

Regulation of the *pspA* Virulence Factor and Essential *pcsB* Murein Biosynthetic Genes by the Phosphorylated VicR (YycF) Response Regulator in *Streptococcus pneumoniae*†

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The VicRK (YycFG) two-component regulatory system (TCS) is required for virulence of the human respiratory pathogen *Streptococcus pneumoniae* (pneumococcus). The VicR (YycF) response regulator (RR) is essential through its positive regulation of *pcsB*, which encodes an extracellular protein that mediates murein biosynthesis. To determine other genes that are regulated by VicR, we performed microarray analyses on a unique $\Delta vicR$ deletion mutant, which was constructed by uncoupling regulation of *pcsB*. Results from these microarray experiments support the idea that the VicR RR exerts strong positive regulation on the transcription of a set of genes encoding important surface proteins, including the PspA virulence factor, two proteins (Spr0096 and Spr1875) containing LysM peptidoglycan-binding domains, and a putative membrane protein (Spr0709) of unknown function. To demonstrate direct regulation, we performed band shift and footprinting experiments using purified unphosphorylated VicR and phosphorylated VicR-P, which was prepared by reaction with acetyl phosphate. VicR and VicR-P bound to regions upstream of *pcsB*, *pspA*, *spr0096*, *spr1875*, and *spr0709*. Phosphorylation of VicR to VicR-P increased the apparent strength and changed the nature of binding to these regions. DNase I footprinting of VicR and VicR-P bound to regions upstream of *pcsB*, *pspA*, *spr0096*, and *spr1875* showed protection of extended regions containing a degenerate sequence related to a previously proposed consensus. These combined approaches did not support autoregulation of the *vicRKX* operon or substantive direct regulation of fatty acid biosynthesis by VicR or VicR-P. However, the $\Delta vicR$ mutant required fatty acids in some conditions, which supports the notion that the VicRK TCS may mediate membrane integrity as well as murein biosynthesis and virulence factor expression in *S. pneumoniae*.

Streptococcus pneumoniae (pneumococcus) is an opportunistic extracellular human respiratory pathogen that causes several serious diseases, including pneumonia, otitis media (ear infection), sinusitis, meningitis, and septicemia (40, 62, 64). Invasive pneumococcal disease results in a high mortality and morbidity rate (as many as 1 million deaths annually worldwide), especially among young, elderly, debilitated, and immunosuppressed individuals (25, 26), and resistance to a range of antibiotics is increasing at an alarming rate among clinical isolates of *S. pneumoniae* (1, 30). As in other bacterial pathogens (17, 23, 57), two-component signal transduction systems (TCSs) are thought to play key roles in pneumococcal colonization and virulence. The genomes of *S. pneumoniae* serotype 2 and 4 strains encode 13 complete TCSs, each containing a presumed cognate response regulator (RR) and histidine kinase (HK) pair, and one orphan response regulator (21, 31, 60, 61). Several of these TCSs have recently been found to be required for full pneumococcal pathogenesis, possibly through the regulation of virulence factor genes. These TCSs include ComDE (quorum sensing and competence) (3, 16, 32), RR04 (PsaBCA manganese transporter) (38), BlpRH (quorum sensing and bacteriocin production) (10), CiaRH (HtrA protease

and competence) (24, 37, 52), RR06 (CpbA surface protein) (56), RitR (PiuBCDA iron transporter) (65), and RR09 (serotype-specific regulation) (4). With the exception of ComDE and BlpRH, which respond to peptides (10, 18, 47), little is known about the signals sensed by these TCSs.

The essential VicRK TCS also plays critical roles in pneumococcal virulence. VicRK is a homologue of the essential TCS found in all low G+C gram-positive bacteria, where it is often designated YycFG based on its initial discovery in *Bacillus subtilis* (13). The VicR (YycF) RR is essential (12, 31, 39, 42, 61, 66), and without functional VicR, pneumococcus cannot grow and act as a pathogen. In addition, phosphorylation of VicR on aspartate residue 52 (D52) is required for pneumococcal growth (see Fig. 1) (42). Changes of aspartate 52 to alanine (D52A), glutamine (D52Q), and asparagine (D52N) are not tolerated (42), whereas a change to glutamate (D52E) results in a mutant VicR with low-level activity, consistent with analogous Asp→Glu mutations that constitutively activate other RRs (29, 54). We also showed previously that the essential target of the VicRK TCS is the *pcsB* gene, which mediates murein biosynthesis and appears to be strongly positively regulated by VicRK under some conditions (41, 42). Aberrant expression of PcsB results in defects in murein biosynthesis and cell division and increased sensitivity to several stress conditions that likely reduce virulence (41, 42). However, to date, it has not been determined whether the regulation of *pcsB* by VicR is direct or indirect.

Beyond the essentiality of VicR and PcsB, the signal transduction by the VicRK TCS itself is important for pneumococ-

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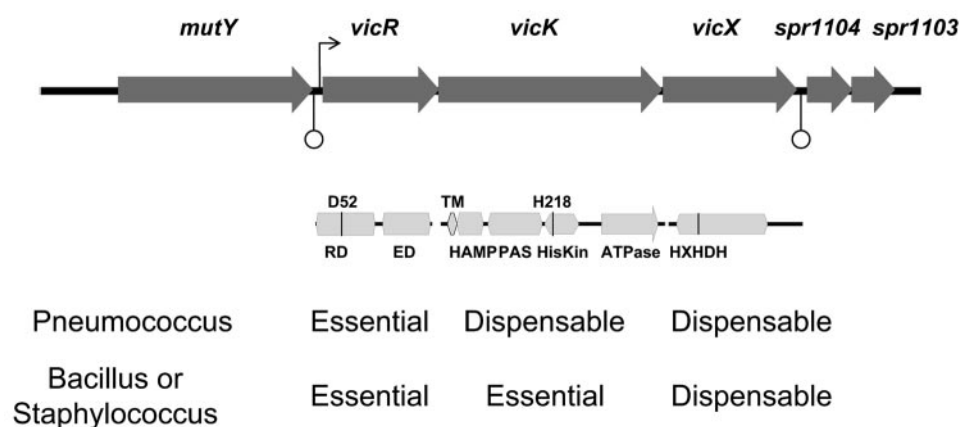


FIG. 1. Genetic organization of the *vicRKX* operon and protein domain features of VicR, VicK, and VicX of *Streptococcus pneumoniae*. Reading frames are depicted as dark gray arrows labeled with corresponding gene names. The thin arrow marks the transcription start site mapped previously by primer extension (66). Inverted lollipop symbols represent putative Rho-independent transcription terminators. Important protein domain features identified from the Pfam database (<http://www.sanger.ac.uk/Software/Pfam/>) and amino acid residues of VicR, VicK, and VicX are illustrated as follows: VicR (RD, receiver domain; ED, effector domain); VicK (TM, transmembrane domain; HisKin, histidine containing phosphotransfer domain; ATPase, histidine kinase ATP binding domain); and VicX (HXHDH, putative metal binding site in β -lactamase fold) (see references 31 and 66). The requirement for growth of each protein in different gram-positive bacteria is indicated.

cal virulence. In contrast to YycG homologues in other gram-positive species (13, 15, 36), mutants containing the deletion for the gene encoding the pneumococcal VicK (YycG) HK grow in laboratory medium (see Fig. 1) (12, 27, 31, 42, 61, 66). However, the VicK HK does become essential when the amount of the VicR RR is reduced from a regulatable promoter (42). This result further supports the contention that phosphorylation of the VicR RR is required for growth. The VicK HK is the only protein in *S. pneumoniae* containing an internal PAS sensing domain (see Fig. 1) (21, 59, 60), and VicK contains a single transmembrane domain compared to the two transmembrane domains in other YycG homologues (43). Furthermore, pneumococcal *vicK* mutants are attenuated for virulence in murine models of infection (27, 42), which further supports an important role for VicRK in pathogenesis.

Microarray analyses of relative transcript amounts in a strain depleted of VicRK suggested that besides *pcsB*, the VicRK TCS positively regulates one known virulence factor gene and several other nonessential genes that may be important in pneumococcal virulence (42). These genes encode the PspA surface virulence factor involved in the evasion of complement during infection (6, 49, 50, 63), the two surface proteins (Spr0096 and Spr1875) in *S. pneumoniae* that contain the LysM peptidoglycan binding motifs, the LytB glucosaminidase involved in a late step in murein biosynthesis (9), and a putative membrane protein (Spr0709) of unknown function. Each of these genes is preceded by a sequence related to the consensus site proposed for YycF RR binding in *Bacillus subtilis* (22) and *Staphylococcus aureus* (11). However, many of these putative VicR binding sites contain one or two mismatches with the already degenerate consensus sequence (11, 22), which consequently is only partly predictive in *S. pneumoniae*.

Another recent microarray study in which the VicR RR was overexpressed gave results consistent with positive regulation of most of the above genes, including *spr0096*, *lytB*, *spr1875*, and *pcsB* (39). This study also suggested that the *vicRKX* operon (Fig. 1) is autoregulated and that overexpression of

VicR causes a change in the fatty acid composition of total membrane lipids extracted from *S. pneumoniae* (39). The latter result is consonant with earlier results in *S. aureus*, suggesting that the YycFG TCS somehow mediates cell membrane integrity (36). However, it was not resolved whether this effect on fatty acid composition was caused by small changes in relative transcript amounts from the fatty acid biosynthetic operon observed when VicR was overexpressed. Moreover, in both of these microarray studies, there was induction of stress response genes and changes in relative transcript amounts from several transport operons (39, 42).

To reconcile these previous studies and further discriminate genes that are strongly and possibly directly regulated by the pneumococcal VicRK TCS, we performed microarray analyses of a $\Delta vicR$ deletion mutant, which can be constructed when the regulation of *pcsB* is uncoupled by fusion to a synthetic promoter and ribosome binding site (42). Our new results support the hypothesis that the VicR RR strongly and positively regulates the transcription of a set of genes encoding surface proteins involved in murein biosynthesis and virulence in *S. pneumoniae*. These microarray analyses were not consistent with *vicRKX* autoregulation or substantial direct regulation of the fatty acid biosynthetic genes and several other genes observed in previous studies (39, 42). However, our results do suggest a role for VicRK in maintaining membrane integrity, in part through its regulation of PcsB. Last, we show that both VicR and phosphorylated VicR (VicR-P) bind to specific regions upstream of key target genes and that phosphorylation of VicR to VicR-P increases this binding. These studies are consistent with direct regulation of these genes by the VicR RR and allow refinement of the VicR (YycF) binding site for *S. pneumoniae*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study were derived from *S. pneumoniae* strain R6, which is an unencapsulated derivative of serotype 2 virulent strain D39 (Table 1). A single-colony isolate of strain R6 was assigned the unique strain designation EL59 to track its isogenic deriv-

TABLE 1. Bacterial strains, plasmid, and oligonucleotides used in this study

PCR component	Genotype	Antibiotic marker ^a	Description or sequence	Source or reference
Strains				
EL59	<i>S. pneumoniae</i> R6		Avirulent, unencapsulated starting parent strain derived from serotype 2 strain D39	21; A. Tomasz
EL1454	EL59 <i>kan</i> -t1t2- <i>P_c-pcsB</i> ⁺	Kan ^r	EL59 transformed with linear <i>mreD</i> -[<i>kan</i> -t1t2- <i>P_c]-pcsB</i> ⁺ amplicon	42
EL1472 ^b	EL1454 $\Delta vicR$ <> <i>ermAM</i>	Kan ^r Erm ^r	EL1454 transformed with linear $\Delta vicR$ <> <i>ermAM</i>	42
IU1545	EL59 <i>P_{fecK}-pcsB</i> ⁺	Kan ^r	EL59 transformed with linear PCR amplicon <i>mreD</i> '-[<i>kan</i> -t1t2- <i>P_{fecK}]-pcsB</i> ⁺ - <i>rpsB</i>	41
IU1602	IU1545 $\Delta vicR$ <> <i>P_c-ermAM</i>	Kan ^r Erm ^r	IU1545 transformed with linear PCR amplicon $\Delta vicR$ <> <i>P_c-ermAM</i>	41
EL27	<i>E. coli</i> BL21(DE3), pLysS (pSP001)	Amp ^r Cm ^r	<i>E. coli</i> strain for overexpression of His ₁₀ -VicR	This study
Plasmid pSP001			pET-16B containing full-length <i>vicR</i> ; expresses His ₁₀ -VicR	This study
Oligonucleotides^c				
WN206			ATGAAGGGAATCACAGGATTCAAGCGTT; forward primer (<i>spr1875</i>)	
WN208			AAATGACAAGGCTACTGTTGACGCTAAT; reverse primer (<i>spr1875</i>)	
WN209			AGGGGCTTGGCTTTAATTGTGGATGAA; forward primer (<i>spr0096</i>)	
WN210			GCAGCTACTCCTGCAAGAGTTGCTTTA; reverse primer (<i>spr0096</i>)	
WN211 ^d			GATTTTAAATTTTTTATGGATTACTGTT; forward primer (<i>pcsB</i>)	
WN212			AGAAACCATTACTGTACTTAATAAAA; reverse primer (<i>pcsB</i>)	
WN213			AATAGCTGATACTTGTCTCCTGAATT; reverse primer (<i>pcsB</i>)	
WN214			TTAGGCCTTTTTTTGGTATACTAGTA; forward primer (<i>pcsB</i>)	
WN235			TTGAAAGGGGCAAAAGTAGTATCTT; forward primer (<i>pspA</i>)	
WN236			TAGCGACGCTGGCTAGACTTGTAA; reverse primer (<i>pspA</i>)	
WN253			CACATTTCTCCAAAATCAGCCATGCTT; forward primer (<i>spr0709</i>)	
WN254			AATGAGGAAGCTGCTGGTTAATTCTT; reverse primer (<i>spr0709</i>)	
WN255			GAGTCATTCCGGAGAGAAGAAGACCTA; forward primer (<i>fabR</i>)	
WN256			CTGGAGCCTTACCGATGACATCAAT; reverse primer (<i>fabR</i>)	
WN257			TGACTTCCATGATTTTTTCAGATAGGGA; forward primer (<i>vicR</i>)	
WN258			CCGAGATTGGTTTCTCATCATCTACAA; reverse primer (<i>vicR</i>)	
WN259 ^d			AGTCTATAGCTTGGTACCGACGAT; forward primer (<i>pcsB</i>)	

^a Antibiotic resistance markers: Kan^r, kanamycin; Erm^r, erythromycin; Amp^r, ampicillin; Cm^r, chloramphenicol.

^b *vicR* reading frame replaced in-frame by the *ermAM* reading frame.

^c Used for preparation of DNA fragments in band shift and footprinting assays. Positions of primers used for PCR are indicated by arrows in Fig. 5, 6, and 8.

^d Primer WN211 or WN259 was used with primer WN212 to generate PCR amplicons used in band shift (Fig. 5) or footprinting (Fig. 9 and 10) assays, respectively.

atives. Mutant construction and confirmation were performed as described previously (41, 42). For final experiments, bacteria were cultured statically in brain heart infusion broth (BHI; Difco Laboratories) or a chemically defined medium (CDM) (58) (formulated by JRH Biosciences) lacking antibiotics at 37°C in an atmosphere of 5% CO₂. Growth was monitored by changes in optical density at 620 nm (OD₆₂₀; 1-cm path length) using a Spectronic 20 spectrophotometer. Overnight cultures used to inoculate cultures were propagated in BHI. For growth in CDM, the starting BHI cultures were briefly centrifuged at low speed. Cell pellets were washed with CDM and resuspended in CDM. The starting density of cultures (OD₆₂₀) used for final experiments was 0.003 to 0.005. L-Fucose (Sigma) was added to overnight cultures and to final cultures at final concentrations of 0.4% (wt/vol) and 0.2% (wt/vol), respectively, when required. A fatty acid mixture of linoleic acid and oleic acid (F7175; Sigma) or each fatty acid (L9530 and O3008, respectively) was added to cultures to give final concentrations of 30 μM linoleic acid, 15 μM oleic acid, and 15 μM carrier bovine serum albumin.

RNA extraction and microarray analyses. Total pneumococcal RNAs were prepared by a rapid lysis procedure described before (42) from cultures growing exponentially at 37°C in CDM (OD₆₂₀ 0.1) or BHI (OD₆₂₀ 0.25). *Streptococcus pneumoniae* microarrays were purchased from MWG-Biotech, Inc. (now available from Ocimum Biosolutions) containing 2,018 50-mer oligonucleotide probes covering most of the *S. pneumoniae* R6 transcriptome. Reverse transcription of RNA with Cy3- or Cy5-labeled CTP, hybridization, and washing were performed according to the protocol provided by MWG-Biotech, Inc. Microarrays were scanned on an Axon 4200 scanner (Molecular Devices) according to the manufacturer's instructions, and results were obtained using GenePix microarray acquisition and analysis software interfaced to the scanner. Data were normalized with GeneTraffic software (Iobion Informatics) using the Lowess (subgrid) method without background subtraction. Experiments were performed at least

three times independently (biological repeats), including dye swapping. Signal ratios were calculated from the normalized signals for the *P_c-pcsB*⁺ $\Delta vicR$ mutant divided by the corresponding signals for the *P_c-pcsB*⁺ *vicR*⁺ parent. Signal ratios from different experiments were averaged and used to calculate the average fold changes reported in the tables. Average signal ratios of >1 are the same as the positive fold changes, whereas average signal ratios of <1 were converted to negative reciprocal values to give negative fold changes. Bayesian *P* values were calculated using the Cyber-T Bayesian statistical framework for paired data (33). The cutoff for significant changes in relative transcript amounts was set at positive or negative 1.8-fold with Bayesian *P* < 0.001. A complete listing of the relative transcript amounts of all the genes in mutant EL1472 (*P_c-pcsB*⁺ $\Delta vicR$) compared to parent EL1454 (*P_c-pcsB*⁺ *vicR*⁺) grown in BHI or CDM can be found in Tables S1 and S2 in the supplemental material, respectively. A compilation of all genes whose relative transcript amounts increased or decreased by at least 1.8-fold (*P* < 0.001) in EL1472 compared to EL1454 in either or both media can be found in Table S3 in the supplemental material. All raw and normalized intensity data will be provided upon request.

Overexpression and purification of N-terminal His₁₀-tagged VicR. *S. pneumoniae* R6 genomic DNA was purified as described previously (21). The complete reading frame of *vicR* was PCR amplified from the genomic DNA using *Pfu* polymerase (Stratagene) according to the manufacturer's instructions. The upstream (AAAAGGTGAACATATGAAAAAATACTAATT) and downstream (AAAATGGTTTGGATCCGTAATCAAGCATT) primers contained NdeI and BamHI restriction sites (underlined) and the start and stop codons of *vicR* (italics), respectively. The resulting PCR amplicon was digested with NdeI and BamHI restriction endonucleases and ligated by standard methods (2, 51) into vector pET-16B (Invitrogen), which had been digested with the same enzymes. The resulting plasmid (pSP001) was transformed into *Escherichia coli* strain

BL21(DE3)pLysS, and the resulting transformant was designated EL27. The sequences of the fusion points and insert were confirmed by DNA sequencing.

Strain EL27 (300 ml) was grown exponentially with shaking at 30°C in Luria-Bertani broth containing 100 µg ampicillin per ml to a density of 50 Klett units (red filter). Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM for 3 h to induce expression of His₁₀-VicR. Cells were collected by centrifugation (3,300 × g for 10 min at 4°C). All remaining steps were carried out at 4°C. Cell pellets were resuspended in 1/10 of the starting culture volume in buffer A (20 mM NaPO₄, 0.5 M NaCl, 20 mM imidazole, pH 7.4) and lysed by passage through a French press cell (20,000 lb/in²). Insoluble material was removed from lysates by centrifugation (16,000 × g for 15 min). Supernatants were loaded onto 1 ml HiTrap chelating HP columns (GE Healthcare) charged with NiSO₄ according to the manufacturer's instructions and equilibrated with buffer A. Columns were washed with 10 ml of buffer A. His₁₀-VicR was eluted by an imidazole gradient (20 to 1,000 mM in 20 mM NaPO₄-0.5 M NaCl, pH 7.4). Fractions containing His₁₀-VicR were pooled and concentrated using an Amicon Ultra-15 centrifugation unit (Millipore). The concentrated protein was diluted to 15 ml with TGED buffer (10 mM Tris-HCl [pH 7.4], 0.1 mM EDTA, 0.1 mM dithiothreitol, 5% glycerol) and concentrated again. This process was repeated twice to remove remaining imidazole. Purified His₁₀-VicR was stored in aliquots at -70°C. Protein concentration was determined by the D_c assay (Bio-Rad, Inc.) using bovine serum albumin dissolved in TGED buffer as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) and C₄ reverse-phase high-performance liquid chromatography (HPLC) (see below) were used to check the purity of the His₁₀-VicR, which was greater than 95% (data not shown). The yield of purified His₁₀-VicR was ~2 mg per liter of starting culture. Purified His₁₀-VicR is referred to as VicR in the remainder of this article.

Phosphorylation of VicR by AcP. VicR (5 µM) was phosphorylated with 10 mM acetyl phosphate (AcP) in 50- to 200-µl reaction mixtures containing 1× AcP buffer (50 mM Tris-HCl [pH 7.4], 50 mM KCl, 2 mM MgCl₂) for 1 h at 37°C based on references (19, 28). We reduced the concentration of MgCl₂ in this reaction from 20 mM to 2 mM, because the higher concentration of MgCl₂ inhibited binding in band shift assays (see below). Unphosphorylated VicR used in subsequent band shift and footprinting assays was incubated in the same reaction mixture used for phosphorylation, except that AcP was omitted. The extent of phosphorylation was determined by analytical reverse-phase HPLC at 25°C on a Phenomenex Jupiter 300A C₄ column (4.6 mm by 250 mm) attached to a Shimadzu 10A HPLC system (see reference 19). Mobile phases A (20% acetonitrile, 0.1% trifluoroacetic acid) and B (60% acetonitrile, 0.1% trifluoroacetic acid) were mixed to form the following gradient at a flow rate of 1 ml per min: 80% phase A-20% phase B to 0% phase A-100% phase B in 40 min. Polypeptides were detected by monitoring A₂₂₀.

Gel mobility band shift assays. Band shift assays were based on conditions used in references (11, 22). DNA fragments used in band shift assays were synthesized by PCR amplification of purified *S. pneumoniae* R6 genomic DNA using *Pfu* polymerase as described above. Primers used for PCR are listed in Table 1. One of the primers used in each PCR was fluorescein-labeled (Sigma-Genosys). The resulting PCR product was purified using a QIAGEN PCR cleanup kit. Binding reactions contained 0.05 to 0.1 pmol labeled DNA, 200 ng poly(dI-dC) (i.e., 10 µg per ml), 25 mM NaPO₄ (pH 7.0), 150 mM NaCl, 0.1 mM EDTA, 2 mM MgCl₂, 1 mM dithiothreitol, 10% glycerol, and varying amounts of VicR or VicR-P (prepared as described above) in a total volume of 20 µl. Reactions were incubated at room temperature for 20 min. DNA-protein complexes were resolved on native gels (7 cm in length) containing 5% acrylamide in 1× TGE buffer (50 mM Tris, 400 mM glycine, 1.73 mM EDTA) (11, 22). Electrophoresis was performed for 45 to 60 min at 100 V. DNA and DNA-VicR complexes were detected by scanning gels directly using a Typhoon Imager (GE Healthcare) at the fluorescein detection settings.

DNase I footprinting assays. DNase I footprinting was performed using conditions similar to those used in references (11, 22). Primers in PCRs used to synthesize DNA fragments are listed in Table 1. One of the fragments in each PCR was first labeled with ³²P by reaction with [γ-³²P]ATP and T4 polynucleotide kinase (New England Biolabs). After PCR, labeled amplicons were purified using a QIAGEN PCR cleanup kit. Binding reactions for footprinting were the same as those described above for band shift assays, except the total volume of the reactions was increased to 60 µl. Five microliters of diluted RQ1 DNase (0.5 U; Promega) was added to each binding reaction and incubated at 25°C for exactly 5 min. Reactions were stopped by adding 180 µl of STOP solution (0.4 M sodium acetate, 50 µg per ml sonicated salmon sperm DNA, 2.5 mM EDTA). Digested DNA was extracted once with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated by the addition of 3 volumes of 100% ethanol. Precipitated DNA was collected by centrifugation in a microfuge at 14,000 × g at 25°C,

and pellets were washed once with 70% ethanol, compacted by centrifugation, and dried under vacuum. Pellets were dissolved in formamide loading dye (20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol, 96% formamide). A Maxam-Gilbert G+A ladder was prepared on the labeled DNA fragments by standard methods (2, 51). Samples of the G+A ladder and footprinting reactions containing ~30,000 dpm were resolved on standard 6% polyacrylamide gels containing 7 M urea and 1× Tris-borate-EDTA. Gels were vacuum dried and autoradiographed on XAR film.

RESULTS

Changes in relative transcript amounts in a $\Delta vicR$ mutant grown in BHI. Depletion or overexpression of the VicR RR stressed the cells in previous microarray analyses of the VicRK regulon of *S. pneumoniae* (39, 42). This stress was manifested by increased transcript amounts from stress genes, such as *clpL*, *hrcA*, *grpE*, and *dnaK*, and by sometimes drastic changes in cell shapes (39, 42). Therefore, it was unclear whether changes in the relative amounts of transcripts from certain genes were due to changes in VicR RR amount or the stress response or both. Moreover, these previous studies only modulated the cellular amount of the VicR RR down (42) or up (39). To characterize the VicRK regulon further, we took advantage of our previous finding that the normally essential *vicR* gene can be deleted ($\Delta vicR$) when the *pcsB* gene is expressed constitutively from an artificial, synthetic promoter ($P_c\text{-}pcsB^+$) (42). In BHI, both the $P_c\text{-}pcsB^+ vicR^+$ strain (EL1454) (Table 1) and the $P_c\text{-}pcsB^+ \Delta vicR$ mutant (EL1472) (Table 1) reproducibly lagged compared to the $pcsB^+ vicRK^+$ R6 starting strain (Fig. 2A), but then all three strains grew at comparable rates in early to middle exponential phase (based on semilog replots of Fig. 2A). Microscopic examination showed that cells of the $P_c\text{-}pcsB^+ vicR^+$ and $P_c\text{-}pcsB^+ \Delta vicR$ strains appeared similar and formed chains as described before (41). We observed a new growth defect of the $P_c\text{-}pcsB^+$ mutant compared to the R6 parent strain in BHI medium. The $P_c\text{-}pcsB^+$ mutant stopped growing and began to autolyze at a considerably lower cell density than the R6 parent strain in BHI (see Fig. 2A). This rapid autolysis phenotype was not strongly affected by the deletion of *vicR* (see Fig. 2A) or the addition of fatty acids to cultures (see below) (data not shown). It was not observed when $P_c\text{-}pcsB^+$ strains were grown in CDM (see below).

We performed a microarray comparison of relative transcript amounts in the $P_c\text{-}pcsB^+ \Delta vicR$ mutant compared to the $P_c\text{-}pcsB^+ vicR^+$ parent growing in exponential phase (OD₆₂₀ ~0.25) in BHI (see Fig. 2B; Table 2; see also Tables S1 and S3 in the supplemental material). Deletion of the VicR RR caused decreases in the relative transcript amounts of a group of genes that encode several surface proteins [PspA (Spr0121), Spr0096, Spr1875], a possible integral membrane protein (Spr0709), and a putative MarR-family transcription regulator (Spr1874; *spr1874* and *spr1875* are possibly cotranscribed) (see below). The relative transcript amounts of each of these genes decreased significantly upon depletion of VicR in previous experiments (42). Relative transcript amounts of these genes also decreased when the bacteria were grown in CDM (see next section) (Table 2). Together, these results are consistent with direct positive regulation of these genes by the VicR RR. As expected, the amount of *pcsB* transcript expressed from the synthetic P_c promoter did not change in the two strains (see

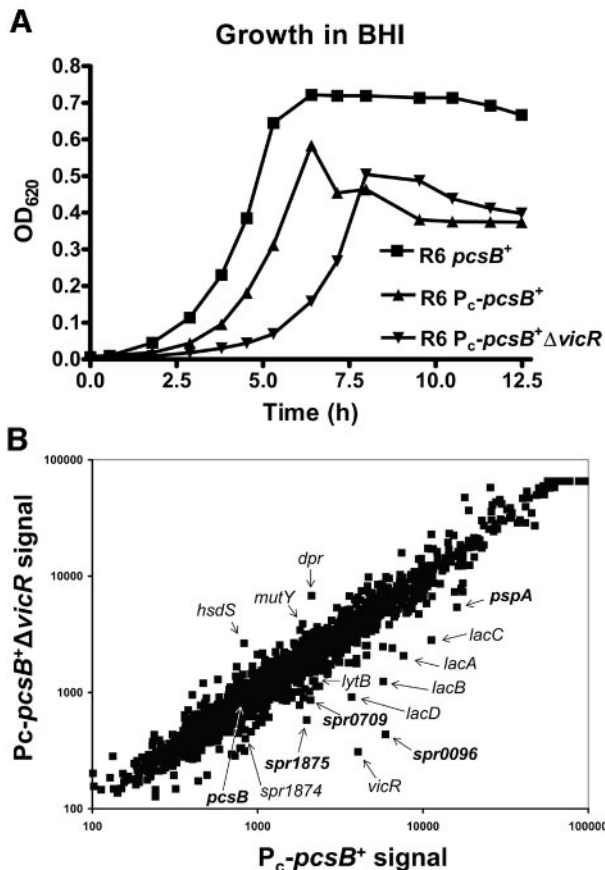


FIG. 2. Microarray analyses of a $\Delta vicR$ strain grown in BHI. (A) Growth characteristics of EL59 ($pcsB^+ vicR^+$), EL1454 ($P_c-pcsB^+ vicR^+$), and EL1472 ($P_c-pcsB^+ \Delta vicR$) in BHI. Growth was monitored by change in OD_{620} at 37°C with 5% CO_2 . (B) Representative log-scale scatterplot of microarray data comparing relative transcript amounts of EL1472 ($P_c-pcsB^+ \Delta vicR$; y axis) with isogenic parent EL1454 ($P_c-pcsB^+ vicR^+$; x axis) grown in BHI. Microarray experiments were performed and analyzed as described in Materials and Methods. Genes above and below the diagonal show higher and lower relative transcript amounts in a $\Delta vicR$ mutant, respectively. Genes marked in boldface were further characterized by band shift and footprinting assays. Fold changes and Bayesian P values (see Materials and Methods) for all transcripts detected in three independent (biological) experiments can be found in Table S1 in the supplemental material.

Fig. 2B, diagonal; Table 2). The microarray data indicate that there was no significant polarity of the $\Delta vicR \langle \rangle ermAM$ in-frame replacement mutation used in these experiments (Table 1) on downstream relative transcript amounts of $vicK$ or $vicX$ (Fig. 1; Table 2). The increases in the relative transcript amounts of $hsdS$ ($spr0448$; putative specificity subunit of type I of restriction endonucleases) and $mutY$ ($spr1108$; DNA repair glycosylase) (Fig. 2B) are considered in the next section.

Notably, we did not detect significant changes (change cutoff of 1.8-fold; $P < 0.001$) (see Materials and Methods) from a number of genes and operons whose relative transcript amounts changed in previous microarray experiments (39, 42), including the $piuBCDA$ iron transport operon ($spr1684-spr1687$), most of the $ptcCBA$ PTS operon ($spr1834-spr1836$), the $pstSCAB-phoU$ putative phosphate transport operon ($spr1895-spr1899$), heat shock and stress genes (e.g., $clpL$, $hrcA$,

$grpE$, and $dnaK$), the putative $fabR$ repressor gene ($spr0376$), and the $fabH-acp-fabKDGf-accB-fabZ-accCDA$ fatty acid biosynthetic gene cluster ($spr0377-spr0387$). When bacteria were grown in CDM (see next section), small changes in the relative transcript amounts from some of these genes did not extend uniformly to other genes within the same operon (Table 2). In some cases, small changes in relative transcript amounts were in the opposite direction in bacteria grown in BHI or CDM (Table 2). Together, these results show that the bacteria were less stressed in these experiments than in those reported previously (39, 42) and suggest that the VicR RR may not strongly regulate these other genes and operons directly. Finally, we detected changes in the relative transcript amounts of certain genes in the $\Delta vicR$ mutant only in BHI (Fig. 2B; see also Table S3 in the supplemental material) (see below). Some of these genes changed expression in previous experiments when VicR amount was depleted (e.g., dpr gene [ferritin-like iron binding protein]) (42) or overexpressed (e.g., $lacABCD$ operon [tagatose 6-phosphate pathway of lactose catabolism]) (39) in bacteria in complex broth media.

Defective growth of the $\Delta vicR$ mutant in CDM and changes in relative transcript amounts. We repeated the above experiment in a CDM in which pneumococcus grows well (58). Unexpectedly, the $P_c-pcsB^+ \Delta vicR$ mutant grew much more slowly in CDM than the isogenic $P_c-pcsB^+ vicR^+$ parent strain (Fig. 3A). We observed the same growth defect for several independent isolates of the $P_c-pcsB^+ \Delta vicR$ mutant (data not shown). We performed comparative microarray analyses for the two strains grown to an OD_{620} of ~ 0.1 (Fig. 3B; Table 2; see also Tables S2 and S3 in the supplemental material). The pattern of relative transcript amounts in bacteria grown in CDM (Fig. 3B) strongly resembled that observed for bacteria grown in BHI (Fig. 2B), except the relative decreases in transcript amounts in the $\Delta vicR$ mutant were considerably greater for bacteria in CDM compared to BHI (Fig. 2B and 3B; Table 2). For example, the normalized change(-fold) for the $spr0096$ transcript dropped from about -20 -fold in BHI to about -60 -fold in CDM (Table 2). This difference reflected a large increase in the hybridization signal strength to the $spr0096$ probe in the $vicR^+$ parent strain for bacteria grown in CDM compared to BHI (compare y axes in Fig. 2B and 3B). No corresponding change was observed in the hybridization signal strength to the $vicR$ probe in the $vicR^+$ strain grown in the two media (Fig. 2B and 3B, y axes). Thus, the magnified effect in CDM could reflect a change in the phosphorylation state of the VicR RR in the $vicR^+$ strain in the two media.

In bacteria grown in CDM, significant decreases in the relative amounts of transcripts from the $pspA$, $spr0096$, $spr1875$, $spr0709$, and $spr1874$ genes were again observed in the $\Delta vicR$ mutant compared to the $vicR^+$ parent (Fig. 3B; Table 2), consistent with possible direct positive regulation of these genes by VicR. In the $\Delta vicR$ mutant grown in CDM, we also detected a decrease in the relative transcript amount from $lytB$ (-3.0 -fold; $P = 1.90E-6$) (Table 2), which encodes an important glucosaminidase involved in a late step in cell separation (9). The relative transcript amount of $lytB$ decreased or increased in previous experiments when the VicR amount was depleted or overexpressed, respectively (39, 42). As noted above, the relative amounts of some transcripts only changed when bacteria were grown in BHI (Fig. 2A), but not CDM. Conversely, the

TABLE 2. Changes in relative transcript amounts in the $P_c\text{-}pcsB^+$ $\Delta vicR$ mutant compared to its $P_c\text{-}pcsB^+$ $vicR^+$ parent grown in BHI and CDM^a

R6 database no.	Gene	BHI		CDM	
		Average fold change	Bayesian <i>P</i>	Average fold change	Bayesian <i>P</i>
Changed in $\Delta vicR$ mutant and when VicR was depleted (42)					
spr2021 ^{b, c}	<i>pcsB</i>	1.1	3.79E-1	1.1	5.29E-1
spr0121 ^c	<i>pspA</i>	-2.8	4.32E-7	-2.4	1.64E-4
spr0096 ^c	<i>spr0096</i>	-16.4	3.63E-9	-51.3	1.99E-10
spr0709	<i>spr0709</i>	-3.0	1.70E-5	-10.1	2.15E-9
spr1875 ^c	<i>spr1875</i>	-3.7	1.09E-8	-28.0	1.53E-11
spr1874	<i>spr1874</i>	-2.0	6.11E-6	-3.8	3.26E-6
Changed in $\Delta vicR$ mutant, but not when VicR was depleted (42)					
spr0446	<i>hsdS</i>	-2.0	2.44E-5	-2.8	1.78E-5
spr0448	<i>hsdS</i>	3.0	1.39E-7	3.5	1.98E-7
spr1108	<i>mutY</i>	2.2	5.76E-6	4.1	2.33E-7
Changed when VicR was depleted (42), but not in $\Delta vicR$ mutant					
spr1684 ^c	<i>piuB</i>	-1.2	2.45E-2	2.6	2.37E-2
spr1685 ^c	<i>piuC</i>	-1.3	4.84E-3	1.5	3.05E-2
spr1686 ^c	<i>piuD</i>	-1.2	2.76E-2	3.2	1.66E-4
spr1687 ^c	<i>piuA</i>	-1.0	6.68E-1	1.3	4.98E-2
spr1834	<i>ptcC</i>	-2.8	1.02E-6	1.2	7.60E-2
spr1835	<i>ptcB</i>	-1.6	5.62E-4	1.1	4.36E-1
spr1836	<i>ptcA</i>	-1.8	4.20E-4	1.2	6.42E-2
spr1895	<i>pstS</i>	1.5	8.51E-3	-1.2	1.91E-1
spr1896	<i>pstC</i>	1.6	5.01E-3	1.2	3.13E-1
spr1897	<i>pstA</i>	1.6	3.50E-3	1.1	8.12E-1
spr1898	<i>pstB</i>	1.5	3.58E-3	1.3	1.72E-1
spr1899	<i>phoU</i>	1.3	9.34E-3	1.3	7.72E-2
spr0307	<i>clpL</i>	-1.1	3.17E-1	2.0	1.19E-3
spr0453 ^c	<i>hrcA</i>	1.1	9.29E-1	1.5	1.54E-2
spr0454 ^c	<i>grpE</i>	1.1	8.00E-1	1.1	5.77E-1
spr0455 ^c	<i>dnaK</i>	1.2	7.26E-1	1.3	1.18E-1
spr0456	<i>dnaJ</i>	1.2	3.10E-1	1.3	7.17E-2
Other genes of interest discussed in the text					
spr0867 ^c	<i>lytB</i>	-1.4	3.12E-3	-3.0	1.9E-6
spr1106 ^c	<i>vicK</i>	1.3	2.16E-2	1.5	4.62E-3
spr1105	<i>vicX</i>	1.2	2.72E-2	1.5	7.88E-3
Genes that mediate fatty acid biosynthesis (see text)					
spr0376 ^c	<i>fabR</i>	-1.4	2.47E-2	-1.1	3.92E-1
spr0377 ^c	<i>fabH</i>	-1.0	7.99E-1	-1.1	5.55E-1
spr0378 ^c	<i>acp</i>	-1.0	9.69E-1	-1.0	9.09E-1
spr0379 ^c	<i>fabK</i>	-1.2	1.00E-1	-1.4	6.11E-2
spr0380 ^c	<i>fabD</i>	-1.2	1.51E-1	-1.5	1.77E-2
spr0381 ^c	<i>fabG</i>	-1.2	1.52E-1	-1.4	6.84E-2
spr0382 ^c	<i>fabF</i>	-1.4	2.45E-2	-1.5	1.09E-2
spr0383 ^c	<i>accB</i>	1.1	6.61E-1	-1.7	1.30E-3
spr0384 ^c	<i>fabZ</i>	-1.1	3.03E-1	-2.0	9.92E-4
spr0385 ^c	<i>accC</i>	-1.1	5.46E-1	-1.4	1.29E-2
spr0386 ^c	<i>accD</i>	-1.2	9.97E-2	-2.0	4.12E-4
spr0387 ^c	<i>accA</i>	1.1	2.36E-1	-1.1	2.56E-1

^a Growth of bacteria, preparation of RNA, microarray analyses, calculation of signal ratios and fold changes, and statistical analyses are described in Materials and Methods. Positive and negative fold changes correspond to increased and decreased transcript amounts, respectively, in the $P_c\text{-}pcsB^+$ $\Delta vicR$ mutant compared to the $P_c\text{-}pcsB^+$ $vicR^+$ parent (see Materials and Methods). Significant changes in relative transcript amounts were considered positive or negative 1.8-fold with Bayesian *P* of <0.001 . Entries that do not meet these criteria are shown for genes of interest based on previous microarray analyses (39, 42) (see text). A several fold change of -1.0 means that there was a very slight decrease in relative transcript amount before rounding and is equivalent to no change (1.0).

^b No change was expected, because *pcsB*⁺ expression was uncoupled from VicR regulation by fusion to the artificial P_c promoter.

^c Genes that showed significant changes in relative transcript amounts when VicR was overexpressed as reported by Mohedano et al (39).

relative transcript amounts of some genes decreased in the $\Delta vicR$ mutant grown in CDM, but not in BHI, including those for several putative integral membrane proteins (e.g., Spr0142, Spr0143, and Spr1548) of unknown functions (Fig. 3B, circles;

see also Table S3 in the supplemental material). In bacteria grown in CDM, we did observe relatively small decreases in the relative transcript amounts of two noncontiguous fatty acid biosynthetic genes (*fabZ* and *accD*) in the $\Delta vicR$ mutant com-

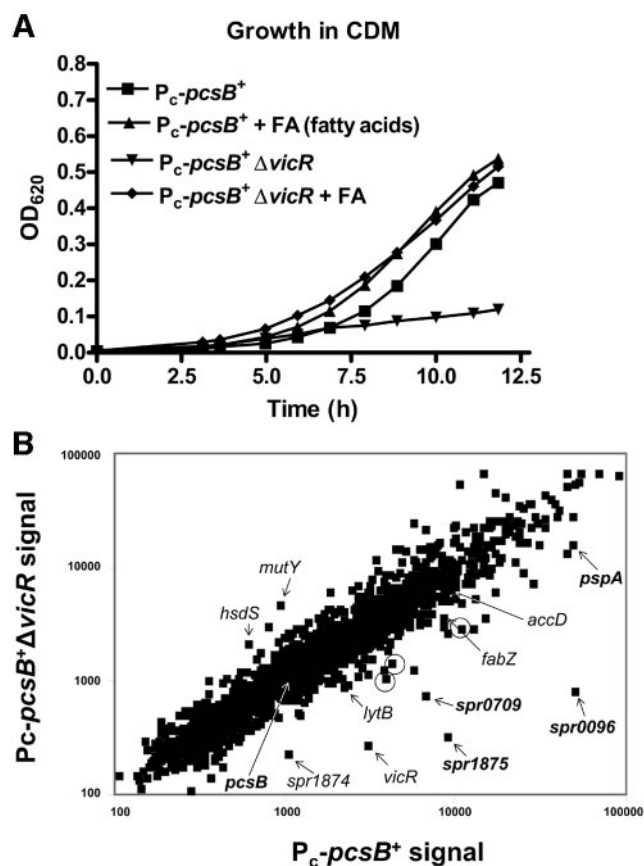


FIG. 3. Microarray analyses of a $\Delta vicR$ strain grown in CDM. (A) Growth characteristics of EL59 ($pcsB^+ vicR^+$), EL1454 ($P_c\text{-}pcsB^+ vicR^+$), and EL1472 ($P_c\text{-}pcsB^+ \Delta vicR$) in CDM in the presence or absence of fatty acid (FA) supplement (see Materials and Methods). Growth was monitored by change in OD₆₂₀ at 37°C with 5% CO₂. (B) Representative log-scale scatterplot analysis of microarray data comparing relative transcript amounts of EL1472 ($P_c\text{-}pcsB^+ \Delta vicR$; y axis) with isogenic parent EL1454 ($P_c\text{-}pcsB^+ vicR^+$; x axis) grown in CDM. Microarray experiments were performed and analyzed as described in Materials and Methods. Genes marked in boldface were further characterized by band shift and footprinting assays. Circled genes encode putative integral membrane proteins of unknown functions. Note that the signal ratios for *spr0096*, *spr0709*, and *spr1875* are further away from the diagonal than those in Fig. 2B due to significantly higher signals observed in strain EL1454 ($P_c\text{-}pcsB^+ vicR^+$) in CDM compared to BHI (see text). Fold changes and Bayesian *P* values (see Materials and Methods) for all transcripts detected in three independent (biological) repetitions of this experiment are given in Table S2 in the supplemental material.

pared to the $vicR^+$ parent (Table 2); however, the relative transcript amounts of *fabR* and the other fatty acid biosynthetic genes did not significantly change (Table 2).

Transcript amounts from comparatively few genes showed large increases in the $\Delta vicR$ mutant compared to the $vicR^+$ parent grown in BHI and CDM (Fig. 2B and 3B; Table 2; see also Tables S1 through S3 in the supplemental material). Relative transcript amounts from *hsdS* (*spr0448*, a possible specificity subunit of type I of restriction endonucleases) and *mutY* (DNA repair glycosylase) increased in the $\Delta vicR$ mutant in both media (Table 2). The increased transcription of *hsdS* (*spr0448*) seemed to be opposed by decreased relative tran-

scription in the $\Delta vicR$ mutant from a second *hsdS* (*spr0446*) gene (Table 2). Changes in the transcript amounts from these two genes had not been detected in previous microarray experiments (39, 42). *mutY* is located immediately upstream from the *vicRKX* operon (Fig. 1). A strong putative transcription terminator separates *mutY* from *vicR* (Fig. 1), and there was no indication of a contiguous *mutY-vicRKX* transcript on earlier Northern blots (66). The $\Delta vicR \langle \rangle ermAM$ allele used in these studies is an in-frame replacement of the *vicR* reading frame except for the last five codons by the *ermAM* reading frame (Table 1), and DNA sequence analysis of strain EL1472 (Table 1) showed that there were no mutations in *mutY*. Although it seems unlikely that the $\Delta vicR \langle \rangle ermAM$ mutation itself is influencing *mutY* transcript amount, we cannot completely rule out this possibility.

Defective growth of the $\Delta vicR$ mutant in CDM can be reversed by addition of fatty acids and depends on expression levels of *PcsB*. Previous studies of a temperature-sensitive mutant of *S. aureus* suggested that the YycF RR mediates membrane integrity (36). Recent results suggest that overexpression of VicR may cause a change in the fatty acid composition of total membrane lipid extracted from *S. pneumoniae* (39). Although the $\Delta vicR$ mutation did not cause uniform or significant changes in relative transcript amounts from the fatty acid biosynthetic genes (Fig. 3B; Table 2), we tested whether a fatty acid mixture of 30 μ M linoleic acid and 15 μ M oleic acid in 15 μ M carrier bovine serum albumin could restore growth of the $P_c\text{-}pcsB^+ \Delta vicR$ mutant in CDM. Indeed, the fatty acid mixture or each of the fatty acids alone restored the growth of the $P_c\text{-}pcsB^+ \Delta vicR$ mutant to that of the $P_c\text{-}pcsB^+ vicR^+$ parent strain (Fig. 3A; also data not shown). A control experiment showed that addition of the fatty acid-free carrier bovine serum albumin alone did not restore growth of the $\Delta vicR$ mutant (data not shown). An additional microarray analysis of the strains grown in CDM containing fatty acids still showed sizable decreases in the relative amounts of the *spr0096*, *spr1875*, *spr1874*, *lytB*, and *spr0709* transcripts in the $P_c\text{-}pcsB^+ \Delta vicR$ mutant compared to the $P_c\text{-}pcsB^+ vicR^+$ parent (data not shown). This result is again consistent with direct positive regulation of transcription of these genes by VicR, whether fatty acids are absent or present and whether the cells are growing poorly or not.

Microarray and Western blot analyses showed that the amount of *PcsB* is about threefold lower in the $P_c\text{-}pcsB^+$ mutant than in the $pcsB^+$ R6 parent in CDM (data not shown). Therefore, it seemed possible that the impaired growth of the $P_c\text{-}pcsB^+ \Delta vicR$ strain in CDM was caused by a combination of reduced *PcsB* expression and decreased transcription of other genes in the VicR regulon. To test this hypothesis, we attempted to modulate *PcsB* amount by expression from another artificial promoter. Similar to the $P_c\text{-}pcsB^+$ construct, fusion of *pcsB^+* to the fucose-inducible P_{fcsK} promoter (7) and *fcsK* ribosome binding site uncouples *pcsB^+* expression from VicR regulation and allows deletion of *vicR* (41). In contrast to the $P_c\text{-}pcsB^+ \Delta vicR$ mutant (Fig. 3A), growth of the $P_{fcsK}\text{-}pcsB^+ \Delta vicR$ mutant (IU1602) (Table 1) was not impaired and was similar to that of its $P_{fcsK}\text{-}pcsB^+ vicR^+$ parent (IU1545) (Table 1) in CDM plus 0.2% (wt/vol) fucose lacking fatty acids (data not shown). A control experiment showed that fucose addition could not account for this difference, because addition of 0.2%

(wt/vol) fucose did not restore growth of the $P_c\text{-}pcsB^+ \Delta vicR$ mutant in CDM (data not shown). Western blot analyses were performed to compare PcsB amounts bound to cells in these experiments. Unexpectedly, the amount of PcsB protein bound to cells was considerably less (~ 3.5 -fold) in the $P_c\text{-}pcsB^+ \Delta vicR$ mutant than that in its $P_c\text{-}pcsB^+ vicR^+$ parent or the $P_{fcsK}\text{-}pcsB^+ vicR^+$ and $P_{fcsK}\text{-}pcsB^+ \Delta vicR$ strains (data not shown). We do not understand the mechanism for this drop in the relative amount of PcsB in the $P_c\text{-}pcsB^+ \Delta vicR$ mutant. Nevertheless, the correlation between reduced PcsB amount and a requirement for fatty acids is consistent with the idea that PcsB may act with other members of the VicR regulon to influence the membrane integrity of *S. pneumoniae* (see Discussion).

AcP is a robust phosphoryl group donor to purified VicR.

The above results show that only a handful of genes that encode cell surface proteins and virulence factors are strongly regulated by the VicR RR under all conditions tested. To learn whether this regulation is direct, we performed a series of band shift and footprinting experiments. We fused a His₁₀ tag to the N terminus of VicR (see Materials and Methods). Other tagged constructs of VicR reported previously aggregated and formed inclusion bodies that were solubilized by denaturation followed by refolding (8, 12). We found that certain standard induction conditions (30°C in *E. coli* for 3 h after IPTG addition) left a significant amount of His₁₀-VicR in soluble form, obviating the need for refolding. His₁₀-VicR was purified to near homogeneity by immobilized metal affinity chromatography fast protein liquid chromatography (see Material and Methods). His₁₀-VicR, which for simplicity will be referred to as VicR in the remainder of this article, was phosphorylated by purified VicK-P HK (data not shown), analogous to reports for two other fusion constructs of VicR (8, 12). In addition, we tested whether the physiologically significant (46, 55), small-molecule phosphoryl group donor AcP phosphorylated purified VicR (see Material and Methods). Extent of phosphorylation was determined by HPLC on a C₄ column (19) (see Materials and Methods). Unphosphorylated VicR ran as a single peak (Fig. 4). Following reaction with AcP in 2 mM or 20 mM MgCl₂, over 80% or 95% of VicR, respectively, was converted to an earlier eluting peak that corresponds to VicR-P (Fig. 4). We infer that the D52 phosphoryl group of VicR-P is stable at 25°C, because we observed marked differences between VicR-P and unphosphorylated VicR in DNA binding experiments (see below).

Unphosphorylated VicR binds upstream of and within *pcsB*.

We performed band shift experiments on four regions upstream of genes whose transcript amounts consistently depended on VicR function in bacteria grown under different conditions (Fig. 2 and 3) (39, 42). We focused on the *pcsB*, *pspA*, *spr0096*, and *spr1875* genes, because each contains a variant of the direct-repeat consensus sequence proposed for YycF binding in *B. subtilis* and *S. aureus* (Table 3) (11, 22) upstream of its translation start codon (Fig. 5; Table 3). One putative VicR binding site with one mismatch compared to the consensus is located upstream of and with its direct repeats in the same direction as *pcsB*, and another with one mismatch and opposite orientation is within *pcsB* (Fig. 5A; Table 3). Putative VicR binding sites with no or two mismatches are located upstream of and in the same direction as *pspA* and *spr0096*, respectively (Fig. 5B and 5C; Table 3). Last, tandem

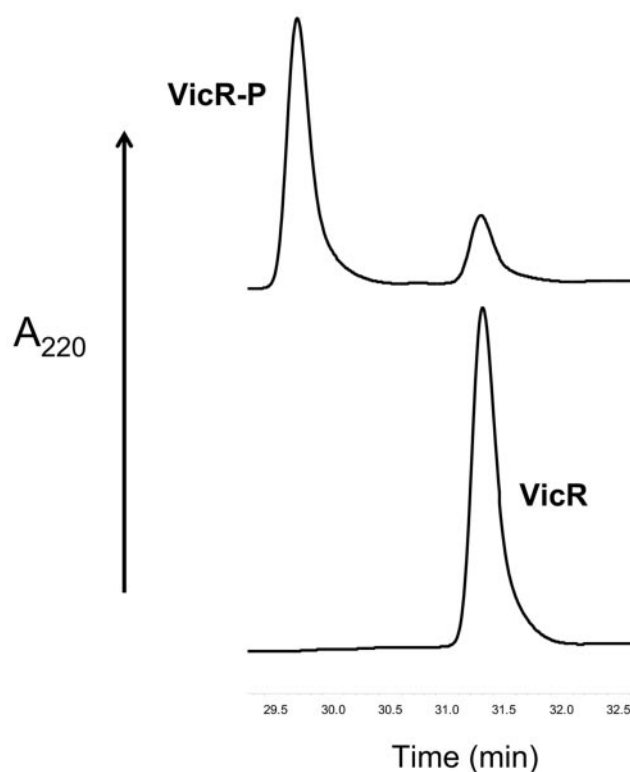


FIG. 4. AcP is a phosphoryl group donor for VicR in vitro. VicR was phosphorylated by AcP in the presence of 2 mM MgCl₂, and the extent of the reaction was analyzed by analytical reverse-phase HPLC on a C₄ column as described in Material and Methods. Bottom trace, unphosphorylated VicR; top trace, VicR reacted with AcP. Based on areas under curves in chromatographs, about 80% of VicR was converted to VicR-P using these reaction conditions. Increasing the MgCl₂ concentration to 20 mM allowed >95% phosphorylation of VicR to VicR-P (data not shown).

putative VicR binding sites, each with two mismatches with the consensus are located upstream of and in the opposite direction from *spr1875* (Fig. 5D; Table 3).

We first examined the binding of unphosphorylated VicR to three regions of *pcsB* by band shift assays (Fig. 6). We used fluorescently labeled PCR primers to prepare labeled DNA fragments (Fig. 6) (see Materials and Methods). VicR bound to the site upstream of *pcsB* (primers WN211-212F) (Fig. 6), and the shifted band moved to a lower mobility with increasing amounts of VicR. The PAGE conditions used in these band shift studies were not optimized to preserve bound complexes, and the actual K_d s for VicR binding could be considerably lower than the low-micromolar range used in these experiments. By comparison, we are adding considerably less (8- to 35-fold) VicR to our band shift reactions than was required in analogous experiments using the YycF homologues of *B. subtilis* or *S. aureus* (11, 14, 22). On the other hand, binding in the low-micromolar range could still be of physiological significance, depending on the amount of a protein present in cells. In a relatively small (1- μ m diameter), ovoid cell, such as *S. pneumoniae*, 500 dimers of a protein corresponds to a total concentration of about 1.6 μ M. As a specificity control, VicR did not bind to the region around the translation start of PcsB

TABLE 3. Sequences upstream of or within pneumococcal target genes related to the YycF consensus binding site proposed for *B. subtilis* and *S. aureus*^a

Site	Sequence
Proposed consensus (<i>B. subtilis</i> and <i>S. aureus</i>)	5'- <u>TGT(A/T)A(A/T/C)</u> >-N ₅ - <u>TGT(A/T)A(A/T/C)</u> >-3'
59 bp upstream of <i>pcsB</i> ^b	5'-TGTAAC>-AAAGG- <u>CGTAAT</u> >-ATTT-3'
125 bp within <i>pcsB</i> ^c	3'-ATTG-<AATTGT- <u>CGTGT</u> -<TGTTGT-5'
85 bp upstream of <i>pspA</i> ^b	5'-TGTAAC>-AAAAA-TGTAAT>-ATAA-3'
44 bp upstream of <i>spr0096</i> ^b	5'-TGT <u>CAA</u> >-GAAAC-GGTAAT>-ATTT-3'
50 bp upstream of <i>spr0709</i> ^{b,d}	5'-TGTAAC>-CTTTC-TGTAAT>-AATT-3'
148 bp upstream of <i>spr1875</i> ^b	3'-AAAA-<CACTGT-CTCTG-<GATTGT-5'
108 bp upstream of <i>spr1875</i> ^c	3'-CAAA-<CAATGT-TCCTT-<ATCTGT-5'
15 bp upstream of <i>fabR</i> ^c	5'-TGTA <u>AA</u> >-AGTTT- <u>TTTGAA</u> >-GAAG-3'
Pneumococcal consensus	5'- <u>TGT(A/C/T)A'(A/G/C)</u> >-(A/C/G)(A/T)(A/C/T)(A/T/G)(A/C/G)- (G/T/C) <u>GT(A/C)A(T/C)</u> >-A(A/T)(A/T)(A/T)-3' 5'- <u>TGTNAN</u> >-N(A/T)NNN-NGT(A/C)A(C/T)>-A(A/T)(A/T)(A/T)-3'

^a Locations are from the 3' ends of the sequence shown and A in the start codons of each gene. Mismatches with the proposed consensus in *B. subtilis* and *S. aureus* (11, 22) are underlined. Orientations of the direct repeats relative to downstream genes are indicated by >> and << for same and opposite directions, respectively. Invariant nucleotides in consensus sequences are in boldface.

^b Used for pneumococcal consensus at the bottom of the table. The consensus-related sequences for these genes are identical in strain R6 (21) and serotype 4 strain TIGR4 (60), except for *pspA* (*spr0121* in R6 and *sp0117* in TIGR4). The sequence upstream of *pspA* in TIGR4 is 5'-TGTAAT>-AAAAA-TGTAAT>-ATAA-3'.

^c Specific binding of VicR or VicR-P was not detected in footprinting experiments (see text).

^d Not tested in footprinting experiments, but the pattern of binding of VicR and VicR-P to the single site upstream of *spr0709* was similar to those shown for other binding sites in Fig. 7.

(primers WN214-212F) (Fig. 6) or to other DNA fragments lacking a sequence related to the consensus (see *vicR* binding, below). Finally, VicR bound to the internal site within *pcsB* (primers WN214-213F) (Fig. 6). However, there was considerably less binding to the internal site than the upstream site at each concentration of VicR and no "super shifting" to lower mobility, which together indicate weaker binding to the internal site compared to the upstream site (Fig. 6). Consistent with this interpretation, footprinting experiments (see below) failed to detect specific binding of VicR or VicR-P to the internal site within *pcsB* (data not shown).

Phosphorylation of VicR to VicR-P increases the strength and changes the nature of binding to regions upstream of *pcsB*, *pspA*, *spr0096*, *spr1875*, and *spr0709*. Unphosphorylated VicR also bound to fragments containing the sequences described above and listed in Table 3 upstream of *pspA*, *spr0096*, and *spr1875* (Fig. 7). Importantly, phosphorylation of VicR to VicR-P noticeably increased the binding to fragments upstream of *pcsB*, *pspA*, *spr0096*, and *spr1875* (Fig. 7), as indicated by the lower concentrations of VicR-P compared to VicR needed to observe binding. Furthermore, the nature of the binding of VicR-P was different from that of VicR. At lower protein concentrations, DNA fragments bound to VicR-P "super shifted" to slower mobilities than those bound to VicR (Fig. 7). This result suggests that more than one VicR-P may bind to each fragment through dimerization or oligomerization or possibly that VicR-P binding bends the DNA fragments. A similar band shift pattern was obtained for binding of VicR and VicR-P to a region containing a putative binding site upstream of *spr0709* (Table 3; also data not shown).

VicR and VicR-P do not bind upstream of *vicRKX*. A recent paper concluded that transcription of *vicK* is autoregulated by VicR in *S. pneumoniae* (39). *vicK* is located in a three-gene operon with *vicR* and *vicX* (Fig. 1) (66), which encodes a protein that may play a role in VicR signal transduction (42). A promoter for the *vicRKX* operon was mapped by primer extension to the intercistronic region between *mutY* and *vicR*

(Fig. 1 and 8) (66). This intercistronic region does not contain a sequence related to the YycF consensus binding site. Band shift experiments did not detect significant binding of VicR or VicR-P to the *mutY-vicR* intercistronic region. As a control, there was again readily detectable binding to the region upstream of *pspA* (Fig. 8). At the highest concentration used (1.5 μM), we detected weak and probably nonspecific binding of VicR to this fragment, and phosphorylation of VicR (VicR-P) eliminated this weak binding (Fig. 8). These results and other considerations (see Discussion) do not support the autoregulation of *vicRKX* by VicR.

Phosphorylation of VicR to VicR-P prevents binding upstream of *fabR*. The VicRK TCS was proposed to directly or indirectly control the expression of the fatty acid biosynthetic pathway in *S. pneumoniae* (39). One mechanism that could contribute to direct regulation is binding of VicR or VicR-P to a site upstream of the *fabR* gene, which encodes a putative repressor of fatty acid biosynthesis (34, 39). A sequence (TG TAAA>-AGTTT-TTTGAA>) that matches a half-site of the consensus (Table 3) is located 15 bp upstream of *fabR* (Table 3) (39). Binding to a fragment containing this site was observed in band shift experiments at higher concentrations of VicR than was required for fragments containing the binding site upstream of *pspA* (Fig. 8). Significantly, phosphorylation of VicR to VicR-P abolished binding to this fragment (Fig. 8). Footprinting experiments (see below) failed to detect protection of this half-site by VicR or VicR-P (data not shown). This result and the microarray experiments presented above argue against a direct role of the VicR RR in regulating fatty acid biosynthesis in pneumococcus (see Discussion).

VicR and VicR-P bind to the same regions upstream of *pcsB*, *pspA*, *spr0096*, and *spr1875*. We first performed DNase I footprinting experiments of VicR and VicR-P to the putative binding site upstream of *pcsB* (Fig. 5A, 6, and 7; Table 3) (see Materials and Methods). VicR-P protected an extended region on both DNA strands that includes the sequence related to the consensus (Fig. 9; Table 3). Moderately greater protection was observed at a lower concentration of VicR-P than VicR (Fig.

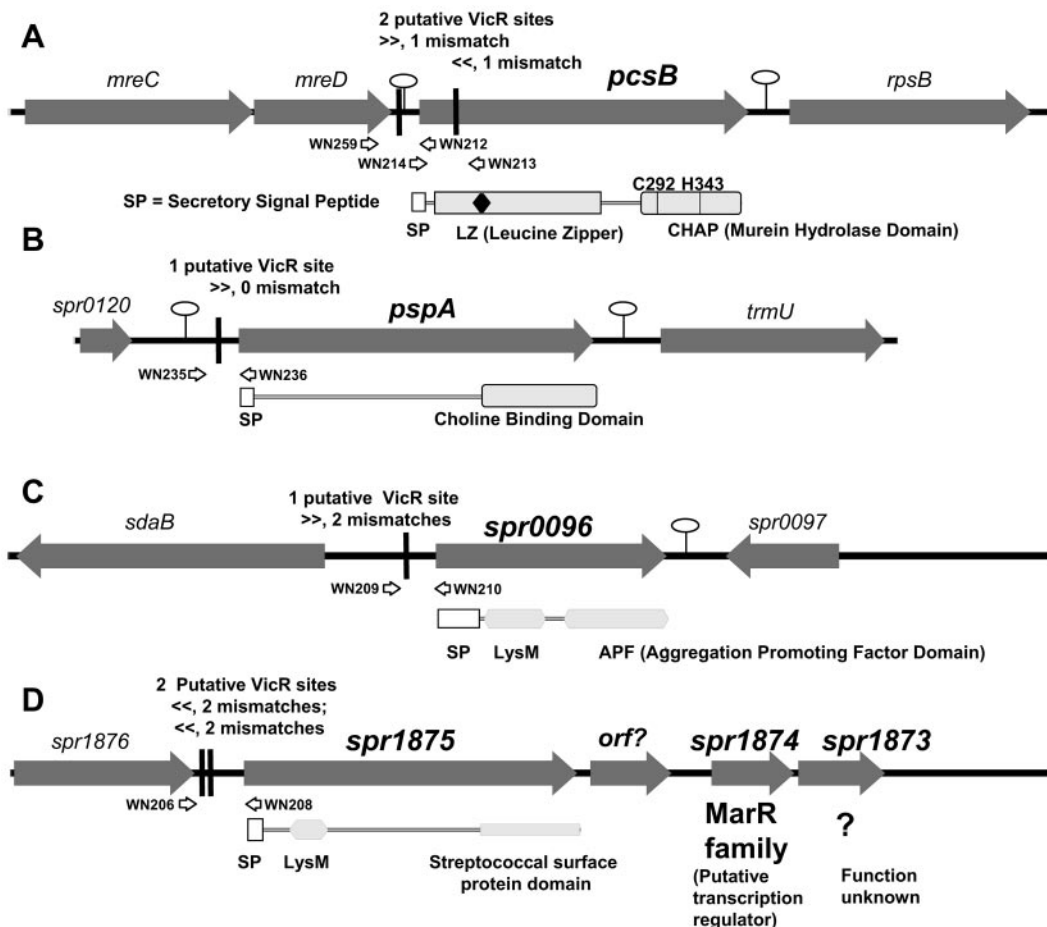


FIG. 5. Genetic organization and protein domain features of four members of the VicRK regulon in *S. pneumoniae* identified in this article. (A) *pcsB*; (B) *pspA*; (C) *spr0096*; (D) *spr1875*. Genes are labeled in boldface and depicted by arrows along with neighboring genes. Lollipop symbols represent putative Rho-independent transcription terminators. VicR binding sites related to the YycF binding consensus proposed for *B. subtilis* and *S. aureus* (Table 3) (11, 22) are indicated by vertical lines along with the orientations of direct repeats (>> or <<) in the site and the number of mismatches, if any, compared to the consensus. The locations of PCR primers used to synthesize DNA fragments for band shift and footprinting assays are indicated (Table 1).

10). This result is consistent with the results of the band shift experiment that phosphorylation of VicR to VicR-P increases its binding (Fig. 7). Only a higher range of VicR concentrations allowed detection of a protected region in these DNase I footprinting experiments (Fig. 10), and the determination of relative binding constants of VicR and VicR-P to this and other regions will need to be done by other methods, such as fluorescence anisotropy measurements (19, 20). Lastly, the footprinting experiments showed that at these higher concentrations, VicR and VicR-P protected the same region on the top (Fig. 10) and bottom DNA strands (data not shown).

Comparable DNase I protection patterns were observed for binding of VicR and VicR-P to the sequences related to the consensus upstream of *pspA* and *spr0096* (Table 3; also data not shown). In addition, VicR and VicR-P protected the consensus-like sequence that was farthest (148 bp) upstream from *spr1875* (data not shown). We did not detect protection of the consensus-related sequence closer (108 bp) to *spr1875* (Table 3; also data not shown); however, this region contained few DNase I cleavage sites, and we may have missed binding to this

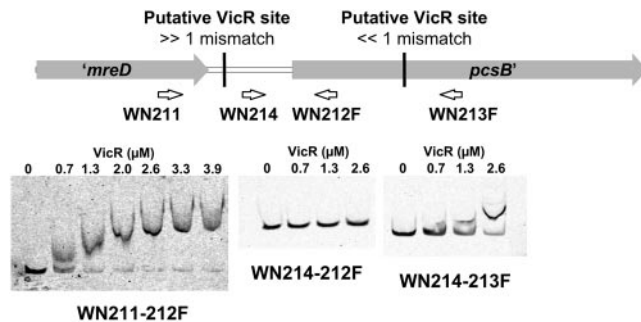


FIG. 6. VicR binds to regions upstream from and within *pcsB*. The locations of putative VicR binding sites upstream from and within *pcsB* (Table 3) are marked with the same designations used in Fig. 5. Unlabeled and fluorescently labeled (designated by F) primers used in PCRs to generate fluorescently labeled DNA fragments are indicated (Table 1) (see Materials and Methods). DNA fragments were incubated with the increasing amounts of VicR indicated, and binding complexes were resolved by native PAGE (see Materials and Methods). Footprinting assays showed specific binding of VicR and VicR-P to the region upstream of *pcsB*, but not to the region within *pcsB* under the conditions used in these assays (see text).

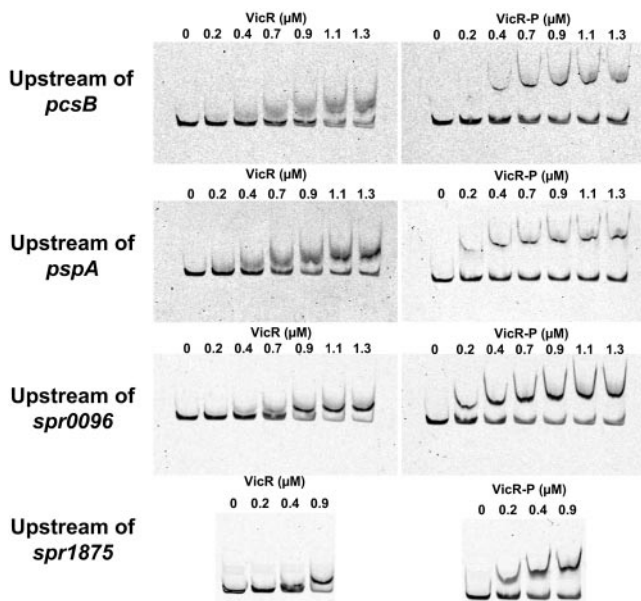


FIG. 7. Phosphorylation of VicR to VicR-P enhances binding to regions upstream of *pcsB*, *pspA*, *spr0096*, and *spr1875*. Fluorescently labeled DNA fragments were synthesized by PCR using primers listed in Table 1 and depicted in Fig. 5 and 6. Each fragment contains a sequence related to the proposed consensus (see Table 3). VicR-P was prepared by reaction with AcP as described in Materials and Methods (Fig. 4). Phosphorylated VicR-P was used immediately in binding assays containing the increasing amounts of VicR and VicR-P indicated (see Materials and Methods) (Fig. 6). Bound complexes were resolved by PAGE on native gels. Similarly enhanced binding of VicR-P compared to VicR was observed for a fragment containing a sequence related to the consensus from upstream of *spr0709* (see text) (Tables 1 and 3; also data not shown).

region. As for the upstream site of *pcsB* (Fig. 9 and 10), VicR-P seemed to bind more strongly than unphosphorylated VicR to extended regions around and including the consensus-related sequences upstream of *pspA*, *spr0096*, and *spr1875* (data not shown). The implications of these binding and protection studies for control by the VicRK TCS in *S. pneumoniae* are considered below.

DISCUSSION

Combined microarray, band shift, and footprinting studies in this article support the conclusion that the VicR RR strongly and directly regulates the transcription of a set of genes encoding important surface proteins in *S. pneumoniae* (Fig. 11). These proteins include essential PcsB, which mediates murein biosynthesis possibly as a murein hydrolase (see references in reference 41) and PspA, which is an important virulence factor that interferes with complement activation on the cell surface (6, 49, 50, 63). PspA is among the most studied proteins in *S. pneumoniae* because of its potential as a vaccine target (5, 6), and PcsB has considerable potential as new target for antibiotic and vaccine development (41). The amount of the *pspA* transcript increased in bacteria in the blood of mouse hosts compared to control bacteria grown in serum-containing broth (44, 45). Our results suggest that the VicRK TCS may contribute to this regulation of *pspA*.

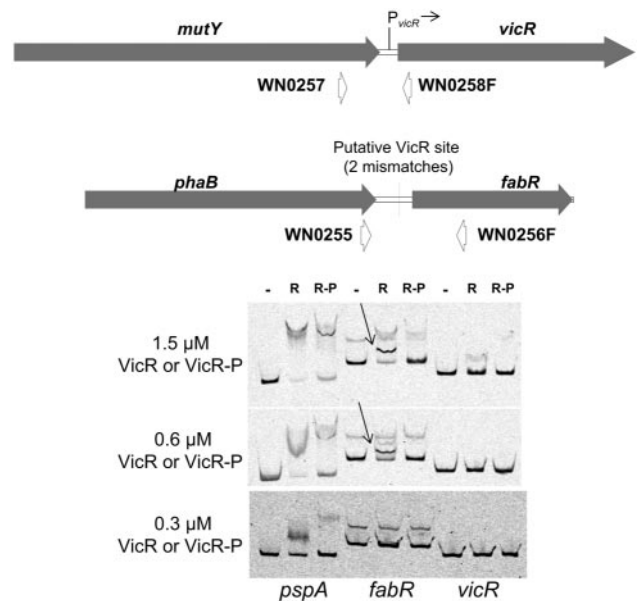


FIG. 8. Lack of binding of VicR-P to regions upstream from *vicR* and *fabR*. *vicR* encodes the VicR RR, and *fabR* encodes a protein that may negatively regulate the fatty acid biosynthetic operon (see text). Band shift assays were performed using fluorescently labeled DNA fragments synthesized by PCR with the indicated primers (Table 1; Fig. 7) (see Materials and Methods). The intergenic region between *mutY* and *vicR* lacks a sequence related to the consensus, whereas the intergenic region between *phaB* and *fabR* contains a sequence related to the consensus with two mismatches in one half site (Table 3). Binding reactions contained the increasing amounts of VicR or VicR-P denoted at left. Lanes are labeled as follows: -, no protein; R, unphosphorylated VicR; R-P, phosphorylated VicR-P. A fragment containing the VicR/VicR-P binding site of *pspA* (Table 3; Fig. 7) was used as a positive control. The arrow marks a shifted band corresponding to binding of unphosphorylated VicR at higher protein concentrations. However, this shifted band was not present in reactions containing VicR-P, and footprinting did not detect specific binding of VicR or VicR-P to the intergenic region upstream of *fabR* (data not shown) (see text).

Besides PcsB and PspA, the only two proteins in *S. pneumoniae* that contain LysM peptidoglycan binding domains (Spr0096 and Spr1875) are members of the VicR regulon, as is a putative membrane protein of unknown function (Spr0709). The VicR RR also seems to regulate a fairly limited number of genes encoding cytoplasmic proteins, including a putative MarR family transcription regulator (Spr1874) that may be produced by the same operon as LysM-containing Spr1875. Regulation of this set of genes by VicR is positive, since deletion and depletion or overexpression of VicR decreases (Fig. 2B and 3B; Table 2) (42) or increases (39), respectively, the relative transcript amounts of these genes, sometimes greatly (30- to 50-fold decreases) (Table 2) (42). Of these genes, only *pcsB* is essential for growth in laboratory media (41, 42) (data not shown), and strong positive regulation of *pcsB* can account for the essentiality of the VicR RR in pneumococcus (Fig. 2 and 3) (41, 42). This is the only instance so far where the essentiality of a YycFG TCS family member can be ascribed to regulation of a single essential gene (see references 41 and 42).

Our findings in *S. pneumoniae* parallel those in *S. aureus*, where a major role of the YycF RR, which is the VicR homo-

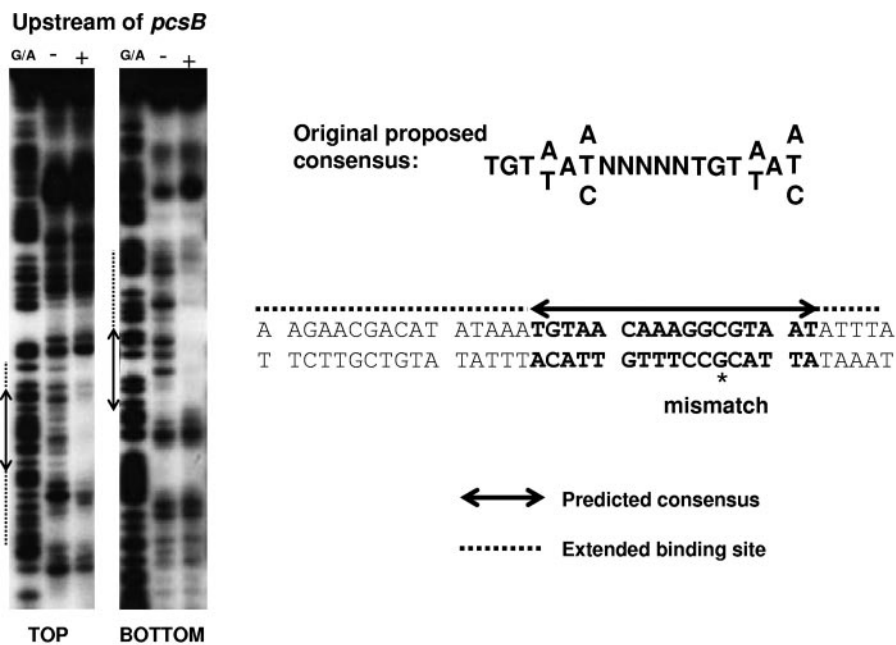


FIG. 9. DNase I footprinting analysis of VicR-P binding upstream of *pcsB*. DNA fragments were synthesized by PCR with either the top strand (primer WN259) or bottom strand (primer WN212) labeled with ³²P (Table 1; Fig. 5) (see Materials and Methods). Labeled DNA fragments were incubated in the same binding reaction mixture used for the band shift experiments (Fig. 6 and 7) either lacking (–) or containing 3 μM VicR-P, which was phosphorylated by AcP as described in Materials and Methods (Fig. 4 and 7). Bound complexes were treated with DNase I, and DNA fragments were resolved by denaturing PAGE alongside a Maxam-Gilbert G+A ladder (G/A) prepared from the same labeled DNA (see Materials and Methods). Regions protected by VicR-P that are related to the direct repeat consensus (Table 3) (11, 22) and extended regions of protection outside the consensus are marked by solid or dashed lines, respectively. The sequence of the protected region upstream of *pcsB* is shown, including the mismatch with the original consensus, which is shown above it (see text) (Table 3).

logue, seems to be regulation of genes involved in cell wall biosynthesis, surface adherence, and virulence (11). In this previous study (11), binding of purified YycF was determined to *S. aureus* genes preceded by a consensus binding site derived from *B. subtilis* (22). This binding has not yet been correlated with changes in relative transcript levels. From these combined studies, it is striking that genes encoding different murein biosynthetic and surface proteins seem to be regulated by YycF homologues in each kind of bacterium (Fig. 11) (11, 22, 39, 42). Remarkably, depletion of YycFG expression in *S. aureus* does not lead to the severe (11), but different, defects in cell morphology observed for *B. subtilis* (13, 14) and *S. pneumoniae* (39, 41, 42). This result underscores that the YycFG TCS family plays different roles in these different species of gram-positive bacteria. This specialization seems to extend even to different species of streptococcus. A recent paper shows that the VicRK TCS regulates biofilms formation and competence development in *Streptococcus mutans* (53). In this instance, part of the regulation likely occurs through the regulation of genes encoding extracellular sugar transferases involved in exopolysaccharide biosynthesis. These *S. mutans* genes are not present in *S. pneumoniae* (21, 60), and microarray data reported here (see Tables S1 and S2 in the supplemental material) and before (39, 42) do not indicate a link between VicRK TCS regulation and changes in transcript amounts of the competence regulon of *S. pneumoniae*. On the other hand, it seems likely that the PcsB homologue of *S. mutans* (GbpB) is positively regulated by the VicRK TCS (53).

Previous work demonstrated that phosphorylation of VicR is

required for its essential function in *S. pneumoniae* (see the introduction) (42). Consistent with this finding, we show here that phosphorylation of VicR to VicR-P increases the strength and changes the nature of binding to a region upstream of *pcsB* (Fig. 6, 7, and 10; Table 3) as well as to regions upstream of *pspA*, *spr0096*, *spr1875*, and *spr0709* (Fig. 7; also data not shown). This is the first report that phosphorylation of a YycF family RR affects binding to target sequences. However, similar to previous reports for YycF in *B. subtilis* (22) and *S. aureus* (11), VicR and VicR-P protected the same regions from DNase I digestion at the higher protein concentrations required in footprinting experiments. Thus, our current data suggest that phosphorylation of VicR to VicR-P affects the strength of binding to target sequences and not the location of binding.

In these experiments, we showed that VicR can readily be phosphorylated in vitro by AcP (Fig. 4), which is an important signaling molecule in some bacteria (reviewed in reference 67). This is the first report that a YycF family member uses AcP as a phosphoryl group donor. Previously, it was reported that AcP failed to activate the YycF RR of *B. subtilis* (14). The phosphorylation of VicR by AcP could partly account for the fact that the cognate VicK HK is not essential in *S. mutans* (53) or in *S. pneumoniae* unless VicR amount is reduced (see the introduction) (12, 27, 31, 42, 61, 66). In addition, phosphorylation of VicR by AcP provides another possible input into the VicR signal transduction pathway. (Fig. 11).

The consensus sequence proposed for YycF RR binding in *B. subtilis* and *S. aureus* is only partly predicative of VicR

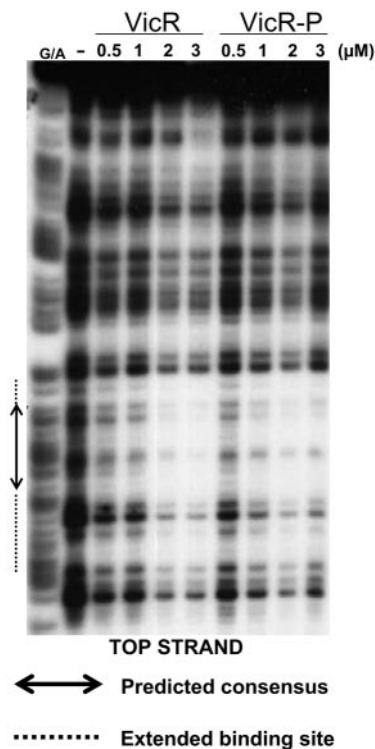


FIG. 10. VicR and VicR-P bind to the same region upstream of *pcsB* at higher protein concentrations. DNA fragments were prepared as for Fig. 9. Labeled DNA fragments were incubated with the increasing amounts of VicR or VicR-P indicated, digested with DNase I, and resolved by denaturing PAGE as described for Fig. 9. Protection of VicR-P was detected at lower concentrations of VicR-P (0.5 to 1 μ M) than VicR, and VicR and VicR-P protected the same region upstream *pcsB* at higher concentrations of protein (2 or 3 μ M). A similar pattern of concentration-dependent protection was observed for binding of VicR and VicR-P when the bottom strand was labeled (data not shown). Analogous protected regions containing sequences related to the consensus listed in Table 3 were detected by footprinting upstream of *pspA*, *spr0096*, and *spr1875* (see text) (data not shown).

binding in *S. pneumoniae*. Of the five genes that were regulated most strongly and consistently in the $\Delta vicR$ mutant, the upstream regions of only two match the consensus exactly (*pspA* and *spr0709*) (Table 3). Of the six other genes preceded within 400 bp by perfect matches in either DNA strand, five (*spr0047*, *spr0564*, *spr1102*, *spr1651*, and *spr2015*) were not regulated in any microarray experiment (see Results) (39, 42), and one (*spr1895*) was only regulated in some experiments (42). The other three strongly regulated genes are preceded by sites related to the consensus with one (*pcsB*) or two mismatches, each in a separate half site of the direct repeat (*spr0096* and *spr1875*) (Table 3). As was noted before for the consensus in *B. subtilis* and *S. aureus* (11, 22), the direct repeats in the VicR binding site can be oriented in the same (*pcsB*, *pspA*, *spr0096*, and *spr0709*) or the opposite (*spr1875*) direction as the regulated gene. The refined pneumococcal consensus based on these five sites is considerably more degenerate than the original consensus and extends downstream from the second direct repeat half site (Table 3).

Moreover, results from this and previous studies (39, 42) suggest that VicR may regulate additional genes by binding to

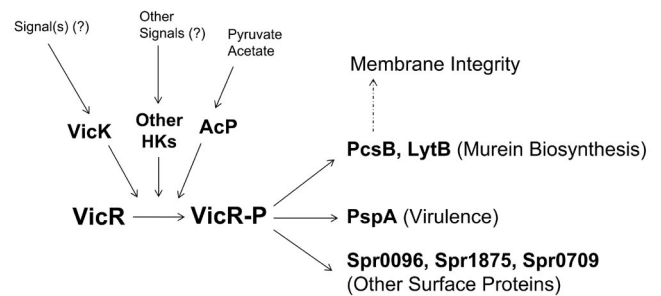


FIG. 11. Signal transduction pathway mediated by the essential VicRK TCS in pneumococcus. Based on data reported here and in previous experiments (41, 42), VicR is activated by phosphorylation by the cognate VicK HK or other noncognate HKs in response to multiple signals, which are currently unknown. AcP may also act as a phosphoryl donor to VicR in the cell (Fig. 4). Activated VicR-P acts as a direct positive transcription regulator of several genes encoding surface proteins that play important roles in murein biosynthesis (*PcsB* and possibly *LytB*), virulence (*PspA*), and other surface functions (*Spr0096*, *Spr1875*, and *Spr0709*). VicRK also mediates membrane integrity (see Results) (36, 39), possibly through a direct or indirect functional relationship between *PcsB* and other members of the regulon (see Discussion). The VicRK TCS may regulate additional genes under some conditions (see Discussion) (see Table S3 in the supplemental material) (39, 41); however, the VicR/VicR-P binding site is sufficiently degenerate (Table 3) that direct regulation cannot be assumed without additional experiments.

even more degenerate binding sites. Besides the five genes listed above, there are 21 other genes in the pneumococcal genome preceded within 400 bp by a degenerate version of the pneumococcal consensus (TGTNAN-N₅-NGTNANA) (Table 3). Of these, the transcript amounts of only two (*hdsS* [*spr0446*]; putative specificity subunit of type I of restriction endonucleases and *htrA* [*spr2045*]; serine protease involved in virulence) changed in microarray experiments (see Table S3 in the supplemental material) (42). To complicate matters further, there are numerous genes outside this set of 21 (compiled in Table S3 in the supplemental material) whose relative transcript amounts changed in the $\Delta vicR$ mutant grown in one medium of the two and changed in previous microarray experiments in which VicR amounts were modulated (39, 42). This set includes additional genes that mediate murein biosynthesis [e.g., *lytB* (*spr0867*)]; glucosaminidase that catalyzes a last step in cell separation (9) and pneumococcal virulence [e.g., *dpr* (*spr1430*)]; ferritin-like iron-binding protein required for oxygen tolerance (48, 68); and *htrA* (*spr2045*; serine protease [24, 37, 52]). In addition, there is a small set of genes whose relative transcript amounts changed in the $\Delta vicR$ mutant in both growth media (Fig. 2B and 3B; Table 2), but are not preceded by an exact match to either consensus (e.g., *mutY* [*spr1108*]; DNA repair glycosylase and *hdsS* [*spr0448*]; second putative specificity subunit of type I of a restriction endonuclease). Each of these genes is preceded within 400 bp by a sequence containing mismatches with the proposed consensus sequences in the conserved bases (see Table S3 in the supplemental material). Because of this extreme degeneracy, binding of VicR and VicR-P will need to be assessed to regions containing these consensus-related sequences by band shift and footprinting.

Data presented here do not support autoregulation of the

vicRKX operon by the VicR RR claimed in reference 39. In that work, VicR RR overexpression from a plasmid led to an increase in the relative amount of transcript from *vicK*, which was presumed to be expressed solely from the chromosome. However, the recombinant plasmid used to overexpress *vicR*⁺ should also overexpress *vicK*⁺, based on the PCR primers reported for its construction (see Materials and Methods in reference 39). Consistent with lack of *vicRKX* autoregulation, an increase in the amount of the downstream *vicX* transcript from the chromosome was not detected when *vicR*⁺ and possibly *vicK*⁺ transcription was induced from the plasmid (39). Lack of *vicRKX* autoregulation is further supported by new results presented here showing that the relative amounts of *vicK* and *vicX* transcripts did not significantly change in the $\Delta vicR$ mutant compared to the *vicR*⁺ parent grown in BHI or CDM (Table 2). Finally, the intercistronic region between *mutY* and the *vicRKX* operon does not contain a sequence related to the consensus (Fig. 1; Table 3). We failed to detect specific binding of VicR or VicR-P to this intercistronic region upstream of the *vicRKX* operon in band shift assays (Fig. 8).

Finally, our results support the notion that the VicR RR mediates membrane integrity. A link to membrane integrity was first noted in characterizations of a temperature-sensitive mutant of *yycF* in *S. aureus*, which was hypersensitive to macrolide and lincosamide antibiotics and to long-chain fatty acids at permissive growth temperatures (35, 36). Recently, Mohehdano and coworkers reported that overexpression of VicR causes changes in the fatty acid composition of total membrane lipids extracted from *S. pneumoniae* (39). During the microarray experiments reported here, we observed that a P_{c-pcsB}^+ $\Delta vicR$ mutant grew poorly in CDM, but not in BHI (Fig. 2A and 3A). Addition of the long-chain fatty acids oleic acid (C_{18:1}), or linoleic acid (C_{18:2}), or both restored growth of the $\Delta vicR$ mutant in CDM (Fig. 3A). The lack of growth of the P_{c-pcsB}^+ $\Delta vicR$ mutant was not likely caused by auxotrophy for fatty acids in CDM compared to BHI. Other experiments showed that BHI does not contain sufficient fatty acids to support the growth of *S. pneumoniae* when transcription of the fatty acid biosynthesis operon is reduced from the P_{fcsK} promoter (data not shown).

Microarray data did not support substantive direct regulation of fatty acid biosynthesis by the VicR RR (Table 2). There was little or no change in the transcript amounts from the putative *fabR* regulator gene or the genes in the fatty acid biosynthesis operon in the $\Delta vicR$ mutant under any growth conditions (Table 2). Although we cannot completely rule out compensatory regulation that would mask changes in transcription patterns, the lack of significant changes in these relative transcript amounts in a mutant devoid of VicR argues against a major role of VicR in the regulation of fatty acid biosynthesis. We did detect binding of unphosphorylated VicR to a region upstream of *fabR* in band shift experiments (Fig. 8); however, we were unable to detect a footprint for this binding under the conditions tested (see Results). In addition, phosphorylation of VicR to VicR-P abolished the binding upstream of *fabR* (Fig. 8).

Results from these different studies support a model in which cell surface proteins regulated by the VicRK TCS system mediate membrane assembly or integrity directly or indirectly through effects on other processes, such as murein biosynthesis

(41). The requirement of the $\Delta vicR$ mutant for fatty acids in CDM seemed to require a decrease in the relative expression of *PcsB* and was suppressed when *PcsB* was expressed at an increased level (see Results). Thus, there may be a functional involvement between *PcsB* and other members of the VicR regulon in maintaining membrane integrity. Consistent with this interpretation, overexpression of the *S. aureus* Ssa protein, which shows limited homology to the CHAP domain region of *PcsB*, restored the *yycF1* temperature-sensitive mutant to wild-type levels of susceptibility to macrolide-lincosamide-streptogramin B (MLS_B) antibiotics (35). Further studies of signal transduction by the VicRK TCS will provide insights into how pneumococcus communicates between its cytoplasm and cell surface to regulate these important surface proteins that mediate murein biosynthesis, cell shape, membrane integrity, and virulence. Further studies on the functions of members of the VicRK regulon will shed light on these fundamental cellular processes in *S. pneumoniae*.

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