**Streptococcus pneumoniae** PstS Production Is Phosphate Responsive and Enhanced during Growth in the Murine Peritoneal Cavity

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Differential display-PCR (DDPCR) was used to identify a *Streptococcus pneumoniae* gene with enhanced transcription during growth in the murine peritoneal cavity. Northern blot analysis and comparative densitometry confirmed a 1.8-fold increase in expression of the encoded sequence following murine peritoneal culture (MPC) versus laboratory culture or control culture (CC). Sequencing and basic local alignment search tool analysis identified the DDPCR fragment as *pstS*, the phosphate-binding protein of a high-affinity phosphate uptake system. PCR amplification of the complete *pstS* gene followed by restriction analysis and sequencing suggests a high level of conservation between strains and serotypes. Quantitative immunodot blotting using antiserum to recombinant PstS (rPstS) demonstrated an approximately twofold increase in PstS production during MPC from that during CCs, a finding consistent with the low levels of phosphate observed in the peritoneum. Moreover, immunodot blot and Northern analysis demonstrated phosphate-dependent production of PstS in six of seven strains examined. These results identify *pstS* expression as responsive to the MPC environment and extracellular phosphate concentrations. Presently, it remains unclear if phosphate concentrations in vivo contribute to the regulation of *pstS*. Finally, polyclonal antiserum to rPstS did not inhibit growth of the pneumococcus in vitro, suggesting that antibodies do not block phosphate uptake; moreover, vaccination of mice with rPstS did not protect against intraperitoneal challenge as assessed by the 50% lethal dose.

Acquisition of inorganic phosphate (P\(_i\)) in *Escherichia coli* is principally carried out by Pst and Pit (34), two independent transport systems that are coregulated as members of the phosphate regulon (34). When P\(_i\) is in excess, the expression of the phosphate regulon is inhibited and phosphate uptake is primarily the result of the low-affinity transporter Pit (30). Conversely, under phosphate limitation, most phosphate regulon genes are upregulated (34), including the high-affinity P\(_i\)-specific transporter Pst (3, 34). The Pst transporter complex is composed of five proteins whose genes, *pstSCAB* and *phoU* (7), are collectively transcribed. *pstS*, the first gene transcribed from the *pst* operon, encodes a phosphate-binding protein belonging to the family of ATP-binding cassette (ABC) transporters, *pstC* and *pstA* encode transmembrane proteins, while *pstB* encodes an ATP-binding protein. Mutations in any of these genes abolishes P\(_i\) uptake (7, 27, 33). Finally, *phoU*, while not required for phosphate transport, is responsible for repression of the phosphate regulon (12, 28).

Recently, in *Streptococcus pneumoniae*, a locus with homology to the Pst system was identified (18). Examination of the locus revealed five genes with characteristics similar to those previously described for *E. coli*. The pneumococcal Pst system includes a phosphate-binding protein (*pstS*), two transmembrane proteins (*pstC* and *pstA*), and an ATP-binding protein (*pstB*), all of which are putatively cotranscribed. Immediately downstream and under the control of a presumed independent promoter is a fifth gene homologous to the *phoU* gene of *E. coli*. Mutational analysis of the pneumococcal *pst* locus revealed that, as in *E. coli* (12, 28), mutagenesis of the ABC gene *pstB* resulted in decreased rates of phosphate uptake, decreased growth rates (18), and reduced pathogenicity in a septicemia model of infection (20). Moreover, mutagenesis of *pstB* resulted in decreased levels of transformation and resistance to penicillin-induced lysis (18).

In this report, we examine the gene expression of *pst* and the protective efficacy of vaccination with purified recombinant PstS (rPstS). Having identified a peritoneally enhanced differential display-PCR (DDPCR) product, we confirm enhanced *pstS* transcription and PstS production during murine peritoneal culture (MPC) (19). Further, we demonstrate that *pst* transcription and Pst production are increased in response to decreasing levels of P\(_i\). Finally, we determine that, while PstS is conserved among multiple pneumococcal isolates, vaccination of mice with rPstS is not protective in a septicemia model and that polyclonal antiserum does not inhibit pneumococcal growth in vitro.

**MATERIALS AND METHODS**

*Streptococcus pneumoniae* strains and in vitro growth conditions. Bacterial strains and plasmids used are listed in Table 1. Pneumococci were grown on tryptic soy agar plates supplemented with sheep blood to a final concentration of 5% (vol/vol) (Becton Dickinson Microbiology Systems, Cockeysville, Md.). For growth in liquid media, bacteria were grown in Todd-Hewitt broth supplemented with 5% yeast extract (wt/vol) (THY). For experiments using assorted concentrations of phosphate, the bacteria were grown in casein hydrolysate media (C+Y medium) supplemented with 1.0, 3.0, 10, and 30 mM P\(_i\) (pH 8.0). C+Y media were prepared as indicated by Lack et al. (13) with the exception of sodium phosphate. Phosphate concentration in the basal media was determined using a *Vitros* 950 System

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TABLE 1. Listing of S. pneumoniae isolates and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D39</td>
<td>Serotype 2; NTC 7466</td>
<td>15</td>
</tr>
<tr>
<td>R6</td>
<td>Serotype 2; D39 derivative</td>
<td>16</td>
</tr>
<tr>
<td>Rfx</td>
<td>Serotype 2; R6 derivative</td>
<td>18</td>
</tr>
<tr>
<td>WU2</td>
<td>Serotype 3</td>
<td>6</td>
</tr>
<tr>
<td>ATCC 6303</td>
<td>Serotype 3; ATCC reference strain</td>
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<td>Seattle</td>
<td>Serotype 3; clinical isolate</td>
<td>This study</td>
</tr>
<tr>
<td>DW1.4</td>
<td>Serotype 4; clinical isolate</td>
<td>This study</td>
</tr>
<tr>
<td>DW11</td>
<td>Serotype 11A; clinical isolate</td>
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</tr>
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<td>DW14.1</td>
<td>Serotype 14; clinical isolate</td>
<td>This study</td>
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<tr>
<td>DW19</td>
<td>Serotype 19F; clinical isolate</td>
<td>This study</td>
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<tr>
<td>Plasmids</td>
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<tr>
<td>pMOBlue</td>
<td>Vector (AmpR)</td>
<td>Amersham</td>
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<tr>
<td>pBad/Thio-Top</td>
<td>Vector (AmpR)</td>
<td>Invitrogen</td>
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<tr>
<td>pWU2PSTs</td>
<td>pBad/Thio-Top derivative carrying pstS from WU2</td>
<td>This study</td>
</tr>
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Cloning of DDPCR product. DDPCR products representing putative MPC-enhanced genes were identified and localized by densitometric analysis of autoradiographs from dried DDPCR gels (Applied Imaging System Densitometer; Applied Imaging Systems, Santa Clara, Calif.). DNA fragments from bands with enhanced expression under both MPC RNA concentrations (100 and 200 ng) were localized by overlaying the autoradiographs and were excised using a clean scalpel blade. Products were eluted in water and reamplified by PCR using the primer specific to each DDPCR. Amplified products were cloned using a TA cloning vector (pMOBlue; Amersham, Little Chalfont, Buckinghamshire, England), according to the manufacturer’s protocol.

Confirmation by Northern dot blot. RNA dot blotting was performed using RNA derived from MPC- and CC-grown pneumococci. RNA from each set of conditions was serially diluted and blotted onto nylon membranes using a slot blot apparatus and vacuum manifold. RNA was fixed to the nylon membranes by soaking in 0.05 M NaOH for 20 min followed by rinsing with 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (4). The membranes were prehybridized with Rapid-hyb buffer (Amersham Pharmacia Biotech Inc., Piscataway, N.J.) for a minimum of 1 h and were hybridized under stringent conditions using the standard protocol (24). Probes were obtained by PCR amplification of pstS from a recombinant plasmid containing the full-length gene and were labeled using Ready-To-Go DNA Labeling Beads (dCTP) (Amersham). To confirm equal loading of RNA, parallel duplicate membranes were hybridized against a probe to the S. pneumoniae DNA gyrase A subunit. Previously, it had been shown that DNA gyrase A is constitutively expressed (10, 11). Analysis of DNA gyrase A transcription is an appropriate control for determination of the equalized RNA load. Northern dot blot analysis of pstS expression was performed in triplicate, with signal intensity determined by comparative densitometry of autoradiographs using a Gel Doc 2000 (Bio-Rad; Hercules, Calif.). Signal intensity was determined for spots in the linear detection range. Statistical significance was determined using an f test to see if the mean ratio exceeds 1, 1 being the no-effect hypothesis.

Sequence analysis of DDPCR products. Plasmids containing putative enhanced genes were grown at one plaques per plate in 10^-6 M cultures and were isolated from the 10^-6 M culture. DNA sequences were determined by automated sequencing available at the Centers for Tropical Disease Core Laboratory (GenBank accession number AY039745).

Expression, purification of PstS, and development of PstS antiserum. Ligation of pstS into pBluescript-KII plasmid created a thioredoxin/PstS histidine tag fusion construct (pPstS) regulated by an arabinose-inducible promoter. Briefly, 100 ml of Luria broth was inoculated with E. coli containing pWU2PSTs and was incubated for 4 h at 37°C. 1-Arabinose (Sigma, St. Louis, Mo.) was added to a final concentration of 0.2%, and the bacteria were incubated for an additional 4 h. After incubation, bacteria were pelleted and the plasmid was collected by using the Express Purification Kit (Invitrogen). Purification of the recombinant protein was performed as indicated by the manufacturer and was confirmed by Coomassie blue staining of a sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) gel loaded with the recombinant protein (data not shown). Antiserum to PstS was developed by intraperitoneal injection of mice with 1 μg of pPstS in 100 μl of Freund’s complete adjuvant, followed by two subsequent injections of 3 and 6 weeks in Freund’s incomplete adjuvant.
Serum was collected from the mice by retro-orbital bleeding prior to immunization and 3 weeks after the third immunization. Following collection, the serum was pooled and tested by Western blot analysis to assure specificity of the antiserum to rPstS and native PstS.

**Western blot analysis.** Western blotting was performed as indicated earlier (4), with antiserum to rPstS used at a dilution of 1:1,000, and secondary antibody was used at a dilution of 1:3,500.

**Quantitative immunodot blot analysis.** WU2 isolated from MPCs and CCs was pelleted and suspended in 500 µl of phosphate-buffered saline (PBS) (Sigma). Bacteria were disrupted by bead beating using 0.1-mm-diameter zirconia glass beads in a Mini-Beadbeater (Bio-spectrum, Bartlesville, Okla.) and centrifuged to remove cell wall debris. The protein concentration of the supernatant was determined by bicinchoninic acid (26) (BCA-200 Protein Assay Kit; Pierce, Rockford, Ill.) with samples of whole-cell lysate (WCL) subsequently diluted to 50 µg/ml in transfer buffer (25 mM Tris, 192 mM glycine, and 20% [vol/vol] methanol, pH 8.3). Samples were blotted onto nitrocellulose in duplicate twofold serial dilution using a 96-well dot blot apparatus. Additionally, purified rPstS was also serially blotted with an initial concentration of 100 ng/ml. Membranes were blocked in PBS supplemented with 0.1% Tween 20 and 5% nonfat dry milk for 1 h, with development of the membranes following standard immunodot blot protocol (4): namely, rPstS murine antiserum (1:1,000) in PBS and horseradish peroxidase-conjugated antibody (1:7,500). Blots were developed using the enhanced chemiluminescence system (Amersham). PstS production under different concentrations of phosphate was then quantified in terms of nanograms per microgram of WCL with the purified rPstS serving as the known standard. Protein concentration was determined using bicinchoninic acid (BCA-200 Protein Assay Kit, Pierce). To further ensure equivalent loading of protein for immunodot blot analysis, protein were visualized on a Coomassie-stained SDS-PAGE gel for each sample (data not shown). Statistical analysis was performed using Student’s t test (two samples, assuming equal variances).

**Determination of phosphate concentration within peritoneal cavity.** To determine phosphate levels within the MPCs and CCs, dialysis bags loaded with RPMI 1640 were surgically implanted into the peritoneal cavity of mice or alternatively submerged in RPMI 1640. After 8 h, bags were collected and supernatant was analyzed. Phosphate levels were determined using a Vitros 950 System and the Vitros Phos Slide (Johnson & Johnson) in the Clinical Chemistry laboratory of John Sealy Hospital. Six samples were collected for each set of culture conditions, and statistical analysis was performed using Student’s t test (paired two samples for means).

**Immunodot blot analysis of PstS production versus phosphate concentration.** PstS production in various concentrations of phosphate was determined using quantitative immunodot blotting. Exponential-phase *S. pneumoniae* strains D39, R6, R6x, WU2, ATCC 6303, DW4.1, and DW14.1 grown in Todd-Hewitt broth supplemented with 5% yeast extract were washed and diluted 1:10 in C+Y media supplemented with 1.0, 3.0, 10, and 30 mM P<sub>i</sub> for 1 h. After incubation for 37°C at 200 rpm, bacteria were pelleted and the supernatant was removed. PstS production was then determined as previously described, with equivalent loading of protein also assessed as previously described. Samples were collected in triplicate for each set of culture conditions, and statistical analysis was performed using Student’s t test (paired two samples for means).

**Northern blot analysis of pst transcript versus phosphate concentration.** Northern dot blot analysis of the *pst* locus in R6x and WU2 was performed using radiolabeled *pst* S. RNA was collected from the bacteria grown in C+Y media supplemented with 3.0, 10, and 30 mM P<sub>i</sub>. For each set of culture conditions, Northern dot blot analysis of the *pst* locus was performed using a prehybridization cocktail consisting of 50%formamide, 5× Denhardt’s solution, 5× SSPE, and 0.05% sodium pyrophosphate. The DNA was denatured by boiling for 5 min and electrophoresed on a 1% agarose gel, transferred to nitrocellulose, and hybridized overnight with 32<sup>P</sup>-labeled *pst* specific oligonucleotides. Hybridizations were performed at 42°C using a Molecular Dynamics hybridization oven and were then washed twice in 2×SSC/0.1% SDS at 65°C for 15 min each. The membranes were then exposed to autoradiographic film (Kodak X-OMAT, Eastman Kodak Co.) at −70°C. Films were scanned at 0.5-mm intervals using a ScanJet 4C scanner (Hewlett-Packard, Palo Alto, Calif.) and processed to generate blue density measurements using Photoshop (Adobe Systems, Mountainview, Calif.).

**RESULTS**

In an attempt to identify *S. pneumoniae* genes expressed during intraperitoneal growth, RNA was collected from WU2 after MPC and CC and was analyzed by DDPCR. Examination of the DDPCR autoradiographs identified several DDPCR products present at higher levels during MPC than during CC. Conversely, products were also identified at higher levels during CC than during MPC. Products present at higher levels during MPC were amplified using PCR, cloned into pMOS Blue.<sup>1</sup> and subjected to automated sequencing. Nucleotide sequences were searched against the TIGR *S. pneumoniae* database using BLASTN analysis that located the fragment within a defined contiguous sequence and identified the complete gene sequence. Subsequently, the full-length gene was then searched against GenBank using BLASTN and BLASTX analyses. Of the multiple products identified, DDPCR product P6-1 was identified as *pstS*, the *S. pneumoniae* phosphate-binding protein and first gene of the *pst* operon. Moreover, because previous reports have indicated that *pst* plays an important role in vivo (12, 18, 20, 28), we decided to examine DDPCR product P6-1 further.

Comparative densitometry of the P6-1 autoradiograph revealed a 3.2-fold increase in signal strength during MPC when compared to CC (Fig. 1). To verify that *pstS* was in fact MPC-enhanced, total RNA from MPC and CC was compared by dot blot analysis using radiolabeled full-length *pstS* as a probe. Densitometric analysis of three Northern dot blots revealed 1.9-, 1.6-, and 2.0-fold increases in *pstS* transcription using equivalent amounts of RNA for analysis (Fig. 2). This difference (−1.8-fold) was determined to be statistically significant (*P* = 1.0 × 10<sup>−4</sup>) using a *t* test to see if the mean ratio of MPC enhanced transcription exceeded 1, 1 being the no-effect hypothesis. PCR amplification of full-length *pstS* from 10 pneumococcal isolates representing seven serotypes amplified a single band at approximately 875 bp for each isolate (data not shown). Diagnostic restriction analysis of these fragments demonstrated a conserved restriction map regardless of isolate or serotype and was consistent with the predicted *pstS* restriction map of P394, the serotype 4 clinical isolate in the TIGR database and GenBank (data not shown). Sequencing of the cloned WU2 PCR fragment revealed that 870 out of 873...
(99.6%) nucleotides were conserved between WU2 and P394 (data not shown). The 3-nucleotide differences in the sequence do not result in alterations in the predicted amino acid sequence. Southern blot analysis of *pstS* using radiolabeled full-length *pstS* as a probe identified only a single copy of the gene within the WU2 chromosome (data not shown). Presently, the WU2 *pstS* sequence is available through GenBank accession number AY039745.

To express and purify the *pstS* gene product, the full-length *pstS* gene sequence was amplified from WU2 and cloned into pBad/Thio-Topo (Invitrogen). Ligation into pBad/Thio-Topo created a thioredoxin::*PstS::*histidine tag fusion construct under the regulation of an L-arabinose-inducible promoter. Pilot studies found optimal protein production at a 0.2% concentration of arabinose and confirmed the size of the recombinant PstS (rPstS) protein at 49 kDa (data not shown). Purification of rPstS was performed on an NiCl$_2$ column following the protocol. A probe to pneumococcal DNA gyrase subunit A served as a control to ensure equal loading of RNA. These experiments were performed in triplicate. *pstS* in MPC and in CC demonstrated a 1.8-fold difference.

A Western blot analysis of pneumococcal WCL demonstrating specificity of the rPstS antiserum to native PstS (33 kDa) (1) and rPstS (49 kDa) (2). The 37-kDa protein (3) is believed to be a degradation product. Kilodaltons are indicated on left. (B) PstS production during growth in 1.0 mM P$_i$ versus that in 30 mM P$_i$ demonstrated statistically significant increases in PstS production during growth in 1.0 mM P$_i$ versus that in 30 mM P$_i$. Additionally D39, ATCC 6303, and DW14.1 showed statistically significant increases in PstS production at 3.0 and 10 mM P$_i$ from that at 1.0 mM P$_i$. Of the six isolates, R6x was the only strain that did not demonstrate a significant increase in PstS production in response to changes in phosphate concentration. To ascertain if this lack of response was strain specific, the R6x parental strain R6 was tested and was found to have statistically significant, phosphate-dependent production of PstS in response to growth at 3.0 mM versus 30 mM P$_i$, with 1.0 mM P$_i$ not tested (data not shown). Likewise, transcriptional analyses of R6x using Northern dot blot analyses determined that *pstS* transcription in R6x did not respond to P$_i$ concentrations, whereas strain WU2, the control, did respond with a 2.1-fold increase in *pstS* RNA levels at 3.0 mM P$_i$ from those at 30 mM P$_i$ (Fig. 5), consistent with the 1.8-fold increase in PstS production observed by immunodot blot analysis.

Western blot analyses using pooled human convalescent-phase sera failed to identify rPstS (data not shown). In order to greater during MPC (n = 4) than during CC (n = 6); this difference was statistically significant using Student’s *t* test (two samples; assuming equal variances) (P = 3.0 × 10$^{-3}$). Quantitative analysis of P$_i$ determined phosphate levels in the murine peritoneum to be half of those present in CC. Specifically, phosphate levels within MPC (n = 6) were determined to be 2 mM P$_i$, whereas those of CCs (n = 6) were found to be 4 mM P$_i$. These results indicate that *pstS* expression and protein production are phosphate responsive. This difference in phosphate levels was found to be statistically significant using Student’s *t* test (paired two samples for means) (P = 8.0 × 10$^{-4}$).

To determine if the P$_i$ concentration affected production of PstS, quantitative immunodot blot analysis was performed using WCL from six pneumococcal isolates: D39, R6x, WU2, ATCC 6303, DW4.1, and DW14.1, grown in media containing 1.0, 3.0, 10, or 30 mM P$_i$ (Fig. 4). Of the six isolates, five demonstrated statistically significant increases in PstS production during growth in 1.0 mM P$_i$ versus that in 30 mM P$_i$. The *pstS* gene sequence was amplified from D39, ATCC 6303, and DW14.1. No significant differences were observed in phosphate-responsive PstS production during growth in 1.0 mM P$_i$ versus 30 mM P$_i$. A significant fold increase in PstS production during growth at 3.0 mM P$_i$ versus 30 mM P$_i$ was observed in ATCC 6303 and DW14.1. Additionally, these isolates demonstrated statistically significant increases in PstS production at 3.0 and 10 mM P$_i$ from that at 1.0 mM P$_i$. Of the six isolates, R6x was the only strain that did not demonstrate a significant increase in PstS production in response to changes in phosphate concentration. To ascertain if this lack of response was strain specific, the R6x parental strain R6 was tested and was found to have statistically significant, phosphate-dependent production of PstS in response to growth at 3.0 mM versus 30 mM P$_i$, with 1.0 mM P$_i$ not tested (data not shown). Likewise, transcriptional analyses of R6x using Northern dot blot analyses determined that *pstS* transcription in R6x did not respond to P$_i$ concentrations, whereas strain WU2, the control, did respond with a 2.1-fold increase in *pstS* RNA levels at 3.0 mM P$_i$ from those at 30 mM P$_i$ (Fig. 5), consistent with the 1.8-fold increase in PstS production observed by immunodot blot analysis.

In summary, we have demonstrated that pneumococcal PstS production is inducible during growth of the bacterium in a phosphate-dependent manner and can be detected consistently using both immune and nonimmune sera. These results provide additional support for the use of pneumococcal PstS as a vaccine candidate.
determine if antibodies to rPstS inhibit the ability of the pneumococcus to grow in vitro, WU2 was grown at various phosphate concentrations in the presence of rPstS antisera at dilutions of 1:100, 1:500, and 1:1,000. Growth was not inhibited by the presence of antiserum. In contrast, cultures exposed to high levels of antiserum (dilutions of 1:100 and 1:500) grew better than those at low antiserum concentrations (1:1,000), possibly indicating that components in the serum stimulated bacterial growth in a dose-dependent manner (data not shown). Phosphate levels did, however, affect growth rates, with WU2 growth rates peaking at 30 and 10 mM \( P_i \) versus that at 3.0 mM \( P_i \) (data not shown), consistent with the findings reported by Novak et al. (18). Along similar lines, LD\(_{50}\) determinations examining protection in mice vaccinated with rPstS did not demonstrate a significant difference either. The LD\(_{50}\) in mice challenged with pneumococci intraperitoneally was determined to be \( 2.0 \times 10^3 \) for rPstS-vaccinated mice, whereas in controls it was found to be \( 2.5 \times 10^3 \). This is despite control studies demonstrating that vaccinated mice developed antibodies to rPstS (data not shown).

**DISCUSSION**

Environmental signals present in vivo such as temperature, nutrient availability, pH, and osmolality have all been shown to influence bacterial virulence gene expression (9). Previously, we have characterized pneumococcal gene expression and protein production during MPC (19). We have demonstrated changes in two-dimensional protein profiles, adhesive capacity, and virulence-associated gene expression. In this report, we focus on the characterization of DDPCR product P6-1, an MPC-enhanced product that encodes \( \text{pstS} \), the phosphate-binding protein of the pneumococcal \( \text{pst} \) phosphate transport system. We demonstrate that \( \text{pstS} \) is highly conserved among \( \text{S. pneumoniae} \) isolates, that both transcription of \( \text{pstS} \) and production of PstS are enhanced in the murine peritoneum, and that PstS production is responsive to environmental levels of \( P_i \).

PstS encodes a phosphate-binding protein/ABC transporter with a high level of homology to the same gene in \( \text{Methanobacterium autotrophicum} \) and \( \text{E. coli} \). Previous studies with \( \text{E. coli} \) have shown that the \( \text{pst} \) locus is a member of the phosphate regulon and plays an important role in the acquisition of phosphate when levels of environmental \( P_i \) are low (31). Recently, studies in \( \text{S. pneumoniae} \) in vitro have demonstrated that mutagenesis of the pneumococcal \( \text{pst} \) locus resulted in decreased uptake of \( P_i \), reduced rates of growth, resistance to penicillin-induced lysis, and an inability of the bacteria to undergo transformation (18). In a separate study, Polissi et al. identified what at the time was an unknown pneumococcal gene homologous to \( \text{pstB} \) in \( \text{Methanococcus jannaschii} \). This gene was determined to be critical for the survival of \( \text{S. pneumoniae} \) in a septicemia model of infection (20). Our findings of both increased \( \text{pstS} \) transcription and increased PstS production in response to growth in the murine peritoneum support the notion that phosphate acquisition is necessary for survival in vivo. Because \( \text{pstS} \) is the first gene transcribed from the \( \text{pst} \) operon, increased \( \text{pstS} \) transcription and PstS production may be interpreted as indicators of transcription of the operon and production of the Pst transport complex. Overall, our findings suggest that the pneumococcus adjusts to the low concentration of phosphate (2 mM \( P_i \) present in the peritoneum by increasing the production of this phosphate transporter.

Previously, Novak et al. (18) attempted to examine \( \text{S. pneumoniae} \) \( \text{pst} \) transcription in response to \( P_i \) levels. These studies using strain R6x determined that \( \text{pst} \) expression did not respond to decreasing levels of \( P_i \), an unexpected result in light of other reports indicating that \( \text{pst} \) expression is phosphate regulated in other bacteria (21, 34). Our analysis of Pst production in seven isolates determined a statistically significant increase in PstS production in six strains in response to decreasing levels of \( P_i \). Analysis of \( \text{pstS} \) transcription in R6x, the nonresponding isolate, established that this lack of response occurred at the transcriptional level. What is more, analyses of D39 and R6, the R6x parental strains, determined that this lack of response is strain specific and that extracellular levels of \( P_i \) regulate most isolates.

Restriction analyses and limited sequence analyses of \( \text{pstS} \) from 10 isolates of \( \text{S. pneumoniae} \) have shown that \( \text{pstS} \) is conserved between isolates at both the nucleotide and predicted amino acid level. Conservation of \( \text{pstS} \) may indicate the metabolic importance of the protein product. Similarly, the conserved nature of the protein and operon among different
bacteria may point toward a conserved evolutionary lineage. Conservation of pst may indirectly point toward the existence of a Streptococcus spp. phosphate regulon, an idea which is supported by the failure of mutagenesis of the locus to completely ablate phosphate uptake (indicating the existence of a second transporter) (18) and by the existence of a phosphate regulon in other pst-conserved bacteria, including the gram-positive Bacillus subtilis (5, 14, 21, 25).

Generally, it is believed that identification and characterization of in vivo-expressed genes will provide insight into the mechanisms that underlie bacterial pathogenesis. Furthermore, because in vivo-produced factors are those likely necessary for bacterial adaptation, survival, and disease progression, it is believed that in vivo-expressed genes are potential targets for antimicrobials, vaccines, and other pharmacological agents (9). Prior to this report, two studies have examined the importance of the Pst transporter for bacterial survival in an infection model. As previously discussed, Polissi et al. demonstrated that mutagenesis of pstB in S. pneumoniae resulted in an attenuated mutant incapable of surviving in a septicemia model of infection (20). Daigle et al. demonstrated that mutagenesis of pstC in E. coli resulted in a serum-sensitive mutant incapable of systemic survival after an intragastric challenge (8). Along this line, studies with Mycobacterium tuberculosis have determined that vaccination of mice with a DNA vaccine encoding the Mycobacterium pstS gene offered protection after intravenous challenge with M. tuberculosis (29).

Because of the requirement for Pst in vivo and the potential for a PstS vaccine, we elected to examine if antibodies to the phosphate-binding protein PstS inhibited bacterial growth in vitro and, more important, offered protection against pneumococcal challenge. Unfortunately, this was not the case, as rPstS antiserum failed to affect the growth rate of WU2. Additionally, mice vaccinated with rPst and controls did not demonstrate a difference in the LD_{50}. These results suggest that either antibody to rPstS does not block P_{i} uptake or that PstS is inaccessible to rPst antibodies; the latter hypothesis is supported in part by the conserved nature of the protein, indicating that PstS is not under antigenic pressure. Presently, it remains unknown if vaccination with PstS is efficacious in an intranasal challenge model.

In summary, DDPCR identified an S. pneumoniae gene with enhanced transcription during MPC. Analysis of the gene identified it as pstS, the first gene transcribed from the pst locus. Characterization of pstS by Northern and Western dot blot analysis confirmed that transcription of pstS and production of its gene product were enhanced during MPC and during growth in low concentrations of P_{i}. While the specific PstS role in virulence has not been specifically determined, previous studies examining mutagenesis of other components in pst indicate that the complete operon is required for phosphate uptake and that mutagenesis of pst is pleiotropic, having multiple effects at multiple levels (12, 18, 20, 28). Unfortunately, our studies indicate that antibodies to rPstS do not appear to be protective, nor do they appear to block phosphate uptake in vitro.

Finally, because pstS is the first gene transcribed from pst, enhanced expression of pstS may indicate that other phosphate-dependent genes may also be enhanced in vivo. It is, however, important that physiological factors other than phosphate may be present within the MPC model and may be capable of inducing pstS. Future studies will be necessary to determine this.

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