39Δspd1836 and D39ΔOM001) showed similar growth profiles to the parental strain D39 (Figure 4.6S).

Western immunoblot analysis with an antibody against Spd1837, showed that D39Δspd1837 and D39ΔOM001 did not produce Spd1837 while D39Spd1837<sub>C8S</sub> still had the mutant form of Spd1837 produced at a level equivalent to the wildtype, as did D39Δspd1838 and D39Δspd1836 (Figure 4.1A). As tyrosine phosphorylation of CpsD is important for the CPS regulation in the pneumococcus [30, 31], we analyzed the overall tyrosine phosphorylation profiles of the mutant strains. All six strains had similar levels of overall tyrosine phosphorylation, specifically of CpsD (Figure 4.1B and 1C), indicating that at least under the growth condition used, Spd1836, Spd1837 and Spd1838 had no detectable effect on protein tyrosine phosphorylation. Additionally, the expression of the other known PTP in the pneumococcus, CpsB was also similar in these strains (Figure 4.1D). As a loading control, the expression of the choline-binding protein A (CbpA) was also checked and this verified that similar amount of proteins were loaded into each lane (Figure 4.1E).
Figure 4.1: Proteins encoded by the OM001 operon do not alter tyrosine phosphorylation of CpsD.

Proteins from whole-cell lysates from D39, D39Δspd1837, D39Spd1837_C8S, D39Δspd1838, D39Δspd1836 and D39ΔOM001 cells were separated by SDS-PAGE, and Western immunoblotting was undertaken with anti-Spd1837 (A), anti-CpsD (B), anti-phosphotyrosine (PY) (C), anti-CpsB (D) and anti-CbpA (E). MW, molecular weight (in kDa). The arrow on (C) indicates a band corresponds to CpsD.

We then investigated whether these mutations modulated the synthesis of the CPS, using the uronic acid assay as described in the Materials and Methods. There was no significant difference in the amount of both total and cell wall-associated CPS produced by D39, D39Δspd1837, D39Spd1837_C8S, D39Δspd1838 and D39Δspd1836 while the operon deletion mutant, D39ΔOM001 had a slightly higher cell wall-associated CPS compared to that of the wildtype D39 strain under aerobic condition (Figure 4.2A). When we investigated CPS biosynthesis in anaerobic conditions, the overall total and cell wall-associated CPS levels of all strains were increased by approximately 20%, as previously
observed [32]. However, there was no significant effect of mutating \textit{spd1836}, \textit{spd1837} or \textit{spd1838} individually or together either on total or cell wall-associated CPS synthesis (Figure 4.2B).

**Figure 4.2: CPS production by D39 and WU2 strains.**

CPS was prepared from equal numbers of bacterial cells of D39, D39\textDelta{spd1837}, D39\textDelta{spd1837}_{C85}, D39\textDelta{spd1838}, D39\textDelta{spd1836}, and D39\textDelta{OM001} grown either aerobically (A) or anaerobically (B) and WU2 and WU2\textDelta{spd1837} grown either aerobically (C) or grown anaerobically (D). The CPS level was determined by uronic acid assay as described in Materials and Methods. The white bars represent the total CPS produced by various mutants as a percentage of total D39 CPS ((A) and (B)) or total WU2 CPS ((C) and (D)). The black bars represent the cell wall-associated CPS produced by mutants as a percentage of total D39 CPS ((A) and (B)) or total WU2 CPS ((B) and (C)). Bars represent means from three independent replicates while the error bars represent the standard error.

Serotype 3 strains produce CPS via synthase-dependent mechanism [33], compared to the Wzy-dependent mechanism in serotype 2 and all other strains except
serotype 37 [34-36]. This implies that Spd1837 is the only identified PTP in this serotype as it does not possess CpsB [33]. Thus, in order to investigate if Spd1837 played a role in CPS biosynthesis in this background, we also constructed a spd1837 deletion in the serotype 3 strain WU2. Similar to in D39, there was no significant difference in CPS levels between WU2 and WU2Δspd1837, either when the bacteria were grown aerobically (Figure 4.2C) or anaerobically (Figure 4.2D). This suggests that Spd1837 plays no role in the regulation of CPS biosynthesis in two serotypes of S. pneumoniae that synthesize CPS via two different mechanisms.

4.4.2 Contribution of Spd1836, Spd1837 and Spd1838 to virulence in mouse model of infection

Previous work has shown that the OM001 operon encoding spd1836, spd1837 and spd1838 plays a role in pneumococcal virulence [12]. We then undertook animal experiments to investigate the contribution of the individual genes of the operon to virulence in mice using an intranasal model. We found that none of the groups challenged with D39Δspd1837, D39Spd1837C8S, D39Δspd1838, D39Δspd1836 and D39ΔOM001 showed statistically reduced number of bacteria recovered from the nasopharynx, pleural lavage and lungs compared to the group challenged with the wildtype D39 (Figure 4.3A and 4.3B). There was however a significant reduction in the number of pneumococci recovered in the blood of mice challenged with D39Δspd1836 compared to the wildtype D39 (Figure 4.3C). Although not reaching statistical significance, a similar trend towards reduced number of bacteria recovered from the nasopharynx, pleural lavage, and lungs was observed for the group challenged with D39Δspd1836 and D39ΔOM001, and D39ΔOM001 from the blood compared to the group challenged with the wildtype D39 (Figure 4.3). Therefore, invasion of the lungs and blood was also compared by Fisher’s exact test. Using this test, we found that significantly fewer mice succumbed with invasive disease of lungs and blood when challenged with D39Δspd1836 and D39ΔOM001 compared to the wildtype D39. Eight out of fifteen mice challenged with D39Δspd1836 and three out of eight mice challenged with D39ΔOM001 had negligible number of pneumococci recovered from their lungs and blood while only one out of sixteen mice challenged with the wildtype D39 did not succumb to invasive disease. Thus, this showed that the contribution of the OM001 operon to pneumococcal virulence was solely due to spd1836.
4.4.3 Spd1836 and Spd1838 may be essential for pneumococcal survival in human saliva

As previous work by Verhagen et al. [13] had suggested that the OM001 operon may play a role in the survival in human saliva, we investigated if our defined spd1836, spd1837 and spd1838 mutants showed less survival in saliva compared to the wildtype D39 strain. Deletion of spd1836, spd1838 and the overall deletion of the operon OM001 resulted in lower bacterial survival when grown in human saliva at 25 °C without
CO₂ compared to the wildtype D39, and complementation of OM001 into D39ΔOM001 restored the survival percentage to wildtype level (Figure 4.4A). In contrast, none of the mutants including the complemented strain showed significant differences in survival when incubated in human saliva at 37°C with CO₂ compared to the wildtype strain (Figure 4.4B). Notably, neither chromosomal deletion nor the active site point mutation of spd1837 (spd1837_C8S) affected pneumococcal survival at 25°C without CO₂ and at 37°C with CO₂ (Figure 4.4A and Figure 4.4B). Overall, the results suggest that deletion in spd1836 and spd1838 reduced pneumococcal survival in human saliva during conditions that mimic transmission (at 25°C without CO₂), but not during conditions that mimic in-host carriage (37°C with CO₂). With evidence that human saliva can be a potential reservoir for the person to person spread of the pneumococcus, this would identify these factors as novel factors potentially important for pneumococcal transmission.

Figure 4.4: Survival of D39 bacteria and mutant derivatives in human saliva.
A starting concentration of 10⁶ CFU mL⁻¹ wildtype or mutant bacteria were incubated with saliva at the two conditions; (A) at 25°C without CO₂ and (B) at 37°C with CO₂. Experiments were performed in duplicate and repeated three times, using independent biological replicates. There was an approximately 0.5-log decrease in viable count for the D39 strain grown at 25°C without CO₂ and approximately 2-log decrease in viable count for the D39 strain grown at 37°C with CO₂. The CFU for D39 at t = 24 at 25°C without CO₂ was 5 × 10⁵ and the CFU for D39 at t = 24 at 37°C with CO₂ was 1 × 10⁴. Data were normalized such that the values represent the survival percentage of the mutant strains relative to the wildtype.
D39 (taken as 100%) ± SEM. Statistical differences between survival of *S. pneumoniae* in multiple dilutions of saliva were assessed by one-way ANOVA and Dunnett’s post hoc tests. **, P < 0.01, ***, P < 0.001.

4.5 Discussion

While the *OM001* operon is highly conserved amongst pneumococcal strains, little is known concerning the roles that the individual genes play in the physiology and virulence of the pneumococcus. Previously we have shown that Spd1837 is a PTP of the LMWPTP family (Chapter 3), and as data suggested importance of the operon in virulence [12], we investigated the individual characteristics of the mutants of these three genes in the operon.

Protein tyrosine phosphorylation in bacteria is now recognized as a critical post-translational regulation of virulence, modulating the pathogenic ability of a range of important human pathogens [9, 37]. The pneumococcus is one of the pathogens for which tyrosine phosphorylation plays an important role, regulating the biosynthesis of its single most important virulence factor, the CPS. The PTP CpsB, has been shown to be required for complete pneumococcal encapsulation [7]. Therefore, we investigated if the LMWPTP Spd1837 and its adjacent co-transcribed proteins, Spd1836 and Spd1838 play a role in the biosynthesis of CPS in the pneumococcus. However, neither Spd1837 nor Spd1836 and Spd1838 modulated tyrosine phosphorylation or CPS production under either aerobic or anaerobic conditions unlike CpsB [38], although the cumulative effects of deleting *spd1836, spd1837* and *spd1838* resulted in a slight increase in the levels of cell wall-associated CPS compared to the wildtype. This is perhaps unsurprising for the LMWPTP Spd1837 as PTPs not co-transcribed with bacterial tyrosine kinases generally have species specific roles (Table 4.4S and Table 4.5S).

As a deletion mutation in the *OM001* operon was previously reported to attenuate pneumococcal virulence in multiple *in vivo* models of infection [12], we investigated the individual contributions of *spd1836, spd1837*, and *spd1838* to pneumococcal virulence. Similar to the previous study, we found that the deletion of the operon *OM001* led to a reduction in *in vivo* virulence with fewer mice succumbing to
invasive disease of the lungs and blood. However, our data suggested that it was *spd1836* absence rather than the combination of *spd1836*, *spd1837* and *spd1838* deletion that led to the reduced invasive capacity of the pneumococcus, with the D39∆*spd1836* mutant showing similar results as the D39∆*OM001* mutant. Our decrease in virulence were not as dramatic an attenuation as seen with the deletion of the operon *OM001* previously [12], however, the prior study utilized different models including gerbils. Regardless, our study identified Spd1836 as a novel virulence factor, playing a role in invasive disease of lungs and blood.

Based on Tn-seq conducted by Verhagen and colleagues [13], the *spd1836* and *spd1837* genes (locus tag SP195_1980 and SP195_1981 respectively in the previous study) were implicated as being potentially important for pneumococcal transmission, however, no testing of individual mutants was reported. Here, we have shown that Spd1836, and Spd1838 along with the operon as a whole play a role in the survival of pneumococci in human saliva, with respective mutants showing statistically significant decreases in CFU when incubated at 25°C without CO₂ but not when incubated at 37°C with CO₂. These results are slightly different to those found by the previous study, as we did not see any difference in D39∆*spd1837* and our differences were only seen in conditions which mimic transmission (25°C without CO₂). However, our study using defined mutants (rather than the Tn-seq) in a different strain (serotype 2 D39 vs serotype 19F) allowed for a more detailed analysis of the characteristics of these mutants. It would be interesting to investigate the precise role of Spd1838 and Spd1836 proteins in transmission via saliva, given their effects on pneumococcal survival in saliva as reported here.

The epidemiological evidence following vaccine administration highlights the importance of studying transmission and colonization which was previously overlooked in favour of virulence and invasion studies. Given the recent advances in pneumococcal transmission studies [39, 40], one can expect more factors important for transmission will be characterized in the future. We are currently working to identify the mechanisms by which genes of this operon modulate virulence and transmission of the pneumococcus. Additionally, Spd1836 emerges from our study to be a previously uncharacterized virulence factor that may be important for progression to invasive pneumococcal disease. Further work is needed to identify the mechanism for this, and to identify whether this presents as a novel target for the development of new antimicrobials.
4.6 References


4.7 Supporting Information

Figure 4.5S: Schematic representation of the OM001 operon.

In the chromosome, the operon consists of spd1838 which encodes for a translocase, YajC (99 amino acids); spd1837 which encodes for a low molecular weight protein tyrosine phosphatase (142 amino acids); and spd1836 which encodes for a Membrane Occupation and Recognition Nexus (MORN) repeats-containing protein (136 amino acids).
Table 4.2S: List of oligonucleotides used. Sequence of oligonucleotides were derived from the chromosomal DNA sequence of *S. pneumoniae* serotype 2 D39 and serotype 3 WU2.

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZA3</td>
<td>5’-ATACTTACGTTATCTGTGG-3’</td>
<td>F, amplify <em>spd1837</em> upstream region</td>
</tr>
<tr>
<td>ZA4</td>
<td>5’-AAGAAGGCCATTGTAACGGTCGGCGG-3’</td>
<td>R, amplify <em>spd1837</em> downstream region</td>
</tr>
<tr>
<td>ZA5</td>
<td>5’-GGAAGGGGCCAGGTCTCTCTGAAAGGAGGTTAGTGAAATAT-3’</td>
<td>F, for overlap extension PCR of <em>spd1837</em>, complimentary to <em>janus cassette</em></td>
</tr>
<tr>
<td>ZA6</td>
<td>5’-CATTATCCATTAAAAATCAACGGCGCATTTCTCTTTATAGAATAACGG-3’</td>
<td></td>
</tr>
<tr>
<td>ZA9</td>
<td>5’-GCTTTTGTAGTCAGCTGGGAATATTTG-3’</td>
<td>R, for overlap extension PCR of <em>spd1837</em>, complimentary to <em>janus cassette</em></td>
</tr>
<tr>
<td>ZA10</td>
<td>5’-CAAAATTTCAGCTGACAAGAC-3’</td>
<td>F, exchange <em>spd1837</em> C8, complimentary to upstream of <em>spd1837</em></td>
</tr>
<tr>
<td>ZA15</td>
<td>5’-GGAAGGGGCCAGGTCTCTATATGGAGGAGGTTATAT-3’</td>
<td>R, exchange <em>spd1837</em> C8, complimentary to downstream of <em>spd1837</em></td>
</tr>
<tr>
<td>ZA16</td>
<td>5’-CATTATCCATTAAAAATCAACGGCGCATTTCTCTTTATAGG-3’</td>
<td>F, for overlap extension PCR of <em>spd1838</em>, complimentary to <em>janus cassette</em></td>
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<tr>
<td>ZA17</td>
<td>5’-CAAAATTTCAGCTGACAAGAC-3’</td>
<td>R, for overlap extension PCR of <em>spd1838</em>, complimentary to <em>janus cassette</em></td>
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<tr>
<td>ZA18</td>
<td>5’-CAATTTCAGCTTCTCTCTTTTCTGTCTTTATAGG-3’</td>
<td>F, delete <em>spd1838</em>, complimentary to upstream of <em>spd1838</em></td>
</tr>
<tr>
<td>ZA19</td>
<td>5’-GGAAGGGGCCAGGTCTCTCTCTTATCAAGGAGGTATAT-3’</td>
<td>R, delete <em>spd1838</em>, complimentary to downstream of <em>spd1838</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td>F, for overlap extension PCR of <em>spd1836</em>, complimentary to <em>janus cassette</em></td>
</tr>
<tr>
<td>Primer</td>
<td>Sequence</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td>ZA20</td>
<td>5’-CATTATCCATTAAAAATCAAACGGCTTAACCTCCTTTTCTAAACGTTC-3’</td>
<td></td>
</tr>
<tr>
<td>ZA21</td>
<td>5’-GAAAAGGAGGAGTTAAGAGGCTCTCTAACCTAAAGGAGGTATTAT-3’</td>
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<td>ZA22</td>
<td>5’-GATAAGGAGGCTCTTAACCTCCTTTTCTAAACGTTC-3’</td>
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<td>ZA24</td>
<td>5’-CAAAGGAGAAACAAGCCTCCTTATCAAAGGAGGTATTAT-3’</td>
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<td>ZA25</td>
<td>5’-GATAAGGAGGCTCTCTTTCTCTTTGTCTTTTACATAGG-3’</td>
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<td>AS113</td>
<td>5’-CCGTTTGATTTTTAATGGATAATG-3’</td>
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<td>5’-TGTTCCCAGCTATTCTTATTCAAGA-3’</td>
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<tr>
<td>AS121</td>
<td>5’-TCTCTTTATCCCCCTTTCTTTATGTC-3’</td>
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</tr>
<tr>
<td>ZA36</td>
<td>5’-CAGCTAAATTACCAACCTTCC-3’</td>
<td></td>
</tr>
<tr>
<td>ZA37</td>
<td>5’-TTTTCAACATAAGCTGGAACGTTC-3’</td>
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Forward and reverse primers are represented by plus (F) or minus (R), respectively.
Figure 4.6S: Growth profiles of D39 strains.
Growth curves of *S. pneumoniae* strains grown in THY. Data are mean ± SEM absorbance measurements from three independent biological experiments.
Table 4.35: The total number of mice and the number of surviving mice at the end of an intranasal challenge experiment with the strains

<table>
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<th>Group</th>
<th>Total number of mice</th>
<th>Number of surviving mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>D39</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>D39Δspd1837</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>D39Spd1837 Δcs5</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>D39Δspd1838</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>D39Δspd1836</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>D39ΔOM001</td>
<td>8</td>
<td>3</td>
</tr>
</tbody>
</table>
Figure 4.7S: Viable bacterial count of the strains at different time points after incubation in human saliva.

A starting concentration of $10^6$ CFU mL$^{-1}$ of wildtype or mutant strain was incubated with saliva at two conditions, at 25 °C without CO$_2$ (A) and at 37 °C with CO$_2$ (B). Samples were taken for CFU count at $t = 0$ and 3 hr. Data are mean ± SEM from three independent biological experiments.
Table 4.4S: LMWPTP-bacterial tyrosine kinase (BY-kinase) pair with a role in capsular polysaccharide (CPS)/exopolysaccharide (EPS) biosynthesis.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>LMWPTP</th>
<th>BY-kinase</th>
<th>Function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> K-30</td>
<td>Wzb</td>
<td>Wzc</td>
<td>Group 1 CPS assembly</td>
<td>[1]</td>
</tr>
<tr>
<td><em>E. coli</em> K-12</td>
<td>Wzb</td>
<td>Wzc</td>
<td>Colanic acid production</td>
<td>[2]</td>
</tr>
<tr>
<td>Enteropathogenic <em>E. coli</em></td>
<td>Etp</td>
<td>Etk</td>
<td>Secretion and assembly of the group 4 CPS</td>
<td>[3, 4]</td>
</tr>
<tr>
<td><em>Acinetobacter iwoffii</em></td>
<td>Wzb</td>
<td>Wzc</td>
<td>Emulsan production</td>
<td>[5]</td>
</tr>
<tr>
<td><em>Acinetobacter johnsonii</em></td>
<td>Ptp</td>
<td>Ptk</td>
<td>Colanic acid/EPS synthesis</td>
<td>[6]</td>
</tr>
<tr>
<td><em>Erwinia amylovora</em></td>
<td>Asml</td>
<td>AsmH</td>
<td>Amylovoran production</td>
<td>[7]</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>Yor5/ Wzb</td>
<td>Yco6/Wzc</td>
<td>CPS production</td>
<td>[8]</td>
</tr>
<tr>
<td><em>Pseudomonas solanacearum</em></td>
<td>EpsP</td>
<td>EpsK</td>
<td>EPS I production</td>
<td>[9]</td>
</tr>
</tbody>
</table>
Table 4.55: LMWPTP with role(s) in processes other than capsular polysaccharide (CPS)/exopolysaccharide (EPS) biosynthesis.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>LMWPTP</th>
<th>Function</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>Porphyromonas gingivalis</td>
<td>Ltp1</td>
<td>Regulate transcriptional activity of the global</td>
<td>[10]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>regulator LuxS</td>
<td></td>
</tr>
<tr>
<td>Burkholderia contaminans</td>
<td>BceD</td>
<td>Biofilm formation</td>
<td>[12, 13]</td>
</tr>
<tr>
<td>E. coli</td>
<td>Etp</td>
<td>Regulate heat shock resistance</td>
<td>[14]</td>
</tr>
<tr>
<td>Mycobacterium tuberculosis</td>
<td>PtpA</td>
<td>Inhibit phagosome acidification and block fusion</td>
<td>[15-17]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>with lysosomes</td>
<td></td>
</tr>
<tr>
<td>Burkholderia cenocepacia</td>
<td>Dpm</td>
<td>Inhibit phagosome maturation</td>
<td>[18]</td>
</tr>
</tbody>
</table>
**Supplementary materials references:**


Chapter Five

Research Article Three: *Streptococcus pneumoniae* protein tyrosine phosphatase Spd1837 confers resistance to hydrogen peroxide and modulates capsular polysaccharide production in an SpxB-dependent manner
## Statement of Authorship

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<th>Streptococcus pneumoniae protein tyrosine phosphatase Spd1837 confers resistance to hydrogen peroxide and modulates capsular polysaccharide production in an SpxB-dependent manner</th>
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| Publication Status | ☐ Published  
☐ Accepted for Publication  
☐ Submitted for Publication  
☑ Unpublished and Unsubmitted work written in manuscript style |
| Publication Details | Intended for submission to Journal of Bacteriology. |

## Principal Author

| Name of Principal Author (Candidate) | Zuleeza Ahmad |
| Contribution to the Paper | Performed all experiments, performed analysis on all samples, interpreted data, constructed all figures, tables, and supplementary, wrote manuscript and acted as corresponding author for the submission. |
| Overall percentage (%) | 100% |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. |
| Signature |  
Date 14/12/17 |

## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate’s stated contribution to the publication is accurate (as detailed above);  
ii. permission is granted for the candidate to include the publication in the thesis; and  
iii. the sum of all co-author contributions is equal to 100% less the candidate’s stated contribution.

| Name of Co-Author | Renato Morona |
| Contribution to the Paper | Supervised development of work, helped in data interpretation, helped to evaluate and edit the manuscript and provision of laboratory and materials. |
| Signature |  
Date 14/12/2017 |
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<tr>
<td>Contribution to the Paper</td>
<td>Supervised development of work, helped in data interpretation, helped to evaluate and edit the manuscript. Will act as the final corresponding author.</td>
</tr>
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Title: *Streptococcus pneumoniae* protein tyrosine phosphatase Spd1837 confers resistance to hydrogen peroxide and modulates capsular polysaccharide production in an SpxB-dependent manner

Running title: Spd1837 and SpxB modulate the pneumococcal capsule

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Phone: 61 468744170
Chapter 5: Research Article 3: *Streptococcus pneumoniae* protein tyrosine phosphatase Spd1837 confers resistance to hydrogen peroxide and modulates capsular polysaccharide production in an SpxB-dependent manner

5.1 Abstract and Importance

Abstract

*Streptococcus pneumoniae* (the pneumococcus) is a major human pathogen, causing significant mortality and morbidity annually. While the predominant virulence factor of the pneumococcus, the capsular polysaccharide is regulated by a phosphotyrosine regulatory system, we recently showed that a previously uncharacterised protein tyrosine phosphatase, Spd1837, played no role in capsular polysaccharide regulation. However, one of the characteristic features of the pneumococcus is its ability to produce large quantities of hydrogen peroxide (H$_2$O$_2$) predominantly via the pyruvate oxidase, SpxB. Interestingly, as with other protein tyrosine phosphatases, here we show that the phosphatase activity of Spd1837 was inhibited by H$_2$O$_2$ *in vitro*, suggesting that SpxB may play a role in regulating Spd1837 activity. Subsequent construction of double mutations in *spxB* and *spd1837* resulted in significant reductions in capsular polysaccharide production indicating a novel cross-talk between SpxB and Spd1837. Furthermore, Spd1837 also confers resistance to H$_2$O$_2$ and SpxB impacts this ability, providing further evidence of this link between these two proteins. Interestingly, a phosphatase-deficient mutant in *spd1837* indicated that Spd1837 may have roles independent of its phosphatase activity. With evidence that *spxB* is downregulated and mutated during human infection, these results suggests that Spd1837 may be critical for the complete encapsulation of the pneumococcus during invasive disease.

Importance

The capsular polysaccharide is the single most important virulence factor of the pneumococcus, essential for the bacterial survival in every niche it encounters. Thus, its regulation is critical for the success of this major human pathogen. Here, we identify a novel link between pyruvate oxidase SpxB, the low molecular weight protein tyrosine
phosphatase (LMWPTP) Spd1837 and capsular polysaccharide regulation. This study provides evidence that indeed, similar to a number of other bacterial LMWPTPs, Spd1837 does in fact modulate capsular polysaccharide production, albeit in an SpxB-dependent manner. Furthermore, we also describe a role for Spd1837 in H$_2$O$_2$ resistance. With recent evidence suggesting SpxB is not required during invasive disease, Spd1837 may potentially act as a safe-guard mechanism which ensures proper encapsulation during this stage of pneumococcal pathogenesis.

5.2 Introduction

*Streptococcus pneumoniae* (the pneumococcus) is a major human pathogen, causing numerous debilitating diseases, including pneumonia, meningitis and bacteraemia. Despite the use of existing vaccines and antibiotics, pneumococcal infections still cause approximately 1.3 million deaths annually especially among children and the elderly (1). Normally a harmless coloniser of the nasopharynx, the ability of the pneumococcus to invade deeper tissues reflects its ability to respond to changes in nutrient and oxygen availability in different niches. The pneumococcus utilises tyrosine phosphorylation to modulate some of its most important virulence factors such as capsular polysaccharide (CPS) and the autolysin, LytA (2, 3).

Recently, we have uncovered a protein tyrosine phosphatase, Spd1837 that may play a role in pneumococcal metabolism (Chapter 3). As with other protein tyrosine phosphatases which harbour the CX$_5$R motif in their active site, Spd1837’s catalytic cysteine is predicted to be deprotonated at physiological pH. This highly positive environment of the cysteine’s thiol group is required for the phosphatase enzymatic activity (4). However, this also potentially renders the phosphatase susceptible to oxidation, leading to its transient inactivation (5). This observation has been documented for many eukaryotic phosphatases with a CX$_5$R active site including PTP-1B, the dual-specificity phosphatase PTEN, and PRL-1 (phosphatase of regenerating liver-1) (6-8).

* S. pneumoniae poses an intriguing paradox as it is known to produce large quantities of hydrogen peroxide (H$_2$O$_2$) (9) while at the same time lacks the typical peroxide-detoxifying enzymes and regulators such as catalase, NADH peroxidase, OxyR and
PerR (10, 11). The H$_2$O$_2$ produced is therefore anticipated to have the ability to oxidise and inactivate phosphatases with a CX$_5$R active site such as Spd1837. The pyruvate oxidase, SpxB, is the main enzyme responsible for H$_2$O$_2$ production in the pneumococcus (12) with studies showing that spxB mutation resulted in only 20% of H$_2$O$_2$ being produced relative to the wildtype (13, 14). Interestingly, aside from the pneumococcus, the spxB gene is only present in some streptococcal species that colonise the oropharynx, such as *Streptococcus gordonii*, *Streptococcus oralis*, and *Streptococcus sanguinis* (15).

The contribution of SpxB to pneumococcal pathogenesis is still unclear. The lack of SpxB was shown to reduce virulence in a number of *in vivo* murine models (12, 16, 17). However, another recent study has shown that mutations of spxB can actually contribute to pneumococcal hypervirulence during invasive disease in mice, and also spontaneous spxB mutants could be recovered from patients with invasive disease (18). The inconsistency in the *in vivo* data is not surprising given the intricate interaction between SpxB and other pneumococcal factors such as pneumolysin (19), the overall colonisation process (17, 20) and metabolism (13).

Similarly, the reported effects of spxB mutation on CPS production have varied from either increased CPS levels (14), decreased CPS levels (13), to no change in CPS levels (13) and this seems to be dependent on the serotype tested and the detection method used. SpxB is recognised as a critical link between CPS biosynthesis and metabolism as reduced acetyl-CoA availability resulting from spxB deletion lead to CPS defects in pneumococcal serotypes possessing CPS with acetylated sugars (13). Additionally, spxB deletion was also shown to alter sugar utilisation pattern in the pneumococcus such that the carbon sources are likely being redirected away from glycolysis to produce more CPS (14).

Our recent study suggested that the tyrosine phosphatase, Spd1837, played no role in CPS biosynthesis (Chapter 4: Figure 4.2). The possibility that the SpxB-produced H$_2$O$_2$ may regulate Spd1837 activity drove us to investigate the hypothesis that Spd1837 may have a role in CPS production in the pneumococcus under specific conditions (Figure 5.1). We found that the strains with a double mutation in *spd1837* and *spxB* had much reduced levels of CPS. For the Δ*spd1837*ΔspxB strain, the effect on CPS was also oxygen-dependent, as growth in anaerobic conditions negated this effect while for Spd1837C8SΔspxB strain, the...
reduced levels of CPS was sustained under anaerobic conditions. Furthermore, Spd1837 also confers resistance to H$_2$O$_2$ and SpxB impacts this ability, providing further evidence of this link between these two proteins. The oxygen-independent effect on CPS observed with Spd1837$^{CBS}\Delta spxB$ and the strains’ extreme sensitivity to H$_2$O$_2$ allude to the possible adverse effects of expressing the phosphatase mutant form of Spd1837 in the cells. Overall, this study reports a new link between SpxB, Spd1837 and the regulation of CPS, the major virulence factor of the pneumococcus.

**Figure 5.1: SpxB-produced hydrogen peroxide (H$_2$O$_2$) may regulate Spd1837 activity.**

In many other protein tyrosine phosphatases with similar active site motif as Spd1837, reactive oxygen species such as H$_2$O$_2$ can oxidise the catalytic cysteine (shown in the thiolate state) leading to its inactivation (shown as different possible oxidation products, SO-$x$).

---

### 5.3 Results

#### 5.3.1 Spd1837 activity is inhibited by hydrogen peroxide

We have previously characterised Spd1837 as a low molecular weight protein tyrosine phosphatase (LMWPTP) in *S. pneumoniae* (Chapter 3). For a number of protein tyrosine phosphatases, their activities have been shown to be regulated by reactive oxygen species e.g. the human LMWPTP (21). While no other bacterial LMWPTPs have been reported to be sensitive to oxidative stress, *S. pneumoniae* has the unique feature of producing H$_2$O$_2$ which is unlike any other bacteria with a characterised LMWPTP (12). We therefore investigated if the H$_2$O$_2$ produced by the pneumococcus would inhibit Spd1837
activity. Using the purified protein (Chapter 3: Figure 3.1), we showed that the Spd1837 phosphatase activity was inhibited by H$_2$O$_2$ (Figure 5.2A). Exogenous addition of catalase could recover 100% of Spd1837 phosphatase activity when 175 ng ml$^{-1}$ or more catalase was added (Figure 5.2B).

![Graphs](image)

**Figure 5.2: Spd1837 phosphatase activity is inhibited by hydrogen peroxide (H$_2$O$_2$) and restored by catalase.**

(A) Effects of H$_2$O$_2$ on the phosphatase activity of Spd1837. The assays were conducted at 37 °C in 100 mM Tris buffer pH 7.0. With the same amount of purified Spd1837 (400 ng) and pNPP concentration (8.0 mM), the H$_2$O$_2$ concentration required to block Spd1837 activity by 50% (IC$_{50}$) was 10 µM. (B) Catalase restored Spd1837 phosphatase activities inhibited by 100 µM H$_2$O$_2$. Results were expressed as a percentage of the phosphatase activity measured in the absence of H$_2$O$_2$, taken as 100%. Data points represent means from three independent replicates while the error bars represent the standard error.

5.3.2 Spd1837 modifies CPS levels in SpxB-deficient backgrounds

We have previously shown that neither *spd1837* deletion nor point mutation inactivating phosphatase activity (Spd1837$_{C8S}$) affects CPS production in the serotype 2 D39 background, either grown aerobically or anaerobically (Chapter 4: Figure 4.2). We introduced a spxB deletion mutation into our existing wildtype and mutant *spd1837* strains resulting in ΔspxB, Δspd1837ΔspxB and Spd1837$_{C8S}$ΔspxB strains. We then investigated whether the mutations modulated CPS biosynthesis using the uronic acid assay. Firstly, we measured the CPS levels of ΔspxB strain and found that this strain produced approximately 50% more total CPS than the wildtype when grown aerobically (Figure 5.3A), similar to a previous study (14). It was also of interest to investigate CPS levels of the mutant strains under anaerobic condition because: i) the effect of CpsB, the only other verified protein tyrosine phosphatase in the pneumococcus on CPS levels is known to vary according to
oxygen levels (22), and ii) SpxB relies on oxygen availability to catalyse its reaction (23). In fact, spxB expression was previously shown to be severely downregulated in anaerobic conditions (24). Indeed, we found that growth in the lack of oxygen restored CPS levels for ΔspxB to wildtype levels (Figure 5.3B).

![Figure 5.3: CPS production by WT and SpxB-deficient strains.](image)

CPS was prepared from equal numbers of bacterial cells of wildtype (WT), ΔspxB, Δspd1837ΔspxB and Spd1837ΔspxB grown either aerobically (A) or anaerobically (B). The CPS level was determined by uronic acid assay as described in Materials and Methods. The white bars represent the total CPS produced by various mutants as a percentage of total WT CPS and the black bars represent the cell wall-associated CPS produced by mutants as a percentage of total WT. (*, P < 0.05; **, P < 0.01***; P < 0.001; one-way ANOVA with Dunnett’s post-hoc test). Bars represent means from three independent replicates while the error bars represent the standard error.

Next, we observed that spd1837 and spxB double deletion resulted in a dramatic decrease in total CPS under aerobic conditions when compared to both wildtype and ΔspxB strains (Figure 5.3A). The levels of cell wall-associated CPS in these three strains were not different, indicating that the difference was specifically in the CPS that was not attached to the cell wall (Figure 5.3A). When CPS levels were then investigated under anaerobic conditions, deletion of spxB and spd1837 did not affect CPS production unlike when the strain was grown under aerobic conditions. This indicates that the in the presence of oxygen, which is the substrate for SpxB reaction, SpxB modulated Spd1837 activity such that this resulted in an effect on CPS levels. In contrast, during growth in anaerobic conditions, because the SpxB protein is very lowly expressed (24) and deprived of oxygen (in wildtype strain), or is missing altogether (in ΔspxB strain), Spd1837 was no longer
regulated by SpxB, hence the CPS levels was restored to the wildtype levels (Figure 5.3B). Interestingly, the strain with a point mutation in spd1837 in combination with a spxB deletion had reduced total CPS production, regardless of whether oxygen is present (Figure 5.3A) or absent (Figure 5.3B). This implies that the catalytically dead, Spd1837\textsubscript{C8S} protein acted as a possible repressor, leading to a prominent loss of CPS not attached to the cell wall, independent of oxygen availability.

In order to determine if CPS differences were due to changes in the levels of CpsB and CpsD, two of the proteins involved in the phosphotyrosine-regulatory circuit, Western immunoblotting analyses using specific antibodies against these proteins were also undertaken. We found that the levels of CpsD, overall tyrosine phosphorylated proteins and CpsB between the wildtype, ΔspxB, Δspd1837ΔspxB and Spd1837\textsubscript{C8S}ΔspxB strains were not different. Additional Western immunoblotting analyses also revealed no changes in the levels of Spd1837 protein itself between the wildtype, ΔspxB and Spd1837\textsubscript{C8S}ΔspxB strains. An anti-CbpA blot was also included as a loading control, and there appeared to be equal amount of proteins loaded into all the wells (Figure 5.4). Therefore, the differences in CPS levels we have observed in Figure 5.3 were not due to altered expression of Spd1837, nor due to varied levels of proteins with major roles in CPS biosynthesis, CpsB and CpsD, and also overall tyrosine phosphorylation levels.
Changes in CPS levels did not result from altered Spd1837, CpsD and CpsB expression and also changes in overall tyrosine phosphorylation levels. Proteins from whole-cell lysates of ΔspxB, Δspd1837ΔspxB and Spd1837ΔspxB cells were separated by SDS-PAGE, and Western immunoblotting was undertaken with anti-Spd1837 (A), anti-CpsD (B), anti-phosphotyrosine (PY) (C), anti-CpsB (D), and anti-CbpA (E). MW, molecular weight (in kDa). The arrow on (C) indicates a band corresponds to CpsD.

5.3.3 Spd1837 contributes to the pneumococcal resistance to hydrogen peroxide

As well as being responsible for the majority of H$_2$O$_2$ production in the pneumococcus, SpxB has also been shown to be essential for the ability of the pneumococcus to survive in the presence of H$_2$O$_2$, its own toxic byproduct (9). Thus, we investigated if deletion or point mutation in spd1837 affected S. pneumoniae sensitivity to H$_2$O$_2$. We found that only 60% of Δspd1837 remained viable after exposure to 15 mM H$_2$O$_2$ compared to 100% of the wildtype strain (Figure 5.5A). This suggests that Spd1837 confers protection against killing from H$_2$O$_2$ exposure in the pneumococcus.
Figure 5.5: Effect of Spd1837 and SpxB mutations on the strains’ hydrogen peroxide (H₂O₂) resistance.

After growth to mid-log phase, cultures of wildtype (WT), Δspd1837, Spd1837CBS (A) and ΔspxB, Δspd1837ΔspxB, Spd1837CBSΔspxB (B) were incubated in THY containing either 15 mM H₂O₂ (A) or 5 mM H₂O₂ for 30 min. Values are the mean of three independent biological replicates representing the change in CFU expressed as a percentage of the culture without H₂O₂ ± the standard error (*, P < 0.05; **, P < 0.01***; P < 0.001; Student’s unpaired t-test (2-tailed)).

Similar to previous observations (9, 25), no viable ΔspxB were detected after exposure to 15 mM H₂O₂ while 100% (2.55 x 10⁶ CFU ml⁻¹) of the wildtype survived. We titrated H₂O₂ to as low as 5 mM in order to detect survival of the ΔspxB strain (Figure 5.5B). At this concentration, we also tested the H₂O₂ sensitivity of our double deletion strain, Δspd1837ΔspxB. Interestingly, we found that spd1837 deletion together with spxB deletion increased the strain’s resistance to H₂O₂ (up to 16% compared to 7% survival for the single spxB deletion mutant strain) (Figure 5.5B). This is in contrast to the decreased resistance to H₂O₂ observed for the spd1837 deletion mutant in the wildtype background (Figure 5.5A). Hence, this observation further corroborates for the existence of a cross-talk between Spd1837 and SpxB proteins in conferring resistance to H₂O₂, in addition to modulating CPS levels as observed earlier (Figure 5.3).

Again, it appears that expressing the catalytically-dead Spd1837CBS protein contributed to detrimental phenotype in the pneumococcus. Only 30% of Spd1837CBS cells were viable after exposure to 15 mM H₂O₂ (Figure 5.5A) and the Spd1837CBSΔspxB strain was even less viable (2% survival) after exposure to 5 mM H₂O₂ (Figure 5.5B). The difference
between the survival of the two \textit{spd1837} mutant strains (\textit{\Delta spd1837} compared to Spd1837\textsubscript{CBS}, and \textit{\Delta spd1837\Delta spxB} compared to \textit{\Delta spxBSpd1837\textsubscript{CBS}}) was also significant which implies that Spd1837 expression and activity is governed by a complex regulatory mechanism.

5.3.4 Role of \textit{spd1837} and \textit{spxB} in cell adherence

While CPS is important for pneumococcal virulence, a high level of CPS can lead to less efficient colonisation due to many adhesins such as CbpA being masked \((26, 27)\). Thus, strains with lower levels of CPS have been shown to have greater adherence to epithelial cells \((2)\). Therefore, we investigated if the changes in CPS levels resulting from \textit{spxB} and \textit{spd1837} mutations would impact adherence levels. We also used a D39\textit{\Delta cpsBCD::Janus} mutant as the CPS negative strain. As expected, the \textit{\Delta cpsBCD::Janus} strain showed 2.5 fold more adherence to A549 cells than the wildtype (Figure 5.6), similar to another study investigating unencapsulated pneumococci \((2)\). Although there was approximately 50\% more CPS produced by \textit{\Delta spxB} strain as shown in Figure 5.4A, this did not translate to detectable changes in adherence levels (Figure 5.6). Furthermore, despite possessing reduced levels of CPS, the two mutant strains \textit{\Delta spd1837\Delta spxB} and Spd1837\textsubscript{CBS}\textit{\Delta spxB} did not exhibit increased adherence to A549 cells compared to the wildtype strain, rather these strains showed slightly less adherence compared to the wildtype strain \((\sim 65\% \text{ compared to 100\% for WT})\) (Figure 5.6).
Figure 5.6: Adherence of wildtype and mutant strains to A549 cells.

A549 cell monolayers were infected with wildtype (WT) pneumococcus or its derivatives for 1.30 hr. Non-adherent pneumococci were washed off, and the number of adherent pneumococci was determined by plating on BA agar. The data are representative of three independent experiments and expressed as percentage of adherent cells relative to WT ± the standard error. (*, P < 0.05; **, P < 0.01, n.s. = not statistically significant; Student’s unpaired t-test (2-tailed)).

5.4 Discussion

One of the unique features of the pneumococcus is its ability to produce high levels of H$_2$O$_2$, mainly via the pyruvate oxidase, SpxB. In eukaryotes, H$_2$O$_2$ has been shown to play a role as an important signalling molecule (28). While H$_2$O$_2$ offers a rapid and efficient way of regulating many biological process due to its being readily generated, its toxic properties could lead to aberrant signalling when its expression is not being tightly controlled (29). Indeed, in the pneumococcus, previous work has shown that H$_2$O$_2$ specifically inhibits FabF activity, an elongation condensing enzyme which contributes to altered membrane fatty acid composition (30). In previous work, we have shown that Spd1837 did not have any effects on the CPS of the pneumococcus (Chapter 4: Figure 4.2). However, based on data showing that many eukaryotic protein tyrosine phosphatases rely on H$_2$O$_2$ to regulate their activity (6-8), we decided to investigate if this may also be the case in the pneumococcus.
In this study, we demonstrated that Spd1837 can modulate CPS biosynthesis in the pneumococcus in an SpxB-dependent manner. We observed reduced levels of CPS from the lack of Spd1837 in SpxB-deficient background only when oxygen was present whereas the CPS levels between ΔspxB strain and ΔspxBΔspd1837 strain were not different when grown anaerobically. This supports our hypothesis that SpxB may regulate Spd1837 activity, possibly via its production of H$_2$O$_2$, as this would not be produced in anaerobic conditions. In contrast, Spd1837$_{C8S}$ΔspxB strain had reduced levels of CPS compared to ΔspxB regardless of whether oxygen was present or not. This implies a possible role of Spd1837$_{C8S}$ as a repressor in the pneumococcal CPS biosynthesis. As the repression could not be observed in a wildtype background (Chapter 4: Figure 4.2), this repressor activity appears to be suppressed by the presence of SpxB.

We also found that a single deletion in spd1837 caused the bacteria to be more susceptible to killing by H$_2$O$_2$. The contributions of other known modulators of H$_2$O$_2$ resistance, SpxB and the lactate oxidase, LctO are very likely due to their role in the pneumococcal central carbon metabolism (LctO reverses SpxB reaction, regenerating pyruvate) (25). Therefore, Spd1837 effects on the pneumococcal ability to resist death by H$_2$O$_2$ exposure may also be due to the involvement of Spd1837 in the pneumococcal metabolism as postulated in Chapter 3, although the precise mechanism of Spd1837 activity is yet to be elucidated. The spxB deletion mutant was, as expected (9), extremely sensitive to H$_2$O$_2$. Interestingly, the strain with a double deletion in spxB and spd1837 exhibited a greater resistance to killing by H$_2$O$_2$ exposure compared to the ΔspxB mutant while a single deletion of spd1837 made the strain more susceptible as mentioned previously. We speculate that Spd1837 may possibly be involved in the H$_2$O$_2$ killing mechanisms, and this is only evident in the absence of SpxB protein.

Linking the results for uronic acid assay from Figure 5.3 and the results for H$_2$O$_2$ sensitivity assay from Figure 5.5, one common observation could be gathered. It appears that expression of the phosphatase inactive form of Spd1837 (Spd1837$_{C8S}$) contributed to phenotypes that can potentially reduce pneumococcal fitness with lower levels of CPS and increased susceptibility to H$_2$O$_2$. We propose that this phosphatase deficient Spd1837 acts as a repressor of these phenotypes by an as yet unidentified mechanism, with this being controlled by SpxB. This suggests that like the other protein tyrosine phosphatase in the pneumococcus CpsB (22), Spd1837 has both phosphatase-dependent as well as
phosphatase-independent roles. Such adverse effects of a phosphatase-deficient mutant also has been documented with another phosphatase, PTEN, whereby PTEN with its catalytic cysteine replaced with serine contributed to earlier onset of cancer (31).

In the pneumococcus, spxB deletion was previously shown to result in reduced adherence (12) or no change in adherence (32) to epithelial cells, although none of these studies investigated the adherence of the encapsulated serotype 2 D39. In our study, we did not see any changes in adherence for ΔspxB strain compared to the wildtype. Additionally, we found that the ΔspxBΔspxB and Spd1837ΔspxB strains had slightly reduced adherence to A549 cells compared to the wildtype, but were not significantly different from the ΔspxB strain, therefore it is difficult to ascertain the importance of this observation. The minimal effects of the spxB and/or spd1837 mutations on adherence levels were perhaps not unexpected as while the mutant strains had less CPS, they possessed similar amount of cell-wall associated CPS. Previous studies have shown that only CPS associated with the cell wall was previously shown to have a role in adherence (33).

Nevertheless, Spd1837 emerges to be yet another LMWPTP that plays a role in CPS biosynthesis in S. pneumoniae, although the contribution of Spd1837 to CPS production is not as direct as other LMWPTPs. Additionally, we found that Spd1837 provided protection against killing by H₂O₂ exposure, a function that is shared with SpxB and LctO, two enzymes which are known to be involved in pneumococcal metabolism (25). Both ΔspxBΔspxB and Spd1837ΔspxB strains also had altered sensitivity to H₂O₂ compared to the ΔspxB strain, further illustrating a link between these two proteins. Combined with the apparent detrimental effects of expressing the catalytically-dead, mutant form of the phosphatase, we believe that Spd1837 expression and activity is governed by a complex regulatory mechanism. Further experiments include constructing the complemented mutant by restoring the wildtype copy of spd1837 into the Δspd1837ΔspxB and Spd1837ΔspxB to eliminate the possibilities of polar mutations and secondary site mutations. The implications of our study could assist in understanding how the pneumococcus transitions from a harmless commensal to an invasive pathogen. This is based on the observations that spxB is severely downregulated in the lungs and blood (24) and the spontaneous deletion of spxB from strains isolated from patients with invasive disease (18). Therefore, Spd1837 may play a role in ensuring that the pneumococcus is
properly encapsulated in the case where \( spxB \) is lost during systemic disease, protecting the pneumococcus from opsonophagocytosis and killing by the host.

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5.5 **Materials and Methods**

5.5.1 **Growth media and growth conditions**

*Streptococcus pneumoniae* strains (listed in Table 5.1) were routinely grown either in Todd-Hewitt broth with 1% Bacto yeast extract (THY) at 37 °C as indicated or on Columbia blood agar (BA) plates supplemented with 5% (v/v) horse blood and grown at 37 °C in 5% CO\(_2\). Where appropriate, antibiotics were supplemented at the following concentrations: streptomycin at 150 µg ml\(^{-1}\), kanamycin at 200 µg ml\(^{-1}\) and chloramphenicol at 6 µg ml\(^{-1}\).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Antibiotic resistance</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Sm</td>
<td>(3)(^2)</td>
</tr>
<tr>
<td>Δ<em>spd1837</em></td>
<td>Sm</td>
<td>(Chapter 3)</td>
</tr>
<tr>
<td>Spd1837(_{CBS})</td>
<td>Sm</td>
<td>(Chapter 4)</td>
</tr>
<tr>
<td>Δ<em>spxB</em></td>
<td>Sm &amp; Cml</td>
<td>This work</td>
</tr>
<tr>
<td>Δ<em>spd1837ΔspxB</em></td>
<td>Sm &amp; Cml</td>
<td>This work</td>
</tr>
<tr>
<td>Spd1837(_{CBS})Δ<em>spxB</em></td>
<td>Sm &amp; Cml</td>
<td>This work</td>
</tr>
<tr>
<td>Δ<em>cpsBCD::Janus</em></td>
<td>Km</td>
<td>This work</td>
</tr>
</tbody>
</table>

\(^{1}\)Sm, streptomycin; Cml, chloramphenicol and Km, kanamycin

\(^{2}\)WT is referred to as D39S in this paper

5.5.2 **Construction of chromosomal mutation and transformation into *S. pneumoniae***

To construct *spxB* deletion mutation, a chloramphenicol resistance cassette with 2 kb homology to upstream and downstream region of *spxB* gene in *S. pneumoniae* serotype 2 D39 genome was amplified using the primers AS253 (5’-TTAGTTGCAGGTAAGCCATATATC-3’) and AS254 (5’-GTCTTTGTAAATGGCATCTCGCAT-3’). The PCR products were then transformed into the WT, Δ*spd1837* and Spd1837\(_{CBS}\) strains to
delete and replace the open reading frame encoding SpxB with the chloramphenicol resistance cassette. The ΔcpsBCD::Janus was constructed by deleting and replacing the open reading frame encoding CpsB, CpsC and CpsD with the Janus cassette (34) that was amplified using overlap extension PCR using the primers AS115 (5’-CATTATCCATTAAAAATCAAACGGTTCTCTACCCATCCATCC-3’) and AS116 (5’-GGAAAGGGGCCCAGGTCTGTCGGGGGATAGAGATGAATG-3’). Transformations were carried out as described previously (3). All the mutations constructed were verified by DNA sequencing (Australian Genome Research Facility Ltd). All oligonucleotides were purchased from Integrated DNA Technologies.

5.5.3 Phosphatase assays

Phosphatase assays were conducted essentially as described previously (Chapter 3). For the H$_2$O$_2$ inhibition assay, H$_2$O$_2$ was added in the range of 0-500 µM. To rescue the phosphatase activity, catalase from Bovine liver (Sigma) in the range of 2-175 ng ml$^{-1}$ was added to the reaction containing 100 µM of H$_2$O$_2$ in a separate assay. In both assays, 400 ng of Spd1837 and 8.0 mM pNPP was used in 100 mM Tris pH 7.0 as buffer at 37 °C.

5.5.4 Hydrogen peroxide sensitivity assays

Hydrogen peroxide sensitivity assays were conducted essentially as described previously (9). Briefly, bacteria were grown until mid-log phase (OD$_{600nm}$ 0.3-0.4), and each culture was added to 100 µl of THY medium or 100 µl of THY medium containing either 15 mM or 5 mM H$_2$O$_2$, followed by incubation at 37 °C for 30 min. Serial dilutions from each tube were then prepared in ice-cold phosphate-buffered saline to minimise Fenton reaction (35), and duplicate aliquots were spotted onto BA plates with half of the plate spotted with the strain treated with H$_2$O$_2$ and the other half without H$_2$O$_2$ treatment. The percent survival was calculated by dividing the CFU of cultures after exposure to H$_2$O$_2$ by the CFU of cultures without H$_2$O$_2$. Results were analysed using Student’s unpaired t-test (2-tailed).
5.5.5 Uronic acid assay

CPS was prepared from the indicated strains grown either aerobically (BA at 37 °C with 5% CO₂) or anaerobically (BA at 37 °C with 5% CO₂ in a BD GasPak™ Anaerobic Jar (Becton, Dickinson and Company)). The uronic acid assay was performed as described previously (2, 36). Levels were related back to a standard curve of D-glucuronic acid (Sigma Aldrich). Differences in CPS levels were analysed by one-way analysis of variance (ANOVA) with Dunnett’s post-hoc test.

5.5.6 SDS-PAGE and Western Immunoblotting

The whole cell bacterial lysates were prepared from cultures grown in THY to an OD₆₀₀ₙₙ of approximately 0.3 and then subjected to SDS-PAGE and Western immunoblotting as described previously (36). The concentrations of primary antibodies used were as follows; mouse anti-phosphotyrosine 4G10 antibodies (Bio X Cell), mouse anti-CbpA and mouse anti-SpxB at 1/5000 dilution, and rabbit anti-CpsD, rabbit anti-CpsB and rabbit anti-Spd1837 at 1/500.

5.5.7 Adherence assays

Human epithelial cell line A549 was maintained in DMEM medium (Gibco) supplemented with 5% foetal calf serum. To achieve approximately 90% confluency so that the adherence sites remained fully exposed, 4.5 x 10⁴ cells/well were seeded in 24-well plates and incubated overnight at 37 °C in 5% CO₂. The strains were grown in THY until the OD₆₀₀ₙₙ of 0.5, washed and resuspended in culture media before being added to four wells per strain at a density of 5 x 10⁶ CFU ml⁻¹. Infected A549 cells were incubated for 1.30 hr at 37 °C in 5% CO₂ followed by three washes in PBS. To detach the adherent bacteria, 100 µl of 0.25% (v/v) trypsin with 0.02% (w/v) EDTA and 400 µl of 0.25% (v/v) Triton X-100 were added to the wells. 100 µl lysate from each well and serial dilutions (up to 10⁻³) thereof were plated onto BA. Adherent pneumococci were then quantified and expressed as percentage of adherent cells relative to WT. Results were analysed using Student’s unpaired t-test (2-tailed).


### Supporting Information

#### Table 5.2S: Non-normalised data for Figure 5.3

<table>
<thead>
<tr>
<th>Stain</th>
<th>[glucuronic acid], µg ml⁻¹</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
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</tr>
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<td></td>
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</tr>
<tr>
<td>WT</td>
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</tr>
<tr>
<td>ΔspxB</td>
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</tr>
<tr>
<td>Δspd1837ΔspxB</td>
<td>44.86</td>
</tr>
<tr>
<td>Spd1837CBSΔspxB</td>
<td>25.33</td>
</tr>
</tbody>
</table>

|                        |               | Anaerobic                         |               |               |               |
|                        | T-CPS        | CW-CPS        | T-CPS        | CW-CPS        | T-CPS        | CW-CPS        |
| WT                     | 121.52       | 32.95         | 100.81       | 43.43         | 109.14       | 20.81         |
| ΔspxB                  | 81.52        | 47.48         | 96.05        | 30.57         | 71.76        | 18.43         |
| Δspd1837ΔspxB          | 70.33        | 20.81         | 72.00        | 23.19         | 109.38       | 28.43         |
| Spd1837CBSΔspxB        | 26.05        | 22.71         | 48.43        | 30.33         | 35.81        | 13.19         |
Chapter Six

OVERALL DISCUSSION AND CONCLUSIONS
Chapter 6: Overall Discussion and Conclusions

Almost 150 years after *S. pneumoniae* was believed to be originally identified by Klebs in 1875 (Ramirez, 2015), this human-specific pathogen remains a major cause of mortality and morbidity worldwide. With the rise in serotype replacement by non-vaccine serotypes and widespread antibiotic resistance, the search for new anti-microbial targets in the pneumococcus is now more vital than ever. Recent research has recognised protein tyrosine phosphatases as novel anti-microbial targets in bacteria, and thus identification and investigation of their roles in the pneumococcus is of the utmost importance (Whitmore & Lamont, 2012).

In bacteria such as *E. coli*, *S. aureus*, *S. coelicolor*, and *M. tuberculosis*, the presence of at least two functionally active protein tyrosine phosphatases has been reported (Vincent *et al.*, 1999, Soulat *et al.*, 2002, Wong *et al.*, 2013, Sohoni *et al.*, 2014). Additionally, it was demonstrated that in Gram positive bacteria such as *B. subtilis* and *S. aureus*, multiple, different PTPs exist. Firstly, a PHP, Mn²⁺-dependent phosphatase encoded in a *cps* or *cps*-like operon and second, at least one LMWPTP encoded distally from the PHP phosphatase (Soulat *et al.*, 2002, Mijakovic *et al.*, 2005, Musumeci *et al.*, 2005). In *S. pneumoniae*, a PHP protein tyrosine phosphatase, CpsB, is involved in CPS biosynthesis (Morona *et al.*, 2002, Geno *et al.*, 2014). As predicted on the basis of DNA sequence homology, here we show that *S. pneumoniae* does harbour a second protein tyrosine phosphatase, Spd1837, and in Chapter 3 we have biochemically characterised Spd1837 as a LMWPTP.

6.1 Spd1837 is a bona fide LMWPTP with a number of interesting distinctions

As discussed in Chapter 3, the kinetic parameters of Spd1837 are similar to other established members of the LMWPTP family. However, we can identify that the $K_m$ of Spd1837 falls closer to the end of the spectrum with phosphatases which possess a few fold lower $K_m$ than most other LMWPTPs (Table 3.4S). This observation possibly implies that Spd1837 acts at different ranges of substrate concentration compared to other LMWPTPs and that Spd1837 has lower affinity for the substrate. Although, as pNPP is not an authentic *in vivo* substrate, the implication of this is not conclusive. Furthermore, it was also noted
previously that the $K_m$ value for the $p$NPP substrate is not a true equilibrium constant that reflects affinity in a simple way (Tolkatchev et al., 2006). Nevertheless, amino acid substitution of the active cysteine to a serine residue resulted in a complete loss of the enzyme activity of Spd1837 which verifies that Spd1837 utilises the same catalytic mechanism as other LMWPTPs.

Again, while Spd1837’s optimum pH matches the optimum pH of many other LMWPTPs, Spd1837 peculiarly exhibited more than 50% activity over a neutral to basic range of pH (pH 6.5 to 9.0) (Table 3.5S). As LMWPTPs are also previously known as acid phosphatases, the tendency of Spd1837 to be more active around neutral to basic pHs appears unusual at first. However, a number of other LMWPTPs remain active around neutral to basic pHs. For instance, *S. coelicolor* Sco3700 (up to 23% activity at pH 9) (Sohoni et al., 2014), *B. cepacia* BceD (~70% active at the highest pH tested, pH 7.5) (Ferreira et al., 2007) and *S. aureus* PtpA and PtpB (70-80% active at the highest pH tested, 6.75) (Soulat et al., 2002). Spd1837 was also relatively more sensitive to vanadate compared to the other LMWPTPs as the IC$_{50}$ of vanadate for other LMWPTPs was 3.0 mM for both *S. aureus* PtpA and PtpB (Soulat et al., 2002), 0.8 mM for *S. coelicolor* PtpA (Li & Strohl, 1996), 10 µM for *C. burnetti* ACP (Hill & Samuel, 2011) and 35 µM for *A. johnsonii* Ptp (Grangeasse et al., 1998) compared to 0.1 µM for Spd1837. Regardless, Na$_3$VO$_4$ still inhibited Spd1837 activity while a recognised inhibitor of serine/threonine phosphatases had no effect. This would suggest that unlike shown for a homologous LMWPTP in *S. pyogenes* (Kant et al., 2015), Spd1837 does not possess activity against serine and threonine substrates.

We also found that Spd1837 exist as monomers in solution. The oligomerisation states of LMWPTPs are known to be as variable as their functions. Based on solved crystal structures, Bovine LMWPTP and *B. subtilis* YwIE form dimers and the interface surrounding the catalytic site is where the dimerisation occurs, subsequently preventing substrates from binding (Tabernero et al., 1999, Akerud et al., 2002, Bernado et al., 2003). Self-association between mammalian LMWPTP have been described whereby the active monomers and the inactive oligomers exist in equilibrium such that the D-loop containing the consecutive double tyrosines from each monomer are inserted into the active site of the other. In the absence of an authentic substrate, phosphorylated Bovine LMWPTP would become its own substrate, leading to dephosphorylated phosphatase, and allowing the regeneration of the inactive oligomeric structure. The term supramolecular proenzymes
has been coined to describe this latent reservoir of phosphatase (Blobel et al., 2009). However, in contrast to classical proenzymes, the inactive form can be spontaneously regenerated when the substrate concentration diminishes. Additionally, alluding to LMWPTP’s exquisite versatility, in the case of *V. cholerae* VcLMWPTP, the phosphatase forms dimers with its catalytic site remaining accessible (Nath et al., 2014). Overall, the oligomerisation of LMWPTP appears to be conserved in both prokaryotes and eukaryotes which a characteristic that is missing for Spd1837, suggesting that Spd1837 may be regulated in a distinct way. In summary, I predict that these few interesting distinctions possessed by Spd1837 in contrast to other LMWPTPs are physiologically relevant and will become apparent once the exact function(s) of Spd1837 is successfully elucidated.

### 6.2 The search for Spd1837’s substrate(s) and function(s) continues

Only one of the thirteen potential substrates identified in this work has been shown to be tyrosine-phosphorylated (L-lactate dehydrogenase), when searched against UniProt database. As summarised in Chapter 3, contrary to our expectations, it appears that the pulled-down proteins from the assays are likely to be interacting proteins as well as phosphatase substrates. To validate this and to obtain more information in regards to Spd1837 substrates(binding partners), an alteration to the current method could be implemented, by modifying our “substrate trapping mutant”. This would involve the substitution of the invariant catalytically essential aspartic acid within the C(X)₅R motif with a serine residue instead of replacing the nucleophilic cysteine. Previous studies have shown that for certain phosphatases, specifically eukaryotic phosphatases, such mutation generated phosphatases with improved substrate-trapping properties compared to active site cysteine mutants (Garton et al., 1996, Buist et al., 2000).

Nevertheless, the knowledge of the identity of the proteins that interact with Spd1837 may prove valuable in order to determine its function in the pneumococcus. As mentioned in Chapter 1, the regulatory mechanism(s) that govern diverse LMWPTP biological functions are not well-understood. The back-to-back tyrosine residues in the D-loop of mammalian LMWPTPs have been shown to be phosphorylated (Tailor et al., 1997, Bucciantini et al., 1999). The second tyrosine is conserved in Spd1837; however the first tyrosine is replaced with a similarly polar tryptophan residue (Figure 1.6). In mammalian
LMWPTPs, the phosphorylation of the second tyrosine in the D-loop results in the recruitment of adapter protein(s). This binding of the adapter protein(s) was also speculated to cause enzyme inactivation or to exclude substrates according to their size as the D-loop is known to fold over the active site (Schwarzer et al., 2006). Therefore, it is possible that Spd1837 can be tyrosine-phosphorylated in the pneumococcus and this phosphorylation in turn leads to the recruitment of some of the proteins we have pulled-down in the described substrate-trapping assays.

A question arises as to why the detected bands were more intense when the lysate was incubated with wild type Spd1837 compared to Spd1837\(_{\text{C8S}}\) if indeed the bands do correspond to interacting proteins that bind Spd1837 away from the active site. Although in theory, the rest of Spd1837\(_{\text{C8S}}\) except from the active site should be identical structurally to Spd1837, replacing the catalytic cysteine to serine is not a simple substitution of SH group to OH group. The cysteine exists as negatively charged thiolate anion at physiological pH (Xie et al., 2002), hence, substitution to a neutral hydroxyl group may affect the overall conformation of Spd1837. This substitution has been shown to affect the structure in substantial way in the case of PTP1B\(_{\text{C215S}}\) mutant (Scapin et al., 2001). Additionally, in the case of yeast Clp1 protein, a similar observation was reported, i.e. some proteins were at least two-fold more enriched in the wild type Clp1 sample compared to Clp1 mutant sample (Chen et al., 2013). This led the authors to conclude that these proteins are interacting proteins or cofactors, i.e. they need the catalytic cysteine to directly regulate Clp1 activity or/and to serve as scaffolding platforms to localise Clp1 activity (Chen et al., 2013). Therefore, we speculate that the cysteine to serine substitution disrupted the dynamic of Spd1837 in such a way that these interacting proteins could not bind mutant Spd1837 as efficiently as to the wild type Spd1837. Additionally, as mentioned in Chapter 3 discussion, it is also worth noting that several PTP substrates interact with other domains of the phosphatase (away from the active site) before dephosphorylation takes place (Blanchetot et al., 2005), thus we cannot discount any of these identified proteins as non-substrates.

Linking the results from Chapter 3 with Chapter 5, it becomes more apparent that indeed, Spd1837 is likely to have phosphatase-dependent and phosphatase-independent roles as the strain with a complete deletion in \(spd1837\) consistently behaved differently than the strain with a point mutation in \(spd1837\) in regards to CPS levels and
sensitivity to H₂O₂ exposure. However, as discussed in Chapter 5, it is currently not possible to attribute the phenotypes that we have observed due to Spd1837 only specifically as we cannot rule out the possibilities of polar effects and secondary site mutations without complementation experiments.

6.3 Proteins encoded from a previously uncharacterised operon in S. pneumoniae are important for virulence and bacterial survival in human saliva.

The next logical step in my project was to try to tease out the function of Spd1837 via virulence studies. This is due to the fact that our attempts at substrate identification did not provide specific answers without conducting major studies such as phosphoproteomic analysis and verifying direct, physical interaction between the pulled-down proteins with Spd1837 in vivo. *spd1837* is encoded in an operon (the *OM001* operon) and this operon was previously implicated to be important for the pneumococcal virulence (Marra *et al.*, 2002). Therefore, animal studies were conducted using defined mutants, including the other genes in the operon (*spd1838* and *spd1836*). As a recent study implicated the *OM001* operon’s importance for pneumococcal survival in human saliva (Verhagen *et al.*, 2014), we also tested if our defined mutants were able to survive in human saliva as this previous study was conducted using Tn-seq. Overall, in Chapter 4, we demonstrated the *OM001* operon as a whole, and specifically Spd1838 and Spd1836 proteins were essential for the ability of the pneumococcus to survive in human saliva in conditions that mimic transmission, and that Spd1836 was also important for pneumococcal virulence in invasive disease.

Transmission via saliva is a controversial subject in the *S. pneumoniae* field. Theoretically, it is possible for the pneumococcus to also reside in the oropharynx as there is no clear anatomical separation or barrier between the nasopharynx and the oropharynx, a characteristic that is displayed by a closely-related species, *S. pyogenes* (Shelburne *et al.*, 2006). These two niches do vary considerably – one may argue that the oral cavity is the harsher environment due to the fluctuations in temperature, pH and nutrient availability (Humphrey & Williamson, 2001). On the other hand, similar to organisms inhabiting the gut, the oral cavity may be considered a more forgiving environment because this niche is
regularly exposed to food consumed by their host. Colonisation of the oropharynx may be advantageous as host actively depletes glucose from the airway (Pezzulo et al., 2011) and the pneumococcus requires neuraminidase to liberate sialic acid from the mucus. Arguably, saliva might be a more efficient medium of transmitting bacteria compared to nasal secretions as healthy individuals are more likely to talk to each other rather than sneezing or coughing to expel contents of the airways.

It is also of interest to expand this study further in the future as we only looked at pneumococcal mono-infection in saliva. Other organisms residing in the oral cavity may affect pneumococcal survival and host response against the bacteria. Studies on colonisation in the nasopharynx showed that pneumococcal colonisation elicited antibodies that cross-react with S. aureus (Lijek et al., 2012) and co-colonisation of mice with H. influenzae and pneumococci led to synergistic increases in neutrophil chemoattractant production and neutrophil influxes (Ratner et al., 2005) which clears pneumococcus while H. influenzae persists (Lysenko et al., 2005). In addition to interspecies interaction, there is also intraspecies competition whereby an individual colonised with a single strain of pneumococcus is less likely to be colonised by a different strain compared to a naïve individual (Kono et al., 2016). We could not confirm the importance of Spd1837 during transmission as suggested by the Tn-seq conducted by Verhagen et al. (2014). This may be due to differences in serotype used or, similar to this previous study, the effect may only be detected using a competition assay and not a single culture assay. Nevertheless, the difference might stem from the saliva source in the first place as the exact composition of human saliva varies considerably between individuals (Humphrey & Williamson, 2001).

Many gaps in knowledge in regards to possible pneumococcal colonisation in the oropharynx remain to be addressed. It is unclear if pneumococci in the oral cavity exist as planktonic bacteria suspended in saliva (similar to in the blood during sepsis) or the bacteria have the ability to attach to epithelial cells in the mouth and to form biofilm similar to closely related species, Streptococcus mitis and Streptococcus mutans (Johansson et al., 2016). One advantage of strong adherence is stable carriage. This however, may lead to less efficient exit and spread to a new host. It is established that pneumococci in the nasopharynx can be rapidly swept away by mucociliary clearance (Fahy & Dickey, 2010), a phenomenon that might apply to pneumococci in the oral cavity due to our food and drink
consumption and oral hygiene habit. Another possible disadvantage of not attaching is the lower transformation rate compared to during planktonic growth in sepsis (Marks et al., 2012). However, being suspended in saliva without any cell attachment might offer a selective advantage as this would bypass the host innate immune response such as Toll-like receptor 2, a pattern recognition receptor critical for macrophage recruitment and type 1 interferon production (Zhang et al., 2009, Parker et al., 2011) and also Toll-like receptor 4, capable of recognizing pneumolysin (Malley et al., 2003). Another future direction is to study the importance of these possible transmission factors across different age group as anti-pneumococcal antibodies may also be present in human saliva, especially in children (Simell et al., 2001, Simell et al., 2002).

The virulence studies in Chapter 4 identified Spd1836 as being a newly discovered virulence factor in the pneumococcus, especially during its progression to cause invasive disease. Very little is known about Spd1836 homolog functions. Thus, more investigation of the MORN motif-containing proteins is needed to assess the exact contribution of Spd1836 to pneumococcal virulence. It is also worth conducting virulence and transmission studies using other serotypes as serotype 2 is shown to be the least shed and had the least colonisation density compared to serotype 4, 6A, 19F and 23F in a mouse infant model (Zafar et al., 2016).

6.4 The complex interplay between the pneumococcal Spd1837, SpxB, CPS biosynthesis and possibly metabolism

In Chapter 5, we show that Spd1837 works together with SpxB to modulate CPS levels, with this being specifically CPS not attached to the cell wall. All but a few of the > 90 CPS types, including for serotype 2 CPS, are negatively charged. As mentioned in Chapter 1, highly-charged CPS may interfere with cell-to-cell interactions with phagocytes (Kozel et al., 1980, Lee et al., 1991, Weinberger et al., 2009) and act as a decoy to neutralise cationic antimicrobial peptides (Llobet et al., 2008). These roles of CPS is specific for released CPS only, i.e. CPS not attached to the cell wall, which is the form that is specifically regulated by SpxB and Spd1837. Additionally, as spxB deletion appeared to lead to CPS defects in pneumococcal serotypes possessing CPS with acetylated sugars only (Echlin et al., 2016), it
is of interest to investigate if the degree of acetylation of pneumococcal CPS determines the final charge of a particular CPS type.

Interestingly, Spd1837_{CBS} showed repressor-like effects when expressed in a spxB-deficient background which was not evident in the wildtype background. This suggests that SpxB is able to control the repressor activity of this mutant protein, and points to the fact that there are likely both phosphatase-dependent and -independent effects of SpxB on Spd1837. Additionally, in Chapter 5 we also showed that the manner by which SpxB regulates Spd1837 activity is also dependent on the oxygen availability during the pneumococcal growth. Therefore, it is of interest to investigate the interplay of SpxB and Spd1837 with two genes that were identified as being responsible for the capacity of pneumococci to grow under ambient air: pca (encoding a carbonic anhydrase) and folC (encoding a dihydrofolate/folylpolyglutamate synthase) (Burghout et al., 2010, Burghout et al., 2013). Studying this interaction may also assist in understanding why despite the predominantly aerobic lifestyle, the pneumococcus lacks many proteins that have been shown to protect against oxidative stress in other bacterial species, such as the global regulators OxyR and PerR or the H_{2}O_{2} scavengers catalase and NADH peroxidase (Tettelin et al., 2002, Hua et al., 2014).

It is yet to be determined if the SpxB protein itself, or H_{2}O_{2} produced by it, or both, modulate Spd1837 activity. Both SpxB and Spd1837 appear to participate in a common process, which is pneumococcal metabolism (Carvalho et al., 2013, Echlin et al., 2016)(Chapter 3). This process is unfortunately still not completely understood at the moment. Therefore, more information is needed to establish the nature of the association between these two proteins. However, given the possible role of Spd1837 in pneumococcal metabolism along with its cross-talk with SpxB, it is likely that Spd1837 may only exert its function under specific conditions such as during specific nutrient limitation. This is the case for another LMWPTP, B. cenocepacia BCAL2200 as the LMWPTP displayed perturbed growth under the lack of amino acid but not glucose (Andrade et al., 2015).

*S. pneumoniae* also undergoes a mainly fermentative metabolism and lacks the cytochromes and heme-containing proteins involved in aerobic respiration (Pericone et al., 2003), therefore it relies entirely on the host for carbon sources and energy generation. The spxB mutants have decreased ability to maintain ATP levels during sub-lethal or lethal
H$_2$O$_2$ stress (Pericone et al., 2003) and a lack of ATP may occur due to inactivation of sugar transport or glycolysis, since both processes are known to be particularly sensitive to oxidative stress (Barrette et al., 1989, Storz & Imlay, 1999). Whether the effect on Spd1837 is due to H$_2$O$_2$ requires further experimentation as our CPS preparations were collected off blood agar plates (it is not possible to isolate CPS not associated with cell wall from pneumococci grown in liquid culture (Morona et al., 2006)). Therefore, the possible effect of H$_2$O$_2$ on Spd1837 might have been neutralised by the catalase in the plates.

Many other proteins which confer protection against H$_2$O$_2$ also modulate H$_2$O$_2$ production in the pneumococcus. Apart from SpxB, these include LctO (Taniai et al., 2008), CarB (Hoffmann et al., 2006), PsaA (Johnston et al., 2004) AhpD (Paterson et al., 2006), TpxD (Hajaj et al., 2012), HtrA (Ibrahim et al., 2004), ClpA (Robertson et al., 2002), NmlR (Potter et al., 2010) and GlpO (Mahdi et al., 2012). Therefore, given that Spd1837 has a role in H$_2$O$_2$ resistance, Spd1837 is potentially another protein in a long list of H$_2$O$_2$ production modulators in the pneumococcus and a future direction is to perform H$_2$O$_2$ production assays on spd1837 mutants using the horseradish peroxidase/phenol red assay described by Okado-Matsumoto & Fridovich (2000).

6.5 Conclusions

In conclusion, this is the first study which describes the existence of a low molecular weight protein tyrosine phosphatase in the pneumococcus. Prior to this, only CpsD and CpsB have been identified to be a BY-kinase and a PTP, respectively, in the pneumococcus. Given that many critical processes such as CPS biosynthesis, cell division and autolysis have been identified to be regulated by tyrosine phosphorylation, the discovery of Spd1837 as another PTP in the pneumococcus will hopefully accelerate the understanding of the importance of tyrosine phosphorylation in this human pathogen. Again, similar to CpsD which functions in multiple pathways in the pneumococcus and CpsB which has phosphatase-dependent and –independent activities, Spd1837 appears to share the same complexity in terms of its function and regulation. Specifically, Spd1837 may be involved in the pneumococcal metabolism and CPS biosynthesis in a physiologically-relevant condition. An updated phosphoproteomic study and validation experiments to verify potential substrates/interacting proteins of Spd1837 via bacterial two-hybrid assay
is the next logical steps to further our understanding of the function of this PTP. Additionally, the other two proteins encoded together with Spd1837 in the OM001 operon, Spd1838 and Spd1836 were shown to be essential for the pneumococcal survival in human saliva with Spd1836 being potentially important during pneumococcal invasive disease too. Therefore, overall this study has provided insights into several stages of pneumococcal pathogenesis, namely transmission, colonisation and invasion.


expansion after antibiotic selection pressure: pneumococcal multilocus sequence types before and after mass azithromycin treatments. *J Infect Dis* **211**: 988-994.


### Appendix A  
*E. coli* and *S. pneumoniae* Strains Utilised/Generated

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant characteristics</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RMA2302</td>
<td>Source of pET-15b, Amp</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>DH5α</td>
<td><em>E. coli</em> transformation strain</td>
<td>Gibco-BRL</td>
</tr>
<tr>
<td>Lemo21 (DE3)</td>
<td><em>E. coli</em> expression strain</td>
<td>New England BioLabs</td>
</tr>
<tr>
<td>XL-10Gold</td>
<td>For site-directed mutagenesis</td>
<td>Agilent Technologies</td>
</tr>
<tr>
<td>ZA1</td>
<td>DH5α with pET-15b::Spd1837, Amp</td>
<td>This work</td>
</tr>
<tr>
<td>ZA2</td>
<td>Lemo21 with pET-15b::Spd1837, Amp</td>
<td>This work</td>
</tr>
<tr>
<td>ZA11</td>
<td>XL-10 Gold with pET-15b::Spd1837&lt;sub&gt;C8S&lt;/sub&gt;, Amp</td>
<td>This work</td>
</tr>
<tr>
<td>ZA12</td>
<td>Lemo21 with pET-15b::Spd1837&lt;sub&gt;C8S&lt;/sub&gt;, Amp</td>
<td>This work</td>
</tr>
<tr>
<td><strong>S. pneumoniae</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ZA24</td>
<td>D39, Sm</td>
<td>(Standish et al., 2014)</td>
</tr>
<tr>
<td>ZA3</td>
<td>D39&lt;sub&gt;spd1837::janus&lt;/sub&gt;, Sm</td>
<td>This work</td>
</tr>
<tr>
<td>ZA6</td>
<td>D39Δ&lt;sub&gt;spd1837&lt;/sub&gt;, Sm</td>
<td>This work</td>
</tr>
<tr>
<td>ZA8</td>
<td>D39&lt;sub&gt;Spd1837&lt;sub&gt;C8S&lt;/sub&gt;&lt;/sub&gt;, Sm</td>
<td>This work</td>
</tr>
<tr>
<td>ZA55</td>
<td>D39Δ&lt;sub&gt;spd1836&lt;/sub&gt;, Sm</td>
<td>This work</td>
</tr>
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<td>ZA58</td>
<td>D39Δ&lt;sub&gt;spd1838&lt;/sub&gt;, Sm</td>
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</tr>
<tr>
<td>ZA88</td>
<td>D39Δ&lt;sub&gt;OM001&lt;/sub&gt;, Sm</td>
<td>This work</td>
</tr>
<tr>
<td>ZA106</td>
<td>D39Δ&lt;sub&gt;OM001::janus&lt;/sub&gt;, Km</td>
<td>This work</td>
</tr>
<tr>
<td>ZA111</td>
<td>D39Δ&lt;sub&gt;OM001::OM001&lt;sup&gt;+&lt;/sup&gt;&lt;/sub&gt;, Sm</td>
<td>This work</td>
</tr>
<tr>
<td>ZA39</td>
<td>WU2, Sm</td>
<td>This work</td>
</tr>
<tr>
<td>ZA43</td>
<td>WU2&lt;sub&gt;spd1837::janus&lt;/sub&gt;, Km</td>
<td>This work</td>
</tr>
<tr>
<td>ZA51</td>
<td>WU2Δ&lt;sub&gt;spd1837&lt;/sub&gt;, Km</td>
<td>This work</td>
</tr>
<tr>
<td>ZA62</td>
<td>D39Δ&lt;sub&gt;spxB&lt;/sub&gt;, Sm and Cml</td>
<td>This work</td>
</tr>
<tr>
<td>ZA63</td>
<td>D39Δ&lt;sub&gt;spd1837ΔspxB&lt;/sub&gt;, Sm and Cml</td>
<td>This work</td>
</tr>
<tr>
<td>ZA90</td>
<td>D39&lt;sub&gt;Spd1837&lt;sub&gt;C8S&lt;/sub&gt;ΔspxB&lt;/sub&gt;, Sm and Cml</td>
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</tr>
<tr>
<td>ZA30</td>
<td>D39Δ&lt;sub&gt;cpsBCD::janus&lt;/sub&gt;, Km</td>
<td>This work</td>
</tr>
</tbody>
</table>

<sup>1</sup>Amp, Ampicillin; Sm, Streptomycin; Km, Kanamycin; Cml, Chloramphenicol
### Appendix B  Oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence</th>
<th>Purpose</th>
</tr>
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<tbody>
<tr>
<td>ZA1</td>
<td>5’-GCCCATATGATGAAAAAATTAGCCTTTGTCTGTCTG-3’</td>
<td>F, amplify <em>spd1837</em>; bolded bases indicate NdeI site</td>
</tr>
<tr>
<td>ZA2</td>
<td>5’-CCGGGATCCCTATTAACCTCTCCTTTTCAAAAGTCTCTAC-3’</td>
<td>R, amplify <em>spd1837</em>; bolded bases indicate BamHI site</td>
</tr>
<tr>
<td>ET5</td>
<td>5’-AATACGACTCAGATTCAGG-3’</td>
<td>F, to sequence <em>spd1837</em> inserted into pET-15b (binds T7 promoter)</td>
</tr>
<tr>
<td>ET6</td>
<td>5’-GCTAGTTATGCTCAAGGG-3’</td>
<td>R, to sequence <em>spd1837</em> inserted into pET-15b (binds T7 terminator)</td>
</tr>
<tr>
<td>ZA3</td>
<td>5’-ATACCTAGTTATCTGTGG-3’</td>
<td>F, amplify <em>spd1837</em> upstream region</td>
</tr>
<tr>
<td>ZA4</td>
<td>5’-AAGAAGGAGTTGTAACCGTCCCAGG-3’</td>
<td>R, amplify <em>spd1837</em> downstream region</td>
</tr>
<tr>
<td>ZA5</td>
<td>5’-GGAAAGGAGGGCCAGTTGTGAAAGATTAAGGAGGGAAATC-3’</td>
<td>F, for overlap extension PCR of <em>spd1837</em>, complimentary to <em>janus cassette</em></td>
</tr>
<tr>
<td>ZA6</td>
<td>5’-CATTATCCAATTAATAACACGGCAATTCCTTTTTCTTTTATAGAAAAACGG-3’</td>
<td>R, for overlap extension PCR of <em>spd1837</em>, complimentary to <em>janus cassette</em></td>
</tr>
<tr>
<td>ZA7</td>
<td>5’-GAAAGGAATGGGAAAAGGAGAGGTTAAGGTGCAAATCC-3’</td>
<td>F, delete <em>spd1837</em>, complimentary to upstream of <em>spd1837</em></td>
</tr>
<tr>
<td>ZA8</td>
<td>5’-CTTTAACCCTCTTTTTCCCATTTCTTTTATAGAAAAACGG-3’</td>
<td>R, delete <em>spd1837</em>, complimentary to downstream of <em>spd1837</em></td>
</tr>
<tr>
<td>ZA11</td>
<td>5’-GAAAAAAATGGTGTCTGGGTAACATTTTGCCGTACCAGCC-3’</td>
<td>F, exchange <em>spd1837</em> C8S in Quikchange® Lightning Site-Directed Mutagenesis</td>
</tr>
<tr>
<td>ZA12</td>
<td>5’-GGGCTACGGGAAAATATTCCAGACTGACAAGACTAAATTTTTTC-3’</td>
<td>R, exchange <em>spd1837</em> C8S in Quikchange® Lightning Site-Directed Mutagenesis</td>
</tr>
<tr>
<td>AS113</td>
<td>5’-CCGTTTGATTTTTAATGGATAATG-3’</td>
<td>F, amplify <em>janus cassette</em></td>
</tr>
<tr>
<td>AS114</td>
<td>5’-AGAGACCTGGGCCCTTTTC-3’</td>
<td>R, amplify <em>janus cassette</em></td>
</tr>
<tr>
<td>AS120</td>
<td>5’-TGTTCCACGTATTTTTATTCAGA-3’</td>
<td>F, amplify <em>rpsl</em></td>
</tr>
<tr>
<td>AS121</td>
<td>5’-TCTCTTTATCCCTTTCTCTGTC-3’</td>
<td>R, amplify <em>rpsl</em></td>
</tr>
<tr>
<td>ZA9</td>
<td>5’-CGCTTTGCTAGTCTGGGAAATATTTG-3’</td>
<td>F, exchange <em>spd1837</em> C8S, complimentary to upstream of <em>spd1837</em></td>
</tr>
<tr>
<td>ZA10</td>
<td>5’-CAATATTTCCAGACTGACAAAAGAC-3’</td>
<td>R, exchange <em>spd1837</em> C8S, complimentary to downstream of <em>spd1837</em></td>
</tr>
</tbody>
</table>
ZA13 5’-TGGACTTTATGAAGCATTGAGTGCAGAAG-3’

ZA14 5’- GGGCCATTACCATTCAGCACGACAAAG-3’

ZA15 5’- GGAAGGGGCGCGCTTGCGGTGTTTGCTCTTTTCTTTTCATAGG-3’

ZA16 5’- CATTATCCCATATAATCAGAGGCTGTTTTTCTCTTTTTCTTTTACTTAAGGCATAGG-3’

ZA17 5’- CAAAGGAGAAACAGAAGGCGCAATTTGAAAATAAGACG-3’

ZA18 5’- CAATTGCGCTTTCTGTGGTTCTGGCCTTTTCTTTTATAAGACG-3’

ZA19 5’- GGAAGGGGCGCGCTTGCGGTGTTTTTCTCTTTTTCTTTTACTTAAGGCATAGG-3’

ZA20 5’- CATTATCCCATATAATCAGAGGCTGTTTTTCTCTTTTTCTTTTACTTAAGGCATAGG-3’

ZA21 5’- CAAAGGAGAAACAGAAGGCGCAATTTGAAAATAAGACG-3’

ZA22 5’- CAATTGCGCTTTCTGTGGTTCTGGCCTTTTCTTTTATAAGACG-3’

ZA26 5’- GCCTCTGGCCCGACACCAAAG-3’

ZA27 5’- CAAGAGTATAATAATTTCTTTTGAA-3’

ZA24 5’- CAAAGGAGAAACAGAAGGCGCAATTTGAAAATAAGACG-3’

ZA25 5’- GGAAGGGGCGCGCTTGCGGTGTTTTTCTCTTTTTCTTTTACTTAAGGCATAGG-3’

ZA36 5’- CAGCTAAATTACCAACCTC-3’

ZA37 5’- TTTTCAACATAAGCTGGAACGTTC-3’

AS253 5’- TTAGTTGCGAAGGCGATATAGTATAC-3’

F, 200 nucleotides downstream of spd1837, sequencing primer
R, 200 nucleotides downstream of spd1837, sequencing primer
F, for overlap extension PCR of spd1838, complimentary to janus cassette
R, for overlap extension PCR of spd1838, complimentary to janus cassette
F, delete spd1838, complimentary to upstream of spd1838
R, delete spd1838, complimentary to downstream of spd1838
F, for overlap extension PCR of spd1836, complimentary to janus cassette
R, for overlap extension PCR of spd1836, complimentary to janus cassette
F, delete spd1836, complimentary to upstream of spd1836
R, delete spd1836, complimentary to downstream of spd1836
F, 200 nucleotides upstream of spd1838, sequencing primer
R, 200 nucleotides downstream of spd1836, sequencing primer
F, delete OM001, complimentary to upstream of spd1836
R, delete OM001, complimentary to downstream of spd1836
F, 1 kb upstream of spd1838, to amplify OM001 for complementation
R, 1 kb downstream of spd1836, to amplify OM001 for complementation
F, delete spxB, insert a chloramphenicol resistance cassette
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>AS254</td>
<td>5’-GTCTTTGTAATGGCATCTCGCAT-3’</td>
<td>R, delete spxB, insert a chloramphenicol resistance</td>
</tr>
<tr>
<td>AS273</td>
<td>5’-CCATGGACTTCATTTACTGGG-3’</td>
<td>F, binds end of spxB, sequencing primer</td>
</tr>
<tr>
<td>AS274</td>
<td>5’-CTTGTCAAGAAATAATGCGAG-3’</td>
<td>R, binds start of spxB, to sequencing primer</td>
</tr>
<tr>
<td>AS115</td>
<td>5’-CATTATCCATTAATAATGACGTTCATCTACCCCTCCATCAGATCACCCATCACATCC-3’</td>
<td>F, for overlap extension PCR of cps locus, complimentary to janus cassette</td>
</tr>
<tr>
<td>AS116</td>
<td>5’-GGAAGGGCCAGGTCTCTGTCGGGGATAGAGATGAATG-3’</td>
<td>R, for overlap extension PCR of cps locus, complimentary to janus cassette</td>
</tr>
<tr>
<td>MycoF</td>
<td>5’-GGGAGGAAACAGGATTAGATAACCT-3’</td>
<td>F, for PCR-based mycoplasma detection of cell line</td>
</tr>
<tr>
<td>MycoR</td>
<td>5’-TGACCCATCTGTACCTGTTAACCTC-3’</td>
<td>R, for PCR-based mycoplasma detection of cell line</td>
</tr>
</tbody>
</table>

Forward and reverse primers are represented by plus (F) or minus (R), respectively.