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RR06 Activates Transcription of spr1996 and *cbpA* in *Streptococcus pneumoniae*▽

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Streptococcus pneumoniae **colonizes at the nasopharynx of humans and is able to disseminate and cause various infections. The hallmark of pneumococcal disease is rapid bacterial replication in different tissue sites leading to intense inflammation. The genetic basis of pneumococcal adaptation to different host niches remains sketchy. In this study, we investigated the regulatory effect of RR06, a response regulator protein, on gene expression of *S. pneumoniae*. Microarray and Northern blot analyses showed that RR06 is specifically required for transcription of spr1996 and *cbpA*. While the function of Spr1996 is unknown, CbpA has been well characterized as a surface-exposed protective antigen and a virulence factor of *S. pneumoniae*. A recombinant form of RR06 was able to bind to a 19-bp conserved sequence shared by the spr1996 and *cbpA* promoter regions. Furthermore, inactivation of *rr06* resulted in loss of CbpA expression as detected by antibody staining and bacterial adhesion. CbpA expression was restored in *trans* by the intact *rr06* gene. However, a mutant, RR06(D51A), with a point mutation in the aspartate residue at position 51 (a predicted major phosphorylation site) of RR06, completely abolished the CbpA expression, suggesting that RR06 phosphorylation is required for transcriptional activation of spr1996 and *cbpA*. Finally, inactivation of *rr06* in additional pneumococcal strains also led to the loss of CbpA expression. These data implicate that RR06 activates the expression of spr1996 and *cbpA* in many other pneumococcal strains.**

Streptococcus pneumoniae naturally colonizes at the nasopharynx of humans and behaves as a commensal in immunocompetent adults. In young children, the elderly, and immunocompromised individuals, the bacterium is able to disseminate to remote tissue sites (lung, middle ear, bloodstream, and brain). Pneumococcal replication in the local environments induces intense tissue inflammation, leading to bacterial pneumonia, acute otitis media, bacteremia, and other complications (38). These observations indicate that *S. pneumoniae* is highly capable of adapting to different host niches during infection, as well demonstrated in many other bacterial pathogens (36). Recent transcriptional studies have identified niche/body site-specific expression patterns for many pneumococcal genes (29, 43). The differentially expressed genes include those involved in capsule biosynthesis and the production of pneumolysin and surface-exposed proteins. The mechanisms of the gene regulation are not well understood except for the well-characterized competence system (44).

Two-component signal transduction systems (TCSs) are common regulatory mechanisms for bacterial responses to various environmental cues (54). Many TCSs are important in bacterial interactions with hosts (12). A typical TCS consists of a membrane-associated histidine kinase and a cytosolic cognate response regulator. Upon receipt of an extracellular stimulus, the histidine kinase is activated by autophosphorylating a conserved histidine residue (54). The phosphorylated histidine kinase further donates the phosphate group to a conserved aspartate residue in its cognate response regulator which, in turn, undergoes a conformational change and regulates gene expression mostly as a transcriptional regulator. There are 13 complete TCSs and 1 orphan response regulator in the complete genome sequences of strains R6 (17) and TIGR4 (56). Eight of the TCSs were reported to contribute to respiratory infection of *S. pneumoniae* (57), although Lange et al. (25) showed that insertion inactivation in the 13 RR tested did not have significant impact on systemic pneumococcal infection in mice.

Among the TCSs in *S. pneumoniae*, CiaR/H, the first pneumococcal TCS identified, is involved in competence and antibiotic susceptibility (13). Recent studies also revealed the down-regulation of the major virulence factor HtrA in *ciaRH* mutants (20, 32, 51). VicRK, also known as YycFG or MicAB, is involved in maintaining integrity of the cell wall (40), fatty acid biosynthesis (37), and expression of pneumococcal surface protein A (PspA) (41). ComD/E was demonstrated to control pneumococcal competence (45) and contribute to virulence (3, 15, 26). BlpR/S regulates the production of bacteriocin-like proteins (9). PnpR/S appears to control the expression of pneumococcal surface antigen A (PsaA) and contributes to pneumococcal virulence and resistance to oxidative stress (33). RitR, the orphan response regulator, represses the *piu* iron uptake system (59). Standish et al. (53) recently showed that RR06/HK06 regulates the expression of choline binding protein A (CbpA). CbpA is required for pneumococcal colonization (2, 24, 47), lung infection (2, 15, 24), and sepsis (18). In vitro studies have also revealed specific interactions of CbpA with human poly-immunoglobulin (Ig) receptor (pIgR), secretory IgA, secretory component, complement factor H, and C3 (7). Some of these biochemical interactions are consistent with the observations that CbpA is necessary for pneumococcal adhesion to and invasion of airway epithelial cells (11, 47, 63).

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TABLE 1. Bacterial strains used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
D39	*S. pneumoniae* isolate, serotype 2, encapsulated; *rr06*$^+$ *cbpA*$^+$; Cm^s	1
R6	D39 derivative, unencapsulated; *rr06*$^+$ *cbpA*$^+$; Cm^s Kan^s Sp^s Erm^s	58
G54	*S. pneumoniae* serotype19F, an isolate from human sputum; *rr06*$^+$ *cbpA*$^+$; Cm^s	46
ST398	R6 derivative; *hk06*::pID701t; R6 transformed with pST393; Cm^r	This study
ST489	R6 derivative; *rr06*::pID701t; R6 transformed with pST382; Cm^r	This study
ST556	*S. pneumoniae* serotype 19F, an isolate from a patient with otitis media; *rr06*$^+$ *cbpA*$^+$; Cm^s	22
ST568	R6 derivative; Δ*cbpA*::Janus cassette; Kan^r	This study
ST796	D39 derivative; *rr06*::pID701t; R6 transformed with pST382; Cm^r	This study
ST797	D39 derivative; *hk06*::pID701t; R6 transformed with pST393; Cm^r	This study
ST815	R6 derivative; R6 transformed with pDL278; Sp^r	This study
ST820	R6 derivative; Δ*rr06*::*ermB*; Erm^r	This study
ST822	R6 derivative; Δspr1996::*ermB*; Erm^r	This study
ST918	ST556 derivative; *rr06*::pID701t; ST556 transformed with pST382; Cm^r	This study
ST920	ST556 derivative; *hk06*::pID701t; ST556 transformed with pST393; Cm^r	This study
ST922	G54 derivative; *rr06*::pID701t; G54 transformed with pST382; Cm^r	This study
ST924	G54 derivative; *hk06*::pID701t; G54 transformed with pST393; Cm^r	This study
ST969	R6 derivative; ST820 transformed with pDL278; Erm^r Sp^r	This study
ST970	R6 derivative; ST820 transformed with pST804; Erm^r Sp^r	This study
ST986	R6 derivative; ST820 transformed with pST982; Erm^r Sp^r	This study
Plasmids		
pID701t	Suicide plasmid; Cm^r	26
pJDC9	Suicide plasmid; Erm^r	5
pMalc2E	Maltose binding protein fusion vector; Ap^r	NEB
pDL278	*E. coli*-streptococcal shuttle plasmid; Sp^r	28
pST382	5′ coding sequence of R6 *rr06* cloned in XbaI site of pID701t; Cm^r	This study
pST393	5′ coding sequence of R6 *hk06* cloned in SmaI site of pID701t; Cm^r	This study
pST804	pDL278::RR06; *rr06* coding sequence with whole intergenic region cloned in BamHI/EcoRI site of pDL278; Sp^r	This study
pST825	pMalE::RR06; coding sequence of R6 *rr06* cloned in BamHI/HindIII site of pMALc2E; Ap^r	This study
pST982	pMalE::RR06; coding sequence of R6 *rr06* cloned in BamHI/EcoRI site of pDL278; Sp^r	This study

[a] Ap, ampicillin; Cm, chloramphenicol; Erm, erythromycin; Kan, kanamycin; Sp, spectinomycin; r, resistant; s, sensitive.

CbpA is among a few pneumococcal proteins that can stimulate antibody production in humans (34, 35) and is able to confer protective immunity against lethal challenge of virulent pneumococci in animal models (2, 4, 42, 47). However, it is unclear if RR06/HK06 regulates additional pneumococcal genes and virulence factors.

In this study, we have analyzed the impact of RR06 on gene expression in *S. pneumoniae.* The transcriptional profile of laboratory strain R6 was compared with an isogenic *rr06*-deficient mutant by microarray. Together with the data from further transcription and protein analyses, RR06 was shown to activate the transcription of its downstream neighbor genes spr1996 and *cbpA* by binding to a conserved sequence motif in the 5′ untranslated regions of genes spr1996 and *cbpA*.

MATERIALS AND METHODS

Bacterial strains and growth media. The bacterial strains and plasmids used in this study are listed in Table 1. G54 is a serotype 19F isolate from human sputum (46) and was kindly provided by Francesco Iannelli. ST556 is a serotype 19F isolate from a patient with otitis media (22) and was kindly provided by Michael R. Jacobs. Pneumococci were routinely grown in Todd-Hewitt broth containing 0.5% yeast extract (THY) or on tryptic soy agar (TSA) plates containing 3% (vol/vol) sheep blood. When necessary, kanamycin (200 μg/ml), spectinomycin (600 μg/ml), chloramphenicol (2 μg/ml), or streptomycin (150 μg/ml) was included in the broth and agar media for selection purposes. *Escherichia coli* strains DH5α and TB1 were used for routine cloning and protein expression, respectively. *E. coli* cultures were grown in Luria-Bertani (LB) broth or on LB agar plates. Antibiotic selection in *E. coli* was performed with the following final concentrations: 100 μg/ml for ampicillin, 100 μg/ml for spectinomycin, 25 μg/ml for chloramphenicol. All ingredients for bacterial culture media and other chemicals used in this work were obtained from Sigma (St. Louis, MO) unless otherwise stated. *E. coli* strains were grown at 37°C with aeration, and pneumococci were incubated at 37°C with 5% CO_2.

Construction of *S. pneumoniae* mutants and plasmids for the complementation study. The *rr06* and *hk06* insertion mutants of *S. pneumoniae* were generated as described elsewhere (62). Briefly, the 5′ coding regions of *rr06* and *hk06* were amplified by PCR from chromosomal DNA of strain R6 using primers Pr245/Pr246 and Pr227/Pr228, respectively. All of the primers were synthesized by Invitrogen (Carlsbad, CA) and are listed in Table 2. PCR amplifications were performed using a high-fidelity DyNAzyme EXT DNA polymerase (MJ Research, Waltham, MA) under the conditions described previously (61). The PCR products were then cloned into the XbaI (for *rr06*) or SmaI (for *hk06*) site of plasmid pID701t (26). The plasmid constructs for insertional inactivation were confirmed by DNA sequencing with vector-based primers. The correct plasmids were transformed into *S. pneumoniae* by natural transformation as described previously (62). The transformants were selected for resistance to chloramphenicol (2 μg/ml) on the TSA plates.

The *rr06* and spr1996 deletion mutants in strain R6 were prepared essentially as described previously (31). The upstream (1,146-bp) and downstream (1,798-bp) flanking regions of *rr06* in R6 were separately amplified from the R6 genomic DNA by PCR using primer pairs Pr722/Pr723 and Pr724/Pr725, respectively. An erythromycin (*ermB*) cassette was amplified from plasmid pJDC9 (5) using primers Pr254 and Pr255. The PCR products of the *ermB* cassette and the *cbpA* flanking sequences were digested by appropriate restriction enzymes as determined by the nested restriction sites (AscI and FseI) at the 5′ end of the primers, purified from agarose gels using the DNA gel purification kit (QIAGEN, Valencia, CA), and ligated using the Quick ligation kit from New England Biolabs (NEB; Beverly, MA). The ligation mixtures were transformed into *S. pneumoniae* to select erythromycin-resistant colonies on blood agar plates as described elsewhere (27). The resultant mutant strains were verified by PCR am-

TABLE 2. DNA sequences and locations of primers used in this study

Primer	Sequence	Location[a]
Pr074	5′-ACAGAGAACGAGGGAAGTACC-3′	4156–4176
Pr227	5′-GAGGGGGAAAAGTTTAATGAGAGC-3′	6161–6184
Pr228	5′-GTTACCTGTTTACCATCTGCTGTAC-3′	5937–5961
Pr231	5′-TTAGCAATGGCGGGAAAGAA-3′	4792–4811
Pr245	5′-CTAGTCTAGACGAGGAAATGATTAGAGAAGGAA-3′	6912–6934
Pr246	5′-CTAGTCTAGATGACAGGAATATCGTGCTTTTG-3′	6735–6756
Pr254	5′-ATTGGCGCGCCGATATAATGGGAGATAAGACG-3′	42–62
Pr255	5′-ATTGGCGCGCCGATATAATGGGAGATAAGACG-3′	989–1011
Pr257	5′-CCTATGGCTGGCGTGACCAACTC-3′	12492–12514
Pr311	5′-ATTGGCGCGCCACCGTTTGATTTTTAATGGATAATG-3′	773–796
Pr312	5′-ATTGGCCGGCCCCTTTCCTTATGCTTTTGGAC-3′	2080–2100
Pr313	5′-ATTGGCGCGCCGTTTATTTCCTTCTATATTTTTTC-3′	9241–9264
Pr314	5′-ACCGGCCGGCCAACCTAATATAACTAGTTAATACTG-3′	11370–11394
Pr722	5′-TGACAGATAGCAAGGGACGCAAGG-3′	8081–8104
Pr723	5′-ATTGGCGCGCCCATCTCTCTCCCTTTCTACTA-3′	6944–6975
Pr724	5′-ACCGGCCGGCCCTGTGAAAAATGTTGGGTATA-3′	6318–6349
Pr725	5′-TGACAGATAGCAAGGGACGCAAGG-3′	4552–4575
Pr726	5′-TTGGCGATATCAGGATGTAACAGT-3′	6563–6586
Pr727	5′-ATTGGCGCGCCCATATTAGTATCCTTTCTTTTAT-3′	4880–4913
Pr728	5′-ACCGGCCGGCCATACAAAATTCGATTTATATACAG-3′	4342–4376
Pr729	5′-TTAGGTAGTACCATGAACCATTGT-3′	2742–2765
Pr739	5′-CGATGGATCCATTGGTGTCAAAGCAGGCCAGTTA-3′	7617–7650
Pr740	5′-CGATGAATTCTAGGATTTTTTATCATAAGCTAAT-3′	6282–6315
Pr750	5′-CCGCGAATTCTTATCTCTTTATGCTTCCATT-3′	4356–4386
Pr758	5′-ACGATCTGTCTTGATGTTTTCTAA-3′	3598–3621
Pr669	5′-CCGCGCATGCCAGTTAAAATTTGATATTGCA-3′	5933–5949
Pr670	5′-CCAACGAATTCGCTATATGAATGACTAGACCAAC-3′	7308–7330
Pr781	5′-CGCGGATCCATGAACATTTTAGTTGCAGATGAC-3′	6934–6966
Pr789	5′-CCGCCGAAGCTTCTAGAATTGCAAAACTTCTTA-3′	6238–6270
Pr801	5′-TCATATCTTGTTTAGGACAGT-3′	4550–4570
Pr802	5′-CTTTTTGATGCAAACATGTTT-3′	4271–4291
Pr803	5′-AATACATGTGCGCCTCTAAGT-3′	5150–5170
Pr804	5′-ACTAAAATAATCGCTAATACC-3′	4871–4891
Pr835	5′-GCAGGCCAGTTAAAATTTGATATT-3′	7605–7628
Pr836	5′-TTCCTCGTCATCTGCAACTAAAAT-3′	6928–6951
Pr855	5′-TCGATTTATATACGGTTCATATTGAA-3′	4331–4356
Pr856	5′-TTCAATATGAACCGTATATAAATCGA-3′	4331–4356
Pr857	5′-CATAAAGAGATAAATACAAAATTCGA-3′	4353–4378
Pr858	5′-TCGAATTTTGTATTTATCTCTTTATG-3′	4353–4378
Pr859	5′-TGAAGTAATATAGTAAGGTTAAAGAA-3′	4309–4334
Pr860	5′-TTCTTTAACCTTACTATATTACTTCA-3′	4309–4334
Pr865	5′-TAGTTTTTTATTAAAGTTCATATAGGG-3′	4943–4969
Pr866	5′-CCCTATATGAACTTTAATAAAAAACTA-3′	4943–4969
Pr867	5′-TTAAGATTAGCTTGTAGACAGATTAGT-3′	4966–4992
Pr868	5′-ACTAATCTGTCTACAAGCTAATCTTAA-3′	4966–4992
Pr869	5′-AGGGTTAACATAAGTGTGTTATTCTTT-3′	4920–4946
Pr870	5′-AAAGAATAACACACTTATGTTAACCCT-3′	4920–4946
Pr990	5′-CAAGATCTCCCTATCCATCTCATGGTATTGGCATTAATG-3′	6799–6837
Pr991	5′-CAATACCATGAGATGGATAGGGAGATCTTG-3′	6808–6837

[a] The numbers indicate primer positions in GenBank accession number AE008564 except for primers Pr254 and Pr255, which are based on accession number Y00116.

plification or direct DNA sequencing of the mutation sites using genomic DNA preparations of the mutant strains. The spr1996 deletion mutant in strain R6 was constructed in a similar manner. The spr1996 flanking regions were PCR amplified using primer pairs Pr726/Pr727 and Pr728/Pr729. The *cbpA* deletion strain ST568 was constructed in R6 by replacing the entire *cbpA*-coding region with the Janus cassette as described previously (31). The upstream and downstream *cbpA*-flanking regions in strain R6 were separately PCR amplified from genomic DNA preparations using primer pairs Pr252/Pr313 and Pr314/257, respectively. The PCR fragments were ligated to the AscI/FseI-digested Janus cassette, which confers kanamycin resistance (200 μg/ml) in *S. pneumoniae* (55). The Janus cassette was PCR amplified with primers Pr311 and Pr312 from the *cbpA*-null mutant ST588 of *S. pneumoniae* strain D39 (31).

To prepare the *rr06* complementation construct, the sequence consisting of the *rr06* coding sequence and its entire 5′ untranslated region was PCR amplified with primers Pr739 and Pr740, digested with BamHI and EcoRI, and cloned into the *E. coli*-streptococcal shuttle plasmid pDL278 (28). The resulting plasmid, pST804, was verified by DNA sequencing and transformed into the *rr06*-null mutant ST820 by natural transformation as described previously (62). The mutant RR06(D51A) allele was generated by a PCR-based strategy (31) in which the aspartate residue at position 51 of RR06 was replaced with an alanine. The GAT (D51) codon of the *rr06* gene was changed to GCA (A51) by amplifying the up- and downstream segments surrounding the 51st codon using primer pairs Pr739/Pr991 and Pr990/Pr740, respectively. Two PCR fragments were fused by overlap extension PCR using primers Pr739 and Pr740 (16) and cloned in the BamHI/EcoRI-digested pDL278. The resultant plasmid, pST982, was verified by DNA sequencing and transformed into ST820 as described above. In parallel, pDL278 was also introduced into various *S. pneumoniae* strains as negative controls.

RNA isolation and microarray analysis. Total RNA extracts were isolated from the cell cultures of the wild-type R6 and *rr06* mutant ST489 grown to an optical density at 620 nm (OD_{620}) of 0.5 in the THY broth with the RNeasy mini kit (QIAGEN) according to the manufacturer's instructions. RNA concentra-

tions were determined by measuring the OD values at 260 and 280 nm, checked for RNA integrity by gel electrophoresis based on rRNA bands, and stored at −80°C until later use. To minimize the impact of growth conditions and RNA preparation on the abundance of specific mRNAs, three RNA samples were prepared for each of the *S. pneumoniae* strains at separate times as biological replicates and analyzed in parallel in the subsequent DNA microarray experiments.

Microarray analysis was performed with the *S. pneumoniae* R6 GeneChip (Affymetrix, Santa Clara, CA), which contained oligonucleotide probes for the 2,043 protein-coding genes and 73 noncoding RNA genes of *S. pneumoniae* strain R6 (17) and additional control oligonucleotides. Probe preparation, DNA hybridization, and data collection were carried out according to the manufacturer's instructions at the Bionomics Research and Technology Center at The Rutgers University (Piscataway, NJ). The hybridization probes consisted of differentially labeled cDNA derived from total RNA isolated from R6 and the isogenic *rr06* mutant ST489. Data analysis was performed using the Affymetrix Microarray Suite software 5.0 with Affymetrix default parameters. The change was calculated by dividing the median of the normalized red channel intensity by the median of the normalized green channel intensity. An average twofold difference in expression level between R6 and ST489 in the three hybridization experiments was used as a cutoff factor. In addition, the statistical program EBarrays was applied to determine if the two gene expression profiles were statistically significant as described elsewhere (23).

DNA and protein sequence analysis. DNA and protein sequence analyses were performed using the DNASTAR Lasergene v6.1 program for Macintosh computers (Madison, WI). The nucleotide sequence of the *rr06-cbpA* locus of strain ST556 was determined by automated DNA sequencing with the primers based on the corresponding DNA sequences of the strains R6 (accession number AE008564) (17) and TIGR4 (accession number AE007507) (56).

Northern blot analysis. Total RNA was extracted from *S. pneumoniae* cultures and quantified as described above. Aliquots of RNA (5 μg) were denatured at 65°C for 15 min and separated by electrophoresis through a denaturing formaldehyde-agarose (1.2%) gel according to standard procedures (48). The RNA was then transferred onto positively charged nylon membranes (GE Healthcare Life Sciences, Piscataway, NJ) and baked at 80°C for 2 h. The membranes were hybridized with the *cbpA* or spr1996 probe. The *cbpA* and spr1996 probe templates were first amplified from the genomic DNA of strain R6 using primer pairs Pr074/Pr758 and Pr231/Pr750, respectively. Radioactive probes were then prepared with [α-^{32}P]dCTP (Perkin-Elmer, Wellesley, MA) using the DECA prime II random priming DNA labeling kit (Ambion, Austin, TX).

Expression and purification of MalE-RR06. The full-length *rr06* coding sequence was amplified using primers Pr781and Pr789 and cloned into the BamHI-HindIII-digested plasmid pMALc2E (NEB). The resulting plasmid, pST825, was used to overexpress the MalE-RR06 fusion protein in *E. coli* strain TB1 as recommended by the supplier. Briefly, the *E. coli* strain was grown in LB at 37°C with aeration. A final concentration of 0.3 mM isopropyl-β-D-thiogalactopyranoside was added to induce the expression of the fusion protein when the OD_{600} reached 0.5. The culture was incubated for an additional 3 h and centrifuged at 8,000 × *g* for 15 min. Cell pellets were resuspended with 50 ml of ice-cold column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 1 mM EDTA) in the presence of 1 mM phenylmethylsulfonyl fluoride. The cell suspension was passed three times through a French press (20,000 lb/in^2) and centrifuged at 10,000 × *g* for 30 min to remove the insoluble debris. The supernatants were diluted 1:5 with the ice-cold column buffer in the presence of 1 mM phenylmethylsulfonyl fluoride and loaded onto a 5-ml amylose column (NEB), which had previously been equilibrated with 40 ml of the column buffer. The column was washed with 120 ml of the ice-cold column buffer. The MalE-RR06 protein was eluted with 10 ml of the column buffer containing 10 mM maltose and collected in fractions of 1.0 ml each. Protein concentrations of the fractions were determined by using the Bio-Rad protein assay reagent (Hercules, CA). The three fractions with the highest protein concentrations were pooled and dialyzed. For storage, glycerol was added to a final concentration of 50%. The fusion protein was purified to homogeneity as verified by Coomassie blue staining of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels.

EMSA. The ability of the MalE-RR06 protein to bind the *rr06*, spr1996, and *cbpA* promoter regions was tested using an electrophoretic mobility shift assay (EMSA). The DNA fragments spanning the 5′ untranslated regions of the *rr06*, spr1996, and *cbpA* genes in strain R6 were amplified with primer pairs Pr835/Pr836, Pr801/Pr802, and Pr803/Pr804, respectively. The double-stranded DNA segments spanning the 5′ untranslated region of *cbpA* were prepared by annealing complementary pairs of oligonucleotides Pr857/Pr858 for Oligo-1, Pr855/Pr856 for Oligo-2, and Pr859/Pr860 for Oligo-3 for 10 min at 90°C in TEN buffer (1 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 100 mM NaCl). The double-stranded DNA oligonucleotides for the spr1996 promoter region were prepared in the same manner by annealing complementary pairs of oligonucleotides Pr867/Pr868 for Oligo-4, Pr865/Pr866 for Oligo-5, and Pr869/Pr870 for Oligo-6. These DNA segments were end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Invitrogen) and used for binding reactions with purified MalE-RR06. For protein-DNA binding, radiolabeled DNA probes (5 ng) were incubated with various concentrations of the MalE-RR06 fusion protein at room temperature for 30 min in 20 μl of binding buffer [20 mM HEPES, pH 8.0, 5 mM $MgCl_2$, 50 mM potassium glutamate, 0.01 mM EDTA, 1 mM NaH_2PO_4, 20 mM NaCl, 1 mM dithiothreitol, 30 μg/ml bovine serum albumin, and 30 mg/ml poly(dI-dC)]. Where indicated, an excessive amount (100 ng) of specific DNA fragments (cold probes) was included in the reaction mixtures for competitive binding. Samples were subjected to electrophoresis in 5% Tris-borate-EDTA nondenaturing Ready gels (Bio-Rad) at room temperature in 0.5× Tris-borate-EDTA buffer with 1.2% glycerol. The gels were dried with a gel dryer and exposed to X-ray films at −70°C for 3 h.

Western blot and immunofluorescence microscopy. Western blot analysis with CbpA was carried out as previously described (31). In brief, pneumococcal strains were grown in THY broth to an OD_{620} of 0.5. Cells were collected by centrifugation and resuspended in lysis buffer (0.1% SDS, 0.1% deoxycholate, and 0.15 M sodium citrate). Protein concentrations were determined using the Bio-Rad protein assay reagent. Samples containing approximately 5 μg of total bacterial proteins were mixed with the standard SDS-PAGE loading buffer (48), boiled for 5 min, and then separated in 10 to 20% Tris-HCl SDS-PAGE gels (Bio-Rad). After semidry electrophoretic transfer of proteins onto the polyvinylidene difluoride membranes (Millipore, Bedford, MA), CbpA was detected with a rabbit antiserum for the full-length CbpA of strain TIGR4 as described previously (31). Following a blocking step in 5% milk (wt/vol), the membranes were reacted with the CbpA antiserum (1:5,000 dilution) and peroxidase-conjugated goat anti-rabbit IgG antibody (1:5,000 dilution). Reactive protein bands were visualized with the enhanced chemiluminescence Western blot kit (Pierce, Rockford, IL). To detect the CbpA variants in strains ST556 and G54 with complement factor H, the blots of bacterial lysates were treated with purified human factor H (Sigma) at a final concentration of 0.4 μg/ml overnight at 4°C. Additional detection and visualization steps were carried out as described elsewhere (31).

Fluorescence staining of live pneumococci with the CbpA antibody was carried out as described previously (31). A final dilution of 1:100 was used for the CbpA antiserum. Bacteria were inspected by standard phase-contrast and fluorescence microscopy using an Olympus BX51 upright fluorescence microscope.

Bacterial adhesion assay. Pneumococcal adherence was determined in a 24-well plate format as described previously (63). Human airway cell lines Detroit 562 (nasopharyngeal cells) and A549 (type II lung epithelial cells) were obtained from the American Type Culture Collection (Manassas, VA) and maintained as described previously (63). Pneumococci (2×10^7 CFU/well) were incubated with confluent cell monolayers of Detroit 562 or A549 cells in 24-well plates for 30 min. After washing three times with phosphate-buffered saline, epithelial cells were detached with trypsin-EDTA, lysed with ice-cold Triton X-100 (0.05%), diluted in phosphate-buffered saline, and plated onto the TSA blood plates for CFU counts. All assays were performed in four to six replicate wells and repeated at least three independent times. The results of representative experiments are presented as means plus standard deviations.

Nucleotide sequence accession number. The nucleotide sequence of the *rr06-cbpA* locus of strain ST556 is contained in GenBank under accession number DQ851866.

RESULTS

Microarray analysis of the *rr06* mutant. A previous study reported that the *rr06/hk06* locus is involved in regulating the expression of *cbpA* (53). We determined if this TCS regulated expression of other pneumococcal genes and virulence factors by transcriptional analysis of an *rr06* mutant in laboratory strain R6 by microarray analysis. The complete genome sequence of R6 has identified 2,043 protein-coding genes and 73 noncoding RNA genes (17). The *rr06/hk06* locus is highly conserved in all the strains with available sequence information at this locus, including strains R6 (type 2), TIGR4 (type 4), G54 (type 19), and ST556 (type 19). The 3′ coding sequence of *rr06* has a 4-bp overlap with the 5′ end of *hk06*, suggesting these two

TABLE 3. Transcription profiles of pneumococcal genes in the *rr06* mutant ST489

Gene name[a]	Function	Fold change[b]			
		Expt 1	Expt 2	Expt 3	Mean
rr06	Response regulator	−127.9	−156.77	−156.99	−147.22
hk06	Histidine kinase	−69.31	−99.44	−79.56	−82.77
spr1996	Hypothetical protein	−11.03	−6.09	−6.43	−7.85
cbpA	Surface-exposed protein	−10.81	−6.65	−7.29	−8.25

[a] The gene names are based on the annotation of the R6 genome (17).
[b] Negative values indicate a reduction in transcription.

genes form an operon. The *rr06* mutant ST489 was constructed by inserting plasmid pID701t in the 5′ coding sequence of *rr06* (see Materials and Methods). Because of the apparent operon structure in the *rr06*/*hk06* locus, this insertion was expected to interrupt the production of both the RR06 and HK06 proteins. Consistent with previous reports (25, 57), ST489 did not show any obvious growth defect in THY (data not shown).

To compare the transcriptional profiles between ST489 and the parent strain R6 by microarray, total RNA extracts were prepared with three independent batches of bacterial cultures for each of ST489 and R6. The RNA preparations were used to prepare the cDNA probes. The differentially labeled cDNA probes of ST489 and R6 were hybridized with the R6 GeneChips representing all of the 2,043 protein-coding regions and other noncoding sequences (intergenic regions and noncoding RNA genes). In this study a gene was considered differentially expressed if (i) hybridization signals between ST489 and R6 differed by a factor of 2 on average and (ii) the calculated probability of differential expression was ≥0.5. A total of 41 genes or intergenic regions had a change of ≥2-fold in three separate biological replicates. To our surprise, only the changes for *rr06*, *hk06*, spr1996, and *cbpA* were consistent in all three separate reactions with statistical significance (Table 3). The rest of the genes were identified in only one of three hybridizations. As a result, the mean fold changes were all below 2 and statistically insignificant.

The transcription levels of *rr06*, *hk06*, spr1996, and *cbpA* were each decreased more than sixfold in ST489 compared with the parent strain R6. A diminished mRNA level of *rr06* in strain ST489 confirmed the *rr06* mutation, whereas the loss of *hk06* transcription was due to a predicted polar effect of the upstream insertion in *rr06*. The *rr06* mutant had similarly reduced levels of expression of spr1996 (7.85-fold) and *cbpA* (8.25-fold). Since ST489 had diminished expression of both *rr06* and *hk06*, the microarray result suggested that the *rr06*/*hk06* TCS is required to activate the transcription of spr1996 and *cbpA*. It should be noted that the mutation in *rr06* completely abolished transcription of *rr06* and *hk06*, but there were still low levels of transcription in spr1996 and *cbpA* in ST489, suggesting that basal levels of transcription occur in spr1996 and *cbpA* even in the total absence of the *rr06*/*hk06* TCS. Surprisingly, the mutation in *rr06* did not result in significantly increased expression of any other pneumococcal genes, suggesting that the *rr06*/*hk06* TCS only activates the expression of spr1996 and *cbpA* under these in vitro culture conditions.

The biological function(s) of the protein encoded by spr1996 remains unknown. Sequence analysis has suggested that Spr1996 is an integral membrane protein with a putative isoprenylcysteine carboxyl methyltransferase activity (data not shown). Spr1996 has a 21.1% amino acid identity with *Saccharomyces cerevisiae* Ste14p, an isoprenylcysteine carboxyl methyltransferase and an integral membrane protein. The sequence and genetic localization of spr1996 is highly conserved among the examined pneumococcal strains. Homologues of spr1996 are also present in the genomes of many other bacteria, including *Streptococcus agalactiae*, *Streptococcus suis*, *Streptococcus pyogenes*, and staphylococci.

To determine whether Spr1996 affects the expression of CbpA, spr1996 was replaced with an erythromycin cassette in the R6 background by allelic replacement. The resulting spr1996 mutant ST822 did not show an obvious growth defect, indicating that Spr1996 is not essential for *S. pneumoniae* growth in vitro (data not shown). Western blotting revealed comparable levels of CbpA production between ST822 and the wild-type strain (data not shown), which argues against a direct role of Spr1996 in CbpA expression. We further tested the adherence and invasion of the spr1996-null mutant in respiratory epithelial cell cultures as shown below in Fig. 6. No obvious difference was observed in the levels of epithelial adherence and invasion between the spr1996-null mutant ST822 and the wild-type strain (data not shown). Extensive characterization of Spr1996 was not further pursued due to the scope of this study.

Cotranscription of *cbpA* with spr1996. Based on the microarray data, we wondered how the *rr06* controls the expression of spr1996 and *cbpA*. As illustrated in Fig. 1A, sequence analysis did not identify an apparent transcriptional terminator in the 78-nucleotide intergenic region between spr1996 and *cbpA*. This raised the possibility that spr1996 and *cbpA* share a single promoter. This idea was tested by Northern blot analysis of various isogenic mutants in the strain R6 background with ^{32}P-labeled probes for coding sequences of spr1996 and *cbpA*. As represented in Fig. 1B, the *cbpA* probe revealed two transcripts in strain R6 with sizes of approximately 2.2 and 2.7 kilonucleotides. Both bands were absent in the *cbpA*-null mutant strain ST568, indicating these were *cbpA*-specific transcripts. The larger transcript had an equivalent size of the combined *cbpA* (2,106 nucleotides) and spr1996 (528 nucleotides), suggestive of cotranscription between spr1996 and *cbpA*. This large transcript was also detected with the spr1996 probe, but the same probe did not detect the 2.2-kilonucleotide transcript (Fig. 1C). We thus conclude that the 2.2- and 2.7-kilonucleotide bands represented a transcript of *cbpA* alone and a bicistronic message of spr1996 and *cbpA*, respectively. In agreement with the previous report (53), the Northern blot results also indicated that the *rr06*/*hk06* locus is not cotranscribed with spr1996 and *cbpA*, because the insertion mutation in *hk06* did not affect the size or amount of the spr1996-*cbpA* transcript (Fig. 1B).

Neither the 2.2- nor 2.7-kilonucleotide band could be detected in the *rr06* mutant ST489 with both the *cbpA* and spr1996 probes (Fig. 1B). The result thus confirmed the microarray data (Table 3) indicating that *rr06* is required for expression of both spr1996 and *cbpA*. Interestingly, inactivation of *hk06* itself (strain ST398) did not lead to obvious alteration in the transcription of spr1996 or *cbpA*, because the mutant and wild-type strains had similar levels of the *cbpA*-spr1996 cotranscript (large band) and the *cbpA* transcript

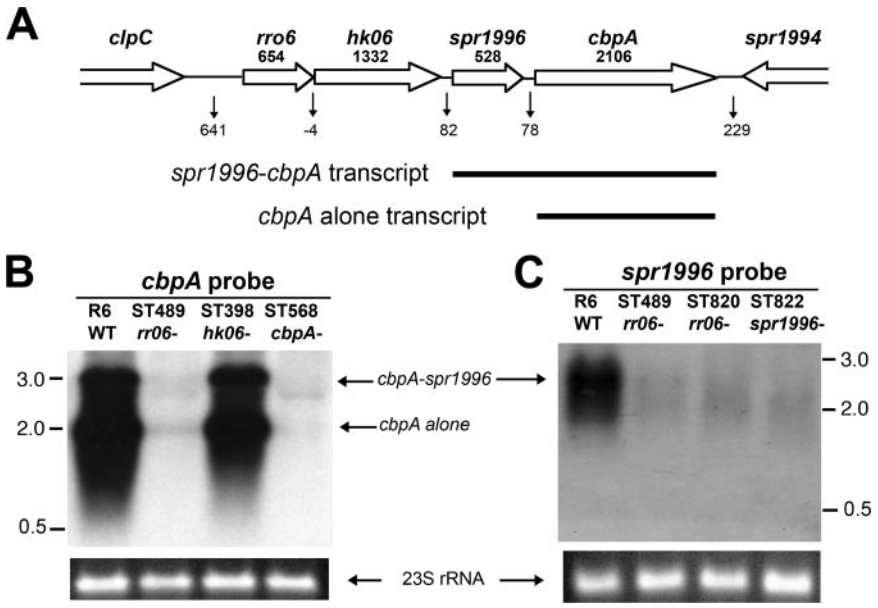

FIG. 1. RR06 is required for transcription of spr1996 and *cbpA*. A. Schematic illustration of the *rr06-cbpA* locus in the chromosome of strain R6 (accession no. AE008564). Open arrows represent the gene directions. The name and length (in bp) of each gene are indicated at the top. The numbers of nucleotides in the intergenic regions are marked below the vertical arrows. Two *cbpA* transcripts as detected in panel B are illustrated with the thick lines. B. Northern hybridization of strain R6 and its isogenic mutants using the *cbpA* probe. Total RNA extractions were separated by agarose electrophoresis, blotted, and hybridized with the radiolabeled *cbpA* probe spanning the 1,065-bp coding sequence at the 5′ region *cbpA*. The sizes of the RNA molecular mass markers are indicated in kilonucleotides. An ethidium bromide-stained 23S rRNA band in an agarose gel is included to indicate equal loading of the total bacterial RNA. C. Same experiment as in panel B, except for the use of the spr1996 probe, which represents a 446-bp coding sequence of spr1996.

(small band) (Fig. 1B). This result suggests that HK06 is not required for the transcription of spr1996 and *cbpA* under these conditions. The same data have also ruled out the possibility that the polar mutation on *hk06* in the *rr06* mutant ST489 contributed to the transcription profile in the microarray experiments (listed in Table 3). A predicted transcript for spr1996 alone (~600 nucleotides in size) was not detected with the spr1996 probe even after extended exposure of the X-ray films (Fig. 1C and data not shown). This observation led to our conclusion that spr1996 is only cotranscribed with *cbpA*. Taken together, the Northern blot data strongly suggest that spr1996 and *cbpA* form an operon, and their expression requires RR06 but not HK06.

Binding of recombinant RR06 to the promoter regions of spr1996 and *cbpA*. A previous study demonstrated that RR06 can bind to the promoter region of *cbpA* (53). Our data described above suggested that RR06 may interact with the promoter regions of both spr1996 and *cbpA*. We tested this possibility by EMSA using a MalE-RR06 fusion protein. The upstream sequences of the *rr06*, spr1996, and *cbpA* coding regions were PCR amplified and allowed to interact with the MalE-RR06 fusion protein. It should be noted that a longer PCR product was used for *rr06* because of a long intergenic region (641 bp) between the coding sequences of *clpC* and *rr06*. As shown in Fig. 2 (middle and right panels), the recombinant RR06 resulted in a dose-dependent mobility shift of the radiolabeled promoter probes for

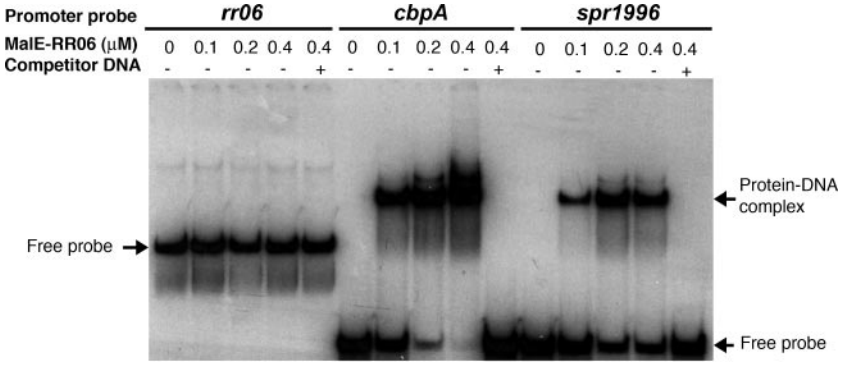

FIG. 2. Purified RR06 binds to the 5′ untranslated regions of spr1996 and *cbpA*. The PCR fragments spanning the 5′ untranslated regions of *rr06* (700 bp), spr1996 (300 bp), and *cbpA* (300 bp) were incubated with purified MalE-RR06 fusion protein for EMSA as described in Materials and Methods. The free probes and protein-DNA complexes are indicated with arrows.

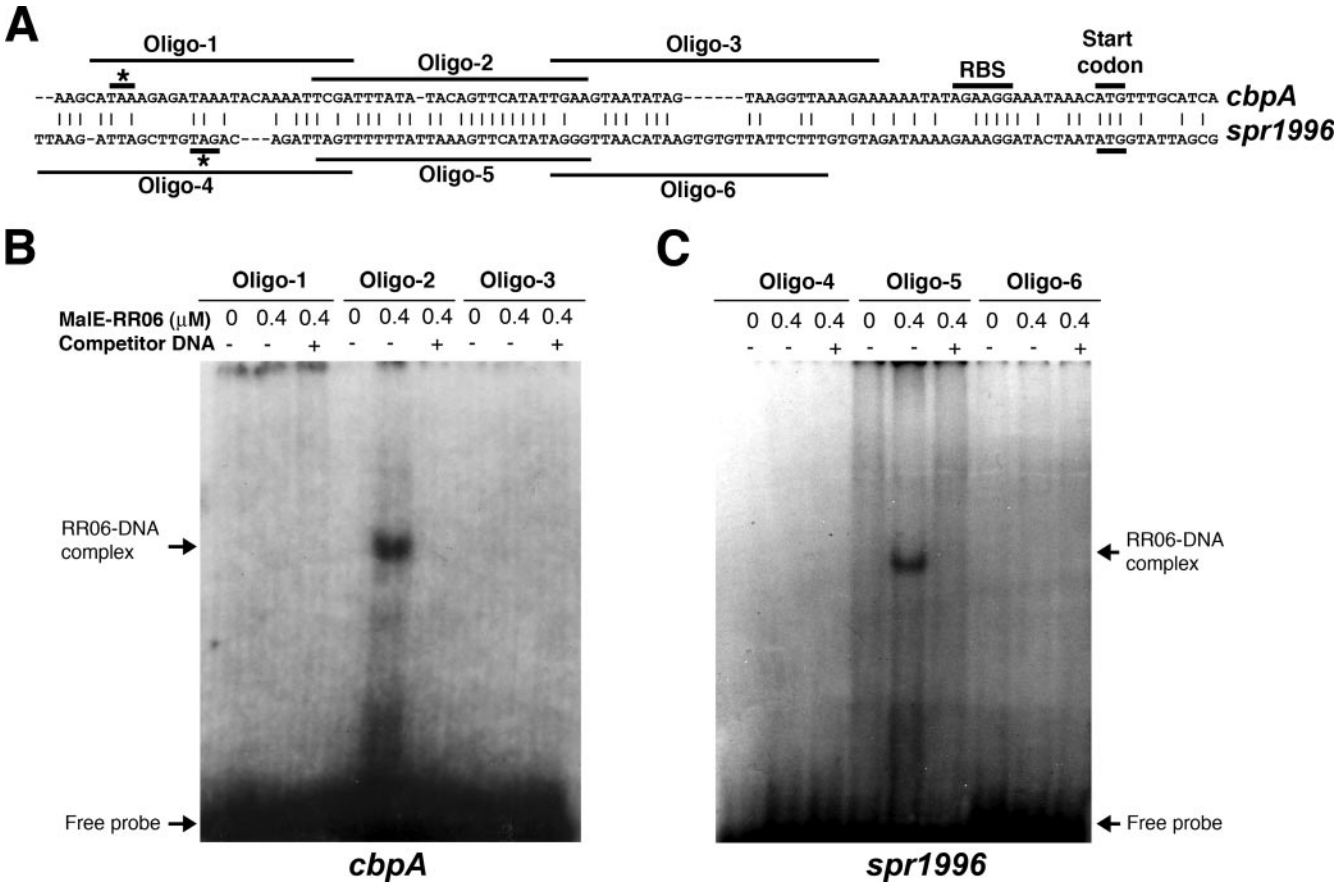

FIG. 3. The RR06 binding capability is localized to a conserved motif in the 5′ untranslated regions of *cbpA* and spr1996. A. Sequence comparison between the 5′ untranslated regions of *cbpA* and spr1996. The appropriate nucleotide sequences from accession no. AE008564 were aligned by the MegAlign program of DNASTAR Lasergene v6.1. Identical sequences are marked with vertical lines. Gaps (indicated by dashes) were introduced to facilitate alignment. The stop codons for spr1996 (top sequence) and *hk06* (bottom sequence) are indicated with asterisks. The positions of the six oligonucleotides (Oligo-1 to Oligo-6) used for mapping the RR06 binding regions in panel B are marked with lines. B. RR06 binding of various segments of the 5′ untranslated regions of *cbpA*. Three pairs of complementary oligonucleotides covering the 5′ untranslated region of *cbpA* (Oligo-1, Oligo-2, and Oligo-3) were individually synthesized and allowed to anneal as described in Materials and Methods. The resulting DNA fragments were used to perform an EMSA with purified MalE-RR06 protein as described for Fig. 2. C. Same experiment as in panel B, except for the use of three pairs of oligonucleotides corresponding to the 5′ untranslated region of spr1996.

both *cbpA* and spr1996. Further, the mobility shift was competed off with the unlabeled PCR products, thus supporting the specificity of the protein-DNA binding interactions. In contrast, the recombinant RR06 protein did not show a detectable mobility shift with its own promoter probe even at the highest protein concentration used in the experiments (0.4 μM), which was suggestive of a lack of autoregulation in the *rr06* locus as reported previously (53). A faint nonspecific band was visible for the *rr06* probe lanes and was also present in the no-protein control lane (Fig. 2, left panel). This observation further supports the specificity of the protein-DNA binding interactions observed with the promoter regions of spr1996 and *cbpA*.

Localization of the RR06 binding motif in promoter regions of spr1996 and *cbpA*. Sequence alignment analysis with the 5′ untranslated regions of spr1996 and *cbpA* revealed a 19-bp conserved motif with 84% DNA sequence identity, although the rest of the two sequences have little homology, as shown in Fig. 3A. We hypothesized that RR06 binds to the spr1996 and *cbpA* promoters by specific interaction with this conserved motif. To test this possibility, a series of complementary oligonucleotides were synthesized which spanned the entire 5′ untranslated regions of spr1996 and *cbpA*. The appropriately annealed double-stranded oligonucleotides were labeled as probes to perform an EMSA. The recombinant RR06 showed detectable binding to the conserved motifs from the 5′ untranslated regions of spr1996 (Fig. 3C, Oligo-5) and *cbpA* (Fig. 3B, Oligo-2). In contrast, the sequences flanking the conserved motifs from both spr1996 (Fig. 3C, Oligo-4 and Oligo-6) and *cbpA* (Fig. 3B, Oligo-1 and Oligo-3) failed to show a mobility shift after incubation with the recombinant RR06. The binding specificity was further confirmed by successful titration of the binding signals with unlabeled oligonucleotides representing the conserved motif for spr1996 (Fig. 3C, Oligo-5) and *cbpA* (Fig. 3B, Oligo-2). Since the sequences flanking the 19-bp conserved motif in spr1996 and *cbpA* did not show detectable binding to the MalE-RR06 fusion protein, we conclude that RR06 specifically binds to this sequence motif in the 5′ untranslated regions of spr1996 and *cbpA*. The 5′ untranslated regions of spr1996 and *cbpA* contain conserved ribosomal

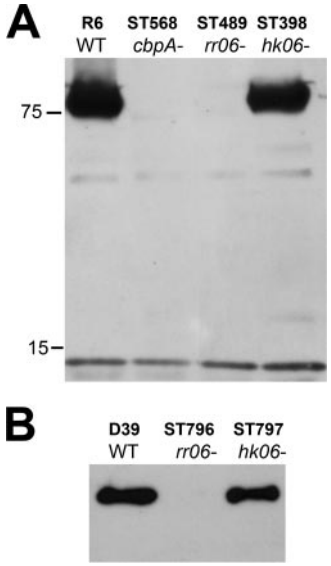

FIG. 4. RR06 is required for expression of the CbpA protein. A. Whole-cell lysates of strain R6 and isogenic mutants in *cbpA*, *rr06*, and *hk06* (approximately 5 μg total protein/lane) were subjected to SDS-PAGE. The blot was reacted in a Western blot assay with a CbpA rabbit antiserum. The sizes of the protein mass markers are marked in kDa. B. Western blot of strain D39 and its isogenic mutants in *rr06* and *hk06*, as described for panel A.

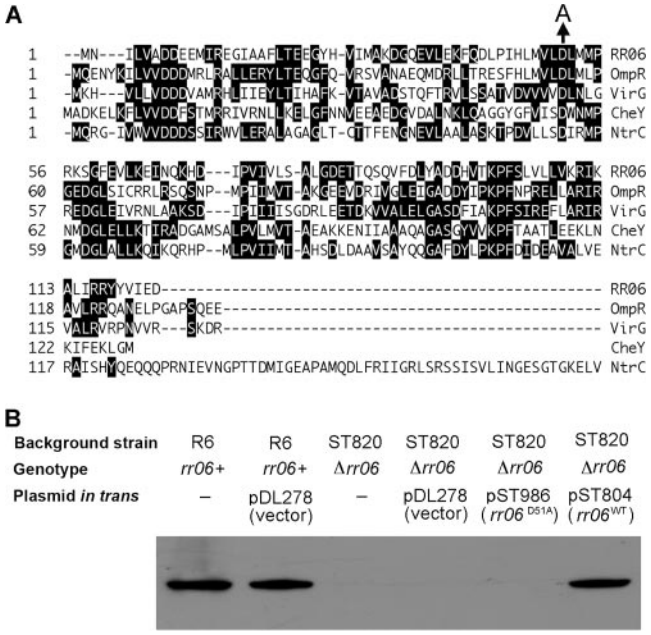

FIG. 5. Functional impact of RR06 phosphorylation. A. Partial sequence alignment of RR06, OmpR (accession no. AAN82619), VirG (accession no. NP_059810), CheY (accession no. AAA23577), and NtrC (accession no. CAA59425) by the Clustal W method in the MegAlign program of DNASTAR Lasergene v6.1. Identical amino acids are shaded. Gaps were introduced for optimal alignment as indicated by dashed lines. Amino acid positions of each sequence are marked on the left side according to the relative distance from the first amino acid. Only the amino-terminal region of NtrC is shown. The D-to-A mutation at Asp-51 of RR06 is marked with an arrow. B. Restoration of CbpA expression in the *rr06*-null strain ST820 by genetic complementation with the wild-type *rr06* or mutant *rr06*(D51A) allele. ST820 was transformed with either pDL278 (vector) or the plasmids harboring the wild-type ($rr06^{WT}$, pST804) or mutant ($rr06^{D51A}$, pST982) *rr06*. The resultant strains were used to detect CbpA by Western blotting as described for Fig. 4A. The wild-type strain R6 was also transformed with the empty vector as a negative control.

binding sites in front of the translational start codon (Fig. 3A). An extensive search of the R6 genome did not identify the RR06 binding sequence elsewhere, supporting the microarray data indicating that RR06 specifically activates the expression of spr1996 and *cbpA*.

Requirement of RR06 but not HK06 for expression of the CbpA protein. We further validated the regulatory effect of RR06 on the *cbpA* locus at the protein level. While CbpA was detected in strain R6 by Western blotting, the protein was undetectable in the *rr06* mutant ST489 (Fig. 4A). As a negative control, CbpA was absent in the *cbpA*-null strain ST568. The result thus confirmed the data from the microarray (Table 3) and Northern blot analyses (Fig. 1). Similar to the Northern blot data (Fig. 1), strain R6 and its isogenic *hk06* mutant ST398 showed a comparable level of CbpA production (Fig. 4A), again suggesting that HK06 is not necessary for CbpA expression. We extended this analysis to strain D39, an encapsulated progenitor of strain R6 by inactivation of *rr06* and *hk06*. The *rr06* mutant ST796 of strain D39 had undetectable CbpA, whereas the isogenic *hk06* mutant ST797 showed comparable levels of CbpA with the wild-type strain (Fig. 4B). Thus, the *rr06* and *hk06* mutants in the D39 background completely mirrored those of strain R6 in terms of the production of the CbpA protein.

We further verified that an *rr06*-null mutant (strain ST820) in the R6 background also had undetectable CbpA as the insertional mutant ST489 (Fig. 5B), thus ruling out the possibility that the diminished CbpA expression of the *rr06* mutant ST489 (Fig. 4A) was due to a polar effect of the insertional mutation in *rr06* on HK06 expression and function. To verify that RR06 directly activates the expression of CbpA, the *rr06* gene including its entire 5′ untranslated region was placed in an *Escherichia coli-S. pneumoniae* shuttle plasmid. The complementation construct pST804 was transformed into the *rr06*-null strain ST820. In contrast to vector controls, the Western blot analysis showed that the *rr06* construct pST804 was able to restore CbpA expression in *trans* in the *rr06*-null mutant ST820 (Fig. 5B). Together, these data indicate RR06 directly activates the expression of CbpA. Based on the transcription analysis data (Table 3 and Fig. 1), the production of the Spr1996 protein is likely to be regulated in a similar manner.

The regulatory activities of many two-component response regulators are controlled by phosphorylation at a highly conserved aspartate residue in the middle regions of the proteins. Sequence analysis indicated the aspartate residue at position 51 (Asp-51) of RR06 as the major phosphorylation site (Fig. 5A) because the corresponding aspartate residues have been demonstrated as a major phosphorylation site in numerous well-characterized RR06 homologues, including OmpR (8), VirG (21), CheY (50), and NtrC (49). To assess the role of phosphorylation in RR06 activity, an *rr06* allele with a D-to-A point mutation at Asp-51 was constructed by site-directed mutagenesis. In contrast to pST804 (carrying the intact *rr06*), pST982 harboring the RR06(D51A) allele did not result in

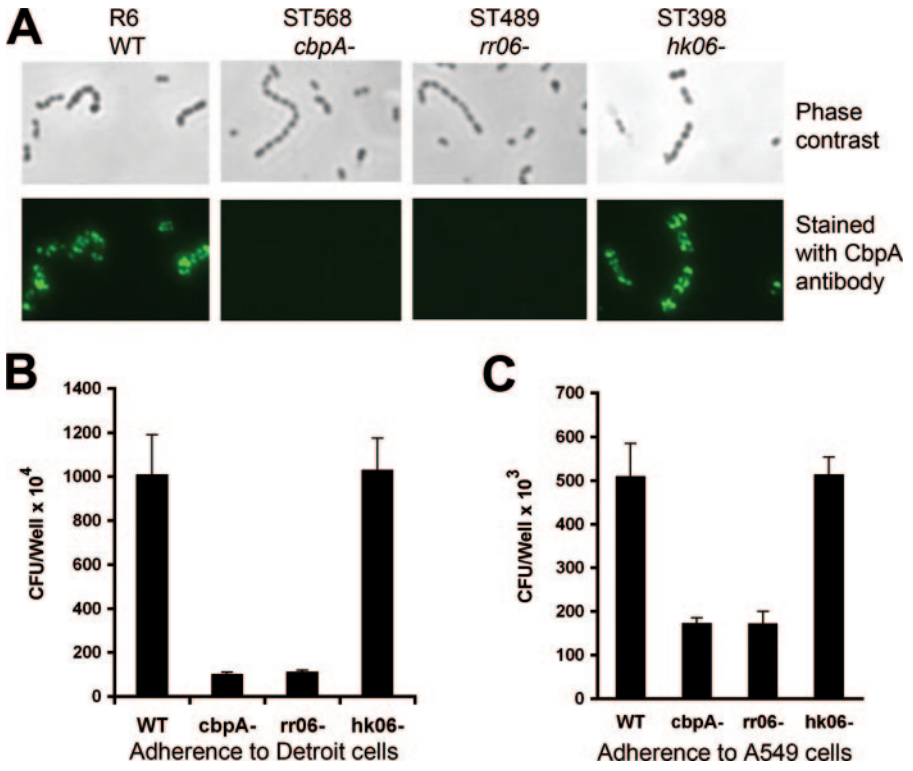

FIG. 6. RR06 is required for pneumococcal adhesion. A. Expression of CbpA at the cell surface of *S. pneumoniae* was detected by immunofluorescence microscopy. Intact organisms of strains R6 and its isogenic mutants in *cbpA*, *rr06*, and *hk06* were visualized under phase-contrast (top panel) or fluorescence (bottom panel) microscopy after being stained with the CbpA antiserum. B. Adherence of R6 and its isogenic mutants to Detroit 562 cells. The results of representative experiments are presented as means ± standard deviations of four duplicate wells. C. Same experiment as in panel B, except for the use of A549 cells.

detectable CbpA when it was transformed in the *rr06*-null strain ST820 (Fig. 5B). This result was highly reproducible, with additional independent ST820 clones transformed with pST982 (data not shown). We concluded that RR06 phosphorylation at Asp-51 is essential for the activation of CbpA expression by this regulator.

Effects of RR06 on pneumococcal adhesion to respiratory epithelial cells. CbpA has been shown to be involved in pneumococcal adhesion to human respiratory epithelial cells by interacting with multiple host receptors (14). Based on the above observation that RR06 is required for CbpA expression (Fig. 4), we reasoned that RR06 might also modulate pneumococcal adhesion. We initially assessed CbpA expression on the bacterial surface of different mutant strains by immunofluorescence microscopy. As shown in Fig. 6A, CbpA was readily detectable in the wild-type strain R6 and the isogenic *hk06* mutant. In contrast, inactivation of *rr06* resulted in the loss of CbpA expression. This result demonstrated that RR06 but not HK06 is required for CbpA expression at the cell surface of *S. pneumoniae*.

We further tested the adhesion of isogenic mutant strains in two human respiratory epithelial cell lines, Detroit 562 (nasopharyngeal cells) and A549 (type II lung cells). Our previous study showed that CbpA dramatically enhances pneumococcal adhesion and invasion in Detroit 562 cell cultures by binding to human pIgR (63). To a lesser extent, CbpA also promotes pneumococcal adhesion to A549 cells, possibly by interacting with host sialic acid, lacto-*N*-neotetraose, and the C3 protein (47, 52). As expected, the *cbpA* mutant showed an approximately 10- and 5-fold reduction in the adhesion levels to Detroit 562 (Fig. 6B) and A549 (Fig. 6C) monolayers, respectively. Consistent with the CbpA expression profile, the *rr06* mutant showed similar levels of decreases in adhesion to both cell lines. In contrast, the *hk06* mutant did not show apparent reduction in epithelial adhesion. It should be noted that RR06 may have substantially lower levels of impact on CbpA-mediated adhesion with encapsulated *S. pneumoniae* strains due to the inhibitory effect of the polysaccharide capsule on pneumococcal adhesion as described previously (53). We only used adhesion of the unencapsulated strain R6 as a marker to verify the impact of RR06 on CbpA expression.

Activation of CbpA expression by RR06 in multiple pneumococcal strains. All *S. pneumoniae* strains tested thus far contain the *cbpA* locus (4, 19), but there are high levels of sequence polymorphisms among various CbpA alleles. This is in sharp contrast with the fact that the regions flanking the *cbpA* locus are highly conserved (19). The complete DNA sequences of *rr06*, *hk06*, spr1996, and *cbpA* are available for strains R6 (17), G54 (10), ST556 (accession number DQ851866), and TIGR4 (56). It is unclear if expression of various *cbpA* allelic variants is regulated in the same manner. There are only a few amino acid sequence differences among the RR06 variants of strains R6, ST556, G54, and TIGR4 (data not shown). Additional sequence alignment also revealed that

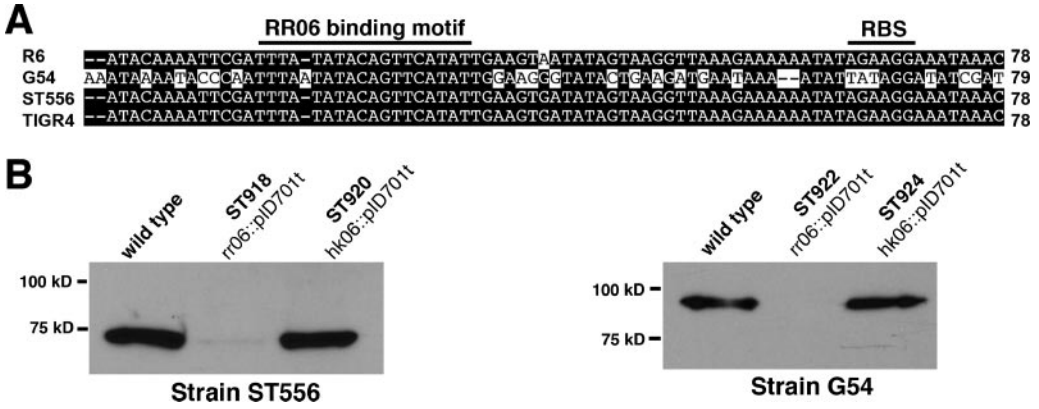

FIG. 7. RR06 is required for CbpA expression in additional isolates of *S. pneumoniae*. A. Alignment of the 5′ untranslated sequences of *cbpA* variants. The nucleotide sequences between the stop codon of spr1996 and the start codon of *cbpA* from strains R6 (accession no. AE008564), G54 (accession no. AL449923), ST556 (accession no. DQ851866), and TIGR4 (accession no. AE007507) were aligned as described for Fig. 3A. The total number of nucleotides in the *cbpA* intergenic region for each allele is indicated at the end of each sequence. B. Expression of CbpA in the wild-type ST556 and isogenic *rr06* and *hk06* mutants. CbpA was detected by Western blotting using purified human complement factor H. C. Same experiment as in panel B, except for the use of cellular lysate from strain G54.

the 5′ untranslated sequences of *cbpA* are identical for all the strains except for G54 (Fig. 7A). Even in strain G54, the 19-bp RR06 binding motif of strain R6 is also preserved (Fig. 7A). This is in sharp contrast to the sequence variations in the *cbpA* coding sequences. CbpA of strain R6 (701 amino acids [aa]) has 85.4%, 32.4%, and 83.7% sequence identities with the CbpA variants of strains ST556 (630 aa), G54 (769 aa), and TIGR4 (693 aa).

To examine the regulatory effect of RR06 on *cbpA* expression in these strains, the *rr06* and *hk06* genes were inactivated in strains ST556 and G54 by plasmid insertion. Similar to a previous report (53), our attempt to disrupt *rr06* in strain TIGR4 was unsuccessful, likely due to the essentiality of RR06 in this strain. Because of sequence diversity in the CbpA variants, the antiserum against the R6 CbpA protein was weakly reactive to many other CbpA allelic variants, including CbpA of strain G54 (data not shown). Based on the strong interaction of CbpA and human complement factor H (31), factor H was used to detect the CbpA variants of ST556 and G54. As shown in Fig. 7B and C, the wild-type ST556 and G54 strains expressed CbpA variants with expected sizes. Similar to the finding in strains R6 and D39 (Fig. 4), CbpA expression was abolished in the *rr06* mutants (ST918 and ST922) of both strain backgrounds (Fig. 7B and C). In contrast, the disruption of *hk06* (ST920 and ST924) did not lead to obvious alteration in CbpA expression in strains ST556 and G54. These data thus showed that RR06 but not HK06 is required for CbpA expression in multiple strains of *S. pneumoniae*.

DISCUSSION

S. pneumoniae is highly capable of adapting to multiple host niches as manifested by its ability to survive and replicate at the mucosal surfaces of the airway, in the bloodstream, and in the brain. In this work, our analyses at both the mRNA and protein levels have demonstrated that RR06, one of the 14 potential DNA binding response regulators, is specifically required for the expression of the immediate downstream genes spr1996 and *cbpA* under standard culture conditions. The narrow specificity of RR06 in gene regulation is in sharp contrast to the rather broad spectra of the other pneumococcal TCSs that have been characterized thus far. A previous microarray analysis of a type 3 *S. pneumoniae* strain 0100993 showed that the CiaRH TCS up- and down-regulates 24 and 22 genes, respectively (51). The orphan response regulator RitR was reported to modulate the expression of 54 genes in strain R800, an R6 derivative (59). Similarly, many pneumococcal genes are affected by the mutations in additional TCSs, including *comDE* (6, 45), *vicRK* (37, 41), *rr04* (33), *ciaRH* (32, 51), and *blpRH* (9). Our data cannot exclude the possibility that RR06 regulates the expression of additional pneumococcal genes under other environmental conditions, such as different tissue sites in humans.

The regulatory activity of RR06 appears to be controlled by phosphorylation. Asp-51 of RR06 is a major phosphorylation site, because the RR06(D51A) allele was defective as a transcriptional activator. The Asp-51 equivalents are the major phosphorylation sites in other well-characterized proteins that are members of the phosphate acceptor family, such as OmpR (8), VirG (21), CheY (50), and NtrC (49). Among these, RR06 has the highest sequence similarity to OmpR. OmpR in *E. coli* modulates the expression of the outer membrane porin proteins OmpF and OmpC in response to changes in the surrounding osmolarity (8). Alanine substitutions in the three aspartate positions 11, 12, and 55 of OmpR, equivalents of positions 7, 8, and 51 of RR06, have identified Asp-55 as a major phosphorylation site, but the OmpR(D55A) allele retains a low level of its regulatory activity (8). Our data have suggested that Asp-51 of RR06 is the only phosphorylation site, because RR06(D51A) lost its activity in activating CbpA expression. In this regard, RR06 behaves like other well-studied bacterial response regulators, such as VirG, CheY, and NtrC. The RR06 Asp-51 equivalents in VirG (21), CheY (50), and NtrC (49) are the only phosphorylation sites to control the activities of these proteins. Further genetic and biochemical analyses are warranted to elucidate the precise mechanism by

which RR06 controls the expression of pneumococcal genes under various conditions.

The impact of HK06 on the expression of spr1996 and *cbpA* is less clear. A previous study showed a fivefold increase in the mRNA level of *cbpA* with an *hk06* mutant of strain D39, suggesting HK06 somehow represses *cbpA* expression (53). However, additional functional data from epithelial adhesion and mouse infection experiments of the same study implicated an opposite role for HK06 (53). Our data indicated that HK06 is not a major contributor to RR06 phosphorylation under these conditions, because the disruptive mutations in *hk06* had no apparent effect on the expression of spr1996 and *cbpA.* Our Northern blot analysis initially showed that *hk06* is not required for the transcription of spr1996 and *cbpA*. Furthermore, the *hk06* mutant did not show obvious alteration in the production of the CbpA protein as determined by Western blotting using the CbpA antibody. In the more sensitive and quantitative adhesion assay, the *hk06* mutant and the wild-type strain had similar levels of adherence in two separate airway epithelial culture systems. Together, our data suggest that RR06 receives a phosphorylation signal from other noncognate histidine kinase(s) or some other phosphate donor if the activity of RR06 is controlled by phosphorylation status of the protein.

CbpA protein was consistently undetectable in multiple *rr06* mutants by Western blotting, despite a detectable transcription of *cbpA* in the *rr06* disruptive mutant in microarray analysis. This discrepancy may reflect the limited sensitivity of our Western blotting-based detection method compared with more sensitive mRNA detection techniques. In this regard, we also noticed an obvious difference between our data and a previous study (53). In contrast to an undetectable level of the CbpA protein in multiple *rr06* mutants of strain D39 in this study, Standish et al. showed a relatively higher level of residual CbpA in an *rr06* deletion mutant of strain D39 (53). The same study also observed a higher background level of *cbpA* mRNA in the absence of RR06 (53). Culture conditions might be a contributing factor, as exemplified by the difference in the amount of yeast extract used in the THY broth (0.5% versus 1%) in the two studies. Consistent with this notion, residual CbpA in the *rr06*-deficient mutant was undetectable by Western blotting when the pneumococci were cultured in serum broth (53).

Cross-talk among different TCSs has been well documented in many bacterial species (54, 60). In this regard, RR06 is reminiscent of another response regulator, VicR, of *S. pneumoniae* (also known as RR02). VicR is essential for pneumococcal survival (57) because it is required to activate the expression of the essential PcsB putative murein hydrolase (39, 40). However, VicK, the cognate histidine kinase of VicR, does not share the same tested regulatory pathways with the VicR. Abrogation of VicK has been shown to have little effect on the expression of PcsB (39, 40), the growth rate of *S. pneumoniae* (39, 40), or the infectivity of the bacterium in the lungs of mice (57). Thus, similar to HK06, VicK is not a major source of phosphorylation for VicR, but the key signal that controls the VicR activity is also unknown. In the chemotaxis system of *E. coli*, a single histidine kinase, CheA, competitively phosphorylates two response regulators, CheB and CheY, in response to changes in glucose concentration (30). It is thus possible that one or more kinases relay the signal(s) to RR06.

We have demonstrated that RR06 activates the transcription of spr1996 and *cbpA* in strain R6, but this regulatory relationship is likely to operate in many additional strains. The coding sequence of the *cbpA* locus is highly variable, likely due to selection pressure from the host immune response against this surface-exposed protein (4, 19). However, the sequence and gene organization of the *cbpA*-flanking regions, including *rr06*, *hk06*, and spr1996, are virtually identical in all tested strains (19). Although the regulatory role of RR06 has not been determined in humans, the natural host of *S. pneumoniae*, some indirect evidence suggests that RR06 is a key in vivo regulator of *S. pneumoniae*. The RR06/HK06 system is one of the eight TCS loci in which deletion mutations have resulted in a significant decrease in *S. pneumoniae* survival in the lungs of mice (57). Differential expression of CbpA has also been well documented. CbpA appears to be present in higher amounts in the transparent colony variants of *S. pneumoniae* compared with opaque colony variants (47). LeMessurier et al. (29) recently reported that pneumococci recovered from the nasopharynx and lungs of mice express higher levels of *cbpA* mRNA than those recovered from the bloodstream. These observations are consistent with other reports that CbpA is required for nasopharyngeal colonization and lung infection in animal models (4, 24, 47). The contribution of CbpA to pneumococcal infection in the bloodstream is not completely clear. Some of the previous studies showed no or a minor effect of CbpA mutations on virulence of *S. pneumoniae* in bacteremia animal models (4, 47), whereas others reported that CbpA-deficient mutants significantly lost virulence in mice after intravenous inoculation (18). It is thus reasonable to postulate that RR06 activates the transcription of spr1996 and *cbpA* in the colonization/commensal stage of *S. pneumoniae* at the mucosal surface of the nasopharynx, while its activity as a transcriptional activator may be repressed once the bacterium enters the bloodstream, resulting in lower levels of spr1996 and *cbpA* expression.

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