

## *Streptococcus pneumoniae* Capsule Biosynthesis Protein CpsB Is a Novel Manganese-Dependent Phosphotyrosine-Protein Phosphatase

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Received 8 August 2001/Accepted 22 October 2001

**The first four genes of the capsule locus (*cps*) of *Streptococcus pneumoniae* (*cpsA* to *cpsD*) are common to most serotypes. We have previously determined that CpsD is an autophosphorylating protein-tyrosine kinase, demonstrated that CpsC is required for CpsD tyrosine-phosphorylation, and shown that CpsB is required for dephosphorylation of CpsD. In the present study we show that CpsB is a novel manganese-dependent phosphotyrosine-protein phosphatase that belongs to the PHP (polymerase and histidinol phosphatase) family of phosphoesterases. We also show that an *S. pneumoniae* strain with point mutations in *cpsB*, affecting one of the conserved motifs of CpsB, is unencapsulated and appears to be morphologically identical to a strain in which the *cpsB* gene had been deleted.**

*Streptococcus pneumoniae* (the pneumococcus) is an important cause of invasive disease in human populations throughout the world, resulting in high morbidity and mortality. An important feature of *S. pneumoniae* is its capacity to produce a polysaccharide capsule which is structurally distinct for each of the 90 known serotypes of the organism (2, 8). The capsular polysaccharide (CPS) is essential for pneumococcal virulence (2); all fresh clinical isolates of *S. pneumoniae* are encapsulated (smooth), and spontaneous nonencapsulated (rough) derivatives of such strains are almost completely avirulent.

The capacity to regulate CPS production in *S. pneumoniae* may be very important for the survival of the pneumococcus in different host environments. Maximal expression of CPS is essential for systemic virulence because of its antiphagocytic properties (2). However, invasive disease is invariably preceded by asymptomatic colonization of the nasopharynx, and the thickness of the capsule influences the degree of exposure of other important pneumococcal surface structures, such as the adhesins, which are required during this initial colonization phase. Pneumococci have been shown to undergo a bidirectional phase variation between two distinct colonial morphologies, described as “opaque” and “transparent.” The transparent phenotype exhibits an enhanced capacity to colonize the nasopharynx of infant rats (22), and the opaque form is associated with massively increased virulence in animal models of systemic disease. This correlates with increased production of CPS relative to cell wall teichoic acid in the opaque phenotype (11).

The first four genes of the *cps* locus (*cpsA* to *cpsD*) are associated with two distinct CPS regulatory mechanisms. These four genes are common to all pneumococcal serotypes, except types 3 and 37 (13, 18). Moreover, homologues of all of these genes are found in the *cps* loci of most streptococci, and ho-

mologues of *cpsB*, *cpsC*, and *cpsD* are found in capsule loci from other gram-positive genera. CpsA has homology to transcriptional regulators, and Cieslewicz et al. (5) have demonstrated that CpsA is a transcriptional activator that regulates the level of *cps* gene expression in *Streptococcus agalactiae*. We have shown that CpsB, -C, and -D are essential for encapsulation and regulate CPS production (17). CpsC and CpsD are predicted to function together in chain-length regulation and export of CPS, in a fashion similar to ExoP in exopolysaccharide (EPS) production in *Sinorhizobium meliloti* (6, 7) and Wzc in *Escherichia coli* (21, 23). We determined that CpsD, like Wzc, is an autophosphorylating protein-tyrosine kinase and demonstrated that CpsC is required for CpsD tyrosine phosphorylation, and we have shown that CpsB is required for dephosphorylation of CpsD (17). We have also shown that autophosphorylation of CpsD at tyrosines located near the carboxy terminus attenuates its activity and reduces the level of encapsulation. Thus, tyrosine phosphorylation of CpsD negatively regulates CPS production. As CpsB has no sequence similarity to known phosphotyrosine-protein phosphatases, such as Wzb, which is associated with gram-negative *cps* loci (21, 23), we have further investigated its role in dephosphorylation of CpsD and in encapsulation.

**Bacterial strains and plasmids.** The *S. pneumoniae* strain Rx1-19F is a derivative of Rx1 (a rough derivative of the type 2 strain D39) expressing type 19F capsule (16). Rx1-19F-B, in which the *cps19fB* gene has been interrupted with pVA891, has been described previously (7). Rx1-19F-B $\Delta$  and Rx1-19F-D $\Delta$ , containing defined in-frame deletion mutations in *cps19fB* and *cps19fD*, respectively, have also been described previously (17). Pneumococci were routinely grown either in Todd-Hewitt broth (Oxoid, Basingstoke, Hampshire, England) with 0.5% yeast extract (Difco Laboratories, Detroit, Mich.) or on blood agar. Where appropriate, erythromycin was added to medium at a concentration of 0.2  $\mu$ g/ml. The *S. pneumoniae* strains were transformed as described previously for strain D39 (3). The production of type 19F capsule by pneumococci was as-

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Cps19fB	<u>MIDIHSHIVFDVDDGPKSREESKALLAESYRQGVRTIVVTS</u> SHRRKGMFETPEEKIAENFL	60
Sagal-CpsB	<u>MIDIHSHIVFDVDDGPKTLEESLSLIEESYRQGVRIIVVTS</u> SHRRKGMFETPEDIIIFKNFS	60
Ssali-CpsB	<u>MIDVHSHIVFDVDDGPKTLEESLDLIGESYAQGVVKIIVVTS</u> SHRRKGMFETPEDKIFANFS	60
Sther-EpsB	<u>MIDVHSHIVFDVDDGPKTLEESLDLIGESYAQGVVKIIVVTS</u> SHRRKGMFETPEDKIFANFS	60
Ssuis-Cps2D	<u>MIDIHSHIIFGVDDGPKTLEESLSLIESEAYRQGVRYIVVTS</u> SHRRKGMFETPEKIIMINFL	60
Saure-Cap8C	<u>MIDIHNHILPNIDDGPTNETEMLDLLKQATTQGVTEIIVVTS</u> HLLHPRYTTPIEKVKSCLN	60
Bsubt-YwqE	<u>MIDIHCHILPAMDDGAGDSADSIEMARAARVQGI</u> RTIILATPHHNGVYKNEPAAVREAAD	60
Llact-EpsC	<u>MIDIHCHILPGIDDGAKTSGDTLMLKSAIDEGITTTTAT</u> PHHPNQFN.NESPLILKVK	59
	I II	
Cps19fB	QVREIAKEVADDLVIAYGAETIYYTLDALEKLEKKE.IPTLNDSRYALIEFSMHTSYRQIH	119
Sagal-CpsB	IVKHETEKRFEHLQILYGGELYTSDMLEKLEKLEKQ.IPTLNNTKFALEIEFSMOTSWKDIH	119
Ssali-CpsB	KVKAEEALYPDLTIYYGGELYTSDIVEKLE.KNLI PRMHNTQFALIEFSARTSWKEIH	119
Sther-EpsB	KVKAEEALYPDLTIYYGGELYTSDIVEKLE.KNLI PRMHNTQFALIEFSARTSWKEIH	119
Ssuis-Cps2D	QLKEAVAEVYPEIRLCYGAELYSKDILSKLEKKV.PTLNGSCYILLEFSTDTDPWKEIQ	119
Saure-Cap8C	HIESLEEVQALNLKFYGGQIRITDQILNDIDRQVITGINDS.RYLLIEFSPNEVPHYTD	119
Bsubt-YwqE	QLNKRLIKEDIPLHVLPGQEBIRIYGEVEQDLAKRQLLSLNDT.KYILIEFPFDHVPYRAE	119
Llact-EpsC	EVQNIIDEHQLPTEVLPQGEVRIYGDLLKEFSEGKLLKAAGTSSYILIEFSPNHVPAYAK	119
	* * * *	
Cps19fB	TGLSNILMLGITPVIAHIERYDALENNEKRVRELIDMGCYTQINSYHVSKPKFFGGEKYKF	179
Sagal-CpsB	TALSNVLMGITPVVAHIERYNALENQKERVKEIINMGCYTQINSFHLKQKLFNDKHKR	179
Ssali-CpsB	SGLSNVLRAGVTPVIAHIERYDALEENADRVEIINMGCYTQVNSSHVLPKPLFGDKDKV	179
Sther-EpsB	SGLSNVLRAGVTPVIAHIERYDALEENADRVEIINMGCYTQVNSSHVLPKPLFGDKDKV	179
Ssuis-Cps2D	EAVNEMTLGLTPVLAHIERYDALAFQSERVEKLIDKGCYTVNSNHVLPKALIGERAKE	179
Saure-Cap8C	QLFPFELQSKGFVPIIAHPERNKAI SQNLDIYDLINKGALSQVTTASLAG....ISGKK	174
Bsubt-YwqE	QLFYDLQLKGYIPIVIAHPERNREIRENPSLLYHLVKEGAASQITSGSLAG....IFGKQ	174
Llact-EpsC	ELFYNIKLEGLQPIVILHPERNSGIENPDILFDFIEQGVLSQITASSVTG....HFGKK	174
	III	
	* * *	
Cps19fB	MKKRARYFLERDLVHVVASDMHNLDSRPPYMQQAYDI IAKKYGAKKAKELFVDNPRKIIM	239
Sagal-CpsB	FKKRARYFLEENLVHFVASDMHNLDRPPFLAEAYKIICRDFGKERANQLFIENAQSILK	239
Ssali-CpsB	RKKRVRVYFLEKNLVHMVASDMHNLGPRPPFMKDAYEIVKKNYGSKRPKNLFIENPKTLIE	239
Sther-EpsB	RKKRVRVYFLEKNLVHMVASDMHNLGPRPPFMKDAYEIVKKNYGPKRAKNLFIENPKTLIE	239
Ssuis-Cps2D	FKKRTRYFLEQDLVHCVASDMHNLYSRPPFMREAYQLVKKEYGEDRAKALFKKNPLLLIK	239
Saure-Cap8C	IRKLAIQMIENNLTHFIGSDAHNTEIRPFLMKDLFNDKCLR.D.YYEDMNGFISNAKLVD	233
Bsubt-YwqE	LKAFSLRLVEANLIHFVASDAHNVTNRNFHTQEALYVLEKEFG.SELPYMLTENAEKLLR	233
Llact-EpsC	IQKLSFKMIENNLTHFVASDAHNVTSRAFKMEKAEFEI IEDSYG.SGVSRLQNNADSVIL	233
	IV	
Cps19fB	DQLI.....	243
Sagal-CpsB	NHYI.....	243
Ssali-CpsB	NQYL.....	243
Sther-EpsB	NQYL.....	243
Ssuis-Cps2D	NQVQ.....	243
Saure-Cap8C	DKKIPKRMPOODYKQKRWFGL	254
Bsubt-YwqE	NQTI FRQPPQVVKRKLFGFF	254
Llact-EpsC	NESFYQEEP I KIKTKKFLGLF	254

FIG. 1. Alignment of Cps19fB with *Streptococcus agalactiae* CpsB (Sagal-CpsB; GenBank accession number AF163833), *Streptococcus salivarius* CpsB (Ssali-CpsB; Y17900), *Streptococcus thermophilus* EpsB (Sther-EpsB; U40830), *Streptococcus suis* Cps2D (Ssuis-Cps2D; AF118389), *Staphylococcus aureus* Cap8C (Saure-Cap8C; U73374), *Bacillus subtilis* YwqE (Bsubt-YwqE; Z92952), and *Lactococcus lactis* EpsC (Llact-EpsC; AF036485). The four conserved motifs are underlined, and identical residues are shaded; the asterisks indicate amino acids predicted to be essential for function. The arrows indicate amino acids mutated in Rx1-19f-B<sub>NO</sub>, and the absence of a residue is indicated by a period.

sessed by quelling reaction with monospecific antisera obtained from Statens Seruminstitut, Copenhagen, Denmark.

**CpsB is a member of the PHP family of phosphoesterases.** A database search using CD-Search (National Center for Biotechnology Information) identified CpsB as a member of the PHP (polymerase and histidinol phosphatase) family of phosphoesterases. Aravind and Koonin (1) divided the members of the PHP superfamily into five distinct protein families, which include phosphoesterases with distinct functions: the N-terminal domains of bacterial DNA polymerase III  $\alpha$  subunits, stand-alone proteins from various bacteria, C-terminal domains of family X DNA polymerases from various bacteria, histidinol phosphatases, and CpsB homologues encoded by *cps* loci from gram-positive bacteria. The only member of this family of proteins for which phosphoesterase activity has actually been demonstrated is the yeast histidinol phosphatase. This enzyme is involved in the ninth step of histidine biosyn-

thesis and cleaves the phosphomonoester bond in histidinol phosphate to release histidinol (10). Aravind and Koonin (1) identified four conserved motifs in all members of the PHP superfamily. Figure 1 shows an alignment of Cps19fB with several homologous proteins with the multiple sequence alignment settings of the program DNAMAN (Lynnon BioSoft, Quebec, Canada). The four conserved motifs and the amino acids predicted to be essential for function are highlighted. Each of the four motifs has conserved histidine residues which were predicted to be involved in metal binding (possibly zinc or nickel). These proteins were predicted to require two coordinated metal cations for enzymatic activity. Indeed, a mutation affecting the conserved histidine in motif 3 in the yeast histidinol phosphatase resulted in a histidine-requiring phenotype (14). The conserved aspartate residues are predicted to be essential for catalysis by participating in electron transfer (1).



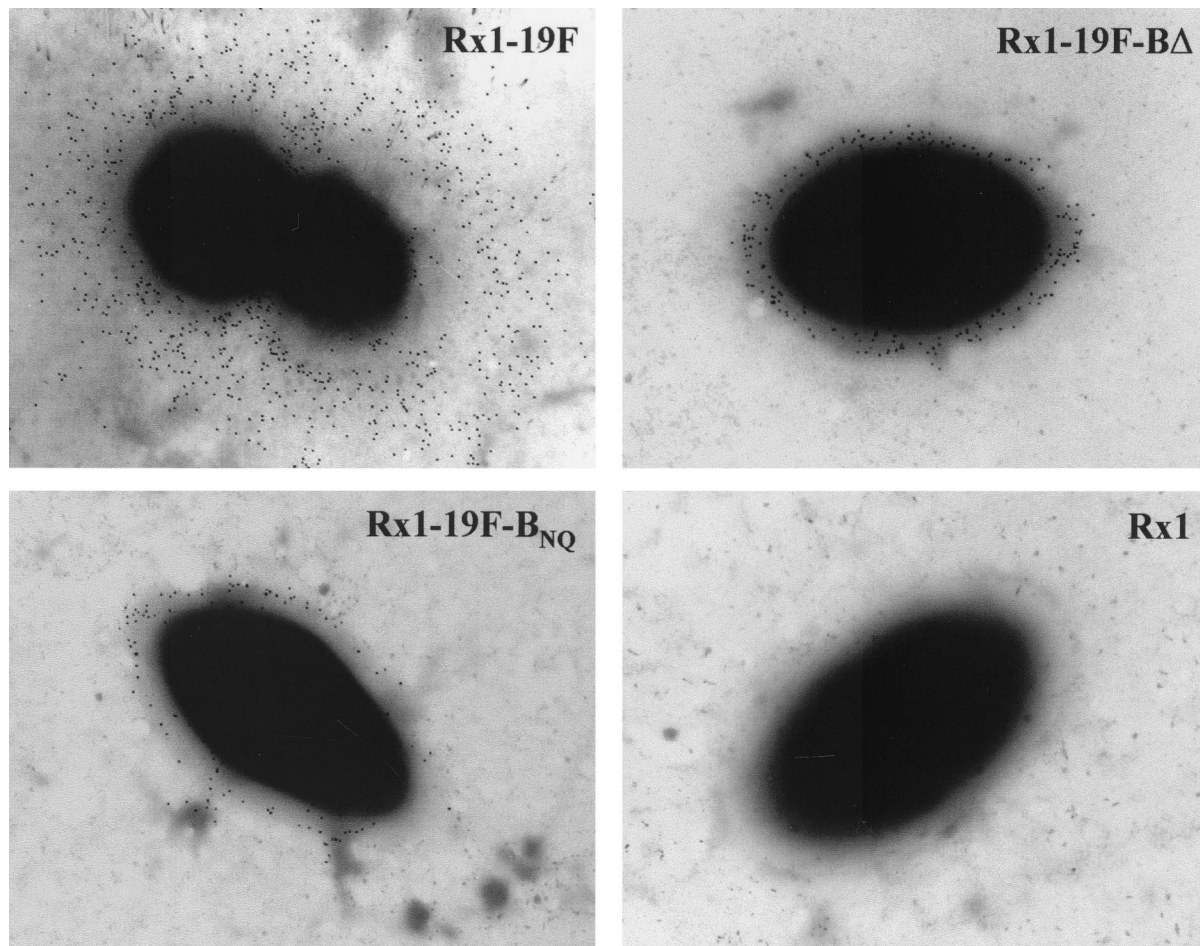


FIG. 2. Detection of CPS on the cell surface of *S. pneumoniae* Rx1-19F and the two *S. pneumoniae* *cpsB* mutants by immunogold electron microscopy. Digital images were taken at  $\times 20,000$  magnification. A single representative bacterium is shown for Rx1-19F, Rx1-19F-B $\Delta$ , Rx1-19F-B<sub>NQ</sub>, and the nonencapsulated strain Rx1.

**Construction of *S. pneumoniae* containing a defined mutation in *cpsB*.** An *S. pneumoniae* strain containing defined mutations in motif 4 of CpsB, in which the aspartate at position 199 was changed to asparagine (D<sub>199</sub>N) and the histidine at position 201 was changed to glutamine (H<sub>201</sub>Q) (Fig. 1), was constructed by using overlap-extension PCR as described by Horton (9). The required changes were incorporated into overlapping primers J194 (GTTGCAAGTAACATGCAGAATTTAGAC; nucleotides [nt] 2201 to 2227 of the *cps19f* sequence, GenBank accession no. U09239) and J195 (GTCTAAATTCTGCATGTTACTTGCAAC; complementary to nt 2201 to 2227). The nucleotide changes relative to the wild-type sequence are underlined. Separate PCRs were set up using primers J195 and CPS5' (TGATGTTCAAGGTATAGGTGTTAA TCA; nt 146 to 169) as well as primers J194 and J6 (TTGGA TATGACCAGACAAGAC; complementary to nt 5704 to 5724). A second round of PCR amplification was then performed using primers CPS5' and J6 and both purified PCR products as a template. The final 6-kb CPS5'-J6 PCR product incorporating the specific mutations was transformed directly into the insertion-duplication mutant Rx1-19F-B (in which the *cps19fB* gene has been interrupted with pVA891). The transformants were initially screened for loss of erythromycin resis-

tance, and the presence of the specific point mutations was confirmed by the sequencing of PCR products. This mutant was designated Rx1-19F-B<sub>NQ</sub>.

The phenotype of Rx1-19F-B<sub>NQ</sub> was then compared with that of Rx1-19F-B $\Delta$ , in which the entire *cpsB* gene has been deleted. When grown on blood agar plates, both strains exhibited a "rough" colony morphology. Further examination of capsule production by immunogold electron microscopy, performed as described elsewhere (19), could not distinguish between Rx1-19F-B<sub>NQ</sub> and Rx1-19F-B $\Delta$ . The bound polyclonal rabbit anti-type 19F antiserum was detected with protein A-gold conjugate (10 nm; Sigma), and the grids were examined with a Phillips CM100 transmission electron microscope operated at 80-kV accelerating voltage. As shown in Fig. 2, both strains have a small amount of anti-type 19F CPS-reactive material covering the cell surface as previously described for Rx1-19F-B $\Delta$  (17).

Western blot analysis of Rx1-19F, Rx1-19F-B $\Delta$ , and Rx1-19F-B<sub>NQ</sub> was also undertaken. Bacterial cell lysates from 1 ml of log-phase culture ( $A_{600} = 0.27$ ; containing  $5 \times 10^8$  CFU/ml) were separated on sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis gels (12) prior to transfer onto nitrocellulose or polyvinylidene difluoride (PVDF) membranes

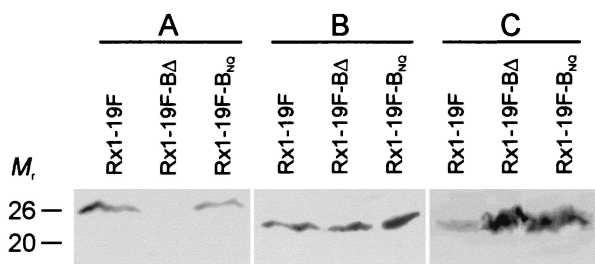


FIG. 3. Western blot analysis. *S. pneumoniae* Rx1-19F, Rx1-19F-B $\Delta$ , and Rx1-19F-B<sub>NQ</sub> lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted onto nitrocellulose (A and B) or PVDF (C) membranes. Filters were probed with mouse polyclonal anti-CpsB antibody (A), mouse polyclonal anti-CpsD antibody (B), or mouse antiphosphotyrosine monoclonal antibody clone 4G10 (C) to detect CpsB, CpsD, and CpsD-P, respectively. The mobility of molecular size markers is also indicated in kilodaltons.

(20). Membranes were probed with either mouse antiphosphotyrosine monoclonal antibody clone 4G10 (Upstate Biotechnology, Lake Placid, N.Y.) used at a dilution of 1:2,000, mouse anti-CpsB antiserum used at 1:500, or mouse anti-CpsD antiserum used at 1:100, followed by the addition of a 1:5,000 dilution of goat anti-mouse immunoglobulin G conjugated to horseradish peroxidase (Bio-Rad Laboratories, Hercules, Calif.). Enzyme-labeled bands were visualized by using Lumi-Light Western blotting substrate (Roche Molecular Biochemicals, Mannheim, Germany). These results confirm that Rx1-19F-B<sub>NQ</sub> produces a mutant CpsB protein, which is detectable by Western blotting, but this protein is absent in Rx1-19F-B $\Delta$  (Fig. 3A). Although all three strains contain similar amounts of CpsD (Fig. 3B), both Rx1-19F-B<sub>NQ</sub> and Rx1-19F-B $\Delta$  contain elevated levels of the phosphorylated form of CpsD (CpsD-P) compared to Rx1-19F (Fig. 3C). These results indicate that mutations D<sub>199</sub>N and H<sub>201</sub>Q collectively inactivate CpsB, dem-

onstrating that the conserved motifs are indeed important for function, as predicted above.

**Detection of phosphatase activity associated with CpsB.** Phosphatase activity was initially investigated in deoxycholate cell lysates of Rx1-19F by assaying its ability to cleave *p*-nitrophenyl phosphate at different temperatures and at different pH values. Deoxycholate cell lysates were prepared by resuspending pneumococcal cells, grown on blood agar plates overnight at 37°C, in 150 mM Tris-HCl (pH 7.0), such that the absorbance at 600 nm ( $A_{600}$ ) was 0.6. The cells were then pelleted in a bench centrifuge and resuspended in a one-fifth volume of 100 mM sodium citrate (pH 6.5), 1 M Tris-HCl (pH 7.0), or 1 M Tris-HCl (pH 8.0), and 100- $\mu$ l aliquots were then used in the assay. The cells were lysed by the addition of 0.1% (wt/vol) deoxycholate (final concentration), followed by incubation at 37°C for 10 min. The reaction was started by addition of 10  $\mu$ l of 5 mg of *p*-nitrophenyl phosphate (Sigma)/ml in 100 mM Tris-HCl (pH 7.0). After incubation for 1 h at 37°C, the cell debris was pelleted, and the  $A_{410}$ s of the supernatants were measured. The phosphatase activity was expressed as units, where one unit is the amount of enzyme that produces 1 nmol of *p*-nitrophenol per h per ml of cell lysate at 37°C (15). No phosphatase activity was detected when the assay was carried out in 100 mM sodium citrate (pH 6.5). In 1 M Tris-HCl (pH 7.0) buffer, phosphatase activity was detectable, but activity was substantially higher when assayed in 1 M Tris-HCl (pH 8.0) (data not shown). Phosphatase activity at 37°C was approximately double that detectable at room temperature (data not shown).

Phosphatase activity in lysates of Rx1-19F, Rx1-19F-B $\Delta$ , and Rx1-19F-B<sub>NQ</sub> was then compared by using 1 M Tris-HCl (pH 8.0) buffer, with incubation at 37°C for 1 h, in the presence or absence of various metal ions (Fig. 4). MgSO<sub>4</sub>, CaCl<sub>2</sub>, MnCl<sub>2</sub>, CoCl<sub>2</sub>, NiSO<sub>4</sub>, CuSO<sub>4</sub>, or ZnCl<sub>2</sub> was added to a final concentration of 1 mM where appropriate. The results of three inde-

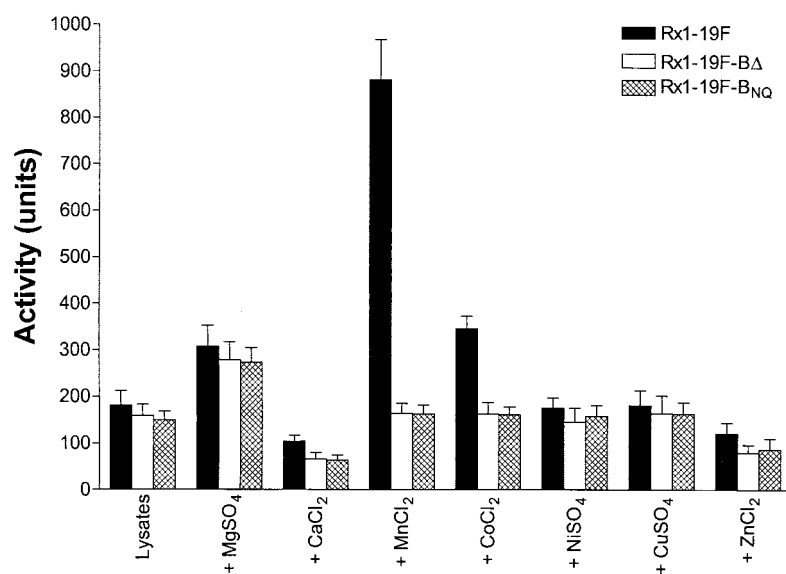


FIG. 4. Phosphatase assay. The ability of deoxycholate lysates from Rx1-19F, Rx1-19F-B $\Delta$ , and Rx1-19F-B<sub>NQ</sub> to cleave *p*-nitrophenyl phosphate in 1 M Tris-HCl (pH 8.0) in the presence or absence of various metal salts was assayed by measuring the  $A_{410}$  and expressing the activity in units (per milliliter of lysate) after incubation at 37°C for 1 h. Data are the means of three independent assays  $\pm$  the standard errors of the means (SEM).

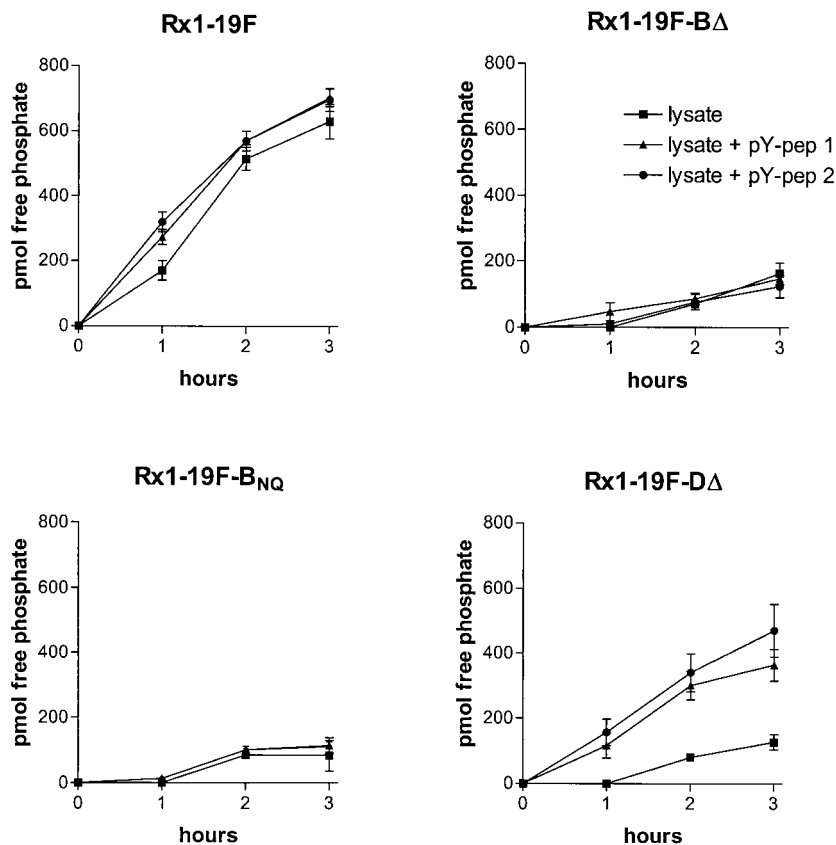


FIG. 5. Phosphotyrosine peptide-based phosphatase assay. The amount (in picomoles) of free phosphate generated at 37°C by 45 μg of cellular protein (50 μl cell lysate) from Rx1-19F, Rx1-19F-BΔ, Rx1-19F-B<sub>NQ</sub>, and Rx1-19F-DΔ was measured at the indicated time points. The phosphopeptides pY-pep 1 (END[pY]INASL) and pY-pep 2 (DADE[pY]LIPQQG) were added at a final concentration of 100 μM to the lysates where indicated. Data are the means of three independent assays ± the SEM.

pendent experiments were analyzed by using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, Calif.). A baseline level of phosphatase activity of up to 200 U was observed for all three strains with or without 1 mM CaCl<sub>2</sub>, NiSO<sub>4</sub>, CuSO<sub>4</sub>, or ZnCl<sub>2</sub>. The addition of 1 mM MgSO<sub>4</sub> stimulated phosphatase activity in lysates from all three strains ca. 1.5-fold. However, in the presence of 1 mM MnCl<sub>2</sub> and, to a lesser extent, 1 mM CoCl<sub>2</sub>, there was a marked and statistically significant elevation of phosphatase activity in the Rx1-19F lysate (ca. 900 and 350 U, respectively), relative to the levels in either of the *cpsB* mutant lysates (<200 U in all cases; *P* = 0.0013 and *P* = 0.0076 for MnCl<sub>2</sub> and CoCl<sub>2</sub>, respectively, as determined by unpaired *t* test in the GraphPad Prism software package). These results suggest that CpsB is a metal-binding phosphatase requiring manganese for optimal activity.

**Detection of tyrosine phosphatase activity associated with CpsB.** Protein phosphotyrosine phosphatase activity in cell lysates from Rx1-19F, Rx1-19F-BΔ, Rx1-19F-B<sub>NQ</sub>, and Rx1-19F-DΔ (a *cpsD* deletion mutant that does not express CpsD) was investigated by using the Tyrosine Phosphatase Assay System (Promega Corporation, Madison, Wis.). Rx1-19F-DΔ was included in this assay because CpsD-P, the natural substrate for CpsB, cannot be produced in this strain, and phosphate should only be released when a synthetic peptide substrate is added to the cell lysate (see below). Cell lysates were prepared

by resuspending pneumococcal cells, grown on blood agar plates overnight at 37°C, in 150 mM Tris-HCl (pH 8.0) such that the *A*<sub>600</sub> was 0.6. The cells were pelleted in a bench centrifuge, resuspended in one-fifth volume of 150 mM Tris-

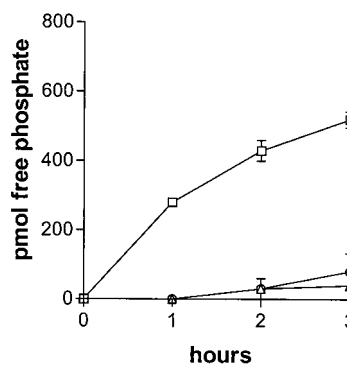


FIG. 6. Dephosphorylation of CpsD-P by CpsB. Cell lysates (50 μl containing 45 μg of protein) from Rx1-19F-BΔ, Rx1-19F-DΔ, or both lysates combined were incubated at 37°C, and the amount of free phosphate (pmol) was determined at the indicated times. Symbols: □, Rx1-19F-BΔ-Rx1-19F-DΔ lysate mix; ○, Rx1-19F-DΔ lysate; △, Rx1-19F-BΔ lysate. Data are the means of three independent assays ± the SEM.



HCl (pH 8.0), and then lysed by using a French pressure cell (deoxycholate interferes with this assay system). The lysates were centrifuged to remove the insoluble cell wall material, and the protein concentration of the supernatant was determined according to the method of Bradford (4). The protein concentrations of the lysates were consistently ca. 900  $\mu\text{g}/\text{ml}$ . The lysates were treated to deplete endogenous free phosphate and ATP according to the manufacturer's instructions. The lysates were then assayed for tyrosine phosphatase activity by incubation at 37°C, in the presence of 1 mM  $\text{MnCl}_2$ , for up to 3 h with or without the addition of the synthetic phosphotyrosine peptides pY-pep 1 (END[pY]INASL) and pY-pep 2 (DADE[pY]LIPQOG). The amount of phosphate released in 50  $\mu\text{l}$  of cell lysate was determined by measuring the  $A_{600}$  after addition of molybdate dye solution (Promega). Free phosphate was quantitated by using a standard curve generated from samples containing known free phosphate concentrations. No phosphate was released in the absence of  $\text{MnCl}_2$  (data not shown).

After 3 h, the wild-type Rx1-19F lysate liberated 600 to 750 pmol phosphate per 45  $\mu\text{g}$  of soluble cellular protein compared to less than 200 pmol for the lysates of CpsB mutants Rx1-19F-B $\Delta$  and Rx1-19F-B<sub>NO</sub> ( $P < 0.0001$ ) (Fig. 5). The addition of phosphotyrosine-containing peptides did not affect the amount of phosphate released by lysates from these strains. All three of these strains contain CpsD-P, which is the natural substrate for CpsB. Thus, the phosphate release observed in the Rx1-19F lysate is likely to be due to CpsB acting on CpsD-P. This activity is greatly reduced in the Rx1-19F-B $\Delta$  and Rx1-19F-B<sub>NO</sub> lysates due to the lack of functional CpsB proteins in these strains. The background activity observed in the Rx1-19F-B $\Delta$  and Rx1-19F-B<sub>NO</sub> lysates is probably due to different phosphatases acting on endogenous cellular substrates. In contrast, when Rx1-19F-D $\Delta$  was assayed, the addition of the phosphotyrosine-containing peptides had a significant impact on the amount of phosphate liberated. The CpsB present in the Rx1-19F-D $\Delta$  lysate was able to liberate phosphate from pY-pep 2 (470 pmol of phosphate) more efficiently than from pY-pep1 (360 pmol of phosphate). In both cases phosphate release was significantly greater than in the absence of substrate ( $P = 0.0154$  and  $P = 0.0121$ , respectively). This demonstrates that CpsB is able to dephosphorylate the synthetic phosphotyrosine-containing peptide substrates in the absence of CpsD-P, its natural substrate.

To directly demonstrate the dephosphorylation of CpsD-P by CpsB, the Rx1-19F-B $\Delta$  and Rx1-19F-D $\Delta$  lysates were mixed and incubated at 37°C. The release of phosphate was significantly greater than that seen with either lysate on its own ( $P = 0.0009$ ) (Fig. 6). Thus, CpsB in the Rx1-19F-D $\Delta$  lysate is able to dephosphorylate the CpsD-P in the Rx1-19F-B $\Delta$  lysate. Together, these results indicate that CpsB is able to dephosphorylate both CpsD-P and synthetic phosphotyrosine-containing peptides and is indeed a phosphotyrosine-protein phosphatase.

**Conclusions.** In this study we have demonstrated that CpsB is a novel phosphotyrosine-protein phosphatase. CpsB is only the second member of the PHP family of putative phosphoesterases for which such enzymatic activity has actually been demonstrated, strengthening the probability that other members of this family also have phosphoesterase activity. The four conserved motifs containing histidine residues present in all

members of the PHP superfamily were predicted to be involved in metal binding (1). We have demonstrated here that CpsB is dependent on the presence of manganese for optimal activity. Moreover, inactivation of CpsB by mutagenesis of motif 4 confirms the important role of these conserved histidine residues.

We have previously hypothesized that CpsC, CpsD, and ATP interact and that tyrosine phosphorylation of CpsD negatively regulates CPS production (17). In the present study we have shown that CpsB is the phosphotyrosine-protein phosphatase required to dephosphorylate CpsD and thus plays an important role in the regulation of CPS production in *S. pneumoniae*. We are currently investigating how the degree of CpsD-P phosphorylation affects CPS synthesis and the role of phosphorylation in CpsC and CpsD function.

This work was supported by grants from the Channel 7 Children's Research Foundation and the National Health and Medical Research Council of Australia (grant 157923).

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