CpsB Is a Modulator of Capsule-associated Tyrosine Kinase Activity in Streptococcus pneumoniae*

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Tyrosine phosphorylation is associated with polysaccharide synthesis in a number of Gram-positive and Gram-negative bacteria. In Streptococcus pneumoniae, CpsB, CpsC, and CpsD affect tyrosine phosphorylation and are critical for the production of a mature capsule in vitro. To characterize the interactions between these proteins and the phosphorylation event they modulate, cps2B, cps2C, and cps2D from the capsule type 2 S. pneumoniae D39 were cloned and expressed both individually and in combination in Escherichia coli. Cps2D purified from E. coli was not phosphorylated unless it was co-expressed with its cognate transmembrane domain, Cps2C. Purified phosphorylated Cps2D had tyrosine kinase activity and could phosphorylate both dephosphorylated Cps2D and an exogenous substrate (poly-Glu-Tyr) in the absence of ATP. Cps2B exhibited phosphatase activity against both purified phosphorylated Cps2D and p-nitrophenyl phosphate. An additional role for Cps2B as an inhibitor of Cps2D phosphorylation was demonstrated in both co-expression experiments in E. coli and in vitro experiments where it blocked the transphosphorylation of Cps2D even in the presence of the phosphatase inhibitor sodium orthovanadate. cps2C and cps2D deletion mutants in S. pneumoniae produced no detectable mature capsule during laboratory culture. Both were avirulent in systemic mouse infections and were unable to colonize the nasopharynx, suggesting that the failure to produce capsule was not dependent on the environment. Based on these results, we propose a model for capsule regulation where CpsB, CpsC, CpsD, and ATP form a stable complex that enhances capsule synthesis.

Production of a polysaccharide capsule is essential for Streptococcus pneumoniae virulence and colonization (1–4). Capsule production allows S. pneumoniae to circumvent host defenses by blocking antibody deposition, reducing complement activation, and attenuating opsonophagocytosis (5–8). To date, 90 serotypes are identified among serologically and structurally distinct capsular polysaccharides. To date, 90

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1 M. H. Bender and J. Yother, unpublished data.
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**TABLE I**

| Strain or plasmid | Relevant properties | Reference or source |
|-------------------|---------------------|---------------------|
| **S. pneumoniae** |                     |                     |
| D39               | Δcys2D, Cps°         | Ref. 43             |
| MB512             | Δcys2D, Cps°         | This study          |
| MB513             | Δcys2D, Cps°         | This study          |
| MB516             | Δcys2C, Cps°         | This study          |
| MB517             | Δcys2C, Cps°         | This study          |

**E. coli**

DH5αF<sup>T</sup> Lacks origin of replication for lac F<sup>T</sup> linked 2) to the first rhamnose in the backbone from the side chain glucose (30). The sequence of the type 2 locus containing the genes required to produce this polymer has been determined, and the type 2 common genes are highly homologous to those in all *S. pneumoniae* capsule types (31). Here, we demonstrate tyrosine kinase activity for Cps2D and phosphatase activity for Cps2B. We also show that Cps2B has two different activities that may modulate capsule production in *S. pneumoniae*. Our results suggest that CpsB may be a central point of control for the capsule-associated phosphotyrosine regulatory system.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Culture Conditions**—The bacterial strains and plasmids used in this work are listed in Table I. Primers are listed in Table II. *S. pneumoniae* strains were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract (Difco) or on blood agar base 2 (Difco) containing 3% sheep red blood cells (Colorado Serum Company). *E. coli* DH5α, BL21(ADE3), and JM109 were grown and maintained in LB medium. *E. coli* BL21-SI was grown and maintained in LBON (LB without the addition of glucose or NaCl) to ensure the repression of the salt-inducible proU promoter (32). Where appropriate, the media were supplemented with erythromycin (0.3 μg ml<sup>−1</sup> for *S. pneumoniae* or 300 μg ml<sup>−1</sup> for *E. coli*), kanamycin (50 μg ml<sup>−1</sup>), and/or ampicillin (100 μg ml<sup>−1</sup>).

**Cloning and Expression of His<sub>g</sub>-tagged Proteins**— Primer sets Cps2-D6/NdeI-Cps2-D5(XhoI), Cps2-C6/NdeI-Cps2-D5(XhoI), and Cps2-B9/BamHI/Cps2-B10/HindIII were used to amplify DNA from the *S. pneumoniae* D39 chromosome for use in the construction of vectors expressing either a Cps2D-His, a Cps2C/Cps2D-His, or a Cps2B-His

Glc-linked α-(1→2) to the first rhamnose in the backbone from the side chain glucose (30). The sequence of the type 2 locus containing the genes required to produce this polymer has been determined, and the type 2 common genes are highly homologous to those in all *S. pneumoniae* capsule types (31). Here, we demonstrate tyrosine kinase activity for Cps2D and phosphatase activity for Cps2B. We also show that Cps2B has two different activities that may modulate capsule production in *S. pneumoniae*. Our results suggest that CpsB may be a central point of control for the capsule-associated phosphotyrosine regulatory system.

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fusion protein, respectively (restriction sites added to facilitate cloning are indicated in parentheses). PCR-amplified products were digested appropriately, electrophoresed through a 0.8% agarose gel, extracted (Gene Clean III kit, Qiogene), and ligated into either pET-20b for Cps2D-His and Cps2C-Cps2D-His or pQE-41 for Cps2D-His. Ligation products were transformed into DH5α (pET-20b derivatives) or JM109 (pQE-41 derivatives). The pMB042 insert encodes a C-terminal His$_{6}$-tagged fusion protein of Cps2D, whereas pMB048 also includes the region upstream of csp2D that contains cpse$_{2C}$. pMB053 contains a His$_{6}$ tag at the N terminus of Cps2B. The presence of the correct inserts was confirmed by restriction enzyme digestion and sequencing (University of Alabama at Birmingham Core Sequencing Facility). pMB042 and pMB048 were transformed into BL21 (DE3) by electroporation, resulting in strains MB043 and MB049. Purification of His$_{6}$ Fusion Proteins—Phosphorylated and unphosphorylated Cps2D (isolated from MB049 and MB043, respectively) were purified during denaturation from 6 M guanidine hydrochloride to both lyse the cells and bind the extract to the nickel-nitrioltriacetic acid column resin (Qiagen), as described in the QIAexpressionist handbook. Denaturing conditions facilitated the separation of Cps2C and Cps2D.

| Primer* | Sequence* | Description |
|---------|-----------|-------------|
| Cps2-B4 (+, AD) | GTCAGGAACTTTTTATAGAC | Cps2B | 5′-3′ |
| Cps2-C4 (+, AD) | CGGCTTCTTTTTATAGAC | Cps2C | 3′-5′ |
| Cps2-D4 (+, AD) | CGGTCTCTTTTTATAGAC | Cps2D | 5′-3′ |
| Cps2-E1 (+, AD) | GTCACAATTTTTATAGAC | Cps2E | 3′-5′ |
| Cps2-B2 (+, AD) | CGGTAATTTTTATAGAC | Cps2B | 3′-5′ |
| Cps2-B5 (+, AD) | CGGACATTTTTATAGAC | Cps2B | 5′-3′ |
| Cps2-C2 (+, AD) | CGGATCTTTTTATAGAC | Cps2C | 3′-5′ |
| Cps2-D1 (+, AD) | CGGACGATTTTTATAGAC | Cps2D | 3′-5′ |

* Forward and reverse primers are represented by plus (+) and minus (−), respectively. Letters represent genes that were deleted using the indicated primers.

Restriction sites are underlined, and some primers have an additional CC to facilitate digestion of the PCR product.

Location of each primer is denoted by the gene name in which the primer resides. Numbers in superscript represent the positions of the start and finish of the homologous region of the primer when compared with the published type 2 sequence (accession number AP026471) (31).

During purification from MB049. Refolding of Cps2D on the column was accomplished by washing with a step gradient of 0–6 M urea in refolding buffer (0.5 M NaCl, 0.1 M Tris, 20% glycerol, pH 7.4) (34). Elution was performed using refolding buffer containing 250 mM imidazole. Eluted fractions were separated by 12% SDS-PAGE and analyzed by Coomassie staining of SDS-PAGE gels and immunoblotted for detection of the His$_{6}$ tag. Cps2B-containing fractions were concentrated and desalted in a Centriprep YM-10 spin concentrator (Amicon) and stored at −20 °C. Cps2B was purified using native column resin (Qiagen) as described in the QIAexpressionist handbook. Native lysis buffer (50 mM Na$_{2}$PO$_{4}$, 300 mM NaCl, 10 mM imidazole, pH 8.0) was supplemented with 1 mM phenylmethylsulfonfluoride to reduce degradation of purified protein. After elution from the nickel-nitrioltriacetic acid column with 250 mM imidazole and a second round of nickel-nitrioltriacetic acid purification, the samples were analyzed by Coomassie staining of SDS-PAGE gels and immunoblotted for detection of the His$_{6}$ tag. Cps2B-containing fractions were concentrated and desalted in a Centriprep YM-10 spin concentrator (Amicon) and stored at −20 °C in lysis buffer supplemented with 20% glycerol and 1 mM phenylmethylsulfonfluoride. Protein concentrations were determined using the Bio-Rad protein assay with sample readings being compared with a standard curve of BSA. Tyrosine kinase activity of purified Cps2D was confirmed by radiolabeling experiments with [γ-32P]ATP using methods identical to those described previously by Vincent et al. (21) in the study of Wzc.

Detection of Transphosphorylation—Tyrosine kinase activity of Cps2D was assessed using the protein-tyrosine kinase kit from Sigma (PTK-101), which utilizes the synthetic substrate poly-Glu-Tyr (31.25 μg ml$^{-1}$) bound to the wells of a microtiter plate. Purified epidermal growth factor receptor (EGFR) was used as a positive control and to quantify activity. Activity was determined in a 100-μl reaction containing 50 mM HEPES (pH 7.4), 20 mM MgCl$_{2}$, 0.1 mM MnCl$_{2}$, 0.2 mM Na$_{2}$VO$_{4}$, 0.3 mM ATP, and varying amounts of Cps2D–P. Wells containing buffer alone were used as a negative control and subtracted out as background. After 30 min, the wells were washed five times with PBS and then reacted with a 1:10,000 dilution of the α-Tyr(P) (PT-66–HRP). Bound antibody was detected using 100 μl of an α-phenylenediamine dihydrochloride substrate solution (Sigma Fast tablets yielding 0.4 mg ml$^{-1}$ α-phenylenediamine dihydrochloride; Sigma), which was allowed to react for 7 min before stopping with the addition of 100 μl of 2.5 mM H$_{2}$SO$_{4}$. The reaction was quantified by measuring absorbance at 492 nm.

Immununological detection of phosphorylated proteins separated by 12% SDS-PAGE (33) was performed using either a monoclonal antibody against phosphorysranine conjugated to horseradish peroxidase (α-Tyr(P)) (PT-66–HRP, Sigma), a monoclonal antibody against phosphoserine (PSR-45; Sigma), a monoclonal antibody against phosphothreonine (PT-8; Sigma), or a monoclonal antibody against tetrahistidine (α-TetraHis; Qiagen). The proteins were transferred onto nitrocellulose membranes using the semi-dry transfer apparatus (Bio-Rad). The membranes were blocked for 1 h at room temperature in either 3% blot-qualified bovine serum albumin (BSA) (Promega) in Tris-buffered saline (100 mM Tris, pH 7.4, 0.9% NaCl) with 0.05% Tween 20 (TBST) for phosphorysranine detection or with 1% BSA in PBS (140 mM NaCl, 3 mM KCl, 5 mM Na$_{2}$HPO$_{4}$, 2 mM KH$_{2}$PO$_{4}$, pH 7.4) with 0.05% Tween 20 (PBST) for detection of the His$_{6}$ tag. The membranes were probed using a 1:15,000 dilution of antibody in either TBST (α-Tyr(P), the monoclonal antibody against phosphoserine, or the monoclonal antibody against phosphothreonine) or PBST (α-TetraHis). After incubation for 1 h, detection of reactive bands was performed either indirectly using a secondary goat α-mouse immunoglobulin conjugated to biotin (G-α-M-Ig-biot) (Southern Biotechnology Associates) and streptavidin conjugated to alkaline phosphatase (SAP) (Southern Biotechnology Associates) or directly using the HRP conjugate. Reactivity was visualized using either 5-bromo-4-chloro-3-indolyl-phosphate and nitroblue tetrazolium (5 and 1 mg ml$^{-1}$, respectively) for SAP detection or Supersignal substrate (Pierce) for HRP detection. Photometric imaging from the HRP-labeled blots was assayed using X-Omat film (Kodak). Relative sizes of the proteins were determined by comparison with a prestained low molecular mass marker (Bio-Rad).

Detection of His$_{6}$ Fusion Proteins—Phosphorylated and unphosphorylated Cps2D (isolated from MB049 and MB043, respectively) were purified under denaturing conditions (6 M guanidine hydrochloride) to both lyse the cells and bind the extract to the nickel-nitrioltriacetic acid column resin (Qiagen), as described in the QIAexpressionist handbook. Denaturing conditions facilitated the separation of Cps2C and Cps2D.
Buffers (50 mM) used to assess Cps2B activity included HEPES (pH 6.0 to 9.0), Tricine-HCl (pH 7.0 to 9.0), succinate (pH 5.0–6.5), Tris acetate (pH 7.5–9.0), GlyGlycine (pH 8.0–9.5), monobasic sodium phosphate (pH 6.0–8.5), and 100 mM citrate (pH 6.5).

Tyrosine dephosphorylation was determined by ELISA assays using Cps2B–His (MB053) on a Bradford plate overnight at 4 °C in PBS as a substrate. After binding, plates were blocked with 1% BSA in PBS for 60 min before being treated with Cps2B in the HEPES buffer described above lacking pNPP. After 60 min, the wells were washed with PBST, and the remaining phosphoryrosines were detected using α-TyrP (1:10,000 dilution). G-α-M-Ig-biot, SAP, and pNPP were used for detection, as described above. All assays were performed in triplicate, and at least three independent assays were performed.

**Dual Induction of Cps2D–P and Cps2B–Cps2D–P and Cps2B were expressed in E. coli BL21-SI using independent induction systems that allowed for sequential expression of the two proteins. BL21-SI harbors the T7 RNA polymerase under control of the E. coli proU promoter, which is inducible by increases in osmolarity and controlled by the concentration of NaCl (maximum induction with 0.3 M NaCl) (32). Both T7 promoter-based constructs (pET-20b) or constructs with IPTG-inducible promoters (pQE-41) can be utilized in the system without cross-induction from either inducing substrate.

To obtain compatible plasmids, cops2B was subcloned from pMB053 (Spel/PvuI) into pREP-4 (SmaI). The resulting plasmid, pMB059, was transformed into BL21-SI (yielding MB063), and Cps2B production was confirmed by IPTG induction and immunoblotting for the His tag. Strain MB063 was then transformed with pMB048 (Cps2C/Cps2D), creating the dual induction strain MB065. Restriction digests and growth on LBON containing both Ap and Km were used to confirm the presence of both plasmids.

To analyze the effects of sequential induction of Cps2C/Cps2D and Cps2B on phosphorylation in MB065, 5-ml cultures were aliquoted from a 500-ml culture at a cell density of 1.5 × 10^8 CFU ml^-1 grown at 37 °C. The primary and secondary inductions of these aliquots were started by the addition of NaCl (0.1, 0.2, or 0.3), H_2O (150 μl for mock inductions), or IPTG (1.0 mM). Each induction was allowed to continue for 2 h at 30 °C with shaking. Between the primary and secondary inductions, a 1-ml sample was removed. The remaining culture was centrifuged (10,000 g, 30 min), washed once with LBON containing Ap and Km, and resuspended in 5 ml of LBON containing Ap and Km. After the second induction another 1-ml sample was removed for analysis. Samples from both the primary and secondary inductions were analyzed by SDS-PAGE. The volumes loaded were normalized to the lowest sample A_600. Monoclonal α-TyrP was used in immunoblots to visualize Tyrosine phosphorylation. As described above, Cps2B were also analyzed by immunoblot using anti-TetraHis and antisera against Cps2B-His (generation described below) to ensure that both proteins were expressed.

To examine the reduction of tyrosine phosphorylation as a function of time, four 10-ml cultures were taken from a 100-ml culture of MB065 at the time, four 10-ml cultures were taken from a 100-ml culture of MB065 at a cell density of 1.5 × 10^8 CFU ml^-1. Two were mock-induced (300 μl of H_2O (150 μl for mock inductions), or IPTG (1.0 mM). Each induction was allowed to continue for 2 h at 30 °C with shaking. Between the primary and secondary inductions, a 1-ml sample was removed. The remaining culture was centrifuged (10,000 g, 30 min), washed once with LBON containing Ap and Km, and resuspended in 10 ml of LBON. A 1-ml sample was taken from each culture at this time (T_i), the incubations were continued, and 1-ml samples were subsequently taken at the times indicated. The samples were analyzed by SDS-PAGE and immunoblotting, as described above.

**Cps2B Binding to Cps2D—**Binding of Cps2B to Cps2D was assessed in an ELISA where Cps2D–P (0.5 μg well^-1) was bound to a microwell plate overnight at 4 °C in PBS. Following blocking with 1% BSA in PBS, half of the wells were dephosphorylated using YOP phosphatase (25 units well^-1 for 1.5 h). The plate was then washed five times with PBST. Cps2B diluted in 50 mM Tris acetate (pH 8.0) was added to both the phosphorylated and dephosphorylated Cps2D-containing wells and incubated for 1 h at room temperature. After washing five times with PBST, binding of Cps2B was assessed by reactivity with a polyclonal antibody against Cps2B developed in rabbit. Induced rabbit antisera, SAP, and pNPP, as described above. Wells coated with BSA alone were used to determine levels of nonspecific interaction and were subtracted from the Cps2B binding. The HEPES buffer used for the phosphatase experiments interfered with the ELISA, resulting in high background levels of nonspecific binding of the proteins and antibodies. As noted above, Cps2B exhibited phosphatase activity in the Tris acetate buffer used here.

**Polynuclear antimur to Cps2B was obtained by subcutaneous injection of 10 BALB/CByJ mice with 0.2 ml of a 1:1 mixture of Freund's complete adjuvant and a solution of 300 μg ml^-1 purified Cps2B-His.** After 7 days, the mice were boosted by intraperitoneal injection of a similar mixture, except Freund's incomplete adjuvant was substituted for Freund's complete adjuvant. Blood was collected 14 days after the boost, and the serum was pooled and filtered through a 0.45-μm filter. The reactivity was verified in immunoblots using purified Cps2B and cell lysates of Cps2B-expressing cells.

**Construction and Characterization of cops2C and cops2C Deletion Mutants—**cops2C and cops2D deletions were generated in *S. pneumoniae* D39 by previously described techniques (3). Briefly, PCR fragments spanning the desired deletions were obtained using D39 chromosomal DNA and the primer pairs Cps2B/Cps2C (unique restriction sites in 3'), MB043 (Cps2D, PvuI/KpnI) and Cps2B2/Cps2D (KpnI/Cps2E1 for the deletion of cops2D and Cps2B2/Cps2D2 (KpnI) and Cps2C2/KpnI/Cps2D1 for the deletion of cops2C (unique restriction sites in parentheses). The resulting fragment pairs were cloned into pGM-T Easy, maintained in DH5α, and then subcloned into the *S. pneumoniae* suicide vector pY14164 (35), creating pMB024 (Δcops2D) and pMB028 (Δcops2C). The presence or the correct inserts was confirmed by restriction enzyme digestion and sequencing. The plasmids were transformed into competent D39 (3), reactions were plated in the absence of selection, and pools of colonies were screened by PCR using primers flanking the desired deletion. Deletion of the desired sequence was confirmed by Southern blotting and sequencing of the complete open reading frame. *S. pneumoniae* derivatives with deletions in either cops2D (MB512, MB513) or cops2C (MB516, MB517) were isolated from two independent transformations. Capsule production was determined in an indirect ELISA. Cultures were grown to ~3 × 10^8 CFU ml^-1 in Todd-Hewitt broth supplemented with 0.5% yeast extract, centrifuged (15,000 × g) washed once in PBS, and heat-killed (65 °C for 30 min). Dilutions of the bacteria were coated onto microtiter plates, and 1:10,000 dilution of a polyclonal antiserum to type 2 polysaccharide (Statens Serum Institut) was used to detect capsule. The assay was developed using G-α-Rabbit-Ig-biot, SAP, and pNPP, as described above. Culture supernatants were also tested but showed no reactivity.

**Mouse Infections—**Female 8–12-week-old BALB/CByJ mice (Jackson Laboratories) were used for systemic infections and colonization studies. *S. pneumoniae* cultures were grown in Todd-Hewitt broth supplemented with 0.5% yeast extract to ~3 × 10^9 CFU ml^-1 and diluted in lactated Ringer's solution. The parent D39 was injected into mice at a dose of 1.5 × 10^7 CFU intraperitoneally and 1.5 × 10^7 CFU intravenously. The deletion strains MB512, MB513, MB516, and MB517 were injected at a dose of 1.5 × 10^7 CFU for both routes of infection. All mice were monitored for 21 days post-infection. For colonization studies, mice were inoculated intranasally with 1.5 × 10^5 CFU and sacrificed.
RESULTS

Phosphorylation of Cps2D Expressed in E. coli Requires Cps2C—Recombinant His₆-tagged derivatives of Cps2B, Cps2D, and Cps2E co-expressed with a nontagged Cps2C were expressed and purified from E. coli (Fig. 1A). The His-tagged proteins were reactive with a monoclonal anti-tetrahistidine antibody (α-TetraHis) (Fig. 1B). Cps2D, however, did not react with α-Tyr(P) unless Cps2C was co-expressed in the same cell (Fig. 1C). As shown in Fig. 1A, the Cps2D-His₆ construct produced a single band (29.5 kDa), whereas a ladder of three bands (29.5, 30.5, and 31.5 kDa) was seen when Cps2C was co-expressed with the Cps2D-His₆ construct. This banding pattern was also observed in the α-TetraHis-probed immunoblot (Fig. 1B), and when reacted with α-Tyr(P), there were two distinct bands (30.5 and 31.5 kDa) (Fig. 1C). To confirm that the 29.5-kDa band on the Coomassie-stained gel was the nonphosphorylated form of the protein, a sample of the purified Cps2D–P was treated with the tyrosine-specific phosphatase YOP and repurified. This treatment resulted in a single band of 29.5 kDa on both the Coomassie-stained gel (Fig. 1A) and the α-TetraHis immunoblot (Fig. 1B), and a loss of reactivity with α-Tyr(P) (Fig. 1C). In similar immunoblots, no reactivity of Cps2D–P with monoclonal antibodies to phosphoserine or phosphothreonine was observed, whereas control phosphoserine- and phosphothreonine-conjugated BSA did react with the antibodies (data not shown).

Cps2D–P Has Tyrosine Kinase Activity—Purified Cps2D–P phosphorylated the exogenous substrate poly-Glu-Tyr in a manner dependent on protein concentration (Fig. 2A). Its activity was equivalent to ~0.7 units μg⁻¹ of the eukaryotic tyrosine kinase EGFR. To determine whether the phosphate was transferred directly from Cps2D–P or from the hydrolysis of ATP, 2 units of Cps2D–P or EGFR were incubated with poly-Glu-Tyr in the presence or absence of ATP. Substrate phosphorylation occurred under both conditions using Cps2D–P but was not observed using EGFR in the absence of ATP (Fig. 2B). As shown in Fig. 2C, Cps2D–P was also capable of transferring its phosphate to YOP-dephosphorylated Cps2D. The transphosphorylated Cps2D–P reacted with α-Tyr(P) (Fig. 2C) but not with monoclonal antibodies to phosphoserine or phosphothreonine (data not shown). In an assay identical to that utilized by Vincent et al. (21) to confirm Wzc tyrosine kinase activity, we observed autophosphorylation of purified Cps2D–P in the presence of [γ⁻³²P]ATP (Fig. 2D). All of the radiolabel contained in either the autophosphorylated Cps2D–P or in transphosphorylated Cps2D–P was removed by YOP treatment, confirming the tyrosine specificity of the phosphorylation reaction (data not shown).

Cps2B Has Phosphatase Activity—We demonstrated phosphatase activity for Cps2B purified from E. coli using pNPP as the substrate. Among a series of buffers (described under “Experimental Procedures”), the highest levels of activity were observed using 50 mM Tris acetate or 50 mM HEPES, with an optimal pH of 8.0 (Fig. 3A). Enhanced activity was obtained by the addition of 20 mM MgCl₂ and 0.1 mM MnCl₂ to the HEPES buffer (pH 7.4) (Fig. 3B). This buffer is identical to the tyrosine kinase assay buffer used above and was therefore used in subsequent experiments. Cleavage of pNPP was inhibited by monitored at 405 nm and is indicated as phosphorylation (OD₄₀₅) on the y axis. D, incorporation of ³²P into Cps2D–P from [γ⁻³²P]ATP. The reactions were stopped at the times indicated above each lane by the addition of 4× SDS-PAGE loading buffer and boiling for 5 min. Cps2D–P was the only radiolabeled band and is shown in the figure.

Fig. 2. Tyrosine kinase activity of Cps2D–P isolated from MB049. In A and B, the ELISA-based tyrosine kinase assay PTK-101 was used to determine the ability of Cps2D–P to phosphorylate the exogenous substrate poly-Glu-Tyr. Phosphorylation was detected using the monoclonal α-Tyr(P) PT-66 conjugated to HRP. Cleavage of o-phenylenediamine dihydrochloride by HRP was detected at 492 nm and is indicated as phosphorylation (OD₄₀₅) on the y axis. A, tyrosine kinase activity relative to Cps2D–P concentration. B, tyrosine kinase activity of Cps2D–P (left panel) or EGFR (right panel) in the presence or absence of 0.3 mM ATP. Reactions contained 2 units of each protein. C, phosphorylation of Cps2D by Cps2D–P. Cps2D–P (0.5 μg) bound to a microtiter plate was either left untreated (lane 1) or was treated with YOP (lanes 2 and 3). Lane 3 was then incubated with 0.5 μg of Cps2D–P for 60 min. Phosphorylation was detected using α-Tyr(P), a biotin-labeled secondary antibody, and SAP. Cleavage of pNPP was after 7 days to determine the number of bacteria colonizing the nasopharyngeal cavity, as previously described (4).
the phosphatase inhibitor Na₃VO₄ (Fig. 3B). No activity was observed in a standard pNPP substrate buffer (0.1 M glycine, 1 mM MgCl₂, 1 mM ZnCl₂, pH 10.4) used for alkaline phosphatase assays or in a 100 mM citrate buffer (pH 6.5) used to assay low molecular mass acid phosphatases (21, 36).

Phosphotyrosine phosphatase activity of Cps2B was demonstrated using Cps2D as the substrate. This reaction was completely inhibited by the addition of Na₃VO₄ (Fig. 3C). Cps2B Inhibits Phosphorylation of Cps2D—To examine the autophosphorylation of Cps2D in an intact system that included Cps2B, Cps2C, and Cps2D, the proteins were co-expressed in E. coli using vectors that allowed for differential induction of the clone expressing Cps2B (pMB059) or the clone expressing both Cps2C and Cps2D (pMB048). The resulting strain, MB065, was induced to express either Cps2B or Cps2C/Cps2D, the inducing substrate was removed, and the alternate protein was induced. When MB065 was induced to express Cps2D before the induction of Cps2B, the levels of Cps2D phosphorylation were the same as those observed when Cps2B was not induced (Fig. 4A, right panel). When Cps2B was expressed prior to Cps2D induction, however, the levels of phosphorylated Cps2D were dramatically reduced as compared with the mock induction (Fig. 4A, center panel). In a second experiment, decreases in the level of phosphorylated Cps2D were analyzed as a function of time. The results were similar to the first experiment, with induction of Cps2B prior to Cps2D resulting in a major reduction in the amount of phosphorylated Cps2D when compared with Cps2B induction after Cps2D (Fig. 4B). The results suggest that Cps2B may have a secondary mechanism for modulating Cps2D phosphorylation that involves inhibition of its initial autophosphorylation.

To examine the possibility that Cps2B can inhibit the donation of a phosphate from Cps2D to Cps2D even when its phosphatase activity is inhibited, we used purified proteins in an ELISA assay. Cps2D→P bound to microtiter plates was first dephosphorylated by treatment with YOP, which was then washed from the plate. As shown in Fig. 5, the dephosphorylated Cps2D could be rephosphorylated by incubation with Cps2D→P (compare lanes 1 and 2), and this reaction was not affected by Na₃VO₄ (compare lanes 2 and 3). When Cps2B was added prior to Cps2D→P, a significant reduction in phosphorylation was seen (compare lanes 2 and 4; p ≤ 0.001). The addition of Na₃VO₄, which completely inhibits the phosphotyrosine phosphatase activity of Cps2B (Fig. 3C), did not restore phosphorylation to the levels seen in the absence of Cps2B (compare lanes 3 and 5; p ≤ 0.01). No inhibition of the transphosphorylation reaction was observed with other proteins (BSA, YOP, and GalU-His₆) under similar conditions (data not shown). Thus, Cps2B appears to affect phosphorylation of Cps2D through both a phosphatase activity and inhibition of the actual phosphorylation event.

Interactions between Cps2B and Cps2D were examined in ELISAs, where the binding of Cps2B to Cps2D→P and YOP-dephosphorylated Cps2D→P was evaluated using a polyclonal antibody against Cps2B. As shown in Fig. 6, Cps2B bound to both the phosphorylated and dephosphorylated Cps2D, but binding to the dephosphorylated form was significantly greater than to the phosphorylated form. Specificity of the interactions was demonstrated in ELISAs, where BSA did not inhibit the binding of Cps2B to Cps2D or Cps2D→P, and GalU-His₆ did not bind Cps2D→P (data not shown).

cps2C and cps2D Are Essential for Virulence and Colonization—Neither the cps2D (MB512/MB513) nor the cps2C (MB516/MB517) deletion mutants of S. pneumoniae D39 produced detectable levels of capsule, as determined in ELISA assays using a type 2-specific polyclonal antiserum (data not shown). To determine whether the failure to make capsule during laboratory culture was reflected in vivo, the mutants were examined in mouse models of pneumococcal virulence, where capsule is known to be required (3, 4). As shown in Table III, both the cps2C and cps2D deletion strains were avirulent when mice were infected either intraperitoneally or intravenously and were unable to colonize the nasopharyngeal cavity of mice challenged intranasally.

**DISCUSSION**

Based on studies performed with the type 19F-Rx1 S. pneumoniae (28) and the purified Gram-negative CpsD homologues (21, 22, 26, 37), CpsD was expected to be an autophosphorylating tyrosine kinase. Our results demonstrated tyrosine kinase activity for Cps2D and showed that Cps2C was required for its
initial autophosphorylation. Once phosphorylated, however, Cps2D alone was an active tyrosine kinase. This result mimicked that seen with the E. coli K30 capsule homologue Wzccps, where the transmembrane domain is essential for phosphorylation, but contrasted with the E. coli colanic acid homologue Wzcca, where phosphorylation occurred independent of the transmembrane domain (22, 38). There are three potential sites of phosphorylation within the phosphotyrosine acceptor domain of Cps2D (28, 31). The banding patterns observed when Cps2C and Cps2D were co-expressed in E. coli indicated that multiple tyrosines were phosphorylated in this system, but complete phosphorylation of the tyrosine-rich domain may not occur.

Cps2DΔ/H11011P purified from E. coli lacked the transmembrane Cps2C, yet it retained tyrosine kinase activity and the ability to transfer a phosphate to an endogenous substrate. The latter was neither dependent on nor affected by ATP, suggesting that
the phosphate was being transferred directly from Cps2D—P. Thus, although Cps2C is required for the initial autoprophosphorylation of Cps2D, it is not a factor in transphosphorylation. Of the three polysaccharide-associated tyrosine kinases that have been purified from Gram-negative bacteria, only Wzc22 min (formerly Etk) has been shown to phosphorylate an exogenous substrate (poly-Glu-Tyr) (26). Ptk, the first member of this family to be characterized, failed to transphosphorylate poly-Glu-Tyr (37), and Wzc_c was not tested (21). It is not known whether these other proteins can transphosphorylate in the absence of their transmembrane domains. The ability of CpsD—P to do so and to localize to the cytoplasm (28) allows for the possibility that it can act as a second messenger with the ability to phosphorylate other proteins in the cell. Experiments in our laboratory are being performed to study this possibility.

Morona et al. (28) showed that deletion of cps19B resulted in an increase in intensity of the CpsD—P band on an anti-phosphotyrosine immunoblot. This result, coupled with the fact that the majority of Gram-negative polysaccharide tyrosine kinase systems have a cognate phosphatase that is encoded by a gene located upstream of the kinase-encoding sequence (21, 22, 29, 36), led Morona et al. to propose a phosphatase function for CpsB (28). CpsB has no homology to other known phosphotyrosine-protein phosphatases nor to any proteins other than its homologues in the streptococcal, staphylococcal, and lactococcal capsule loci (39–42). In contrast to the enzymes in Gram-negative bacteria, which are low molecular mass acid phosphatases, CpsB has a higher molecular mass (28 kDa) and exhibits optimum phosphate activity at a higher pH (8.0 versus 6.5) (21, 29, 36). A domain search of the NCBI data base (www.ncbi.nlm.nih.gov) revealed limited homology to a family of phosphoesterases, but the resulting alignments failed to define regions with functional homology (data not shown). In addition to its phosphatase activity, CpsB2B bound Cps2D and CpsD—P and inhibited the transfer of a phosphate from Cps2D—P to Cps2D, even when its phosphatase activity was eliminated. CpsB thus appears to be a novel phosphatase with two mechanisms by which it can affect capsule synthesis, i.e., removal of phosphates from CpsD—P and prevention of the phosphorylation event.

The mechanism(s) by which CpsC/CpsD and their homologues affect polysaccharide synthesis is not known. Phosphorylation appears to be a central controlling point in all of the systems, although opposite effects have been reported. In S. pneumoniae capsules and E. coli colanic acid synthesis, phosphorylation inhibits polysaccharide production, whereas assembly of the E. coli K30 capsular polysaccharide is enhanced by phosphorylation (22, 28, 29). Our results with the cloned pneumococcal proteins indicate that interaction of CpsC and CpsD with concomitant phosphorylation of CpsD is the default state that occurs in the absence of any other regulatory control. This observation, along with the fact that capsule expression in S. pneumoniae occurs in the absence of CpsD phosphorylation (28), suggests that another protein may ultimately control phosphorylation associated with CpsD. Based on the activities of CpsB, we suggest that it may fulfill such a role. A current model of S. pneumoniae capsule production suggests that CpsD and CpsB are involved in a cycle of ATP-dependent phosphorylation and dephosphorylation that modulates CpsD—P levels. The results of Morona et al. (28) showed that CpsB, CpsC, CpsD, and a functional ATP binding site in CpsD are required for capsule synthesis, but phosphorylation is not necessary. Their model proposed that capsule production is enhanced when a complex that includes CpsC, CpsD, and bound ATP is formed. To that, we would add CpsB. The requirement for CpsB in the complex readily explains the reduced encapsulation of the CpsB mutants, provides a more efficient mechanism for controlling capsule synthesis, and is consistent with the observation that phosphorylation of Cps2D in E. coli is inhibited by the presence of Cps2B.

Although the specific steps at which tyrosine kinases affect polysaccharide synthesis are not known, data from both the Gram-positive and Gram-negative systems suggest that polymerization of repeat units may be involved (20, 22, 25, 28). Formation of the putative CpsB-CpsC-CpsD-ATP complex may allow for efficient synthesis of high molecular mass polymer by either directly or indirectly enhancing the activity of the polymerase. Dissociation of the complex, presumably resulting from some environmental signal, would result in autophosphorylation of CpsD and a reduction in polymerization. Whether CpsD—P directly affects the polymerase or any other functions remains to be determined. The signal that might initiate dissociation of the complex is likewise unknown; however, the membrane-associated proteins CpsA and CpsC are potential candidates for sensors in the pathway. This mechanism for modulating capsule polymerization has direct relevance during infection and colonization. It has been shown that only low levels of capsule are required to colonize, but invasive infections require a substantially larger amount of capsule (4). The capsule-associated tyrosine kinase system represented by CpsB, CpsC, and CpsD has the potential to control the amount of capsule produced and therefore regulate the switch from a colonizing strain to an invasive one. Studies to directly test our model regarding the mechanism(s) of regulation associated with the tyrosine kinase system are underway. The results of those studies should allow us to further define the capsule synthesis complex and possible downstream phosphorylation events modulated by CpsD—P.

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S. pneumoniae Tyrosine Kinase Activity

Table III

| Strain | Dead/total (intraperitoneal) | Dead/total (intravenous) | Colonized/total (intraperitoneal) |
|--------|-----------------------------|--------------------------|----------------------------------|
| D39    | 5/5                         | 7/10                     | 5/10                             |
| MB512 (∆D) | 0/7                         | 0/7                      | 0/7                              |
| MB513 (∆D) | 0/7                         | 0/7                      | 0/7                              |
| MB516 (∆C) | 0/7                         | 0/7                      | 0/7                              |
| MB517 (∆C) | 0/7                         | 0/7                      | 0/7                              |

* Intraperitoneal infections were performed using 1.5 × 10⁶ CFU for the parent D39 and 1.5 × 10⁷ CFU for the mutants. The intraperitoneal LD₅₀ of D39 is approximately 5 × 10⁷ CFU.

* Intravenous infections were performed using 1.5 × 10⁷ CFU for both the parent and mutant strains. The intravenous LD₅₀ of D39 is approximately 5 × 10⁹ CFU.

* Intranasal inoculations were performed using 1.5 × 10⁶ CFU for both the parent and mutant strains.

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