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

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**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.

**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 (Fig. S1, available in the online version of this article). 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.

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Abbreviations: BY-kinase, bacterial tyrosine kinase; CBS, cysteine 8 to serine; CPS, capsular polysaccharide; EPS, exopolysaccharide; LB, Lysogeny Broth; LMWPTP, low molecular weight protein tyrosine phosphatase; NaN₃VO₄, sodium orthovanadate; pNP, p-nitrophenol; pNPP, p-nitrophenyl phosphate; PTP, protein tyrosine phosphatase; THY, Todd Hewitt broth with 1% Bacto yeast extract.

Supplementary material is available with the online version of this article.
**METHODS**

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

*S. pneumoniae* and *E. coli* strains (listed in Table S1) 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 S2. The gene encoding Spd1837 was amplified from *S. pneumoniae* D39 genomic DNA with primers ZA1 and ZA2. The PCR product was digested with *Bam*HI and *Nde*I, the restriction sites for which were included in ZA1 and ZA2. This digested 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).

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-Spd1837_C8S). Markerless, non-polar, in-frame deletion in *spd1837* was constructed in a Serotype 2 D39 streptomycin-resistant strain essentially as previously described [4].

**Purification of Spd1837 and Spd1837_C8S**

Spd1837 and Spd1837_C8S proteins were expressed in Lemo21 (DE3), grown at 37°C for 16 h in Lysogeny Broth (LB), sub-cultured 1/20 in 1 l LB at 37°C for 2 h with expression of recombinant protein then induced with 0.1 mM IPTG for 3 h. Spd1837 and Spd1837_C8S 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 Spd1837_C8S was obtained from 1 l of bacterial culture.

**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 inhibitor 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.

**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 D39Aspd1837 strain and grown for 6 h until the OD600nm was approximately 0.2. 1 mM perrvanadate 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 (GeneScript) 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.SpD1837, His.C8S.SpD1837_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, 1 mM sodium orthovanadate (Na3VO4) 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 h 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 (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. Stepwise 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.

**Liquid chromatography – electrospray ionization 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 ionization 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.
most intense precursor ions were 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 \( \geq 2+ \) 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 a decoy database for false discovery rate calculations (approximately 1 %). Peptides with \( P < 0.05 \) are reported.

**SDS-PAGE and Western immunoblotting**

Samples from the 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.

**RESULTS**

**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_C8) were purified from E. coli as described in Methods (Fig. 1a). Using size exclusion chromatography as the final purification step, the apparent molecular mass of Spd1837 was approximately 15.8 kDa (Fig. S2), 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_C8 did not have activity against pNPP (Fig. 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_C8 lacked any activity against these two phosphotyrosine-containing phosphopeptides (Fig. 1c).

We also demonstrated that Spd1837 dephosphorylated pNPP according to Michaelis–Menten kinetics (Fig. 2a), with a $K_m$ of 8.0 mM and a $V_{max}$ of 1.34 µmol min$^{-1}$ mg$^{-1}$. These $K_m$ values are within the range of $K_m$ reported for other LMWPTPs (Table S3). Spd1837 showed optimum activity at 37°C (Fig. 2b) and pH 7.0 (Fig. 2c), both of which are similar to the optimum conditions for most LMWPTPs (Table S4). The strict specificity of Spd1837 for phosphotyrosine residues was confirmed by analysing the effect of Na$_3$VO$_4$, which specifically inhibits tyrosine phosphatases. Na$_3$VO$_4$ strongly inhibited Spd1837 phosphatase activity (IC$_{50}$ = 0.1 µM) (Fig. 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 (Fig. 2d).

**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 enzymes.
Spd1837. Three unique bands of ~37, 35 and 25 kDa were present in Coomassie-stained SDS-PAGE gels when Spd1837 and Spd1837\textsubscript{CRS} 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 (Fig. 3a). Putative substrates identified by mass spectrometry analysis are listed in Table 1 while specific proteins identified from each band and their tryptic peptides are listed in Table S5.

The intensity of bands in the wild-type His\textsubscript{6}-Spd1837 were greater than when His\textsubscript{6}-Spd1837\textsubscript{CRS} incubated with lysate (compare lanes 1 and 2, and lanes 5 and 6 in Fig. 3a). To further investigate if the identified proteins are actual phosphatase substrates, pulled-down proteins were probed with a specific anti-phosphotyrosine antibody (Fig. 3b, top panel). Bands with apparent molecular weight of ~15, ~25, ~40 and ~50 kDa 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 (Fig. 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. 3(b), top panel, the proteins pulled-down with His\textsubscript{6}-Spd1837\textsubscript{CRS} did not appear to be more tyrosine-phosphorylated than those pulled-down with His\textsubscript{6}-Spd1837.

**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

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**Fig. 3.** Substrate-trapping assay using wild-type and mutant Spd1837. (a) A representative Coomassie-stained gel of pulldowns was submitted for tryptic digest and mass spectrometry analysis. Wild-type Spd1837, Spd1837\textsubscript{CRS} or BSA were coupled to the beads, and beads alone were incubated with buffer only. Beads were incubated with lysate from *S. pneumoniae* D39\textsubscript{spd1837} pre-treated with pervanadate. Samples were subjected to SDS-PAGE and Coomassie-stained. Bolded numbers 1–6 indicate bands excised for mass spectrometry analysis. (b) Western immunoblot of *S. pneumoniae* D39\textsubscript{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.
Substrate-trapping studies were subsequently undertaken in vitro. Interestingly, while purified Spd1837 was an active phosphatase, 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 1). These findings are perhaps unsurprising given many ribosomal proteins are tyrosine phosphorylated [24, 25], while enzymes involved in the 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 pneumococcus to survive in the different niches it encounters during human infection.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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