Streptococcus pneumoniae Phosphotyrosine Phosphatase CpsB and Alterations in Capsule Production Resulting from Changes in Oxygen Availability

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Streptococcus pneumoniae produces a protective capsular polysaccharide whose production must be modulated for bacterial survival within various host niches. Capsule production is affected in part by a phosphoregulatory system comprised of CpsB, CpsC, and CpsD. Here, we found that growth of serotype 2 strain D39 under conditions of increased oxygen availability resulted in decreased capsule levels concurrent with an ~5-fold increase in Cps2B-mediated phosphatase activity. The change in Cps2B phosphatase activity did not result from alterations in the levels of either the *cps2B* transcript or the Cps2B protein. Recombinant Cps2B expressed in *Escherichia coli* similarly exhibited increased phosphatase activity under conditions of high-oxygen growth. *S. pneumoniae* D39 derivatives with defined deletion or point mutations in *cps2B* demonstrated reduced phosphatase activity with corresponding increases in levels of Cps2D tyrosine phosphorylation. There was, however, no correlation between these phenotypes and the level of capsule production. During growth under reduced-oxygen conditions, the Cps2B protein was essential for parental levels of capsule, but phosphatase activity alone could be eliminated without an effect on capsule. Under increased-oxygen conditions, deletion of *cps2B* did not affect capsule levels. These results indicate that neither Cps2B phosphatase activity nor Cps2D phosphorylation levels *per se* are determinants of capsule levels, whereas the Cps2B protein is important for capsule production during growth under conditions of reduced but not enhanced oxygen availability. Roles for factors outside the capsule locus, possible interactions between capsule regulatory proteins, and links to other cellular processes are also suggested by the results described in this study.

In spite of vaccines of increasing complexity and coverage, the Gram-positive human pathogen *Streptococcus pneumoniae* (pneumococcus) remains a significant cause of morbidity and mortality worldwide. All available pneumococcal vaccines are directed against the organism's capsular polysaccharide (CPS), which is essential for virulence and colonization. To survive in distinct niches within the host, *S. pneumoniae* must alter the expression of CPS. Decreased CPS levels are beneficial in the nasopharynx to allow exposure of adhesins and other surface structures that aid in colonization. While some CPS is necessary for nasopharyngeal colonization (1, 2), strains that express CPS at levels higher than parental levels can be defective in their abilities to colonize (3). Once the bacterium escapes the nasopharynx, increased CPS levels are necessary to mask potential surface antigens, prevent immune system recognition of surface-deposited complement, and, to a lesser degree, reduce complement deposition (4–7).

To date, there are >90 known, structurally distinct serotypes of *S. pneumoniae* CPS (8). All but two serotypes (9) are synthesized by a Wzy-dependent mechanism, in which a repeat unit is synthesized on the inner face of the membrane, transported to the outer face, and incorporated into the growing CPS chain by the addition of the long chain to the new repeat unit. The mature polysaccharide is covalently bound to the cell by linkages to the membrane and the peptidoglycan (3, 10). Genetic loci of the Wzy serotypes are characterized by a region of common genes that are highly homologous among serotypes, followed by a region of type-specific genes, whose products are responsible for the synthesis of the component sugars and the formation of the specific linkages unique to a given serotype (9, 11–13). The common genes encode

CpsA, CpsB, CpsC, and CpsD, which appear to be involved in the modulation of CPS synthesis.

CpsB, CpsC, and CpsD combine to function as a phosphoregulatory system, and homologs or functional equivalents are present in polysaccharide-associated loci of many Gram-positive and Gram-negative bacteria (14–21). In *S. pneumoniae*, the loss of any of the proteins results in reduced virulence and colonization in animal models (3, 22–24). CpsC and CpsD comprise the N-terminal integral membrane activation domain and C-terminal cytoplasmic kinase domain, respectively, of an autophosphorylating tyrosine kinase homologous to the *Sinorhizobium meliloti* ExoP protein, which regulates exopolysaccharide chain length in that system (19). *S. pneumoniae* CpsD proteins possess three or four Tyr residues at the C terminus that may be phosphorylated. When expressed as recombinant enzymes in *Escherichia coli*, CpsC is required for CpsD phosphorylation (22), as is also the case for the native *S. pneumoniae* system (25). Using recombinant enzymes *in vitro*, the C-terminal cytoplasmic loop of the *Staphylococcus aureus* CpsC homolog CapA was sufficient for phosphorylation of the CpsD homolog CapB (26). In *S. pneumoniae*, increased CpsD

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phosphorylation has been shown to correlate positively with CPS production in clinical isolates, and growth under atmospheric-oxygen conditions reduces both CpsD phosphorylation and CPS amounts in opaque-colony variants (27). Deletion of *cpsD* results in the loss of the vast majority of CPS (3, 24, 25). What remains is of low molecular weight, but the relative amounts retained on the membrane and transferred to the peptidoglycan are unchanged from those of the parent (3). The precise roles of CpsC and CpsD remain to be defined.

CpsB is a manganese-dependent phosphatase capable of dephosphorylating phosphorylated CpsD (CpsD~P) (22, 28). Unlike its Gram-negative counterpart, Wzb, which belongs to the family of low-molecular-weight phosphotyrosine phosphatases, CpsB belongs to the polymerase and histidinol phosphatase (PHP) superfamily of phosphoesterases (29). PHP superfamily enzymes are present in all domains of life as part of DNA polymerases and histidinol phosphatases as well as stand-alone enzymes (29). Aravind and Koonin divided PHP phosphoesterases into five families based on similarity; CpsB belongs to family 5, which is comprised of PHP enzymes encoded in CPS operons of streptococci, staphylococci, and *Bacillus subtilis* (29).

Unlike low-molecular-weight phosphotyrosine phosphatases, which utilize a catalytic cysteine residue in their active sites (30, 31), PHP proteins utilize a series of coordinated histidine and aspartic acid residues in their catalysis (15, 17, 29, 32–34). These residues are coordinated through four characteristic PHP motifs (29). Mutations in residues in the conserved histidines or aspartic acids have been demonstrated to result in a loss of activity for CpsB (28), its homolog Wzb in *Lactobacillus rhamnosus* (not to be confused with Gram-negative Wzb) (15), and its *B. subtilis* homolog, YwqE (17). The crystal structures of the *S. pneumoniae* CPS serotype 4 enzyme Cps4B confirmed the importance of these conserved histidines for CpsB activity while demonstrating that two additional non-PHP motif residues, glutamic acids at positions 80 and 108, are also integral to CpsB activity (32, 33). Two arginine residues, one each in motifs 3 and 4, were demonstrated to be directly involved in phosphotyrosine binding (32). Deletion of *cpsB* in *S. pneumoniae* has consistently been reported to result in an increase in phosphorylation of CpsD (CpsD~P), but the CPS phenotype coinciding with this increase has varied (3, 25, 28).

Given the need to modulate CPS expression in different niches within the host, it is logical that environmental factors such as nutrient availability or other triggers would influence this regulation. *S. pneumoniae* is an aerotolerant anaerobe that encounters a range of oxygen pressures in the host. In sites such as the middle ear or pleural fluid, oxygen pressure may be 20 mm Hg or lower (35). *In vitro*, similarly low oxygen levels have been shown to cause increased CPS levels in clinical isolates of various serotypes compared to the same isolates grown in atmospheric oxygen (159 mm Hg) (27, 35). Because of their ubiquity in Wzy-dependent pneumococcal serotypes, the *cpsBCD* gene products are a potential point of regulation in the observed oxygen-dependent CPS alterations, and CpsD phosphorylation has been demonstrated to vary directly with CPS production under different atmospheric conditions (i.e., decreased oxygen results in increases in both CpsD phosphorylation and CPS) (27).

Here, we demonstrate that Cps2B phosphatase activity responds to culture oxygen availability, and phosphatase activity and Cps2D phosphorylation are inversely correlated. However, neither phosphatase activity nor Cps2D phosphorylation levels

correlate with CPS levels, and Cps2B is dispensable for parental CPS production under conditions of growth with increased oxygen availability. Our results allow for new models regarding the role of CpsB in CPS synthesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids are listed in Table 1 and Table S1 in the supplemental material. Primers are listed in Table S2 in the supplemental material. *S. pneumoniae* was cultured in Todd-Hewitt broth (BD Biosciences) supplemented with 0.5% yeast extract (THY) at 37°C. For growth under conditions of low oxygen availability, bacteria were cultured in 20 ml THY in a 25-ml test tube in which the medium had been autoclaved in its vessel. Increased oxygen availability was provided by culturing in 12.5 ml THY contained in a 125-ml baffled flask with gentle shaking (50 rpm). As described in Results, the two growth conditions resulted in indistinguishable doubling times. For culture on solid medium, pneumococci were grown at 37°C in a candle jar on either tryptic soy agar with 5% sheep's blood (BBL), blood agar base (BBL) containing 3% defibrinated sheep blood (Colorado Serum Company), or Todd-Hewitt broth with 0.5% yeast extract and 1.5% agar overlaid with 4,000 U of catalase. When appropriate, plates were overlaid with 40 μ l of a 40-mg/ml solution of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal). For *S. pneumoniae*, antibiotics were used at the following concentrations: chloramphenicol (Cm) at 2.5 μ g/ml, erythromycin (Em) at 0.3 μ g/ml, and kanamycin (Km) at 250 μ g/ml. *E. coli* was cultured in LB medium (1% tryptone [BBL], 0.5% yeast extract [BBL], 0.5% NaCl, and 0.1% glucose) at 37°C with shaking at 200 rpm, except as noted. For culture on solid medium, LB medium plus 1.5% agar was used. For *E. coli*, antibiotics were used at the following concentrations: ampicillin (Ap) at 100 μ g/ml, Km at 50 μ g/ml, Em at 15 μ g/ml for DB11 derivatives and 300 μ g/ml for all others, and Cm at 25 μ g/ml.

Phosphatase assays. For *S. pneumoniae*, cultures were grown to $\sim 3 \times 10^8$ CFU/ml (optical density at 595 nm [OD₅₉₅] of ~ 0.2). A 10-ml aliquot of culture was centrifuged at $8,000 \times g$ for 10 min at 4°C. Cultures were concentrated 20-fold by suspension in assay buffer (1 M Tris-HCl, 0.5 M NaCl [pH 8.0]) and normalized to the lowest OD₅₉₅ at harvest. Samples were dispensed in triplicate (100 μ l/well) into the wells of microtiter plates. Permeabilization was initiated by the addition of 5 μ l chloroform and 5 μ l 0.1% SDS per well. The plate was shaken at 37°C for 5 min to ensure thorough permeabilization. The reaction was started by the addition of 100 μ l assay buffer supplemented with 6 mg/ml *p*-nitrophenyl phosphate (pNPP) and 100 mM MnCl₂, prewarmed to 37°C. Absorbance was observed at 405 nm for 10 min at 37°C in a microtiter plate reader. Rates were calculated as $\Delta A_{405}/\text{min}$. Rates were normalized to the D39 rate under low-oxygen conditions, which was arbitrarily assigned a value of 1. AG554 (D39 Δ cps2B) (see below) was used as a negative control and exhibited activity that was $16.2\% \pm 3.7\%$ of the activity of D39 under low-oxygen conditions. The background activity observed with AG554 under high-oxygen conditions was not significantly different from that observed under low-oxygen conditions.

To examine the effects of increased oxygen availability on Cps2B expressed in *E. coli*, MB053 and a vector control strain (JM109 containing pQE-41) were grown in parallel with or without shaking at 200 rpm. Cultures grown overnight were diluted to an OD₅₉₅ of ~ 0.1 into fresh LB medium under the same conditions. Cps2B expression was induced with isopropyl-thio- β -galactoside (IPTG) at a final concentration of 1 mM for 30 min. After induction, 200- μ l culture aliquots were added to 200 μ l 5 $\times$ assay buffer (1 $\times$ assay buffer is 1 M Tris-HCl [pH 8] plus 0.5 M NaCl), 50 μ l H₂O, 25 μ l 0.1% SDS, and 25 μ l chloroform. This mixture was vortexed to permeabilize the cells. For each technical replicate, 100 μ l of this mixture was transferred onto a 96-well plate and warmed to 37°C. The reaction was started as described above, except that 10 mM MnCl₂ was used. Rates were calculated based on the $\Delta A_{405}/\text{min}$. To adjust for the higher level of protein expression under increased-oxygen conditions, Western blots were performed with antiserum specific for Cps2B (see

TABLE 1 Strains and plasmids used in this study^a

Strain or plasmid	Relevant property(ies) ^b	Reference or source
Strains		
<i>S. pneumoniae</i>		
D39	CPS serotype 2 parent	52
AB1013	D39 Δ <i>spxB</i> (allelic replacement of <i>spxB</i> with <i>aphA-3</i>); Km ^r	Our laboratory (our unpublished data)
AG538	D39 <i>cps2B</i> ::pAG216; blue phenotype on X-gal; Cm ^r	This study
AG539	Single-colony isolate of AG538; Cm ^r	This study
AG540	pAG177 $\times$ AG539; repairs AG539 to parental Cps2B [the restored <i>cps2B</i> contains a same-sense mutation, <i>cps2B</i> ^{3259T→C} ; D39(Cps2B ^{D117D})]	This study
AG547	pAG208 $\times$ AG539; <i>cps2B</i> ^{3294G→A} D39(Cps2B ^{G129E})	This study
AG548	pAG242 $\times$ AG539; <i>cps2B</i> ^{2973G→A} D39(Cps2B ^{S22N})	This study
AG550	pAG238 $\times$ AG539; <i>cps2B</i> ^{2964G→A} D39(Cps2B ^{R19K})	This study
AG554	pAG240 $\times$ AG539; D39 Δ <i>cps2B</i>	This study
AG557	pAG255 $\times$ AG539; <i>cps2B</i> ^{3314C→G,3315A→C} D39(Cps2B ^{H136A})	This study
AG562	pAG269 $\times$ AG554; <i>aliA</i> ::pAG269 <i>aliA</i> ⁺ Em ^r ; D39 Δ <i>cps2B</i> vector control (Δ B/VC)	This study
AG563	pAG271 $\times$ AG554; <i>aliA</i> ::pAG271 <i>aliA</i> ⁺ <i>cps2B</i> ⁺ Em ^r ; complemented D39 Δ <i>cps2B</i> (Δ B/B ⁺)	This study
AM1000	D39 Δ (<i>cps2A-cps2H</i>); CPS [−]	1
<i>E. coli</i> MB053		
	JM109 pMB053 Cps2B-His (Cps2B with an N-terminal His tag); Ap ^r	22
Plasmids		
pAG177	pJY4164::EcoRI-digested product of Cps2–A24/Cps2–C34 PCR; contains <i>cps2B</i> ; used to repair Δ <i>cps2B</i> ; recipient vector for mutant <i>cps2B</i> alleles (<i>cps2B</i> contains a same-sense mutation, <i>cps2B</i> ^{3259T→C} [D117D], as a result of a PCR error)	This study
pAG208	pAG177 containing <i>cps2B</i> ^{3294G→A} (G129E)	This study
pAG216	pEVP3 containing <i>cps2B</i> between SphI and BamHI sites	This study
pAG238	pAG177 containing <i>cps2B</i> ^{2964G→A} (R19K)	This study
pAG240	pJY4164::EcoRI-digested product of overlap extension PCR using primer sets Cps2–A24/Cps2–A43 and Cps2–C48/Cps2–C34 stored in pCR2.1 TOPO; used to generate Δ <i>cps2B</i>	This study
pAG242	pAG177 containing <i>cps2B</i> ^{2973G→A} (S22N)	This study
pAG255	pJY4164::EcoRI-digested product of overlap extension PCR using primer sets Cps2–A24/Cps2–B28 and Cps2–B27/Cps2–C34; contains <i>cps2B</i> (H136A) with flanking sequences	This study
pAG269	pJY4164::EcoRI/BamHI-digested product of primer set AliA-1/AliA-2	This study
pAG271	pAG269::SpeI/SphI-digested product of primer set Cps2–B57/Cps2–B62; inserts <i>cps2B</i> after <i>aliA</i> ; Cps2B ⁺ ; Em ^r	This study
pEVP3	Multiple-cloning site creates transcriptional fusions to downstream <i>lacZ</i> ; Cm ^r	53
pJY4164	Lacks <i>S. pneumoniae</i> origin of replication; Em ^r	54
pMB053	pQE-41 encoding His-Cps2B	22
pQE-41	IPTG-inducible expression vector; Ap ^r	Qiagen

^a See Table S1 in the supplemental material for additional strains and plasmids.^b Superscript nucleotide numbers correspond to those reported under GenBank accession no. AF026471.3.

below). Densitometry analyses were performed by using ImageJ (<http://rsbweb.nih.gov/ij/>), and calculated rates were adjusted accordingly. Data were then normalized to MB053 values under low-oxygen conditions. The background from the vector control strain was negligible.

For screening of *cps2B* hydroxylamine mutants (see “Mutagenesis of *cps2B*,” below), Ap-resistant colonies were inoculated into 200 μ l LB medium in 96-well microtiter plates. Cultures were grown for 2 h at 37°C with shaking at 200 rpm prior to induction with 1 mM IPTG for 2 h, with continued shaking. After induction, cultures were centrifuged at 2,000 $\times$ g for 10 min at 4°C. Cells were suspended in 100 μ l of 2 $\times$ assay buffer. Five microliters of 0.1% sodium dodecyl sulfate (SDS) and 5 μ l of chloroform were added to each well to permeabilize the cells. The plate was shaken for 5 min at 37°C at 200 rpm. The assay was started by the addition of 100 μ l of pNPP solution to a final concentration of 3 mg/ml containing MnCl₂ at a final concentration of 50 mM. The absorbance at 405 nm was monitored at fixed intervals for 10 min, and activities were calculated as the increase in A₄₀₅/min. Isolates exhibiting low phosphatase activity were assayed a second time. For those isolates with less than one-third of the activity of *E. coli* Cps2B⁺ strain MB053, Cps2B protein expression was assessed by Western blotting using a monoclonal antibody against the poly-His tag (see below).

For purified enzymes, 0.5 μ g Cps2B was prepared in 100 μ l assay buffer (see above) in triplicate in a 96-well microtiter plate. The plate was warmed to 37°C for 5 min prior to the addition of 100 μ l of an assay buffer solution containing 6 mg/ml pNPP and 10 mM MnCl₂ (yielding final concentrations of 3 mg/ml and 5 mM, respectively). The absorbance at 405 nm was monitored for 5 min, and rates were determined as the change in the A₄₀₅/min. Assay buffer was used as a blank.

Mutagenesis of *cps2B*. For random mutagenesis, pMB053, a pQE-41 derivative carrying *cps2B* with an N-terminal poly-His tag under the control of an IPTG-inducible promoter (22), was mutagenized with hydroxylamine using a protocol reported previously (36). Briefly, 2 μ g of pMB053 was incubated with 0.8 M NH₂OH in a final volume of 100 μ l at 37°C; samples were withdrawn every 6 h through 42 h of incubation. Plasmids were purified by using the QIAquick gel extraction kit (Qiagen) and electroporated into *E. coli* JM109 with selection on LB agar containing Ap. The transformation efficiency fell steeply for the samples at the 30-h to 36-h time points, and this was interpreted to be indicative of mutagenesis of the pQE-41 vector that affected either the Ap resistance gene or plasmid replication. Transformants from the samples at the 36-h and 42-h time points were screened for a loss of phosphatase activity and identified as described above. Thirty-five isolates exhibiting reduced phosphatase ac-

tivity were identified. Seven of the isolates expressed no Cps2B detectable by Western blotting. The *cps2B* alleles from the remainder were sequenced by using purified plasmids and primers Cps2-B9 and Cps2-B10 (primer sequences are presented in Table S2 in the supplemental material). Fourteen isolates contained mutations in *cps2B*, and these sequences were subcloned to clean pQE-41 by digestion with BamHI and HindIII and ligation into the complementary sites in pQE-41. The ligation mixture was transformed into *E. coli* JM109 with selection for Ap. DNA sequencing was performed by the Heflin Center for Human Genetics, University of Alabama at Birmingham, or the Center for AIDS Research, University of Alabama at Birmingham.

For site-directed mutagenesis to change the His-136 residue of Cps2B to Ala (Cps2B^{H136A}), complementary primers that encoded the desired mutation (Cps2-B27/Cps2-B28) were generated. These primers were used with primers Cps2-B10 and Cps2-B9, respectively, to amplify separate 5' and 3' fragments (with respect to *cps2B*) encoding the mutated residues. These products were gel purified by using the Gel/PCR DNA Fragments Extraction kit (IBI Scientific) and mixed in an equal volume for subsequent PCR amplification using primers Cps2-B9 and Cps2-B10 to generate full-length *cps2B* encoding the H136A mutation and containing BamHI and HindIII restriction sites at the 5' and 3' ends, respectively. This product was gel extracted and cloned into pCR2.1-TOPO by using the TOPO TA cloning kit (Invitrogen). Mutant *cps2B* alleles were subcloned into pQE-41 as described above. Sequences encoding the Cps2B^{C158A} and Cps2B^{H201A} mutants were generated in the same manner, using primer pairs Cps2-B40/Cps2-B41 and Cps2-B29/Cps2-B30, respectively, in lieu of Cps2-B27/Cps2-B28.

Construction of *S. pneumoniae cps2B* mutants. To construct vectors to introduce *cps2B* mutations into *S. pneumoniae*, *cps2B* and approximately 550 bp of upstream and downstream sequence were amplified from D39 chromosomal DNA by using primer pair Cps2-A24/Cps2-C34 to generate pAG177. Naturally occurring BsaHI and ClaI sites are present early in the *cps2B* and *cps2C* sequences, respectively, allowing mutations to be constructed without additional alterations of the coding sequence. To accomplish this, *cps2B* was amplified from plasmids encoding mutant Cps2B enzymes by using primer pair Cps2-B48/Cps2-C36. PCR products were cloned into pCR2.1 TOPO, excised by digestion with BsaHI/ClaI, and ligated into the complementary sites of pAG177.

To facilitate screening, an *S. pneumoniae* strain was created with a *lacZ* transcriptional fusion to an insertion in *cps2B*. *cps2B* was amplified from D39 by using primers Cps2-B55 and Cps2-B56 and cloned into pCR2.1 TOPO to generate pAG211. *cps2B* was excised by the digestion of pAG211 with SphI/BamHI and ligated into the complementary sites in pEVP3, resulting in pAG216, which inserts into *cps2B* to impart Cm resistance and a blue phenotype when grown on medium supplemented with X-gal. D39 was transformed with pAG216 as previously described (37), with selection for Cm resistance. D39 and derivatives were observed to have a narrow range of resistance to Cm; D39 grew on plate media with 2 µg/ml Cm, but a resistant strain would not grow on 3 µg/ml Cm. Even at the specified 2.5-µg/ml concentration, D39 growth was observed after longer incubations. To reduce the possibility of any potential contamination with D39, a single-colony isolate (AG538) from the original transformation was streaked and purified (by again picking a single colony) on Cm, yielding the final isolate AG539. AG539 exhibited a blue phenotype on THY agar plates supplemented with catalase and X-gal. Plasmids containing the desired *cps2B* mutations with flanking sequences were transformed into AG539; transformations were plated without selection and screened for a white phenotype. White, Cm^r colonies were verified for the reconstitution of *cpsABC* by PCR using primer pair Cps2A-24/Cps2C-34. Positive PCR products were sequenced by using primer Cps2-B10 to verify the incorporation of the mutation. Chromosomal DNA prepared from isolates containing the mutation was used to generate PCR products that were sequenced for *cps2ABCDE*. No mutations other than the intended ones were observed in any of the constructs. As a control, the parental *cps2B*

sequence was restored to AG539 by transformation with pAG177, with subsequent steps as described above for the mutant *cps2B* alleles.

Deletion of *cps2B*. To construct a complete, markerless *cps2B* deletion, overlap extension PCR was performed with D39 chromosomal DNA by using primer pairs Cps2-A24/Cps2-A43 and Cps2-C48/Cps2-C34 (with a subsequent reaction with the A24/C34 primer set). This product was cloned into pCR2.1 TOPO to yield pAG212. The plasmid was digested with EcoRI, and the 1,833-bp fragment was cloned into the complementary site in pJY4164 to yield pAG240. This plasmid was subsequently transformed into AG539, allowing blue-white screening to detect recombinants, as described above. The presence of the deletion and the lack of other mutations for the resulting strain (AG554) were confirmed by PCR and sequencing. The construction of D39 and Rx1 derivatives in which *cps2B* was deleted by allelic replacement with *aphA-3* (encoding Km resistance) is described in the supplemental material.

Complementation of $\Delta cps2B$. *cps2B* deletion mutant strain AG554 was complemented by placing the parental *cps2B* gene after *aliA*, which is located immediately after the *cps* operon and expressed from its own promoter. To generate the complementation construct, *cps2B* was amplified from D39 by using primers Cps2-B57/Cps2-B62 and cloned into pCR2.1 TOPO. This product was digested with SpeI/SphI and ligated into complementary sites in pAG269, resulting in pAG271. This plasmid was transformed into AG554 with selection for Em resistance, yielding AG563. The construct inserts into and reconstitutes the 3' end of *aliA*, placing the ribosome binding site (RBS) from an *aphA-3* Km resistance gene and *cps2B* downstream (the RBS was obtained from pSF151 and located within primer Cps2-B57). The next gene on the chromosome, SPD_0335, is ~300 bp downstream and has its own promoter, as predicted by BPROM (Softberry), making polar effects from the insertion unlikely. To construct a vector control, the final 600 bp of *aliA*, ending with the stop codon, was amplified from D39 chromosomal DNA by using primers AliA-1 and AliA-2. The EcoRI/BamHI-digested *aliA* product was ligated into EcoRI/BamHI-digested pJY4164 to give pAG269, which was transformed into AG554, yielding vector control strain AG562.

Transcription assays. *S. pneumoniae* strains were grown to $\sim 3 \times 10^8$ CFU/ml under the indicated conditions. A 10-ml aliquot was centrifuged at $8,000 \times g$ for 10 min at 4°C. The cell pellets were immediately frozen at -80°C. RNA was harvested by using the UltraClean microbial RNA isolation kit (Mo Bio Laboratories) according to the manufacturer's protocol, with on-column DNase treatment using the On-Spin Column DNase I kit (Mo Bio Laboratories). cDNA was synthesized by using the iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCRs were prepared by using iQ SYBR green Supermix (Bio-Rad) and the indicated probes (see Table S2 in the supplemental material for sequences). Reactions with 16S rRNA (*rrsA*) probes 16S-1 and 16S-2 and no-reverse-transcriptase controls yielded C_T (threshold cycle) values at least 9 cycles higher than those of the corresponding cDNA reactions (i.e., contaminating DNA accounted for <0.2% of the starting template). Relative expression values were calculated by using the $-\Delta\Delta C_T$ method in the Bio-Rad CFX Manager software package.

Analysis of Cps2B, Cps2D, and Cps2D~P production. Western immunoblots were performed by using monoclonal antibodies against polyhistidine epitopes (Tetra-His; Qiagen) at a 1:5,000 dilution or phosphorylated Tyr residues (PT-66 antibody; Sigma) at a 1:12,500 dilution. Polyclonal rabbit sera prepared against Cps2D and Cps2B were absorbed by using CPS-negative strain AM1000 and diluted 1:1,250 and 1:5,000, respectively, for use in immunoblotting. For *S. pneumoniae*, cultures were grown to a cell density of $\sim 3 \times 10^8$ CFU/ml, concentrated 100-fold, boiled for 10 min, and assayed for protein concentration using the Bio-Rad protein assay (Bradford method). Samples were normalized for total protein and boiled in SDS-PAGE loading buffer, and the proteins were separated by SDS-12% PAGE. Following transfer onto nitrocellulose, the membranes were blocked in 3% blot-qualified bovine serum albumin (BSA) (Sigma) in Tris-buffered saline (0.1 M Tris, 0.154 M NaCl [pH 7.4]) with 0.05% Tween 20 when the PT-66 antibody was to be used or in 5%

FIG 1 CPS is reduced with high-oxygen growth of *S. pneumoniae* D39. (A) CPS production under low- and high-oxygen conditions. Strains were as follows: D39 (parent type 2), ΔB (AG554), ΔB/VC (vector control, AG562), and ΔB/B⁺ (*cps2B* complemented strain, AG563). Results are means $\pm$ standard errors for at least 3 independent cultures for each strain. ***, $P < 0.001$ by ANOVA with Tukey's posttest for the given comparison. (B) Immunoblots with antisera specific for phosphotyrosine (D~P), Cps2D (D), and Cps2B (B). Relative band intensities were determined by densitometry. Values are the means of the D~P/D ratios under low-oxygen conditions divided by those under high-oxygen conditions for the indicated comparisons (2 ± 0.2 for D39 and 0.9 ± 0.08 for ΔB, from 3 independent experiments; $P = 0.01$ by Student's *t* test). The ΔB/D39 ratios for D~P/D were 3.5 ± 0.5 ($n = 3$) under low-oxygen conditions and 6.4 ± 0.4 ($n = 2$) under high-oxygen conditions. The relative amounts of Cps2B and Cps2D were not significantly different between conditions or strains. Cps2D and Cps2D~P were probed on the same blot; Cps2B was probed on a separate blot. Equivalent amounts of total protein were loaded for each sample. In separate blots, sample dilutions were used to confirm the lack of any saturation effects. (C) Real-time PCR of *cps2ABCDE*T under low- and high-oxygen growth conditions. Results are the means $\pm$ standard errors from three independent cultures under each condition and were not significantly different.

powdered milk in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 5.4 mM Na₂HPO₄, 1.8 mM KH₂PO₄ [pH 7.4]) with 0.05% Tween 20 for all others.

Cps2B purification. The preparation of Cps2B enzymes for circular dichroism studies is described in the supplemental material. For other *in vitro* experiments, cultures grown overnight were diluted 1:100 into 25 ml medium and grown for 2 h before induction with 1 mM IPTG for an additional 2 h. Cells were harvested at $2,000 \times g$ for 10 min at 4°C, and pellets were frozen at -80°C . Pellets were thawed on ice prior to the addition of 10 ml lysis buffer. Suspensions were sonicated as described above. Debris was removed by centrifugation at $8,000 \times g$ for 10 min at 4°C. His-tagged proteins were purified from the clarified lysates under native conditions by incubation with 500 μl of a Ni-nitrilotriacetic acid (NTA) agarose slurry (Qiagen) for 2 h at room temperature with rotation. Ni-NTA-lysate solutions were applied onto plastic chromatography columns (Bio-Rad) and allowed to drain by gravity flow. Columns were washed with 2 bed volumes of wash buffer. To remove residual imidazole from the wash buffer, the columns were washed with 2 bed volumes of elution buffer (without imidazole and prepared with 20% glycerol). Protein was then eluted with 4 bed volumes of elution buffer (without imidazole, prepared with 20% glycerol, and with the pH adjusted to 4.0 with HCl). Column eluates were restored to alkaline pH by the immediate addition of 1 bed volume of a buffer containing 2 M Tris (pH 8), 2 M NaCl, and 20% glycerol. Protein concentrations were determined by using the Bio-Rad protein assay against a BSA standard or by the absorbance at 280 nm. Samples were used immediately or stored at -20°C .

CPS analyses. Relative CPS levels were determined by using indirect enzyme-linked immunosorbent assays (ELISAs), as previously described (38), with minor modifications. Briefly, cultures grown to a density of $\sim 3 \times 10^8$ CFU/ml were centrifuged ($20,000 \times g$ for 10 min), and the pellet was suspended to the original culture volume by using phosphate-buffered saline (see above). Samples were heat fixed (56°C for 20 min) and normalized to the same optical density at 595 nm. Dilutions were used to coat microtiter plates, which were subsequently reacted with polyclonal type 2 antiserum (Statens Serum Institute) that had been absorbed with CPS-negative strain AM1000. Bound antibodies were detected following incubation with biotin-conjugated goat anti-rabbit immunoglobulin and streptavidin-alkaline phosphatase, with spectrophotometric detection of pNPP cleavage at 405 nm. The pNPP buffer contained 1 mM ZnCl₂ rather than the previously reported 0.1 M concentration (38).

CPS immunoblots were done as previously described (3). Briefly, cells

from cultures grown to a density of $\sim 3 \times 10^8$ CFU/ml were incubated overnight in protoplast buffer (20% sucrose, 50 mM MgSO₄, 50 mM Tris [pH 7.4]) containing mutanolysin (40 units/ml) to generate cell walls (peptidoglycan containing) and protoplasts (membrane containing), which were separated by centrifugation (3). The *S. pneumoniae* autolysin is also active under these conditions (39). Samples were normalized by the OD₅₉₅ or total protein (Bio-Rad protein assay, Bradford method) of the protoplast fraction, which gave equivalent results. Fractions were then protease treated (Qiaprotease; Qiagen) and separated by using SDS-10% PAGE, followed by transfer onto nitrocellulose membranes. CPS was detected by using polyclonal type 2 antiserum absorbed with CPS-negative strain AM1000.

RESULTS

***S. pneumoniae* D39 exhibits reduced capsule production and reduced Cps2D phosphorylation with increased oxygen availability.** Previous studies demonstrated decreased CPS production with increased oxygen availability by isolates of serotypes 6A, 6B, 9V, and 18C (27). Differences were observed by comparing CPS production during growth under anaerobic, microaerophilic, and aerobic conditions and were demonstrated to be due to changes in the availability of oxygen rather than carbon dioxide. Using methods similar to those described in that study, we tested the effect of oxygen availability on CPS production in serotype 2 strain D39. Reduced oxygen availability (here referred to as low oxygen) was obtained by growth under static conditions using filled culture vessels in which the medium had been autoclaved within its vessel. Growth under conditions with atmospheric oxygen levels (high oxygen) utilized 10:1 vessel-to-medium ratios with gentle shaking at 50 rpm, a condition that resulted in barely perceptible movement of the culture medium. The doubling times under the two conditions were not significantly different (means $\pm$ standard errors of 42 ± 1 min for high oxygen and 40 ± 1 min for low oxygen, determined from 4 independent experiments). Under high-oxygen conditions, CPS was approximately 60% of the level exhibited under low-oxygen conditions (Fig. 1A). Consistent with previous results (27), growth of D39 with high oxygen levels resulted in a decrease in the phosphorylation of Cps2D without a detectable change in the amount of Cps2D protein (Fig. 1B). Using real-time

FIG 2 Cps2B phosphatase activity increases with growth under high-oxygen conditions. (A) Phosphatase activities of D39 and derivatives harboring *cps2B* mutations. Permeabilized cells were assayed for the ability to cleave pNPP. The background from AG554 (D39 Δ *cps2B*) was subtracted before normalization and represented $\sim 15\%$ of the D39 activity under low-oxygen conditions. Results are the means $\pm$ standard errors for at least three independent cultures for each strain. *, $P < 0.05$; **, $P < 0.01$ (determined by Student's *t* test for the indicated comparison). +, $P < 0.05$; ++, $P < 0.01$; +++, $P < 0.001$ (determined by Student's *t* test against D39 grown under low-oxygen conditions in an independent experiment). Levels of S22N and H136A mutants under high-oxygen conditions were significantly different from those of D39 under high-oxygen conditions ($P < 0.001$) (XXX). Strains were as follows: R19K (AG550), S22N (AG548), G129E (AG547), and H136A (AG557). (B) Cps2B activity from recombinant *E. coli*. Cps2B expression in MB053 was induced for 30 min prior to permeabilization and assays for phosphatase activity; activities were normalized to Western blot densitometries of Cps2B for each sample. The background phosphatase activity from the control *E. coli* strain was negligible. Results are the means $\pm$ standard errors for two (low-oxygen) and three (high-oxygen) independent cultures. **, $P < 0.01$ by Student's *t* test.

PCR, we found that transcription through the first six genes of the *cps* locus did not differ significantly between the growth conditions (Fig. 1C).

Cps2B activity increases under increased-oxygen growth conditions. To determine whether Cps2B activity might correlate with the changes in Cps2D~P observed between growth conditions, phosphatase activity from D39 was measured. As shown in Fig. 2A, activity increased approximately 5-fold under high-oxygen growth conditions, in contrast to the ~ 2 -fold decrease in Cps2D phosphorylation (Fig. 1B). Immunoblotting demonstrated that Cps2B production in D39 was relatively unchanged between the conditions (Fig. 1B), suggesting that Cps2B activity responds, either directly or indirectly, to changes to the environment. One possible effector is the oxidizing agent hydrogen peroxide (H_2O_2), which *S. pneumoniae* produces at high levels (up to 1 mM) during growth under aerobic conditions as a result of SpxB-mediated pyruvate oxidase activity (40, 41). Both H_2O_2 levels and alterations in SpxB correlate with changes in colony mucoidy and opacity (42, 43). To determine whether H_2O_2 might affect Cps2B, we tested the phosphatase activity of the purified recombinant enzyme in the presence of H_2O_2 and in a defined *spxB* mutant of D39 that produces < 0.1 mM H_2O_2 during growth with high oxygen. Using concentrations of up to 64 mM H_2O_2 , we observed no effect on Cps2B *in vitro* activity (data not shown). Similarly, the *spxB* mutant exhibited the same increase in phosphatase activity with aerobic growth as that observed for the parent (data not shown). The oxygen-dependent increase in Cps2B phosphatase activity is thus not due to increased H_2O_2 levels, an effect of the SpxB protein itself, or any subsequent parts of the SpxB-related pathway.

To assess the activity of Cps2B in the absence of other *S. pneumoniae* factors, an *E. coli* derivative (MB053) that expresses a His-tagged Cps2B was grown statically or with shaking to provide low or high oxygen availability, respectively. Cps2B protein expression was then induced for 30 min prior to assaying for phosphatase activity. As shown in Fig. 2B, activity was significantly increased under high-oxygen conditions. The smaller magnitude of the increase than that in *S. pneumoniae* (~ 2 -fold in *E. coli* versus ~ 5 -fold in *S. pneumoniae*) is likely due to the high expression level of Cps2B during growth with high oxygen levels in *E. coli*, as we observed decreasing Cps2B activity per unit protein with longer

induction times (data not shown). The increase in Cps2B phosphatase activity with increased oxygen levels thus appears to be an inherent property of the protein that is independent of other *S. pneumoniae* proteins or H_2O_2 .

Deletion of *cps2B* reduces CPS levels only during growth with low oxygen, but the effects are not related to Cps2D phosphorylation levels. In previous studies, deletion of *cps2B* in D39 has been reported to either decrease (24) or increase (3) CPS production. Reexamination of the construct used to generate the *cps2B* deletion used in our studies (3) revealed that it resulted in the fusion of the remaining 18 C-terminal residues of Cps2B to the C-terminal end of Cps2A (also eliminating the final 2 residues of Cps2A and adding 6 residues before the Cps2B residues). Sequencing of the *cps2A-cps2C* junction in D39 Δ *cps2B* strain MB526 confirmed this; we therefore constructed another D39 derivative containing a complete, markerless deletion of the *cps2B* open reading frame. In contrast to our original mutant, which showed an approximately 20% increase in CPS levels (Δ B_{MB526}) (see Fig. S1A and S1B in the supplemental material) (3), the newly constructed *cps2B* deletion mutant demonstrated a reduction in CPS levels of $\sim 40\%$ compared to the levels of the parent under low-oxygen conditions (Δ B) (Fig. 1A). Complementation with *cps2B* restored the parental CPS phenotype (Δ B/VC [vector control strain] and Δ B/B⁺) (Fig. 1A). In contrast, when the availability of oxygen was high, the Δ *cps2B* mutant produced the same amount of CPS as D39, and this level was the same as that produced by the mutant under low-oxygen conditions (Δ B and Δ B/VC) (Fig. 1A). An additional construct in which *cps2B* was replaced with a Km marker (*aphA-3*) further confirmed the effect on CPS under low-oxygen conditions (Δ B_{KM}) (see Fig. S1C in the supplemental material).

Deletion of *cps2B* resulted in increased Cps2D phosphorylation under both low- and high-oxygen conditions (3.5- and 6.4-fold increases, respectively, compared to parent strain D39) (Fig. 1B). For the mutant, the levels of phosphorylation under both conditions were the same, indicating that the decrease in Cps2D phosphorylation that occurs in the parent with increased oxygen availability is Cps2B dependent. Despite the differences observed in CPS amounts for our original *cps2B* deletion mutant and the new construct described here, both strains demonstrated similar increases in Cps2D phosphorylation under low-oxygen condi-

FIG 4 Immunoblot analyses of *S. pneumoniae* *cps2B* mutants. Proteins were detected by using antisera specific for phosphotyrosine residues (D~P), Cps2D (D), and Cps2B (B). Relative band intensities were determined by densitometry. Values are the means of the D~P/D ratios from 3 independent experiments. A blot showing the results from one of the other experiments is shown in Fig. S3A in the supplemental material. The relative levels of Cps2B and Cps2D in the mutant strains were not significantly different from those in D39. Cps2D and Cps2D~P were probed on the same blot; Cps2B was probed on a separate blot. Equivalent amounts of total protein were loaded for each sample. In separate blots, dilutions of the samples were used to confirm the lack of any saturation effects. All cultures were grown under low-oxygen conditions. Strains (from left to right) were as follows: ΔD (MB512), $\Delta A-H$ (AM1000) (CPS⁻), ΔB (AG554), D39 (parent type 2) (CPS⁺), R19K (AG550), S22N (AG548), G129E (AG547), and H136A (AG557).

would be predicted to abrogate phosphatase activities due to similar local interference.

Cps2D phosphorylation correlates with Cps2B activity. Four Cps2B mutant enzymes with various phosphatase activities (up to ~32% of the parental Cps2B activity in *E. coli*) (underlined mutations in Table 2) were transformed into the D39 background via AG539, which contains a *lacZ* insertion in *cps2B* that allows for the identification of recombinants by screening for white colonies. Repair of AG539 with a *cps2B* allele encoding parental Cps2B restored the parental CPS phenotypes (see below), and sequencing of *cps2ABCDE* for each of the mutants demonstrated only the intended mutation. Examination of the Cps2B and Cps2D proteins in multiple experiments demonstrated no significant differences between the parent and mutant strains, although we frequently observed a modest reduction in Cps2B levels for the S22N mutant (~30% lower) (Fig. 4; see also Fig. S3A in the supplemental material). This reduction was not, however, sufficient to account for the nearly complete loss of Cps2B phosphatase activity (Fig. 2A). Under conditions of growth with low oxygen availability, Cps2B phosphatase activity was significantly reduced in comparison to that for D39 for all strains except the R19K-containing mutant (Fig. 2A), and there was a significant correlation between the levels of Cps2B phosphatase activity and Cps2D phosphorylation ($r^2 = 0.85$; $P < 0.05$) (data are from Fig. 2A and 4; see Fig. S3 in the supplemental material for linear regression graph). All mutants exhibited similar relative increases in activity when low- and high-oxygen conditions were compared, with the R19K and G129E mutants retaining higher levels of activity than the S22N and H136A mutants (Fig. 2A).

CPS does not correlate with Cps2B activity or Cps2D phosphorylation levels. The effects of the *cps2B* point mutations on CPS production, along with the relative Cps2B phosphatase activities and Cps2D phosphorylation levels, are shown in Fig. 5. Like the parent, the S22N, G129E, and H136A mutants produced more CPS under low-oxygen than under high-oxygen conditions. Under high-oxygen conditions, the CPS levels in these mutants, the

cps2B deletion mutant, and the parent were the same, despite the observation of a range of phosphatase activities (i.e., none to maximal for these studies). These results indicate that neither the Cps2B protein nor the level of phosphatase activity affects CPS under high-oxygen conditions.

When grown under low-oxygen conditions, CPS levels for the S22N and G129E mutants, which exhibited low phosphatase activity, were reduced in comparison to those of the parent but were higher than those of the *cps2B* deletion mutant. The H136A mutant, like the *cps2B* deletion mutant, had no detectable phosphatase activity; unlike the *cps2B* deletion mutant, however, it produced parental levels of CPS. This result suggests that the Cps2B protein, but not the phosphatase activity, is essential for increasing CPS levels under low-oxygen conditions. The phenotypes of the S22N and G129E mutants may likewise result not from the reductions in phosphatase activity but from affecting an alternate function of the Cps2B protein. The R19K mutant presented a different scenario: here, CPS levels were decreased under low-oxygen but increased under high-oxygen conditions, resulting in similar levels under both conditions despite very different levels of phosphatase activity. We interpret this to be a near-constitutive mutant that allows for the nonphosphatase Cps2B activity to function essentially equivalently under both oxygen conditions, thereby resulting in increased CPS levels under high-oxygen but decreased CPS levels under low-oxygen conditions.

In contrast to the correlation between phosphatase activity and Cps2D phosphorylation, the results with the D39 derivatives containing *cps2B* point mutations demonstrated no significant correlations between Cps2B *in vivo* phosphatase activities and CPS levels, whether considering the mutants collectively or only under oxygen availability conditions (r^2 values of <0.2 and P values of >0.5 , based on data summarized in Fig. 5). Similarly, there were no significant correlations between Cps2D phosphorylation levels and CPS levels.

Cps2B mutants do not affect CPS polymer localization. In D39, CPS is localized to the membranes and cell wall, with negligible polymer being detectable in the culture medium (3). The Cps2B mutants did not exhibit notable alterations in CPS cellular localization or size distribution, as determined by CPS immunoblotting (Fig. 6), and no CPS was detected in the culture medium by ELISAs (data not shown).

DISCUSSION

Regulation of polysaccharide synthesis involves multiple complex mechanisms. For Wzy capsules, a number of observations have indicated roles for a tyrosine phosphoregulatory system that, in *S. pneumoniae*, is comprised of CpsB, CpsC, and CpsD. Previous studies with opaque variants of *S. pneumoniae* clinical isolates demonstrated decreases in levels of CPS production and CpsD phosphorylation during growth with high compared to low oxygen availability (27). Our results agree with and extend those findings and also demonstrate that CpsB phosphatase activity increases under high-oxygen conditions. However, while the phosphatase activity was clearly correlated with reductions in levels of CpsD phosphorylation, we found no correlation between the levels of CPS and these properties. The results show instead that the CpsB protein, but not its phosphatase activity, is necessary for full encapsulation under low-oxygen conditions, whereas CpsB is dispensable for CPS production under high-oxygen conditions. The CPS produced under high-oxygen condi-

FIG 5 CPS production by strains containing *cps2B* mutations. Indirect ELISAs were performed on D39; the repaired recipient strain (AG540); strains producing the R19K (AG550), S22N (AG548), G129E (AG547), and H136A (AG557) mutant Cps2B enzymes; and Δ*cps2B* strain AG554 grown under low- and high-oxygen conditions. Values are expressed as a fraction of D39 CPS production under low-oxygen conditions. One-way ANOVA and Tukey posttests were used for comparisons of CPS levels between conditions for mutant strains and for comparisons of mutant strains to D39 under high-oxygen conditions. Student's *t* test was used to detect differences between the low- and high-oxygen cultures of the same strain and under low-oxygen conditions between each mutant and D39, as each of these was an independent experiment. Results are the means ± standard errors from at least three independent cultures of each strain. *, *P* < 0.05; **, *P* < 0.01; ***, *P* < 0.001 (versus D39 under low-oxygen conditions). +, *P* < 0.05; ++, *P* < 0.01; +++, *P* < 0.001 (versus D39 under high-oxygen conditions). X, *P* < 0.05; XX, *P* < 0.01; XXX, *P* < 0.001 (for comparisons to low-oxygen conditions for this strain). ND, not determined. ^a, phosphatase activities of *S. pneumoniae* strains from Fig. 2A relative to that of D39 under low-oxygen conditions (set to a value of 1). ^b, Cps2D~P levels from Fig. 4, expressed as a fraction of D39 under low-oxygen conditions. Because there was no difference in Cps2D~P levels between D39 and the Δ*cps2B* mutant under high-oxygen growth conditions, these values were not determined for the strains with point mutations.

tions, or in a *cpsB* deletion mutant under low-oxygen conditions, therefore appears to represent baseline CPS levels for a bacterium with otherwise intact CPS machinery. The data are suggestive of models in which CpsB interacts with another protein or proteins to modulate CPS synthesis under low-oxygen growth conditions. Because the loss of CpsB results in decreased CPS levels under low-oxygen conditions, the interaction is predicted to increase CPS levels by either enhancing the effects of an activator or allevi-

ating the effects of a repressor. Mutations such as S22N and G129E may reduce this interaction, thereby reducing CPS synthesis, in addition to any effects on phosphatase activity. The H136A mutation, which eliminates phosphatase activity, may be unaffected in binding interactions, resulting in a CPS phenotype that is unchanged from that of the parent. The interactions are not expected to occur in the parental strain under high-oxygen conditions; however, the phenotype of the R19K mutant suggests that this mutation may allow for a constitutive weak interaction(s) that can now take place under both low- and high-oxygen conditions, resulting in similar levels of CPS under both conditions. CpsB-independent factors may also be important for the reduced CPS levels observed under high-oxygen conditions, and in a subsequent communication, we will describe a mechanism by which this may occur (J. R. Hauser and J. Yother, unpublished data).

Our observations may partly explain why differing conclusions regarding CpsD phosphorylation and CPS levels have been reached; i.e., a positive correlation is observed when parental strains grown under high- and low-oxygen conditions are compared (27), but an inverse relationship is observed when mutants devoid of CpsB are compared to the parent under low-oxygen conditions (25, 28). The results presented here show that the levels of Cps2D phosphorylation are not, in and of themselves, a determinant or a reflection of CPS levels. This conclusion is consistent with previous observations that showed that (i) phosphorylation of recombinant Cps2D occurs in *E. coli* despite the lack of CPS expression or the presence of any parts of the CPS system other than Cps2C (22), (ii) reduced but similar levels of CPS are present in D39 derivatives with either reduced or no phosphorylation of CpsD due to mutations affecting the tyrosine residues (23), (iii) increased CPS levels are present with elevated levels of phosphorylated Cps2D when a *cps2B* deletion is accompanied by an alteration of Cps2A in D39 (3; this study), and (iv) a nearly complete loss of CPS is present with elevated levels of phosphorylated CpsD when *cpsB* is deleted in Rx1 derivatives expressing serotype 2 or 19F CPS (3, 25, 28) (see Fig. S1D in the supplemental material). Rx1 is a highly passaged and manipulated derivative of D39 that exhibits multiple genetic changes (44–46). The profound reduction in the level of CPS in this background compared to that of

FIG 6 Immunoblot analysis of CPS. Cultures were grown under low-oxygen conditions. Cell wall (CW) fractions were loaded relative to the respective protoplast (PP) (membrane-containing) fractions. Results are not quantitative between strains but rather demonstrate relative banding patterns and proportions of CPS in the PP and CW fractions. CPS-negative strains exhibited no reactivity in the assay (data not shown) (3). Protein standards (See Blue Plus 2; Invitrogen) are for comparisons between gels and may not reflect the actual polymer mass. *, AG540 is the recipient strain used for *cps2B* mutations that has been repaired with wild-type (WT) *cps2B*.

D39 is strong evidence that factors outside the CPS locus, and defective in Rx1, are important for CPS regulation. The results of studies conducted in the Rx1 background (25, 28, 47) are therefore confounded by this variable. Our previous observation that CPS levels increased with the deletion of *cps2B* in D39 (3) now appears to result from the alteration in *cps2A*, which affected the C terminus of Cps2A. In a subsequent communication, we will describe the effects of different mutations in *cps2A*, some of which result in increases in CPS levels (K. Gupta and J. Yother, unpublished data). In each of our studies, mutations in the *cps* genes have been observed to alter CPS levels, but none of the mutations in *cps2A*, *cps2B*, *cps2C*, or *cps2D* affected either the ability to transfer CPS to the cell wall or the fraction of total CPS that was localized to the membranes and cell walls (3; this study).

Although CpsB is necessary for parental CPS synthesis under low- but not high-oxygen growth conditions, CpsD is essential under both conditions; i.e., a *cps2D* deletion mutant exhibits severely reduced CPS levels compared to those exhibited by the parent strain D39 under high-oxygen conditions (our unpublished observations), which is similar to that observed for the same mutant under low-oxygen conditions (3). Our results also do not preclude a role for the phosphorylation of CpsD in CPS synthesis; rather, they indicate that a correlation between the levels of the two is not evident. In the *E. coli* K30 (group 1) system, mutations that alter the tyrosine residues and thus eliminate phosphorylation result in a loss of CPS production (48), whereas similar mutations in *S. pneumoniae* D39 result only in a reduction of CPS levels (23). A recent study of *S. pneumoniae* D39 using a novel phosphatase inhibitor described increased Cps2D phosphorylation and reduced CPS levels when bacteria were incubated with the inhibitor at a level just below the MIC for growth (49). Inhibition appeared to occur by direct binding to Cps2B, an effect that may not be inconsistent with altering the ability of CpsB to bind other proteins, thereby influencing CPS production. For the K30 CPS in *E. coli*, it has been proposed that phosphatase activity allows for cycling between phosphorylated and nonphosphorylated forms of the CpsCD homolog Wzc, and this is critical to maintaining high-molecular-weight CPS (48). A failure to cycle due to defects in phosphatase activity likely does not explain our results, given the lack of correlation between phosphatase activities and CPS levels. In the K30 system, a point mutation in an essential catalytic site eliminated both phosphatase activity and CPS production (50), an effect that we observe in *S. pneumoniae* only when *cpsB* mutations are made in the Rx1 background. The Gram-negative Wzb phosphatase and the Gram-positive CpsB phosphatase are members of different families (low-molecular-weight phosphotyrosine phosphatase and PHP, respectively), which could result in overall differences in the systems. It may also be of some relevance that studies with *E. coli* generally use bacteria grown with high oxygen levels, a point that could impact the outcome should similarities between the systems extend to oxygen effects.

The observation that CpsB phosphatase activity is increased during high-oxygen growth suggests that this may be the condition under which this function is most important. Because CpsB is not necessary for parental CPS expression under these conditions, the phosphatase activity may affect functions unrelated to CPS synthesis. Although other targets for CpsB phosphatase activity and CpsD-mediated phosphorylation have yet to be identified in *S. pneumoniae*, it is not unreasonable to consider that CpsB and CpsCD may provide a link between the regulation of CPS and that

of other cellular pathways. A phosphoproteomic analysis of *S. pneumoniae* D39 grown in the same medium used here and with reduced oxygen revealed 85 phosphorylated proteins with 163 phosphorylation sites, 14 of which were on Tyr residues located in 13 different proteins (51). Cps2D was not among the proteins identified using titanium oxide to enrich for phosphopeptides, suggesting that additional phosphorylated proteins may be identified in the future. Conditions enriching the phosphorylation of CpsD, such as reduced-oxygen conditions or *cpsB* deletions and point mutations, may prove valuable in identifying non-CpsD targets of CpsD~P-mediated phosphorylation.

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