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Genetic Analysis and Functional Characterization of the *Streptococcus pneumoniae vic* Operon

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The *vic* two-component signal transduction system of *Streptococcus pneumoniae* is essential for growth. The *vic* operon comprises three genes encoding the following: VicR, a response regulator of the OmpR family; VicK, its cognate histidine kinase; and VicX, a putative protein sharing 55% identity to the predicted product (YycJ) of an open reading frame in the *Bacillus subtilis* genome. We show that not only is *vic* essential for viability but it also influences virulence and competence. A putative transcriptional start site for the *vic* operon was mapped 16 bp upstream of the ATG codon of *vicR*. Only one transcript of 2.9 kb, encoding all three genes, was detected by Northern blot analysis. VicK, an atypical PAS domain-containing histidine kinase, can be autophosphorylated in vitro, and VicR functions in vitro as a phospho-acceptor protein. (PAS is an acronym formed from the names of the proteins in which the domains were first recognized: the *Drosophila* period clock protein [PER], vertebrate aryl hydrocarbon receptor nuclear translocator [ARNT], and *Drosophila* single-minded protein [SIM].) PAS domains are commonly involved in sensing intracellular signals such as redox potential, which suggests that the signal for *vic* might also originate in the cytoplasm. Growth rate, competence, and virulence were monitored in strains with mutations in the *vic* operon. Overexpression of the histidine kinase, VicK, resulted in decreased virulence, whereas the transformability of a null mutant decreased by 3 orders of magnitude.

Prokaryotic organisms commonly sense and respond to changes in their environment using two-component regulatory systems (TCRS). Such systems typically comprise a membrane-associated sensory kinase and a cytoplasmic response regulator. A stimulus is perceived by the sensory domain of the histidine kinase, resulting in its autophosphorylation. Further transmission of the signal is achieved by the phosphorylation of its cognate response regulator. The phosphorylated form of the response regulator binds to promoter regions and thus regulates transcription of genes under its control (22, 31). The TRCS of pathogens have been implicated in detecting conditions favorable for host invasion and activating virulence regulons (29). Virulence of *Salmonella enterica* serovar Typhimurium is regulated by the *phoPQ* TCRS. The regulated genes are crucial for survival in macrophages and confer resistance to cationic antimicrobial peptides (19). Several divalent cations have been shown to activate this TCRS, with Mg^{2+} being the most efficient ion (15). Other TCRS are essential for bacterial growth under laboratory conditions (14, 21, 26). One example is the *divJK* system, first identified in the gram-negative aquatic eubacterium *Caulobacter crescentus* (21). This TCRS controls CtrA, a response regulator essential for transcription of cell cycle-regulated genes and interacting with the principal vegetative sigma factor (σ^{73}) of *C. crescentus* (42).

Two-component systems have been recently identified in *Streptococcus pneumoniae*, a major cause of community-acquired pneumonia (3). One of the best-studied two-component systems in *S. pneumoniae* is *comDE*, which is a key regulator of natural competence (9, 33). This system belongs to the quorum-sensing family of two-component systems. A small heptadecapeptide coexpressed with *comD-comE* was proposed to be the signaling molecule (20, 33). A second TCRS, *ciaRH*, is also involved in regulating competence of *S. pneumoniae* (18). Recently, the predicted response regulator genes of 13 TCRS found in the nearly complete *S. pneumoniae* genome sequence were disrupted (26, 42). No viable response regulator knockout mutants could be obtained for the *vic* system. The only other essential TCRS known so far was implicated in cell cycle control (24).

In this work, we describe the transcriptional organization of the *vic* operon and its high degree of conservation among gram-positive bacteria. We also demonstrate that VicR functions as a phospho-acceptor protein and that VicK can be autophosphorylated in vitro. *vic* operon mutants were constructed and systematically analyzed for effects on growth, competence, and virulence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table 1 and Fig. 1. *Escherichia coli* was routinely grown in Luria-Bertani medium (36). *S. pneumoniae* strains were cultivated in liquid Todd-Hewitt medium (Difco Laboratories) or on sheep blood agar plates at 37°C and in an atmosphere consisting of air with 10% (vol/vol) CO_2, regulated by an infrared-sensing device (incubator from Forma Scientific). *E. coli* plasmids were selected using ampicillin (100 μg/ml), kanamycin (25 μg/ml), and

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TABLE 1. Plasmids and strains used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Plasmids		
pJDC9	Nonreplicative *E. coli-S. pneumoniae* shuttle vector	8
pAS1	pJDC9 derivative	30
pASPB2	pAS1 with B2 promoter, cloned in *Xho*I/*Eco*RI sites	This study
pDSNde	pDS derivative	39
pDSNde-6his	pDSNde; creates a six-histidine fusion tag	39
pGK13-KAN	pJDC9 and pGK13 derivative; *S. pneumoniae* replicative plasmid with kanamycin resistance	26
pNR2	pDSNde containing *vicR,* cloned in *Nde*I/*Bam*HI	This study
pvicK	pDSNde containing *vicK,* cloned in *Nde*I/*Bam*HI	This study
pKOHK2	pAS1 containing the 5′ end of *vicK,* cloned in *Xho*I/*Eco*RI	This study
pKO423	pAS1 containing the 5′ 1,270 nucleotides of *vicK,* cloned in *Eco*RI/*Bam*HI	This study
pKOOX2	pAS1 containing the 5′ end of *vicX,* cloned in *Xho*I/*Eco*RI	This study
pOBRR2	pASPB2 containing the 5′ end of *vicR* plus a ribosomal binding site, cloned in *Eco*RI and *Bam*HI	This study
pOBHK2	pOBRR2 containing the 5′ end of *vicK,* cloned in *Nde*I and *Bam*HI	This study
pOBOX2	pOBRR2 containing the 5′ end of *vicX,* cloned in *Nde*I and *Bam*HI	This study
pHK2KOP	pASPB2 containing the 5′ end of *vicK,* cloned in *Eco*RI/*Bam*HI	This study
pMBP-VanS	pMAL-c2 containing VanS	41
pMBP-VanR	pMAL-c2 containing VanR	41
Strains		
S. pneumoniae		
R6	*S. pneumoniae* laboratory strain	7
KNR. 7/87 14.8.91	Serotype 4 strain, sequenced by Human Genome Sciences	1
Sp1	Serotype 22 strain, clinical isolate	Roche strain collection
Sp1711	Serotype 3 strain, clinical isolate	Roche strain collection
vicKKO	Knockout of *vicK* in R6 with pKOHK2	This study
vicXKO	Knockout of *vicX* in R6 with pKOOX2	This study
SOBvicR	Overexpression of *vic* operon, obtained with pOBRR2	This study
SOBvicK	Overexpression of *vicK* and *vicX,* obtained with pOBHK2	This study
SOBvicX	Overexpression of *vicX,* obtained with pOBOX2	This study
vicKKOP	Overexpression of *vicX,* obtained with pHK2KOP	This study
E. coli		
SURE strain	Host strain for *E. coli* cloning steps	Stratagene
M15[REP4]	Qiagen expression host strain	39

erythromycin (500 μg/ml). Recombinant *S. pneumoniae* strains, containing integrated pAS1 derivatives (Table 1), were selected with erythromycin (500 ng/ml), and those containing pGK13-KAN were selected with kanamycin (750 μg/ml).

Genetic and molecular techniques. Standard procedures for PCR and cloning were employed (6). Plasmid DNA was prepared using a QIAfilter Maxi kit (Qiagen). Sequencing was performed with dye terminator cycle sequencing technology on an ABI PRISM 320 sequencer (Perkin-Elmer, Foster City, Calif.).

Computational analysis and sequence data. Genetics Computer Group (Madison, Wis.) programs (version 9.1) were routinely used for sequence comparison. Transmembrane domains were predicted using TMpred software (http://www.isrec.isb-sib.ch/software/TMPRED_form.html) (24) and ALOM software (25). The program SMART (http://smart.embl-heidelberg.de) (39) was used to detect the PAS in *S. pneumoniae* VicK, the prediction of which was verified by detailed comparison at the sequence level. (PAS is the acryonym formed from the names of the proteins in which the domains were first recognized: *Drosophila* period clock protein [PER], the vertebrate aryl hydrocarbon receptor nuclear translocator [ARNT], and *Drosophila* single-minded protein [SIM].) The *Streptococcus pyogenes* genome sequence was obtained from the University of Oklahoma Advanced Center for Genome Technology (http://www.genome.ou.edu/strep.html). The *S. pneumoniae* sequence information was obtained as described previously (26). Genomic sequence databases for *Staphylococcus aureus* and *Enterococcus faecalis* were obtained through Human Genome Sciences, Inc. (Rockville, Md.). Both databases cover about 98% of the genome. Ambiguous sequences of interest were resequenced.

Promoter mapping and detection of operon transcripts. RNA extraction and Northern blot analysis were carried out as described previously (11). The Promega (Madison, Wis.) primer extension system was used for promoter mapping. A dideoxy cycle sequencing reaction (SequiTherm Cycle sequencing; Epicentre Technologies, Madison, Wis.) was used as a sizing standard.

Expression and purification of VicR. The *vicR* gene was cloned into a pDS56 RBSII six-His] derivative (39). First, the *Nde*I site of this vector (at position 1378) was eliminated by cutting with *Nde*I, filling in the recessed 3′ ends, and recircularization. This plasmid was cut with *Bam*HI and *Hin*dIII, and a polylinker with sites for *Cla*I, *Nde*I, *Sal*I, *Bam*HI, and *Hin*dIII was introduced. A coding region with the start codon in the *Nde*I site (CATATG) introduced in this vector results in an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible gene, encoding a protein with an amino-terminal tail consisting of six histidines (39). The sequence between the Shine-Dalgarno sequence (AGGAG) and the stop codon (TAG) is AGGAG AAATTAACT ATG AGA GGA TCT CAT CAC CAT CAC CAT CAC GGG ATC GAT CAT ATG GTC GAC GGA TCC AAG CTT AAT TAG. The vector (pDSNde) was then obtained by cutting pDSNdeHis with *Eco*RI and *Bam*HI and recircularizing using the linkers S/D-1 (AAT TAA AGG AGG GTT TCA TAT GAA TTC G) and S/D-2 (GAT CCG AAT TCA TAT GAA ACC CTC CTT T).

A gene with the start codon in the *Nde*I site (CATATG) can be expressed with no amino-terminal extension. VicR was cloned into pDSNde between *Nde*I and *Bam*HI restriction sites, leading to pNR2, and overexpressed in *E. coli* M15[pREP4] (Qiagen) using standard procedures. About 100 mg of VicR was obtained from a 2-liter culture. Details of VicR purification and refolding will be described elsewhere. Briefly, *E. coli* cells were lysed by pressure treatment with a homogenizer (model MINI-Lab; Rannie, Denmark) and the recombinant protein was precipitated with 0.6% (wt/vol) polyethylenimine. After extraction of the precipitate with 1 M NaCl, VicR was precipitated again by reducing the salt concentration and then resolubilized under denaturing conditions (6 M urea). Some protein contaminants were removed by anion-exchange chromatography (Q-Sepharose) in the presence of 6 M urea at a salt concentration (100 mM NaCl, 10 mM $MgCl_2$) at which VicR did not bind to the column. VicR was refolded by binding it to the same ion-exchange column at a lower salt concentration, washing with buffer (from which urea was omitted), and elution with a salt gradient. Fractions found to be homogeneous by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and by quasi-elastic light scattering analysis (37) were pooled and further purified by gel filtration chromatography in the presence of 1 M NaCl. Homogeneous fractions obtained from the sizing column were pooled, adjusted to 1.5 mg of protein/ml, dialyzed against 50 mM

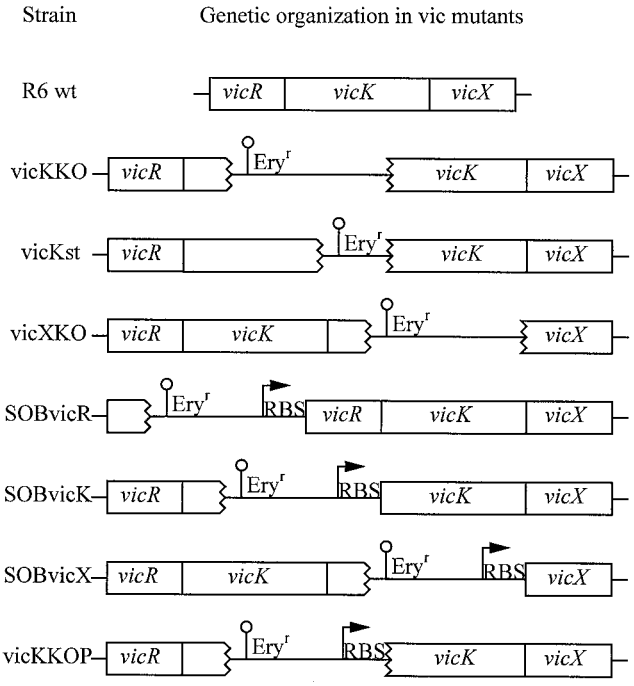

FIG. 1. Genetic organization in different *S. pneumoniae* mutant strains used for studying the *vic* operon. Strains whose designations end with the suffix "KO" are null mutants, while that ending in the suffix "KOP" is a null mutant in which the downstream gene is transcribed by a synthetic promoter. Strains whose designations begin with the prefix "SOB" are *S. pneumoniae* overexpressing strains. The erythromycin resistance (Ery^r) cassette of pJDC9, the synthetic promoter, is described in Materials and Methods and is represented by an arrow on top of a vertical bar. Consensus ribosomal binding sites (RBS) (see Materials and Methods) are also shown. Genes followed or preceded by a vertical zigzag line end or start with an in-frame stop codon, respectively. The strong transcriptional terminators of pJDC9 are represented by an open circle on top of a vertical bar.

Tris-HCl (pH 8.0)–500 mM NaCl–10 mM $MgCl_2$ and filtered (pore size, 0.2 μm). The authenticity of the purified VicR protein was verified by mass spectroscopy, N-terminal sequencing, and amino acid analysis. Aliquots were stored at −80°C.

Expression and preparation of VicK in membrane vesicles. The *vicK* gene was also cloned into pDSNde, leading to pvicK, and overexpressed in *E. coli* M15[pREP4] (Qiagen) using standard procedures. Pellets from 1-liter expression cultures were resuspended in 20 ml of a solution containing 100 mM HEPES (pH 8), 10% glycerol, 1 mM $MgSO_4$, 150 mM NaCl, and Benzonase (250 U/μl; Boehringer Mannheim) and sonicated in a water bath for 18 min while cooling the sample to 4°C every 3 min. The sonicated sample was centrifuged for 20 min at 3,000 × *g* and 4°C. The supernatant of this centrifugation was subjected to ultracentrifugation at 150,000 × *g* and 4°C for 1 h. The pellet was resuspended in 100 μl of 50 mM Tris-HCl (pH 7.5)–50 mM KCl–5 mM $MgCl_2$–1 mM dithiothreitol (DTT), yielding a protein concentration of about 18 mg/ml.

Phosphorylation assays. Fusion proteins of maltose binding protein (MBP) and the cytoplasmic domain of VanS (MBP-VanS) and of MBP and VanR (MBP-VanR) were expressed and purified using the pMAL protein fusion and purification system (New England Biolabs, Inc., Beverly, Mass.), but no EDTA was added to the elution buffer. The constructs for MBP-VanS and MBP-VanR were kindly provided by C. Walsh (41). MBP-VanS (180 μg) was added to 9.25 GBq of [γ-^{32}P]ATP and 0.5 mM ATP in a reaction buffer containing 50 mM HEPES (pH 7.4), 50 mM KCl, and 50 mM $MgCl_2$. The mixture was incubated at 25°C for 1 h. Then the autophosphorylated protein was separated from ATP on a 1-ml Sephadex G-50 spin column (Pharmacia). Phosphotransfer from histidine kinase to response regulator was performed in the same reaction buffer at room temperature for 5 min. The reaction was stopped by adding concentrated gel loading buffer containing the following (final concentration): 10 mM DTT, 2% SDS, 10 mM Tris-HCl (pH 6.8), 12.5% glycerol, 2.5 mM Na_2HPO_4, 5 mM EDTA, and 0.05% bromophenol blue. Samples were then subjected to SDS-polyacrylamide gel electrophoresis using 10-to-20% gradient gels at 120 V.

VicK autophosphorylation assays were performed in a buffer containing 50 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.33 mM NADH, 2.5 mM phosphoenolpyruvate, 1 mM ATP, 0.165 U of pyruvate kinase, 0.25 U of lactate dehydrogenase (from rabbit muscle; Sigma), 555 kBq of [γ-^{32}P]ATP, 3.8 mM *para*-nitrophenyl phosphate, 0.5 mM *o*-phospho-L-threonine, 5 mM *o*-phospho-L-serine, 50 mM sodium orthovanadate, and 80 μg of VicK membrane preparation. After incubation at 30°C for 7 min, the reaction was stopped by the addition of SDS loading buffer comprised of (final concentration) 50 mM DTT, 4% SDS, 50 mM Tris-HCl (pH 6.8), 12.5% glycerol, 2.5 mM NaH_2PO_4, 0.05% bromophenol blue, and 5 mM EDTA. The 10-to-20% gel was run at 120 V and blotted on a polyvinylidene difluoride membrane in a semidry transfer apparatus (Hoefer). Autoradiography was carried out using Kodak Biomax MS films.

Gene disruption and overexpression in *S. pneumoniae*. *vicK* and *vicX* gene disruptions in pneumococcal strains R6, sp1, and sp1711 (Table 1) were obtained by transformation using pAS1-based plasmids (29) pKOHK2 (*vicK*) and pKOOX2 (*vicX*). Correct integration of the constructs was checked by PCR. A synthetic promoter, called B2, designed for overexpression in *S. pneumoniae*: ctc gag TCA GAA AAT TAT TTT AAA TTT CCT CTT GAC ATT TTT GGT TTT TTA TGA TAT AAT AGT TTT AAG tct aga att c (restriction sites [*Xho*I, *Xba*I, *Eco*RI] are underlined, and the *vicR*-specific sequence is shown in lowercase type) was based on the rRNA binding protein 1 promoter from *Bacillus subtilis* (nucleotides 7 to 31), −35 and "extended −10" consensus boxes (nucleotides 32 to 37 and nucleotides 50 to 61) (27), the sequence from the spacing region of the 5′-phosphoribosyl-5-aminoimidazole-4-*N*-succinocarboxamide synthetase from *S. pneumoniae* (*purC*) (nucleotides 38 to 49), and the *repA* promoter sequence (from the −10 box to the initiator site) (nucleotides 62 to 69) (10). The promoter was cloned into pAS1 (*Xho*I/*Eco*RI), leading to pASPB2. This new vector was used to convert pASPB2 into an integrative *S. pneumoniae* expression vector by adding a synthetic ribosome binding site as an extension to the 3′ primer used in PCR amplification of the insert, described below. For expression of the complete *vic* operon, a PCR product of 522 bp was synthesized from primers AP-97 and AP-98. AP-97 is GAC GAA TTC **AAA GGA GGG TTT** CAT atg aaa aaa ata cta att gta g, where restriction sites (*Eco*RI and *Nde*I) are underlined, the ribosome binding site is shown in boldface type, and the *vicR*-specific sequence is shown in lowercase type. AP-98 is AAA GGG ATC CTA acg atg ggt taa gtc tag ttc (nomenclature is the same as that for AP-97). This PCR fragment was cloned into pASPB2 using *Eco*RI/*Bam*HI, leading to pOBRR2. Subsequent cloning of PCR fragments of 416 and 493 bp extending from the start codon of *vicK* and *vicX* into *Nde*I/*Bam*HI restriction sites of pOBRR2 led to pOBHK2 and pOBOX2, respectively. After transformation and integration into the *S. pneumoniae* genome, the respective plasmids produced strains SOBvicR, SOBvicK, and SOBvicX, overexpressing the complete *vic* operon, *vicK* and *vicX*, or only *vicX*, respectively. A *vicK* gene disruption which also introduced a B2 downstream promoter was obtained by cloning a 5′ *Eco*RI/*Bam*HI fragment of *vicK* into pASPB2, leading to pHK2KOP (after *S. pneumoniae* transformation to strain vicKKOP).

Competence in mutant strains. *S. pneumoniae* was transformed essentially according to the method of Apfel et al. (4). When transforming mutant strains with the replicative plasmid pGK13-KAN (Table 1), erythromycin selection was maintained during the entire procedure. The number of colonies resistant to both erythromycin and kanamycin was compared to the number of kanamycin-resistant colonies obtained in a control transformation in *S. pneumoniae* R6.

Experimental virulence. Null and overexpressing *vic* operon mutants were also constructed in two different virulent strains of *S. pneumoniae* (Sp1 and Sp1711 [Table 1]). Control and mutant strains were grown in Todd-Hewitt medium as indicated above, but with erythromycin (250 ng/ml) plus 5% fetal calf serum (Life Technologies, Rockville, Md.). During exponential growth phase, 2 ml of bacterial culture was harvested and diluted with phosphate-buffered saline to a calculated dose of 4×10^6 CFU (Sp1) and 10^3 CFU (Sp1711). Aliquots of the suspensions of each mutant strain were each injected intraperitoneally into five female mice (C57BL6/J [18 to 20 g]; Bethesda Research Laboratories). Mean survival time was calculated as the time from injection of bacteria until death of the infected mouse or as 15 days for survivors. Attenuation in virulence was recorded as an increase in mean survival time. These studies were performed in full compliance with local Swiss regulations for animal experimentation.

Nucleotide sequence accession numbers. The sequences of the following *vic* operon genes have been submitted to the EMBL nucleotide sequence database under the indicated accession numbers: *S. pneumoniae vicR* and *vicK*, AJ06392; *S. pneumoniae vicX*, AJ012049; *E. faecalis vic* operon and flanking genes,

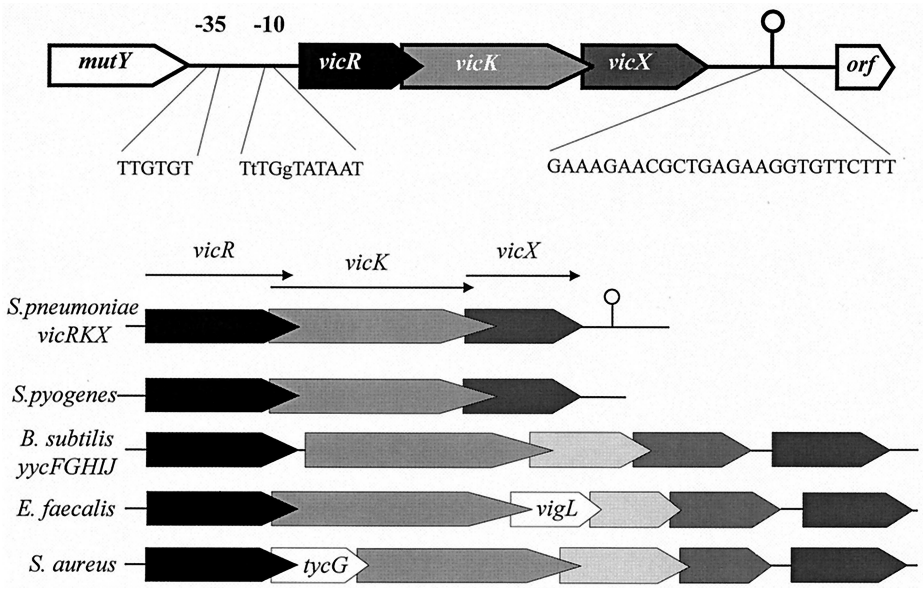

FIG. 2. Gene organization in the *vic* operon. Direction of transcription and translation for the three genes in the three open reading frames is indicated by arrows. The putative terminator is indicated by an open circle on top of a vertical bar. The −10 and −35 boxes are indicated. They were identified by primer extension experiments. The organization of four *vic* operon homologues—those of *S. pyogenes*, *B. subtilis*, *S. aureus*, and *E. faecalis*—is shown at bottom. Identical shading was used for homologous genes.

AJ012050; *S. pyogenes vic* operon and flanking genes, AJ012051; *S. aureus vic* operon and flanking genes, AJ012052.

RESULTS

Promoter mapping and detection of operon transcripts. Primer extension experiments located a putative transcription start site of the operon 16 nucleotides upstream of the *vicR* ATG start codon. The deduced −10 region (TTTGGTATA AT) corresponded to the consensus sequence defined for the extended −10 class of bacterial promoters (34). The proposed −35 hexamer (TTGTGT) shows three out of six matches with the consensus sequence (TATAAAA)(Fig. 2; Table 2).

Northern blot experiments with RNA prepared from *S. pneumoniae* cells grown to early exponential phase or stationary phase in Todd-Hewitt medium and a *vicR* probe did not allow detection of the *vic* operon transcript (Fig. 3B, lane R6). However, when RNA was prepared from the SOBvicR strain (overexpressing the *vic* operon), a strong signal of 2.9 kb, corresponding to the predicted size of a transcript encoding the three genes *vicRKX*, was detected with either a *vicR* probe or a *vicX* probe (Fig. 3).

VicK autophosphorylation. A band corresponding in size to phosphorylated VicK was observed when membrane vesicles prepared from SOBvicR, the strain overexpressing the *vic* operon, were used in the autophosphorylation assay (Fig. 4A). The kinase domain, devoid of the predicted transmembrane domain, fused with MBP, could not be autophosphorylated. Thus, VicK kinase activity may require a conformation of the protein that is formed only in a membrane-integrated state.

VicR phosphorylation. Experiments with VicK and VicR generated no phosphorylated products, although experiments with the two components of the *E. faecalis* VanR/VanS system

TABLE 2. Alignment of part of the VicX sequence with its closest homologues[a]

Identification or accession no.	Position of Zn binding motif (nucleotide position)	Sequence
AJ012050	51–80	RKPED.LDAI LVT**HE**H**RD**HI HGVGVLARKY
VicX	49–78	RKPED.LDAI LIT**HE**H**SD**HI HGVGVLARKY
YycJ	45–74	RKLDD.VDGI FVT**HE**H**SD**HI KGLGVVARKY
AJ012052	51–80	RNIQD.LNGI LVT**HE**H**ID**HI KGLGVLARKY
BLA2_BACCE	102–131	KKFQKRVTDV IIT**HA**H**AD**RI GGIKTLKER
BLAB_BACFR	85–114	DSLHAKVTTF IPN**HW**H**GD**CI GGLGYLQRK
BLAB_SERMA	82–111	ERGY.KIKGS ISS**HF**H**SD**ST GGIEWLNSR
Consensus		i t**H H D** i G g l rk

[a] Alignment of a part of the VicX sequence with its closest homologues from *B. subtilis* (YycJ), *E. faecalis* (AJ012050), and *S. aureus* (AJ012052) and with β-lactamase precursor proteins from *Bacillus cereus* (BLA2_BACCE), *Bacteroides fragilis* (BLAB_BACFR), and *Serratia marcescens* (BLAB_SERMA). The β-lactamase fingerprint (Prosite documentation) is shown in boldface type. The position of the zinc binding motif of a sequence is indicated by nucleotide position. Numbering refers to the database entry. A consensus sequence is derived from this motif alignment and is shown according to the following rules: capital letters, amino acids conserved in all seven proteins; lowercase letters, amino acids present in at least five protein sequences.

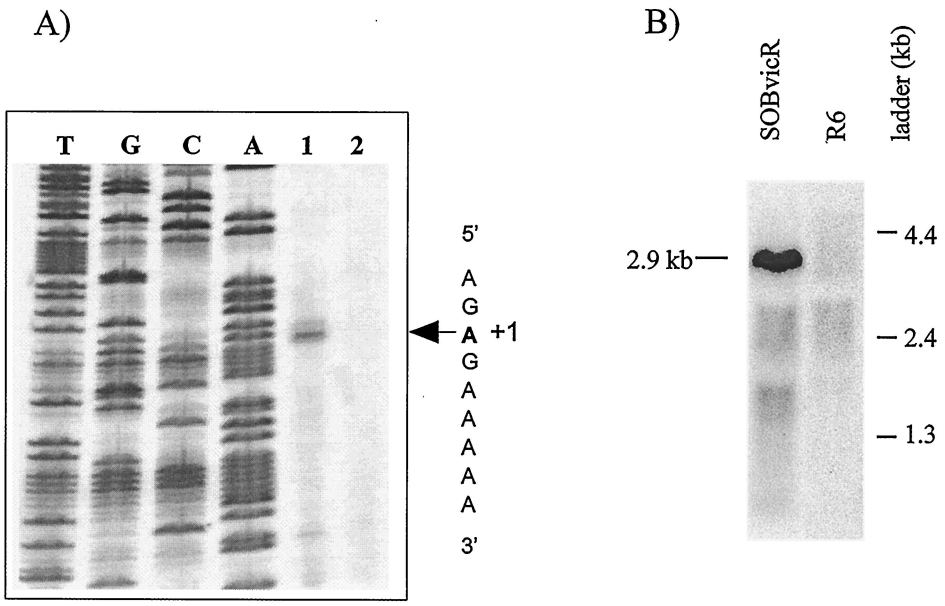

FIG. 3. Transcriptional organization of the *vic* operon. (A) Autoradiography of a primer extension experiment. Dideoxy DNA sequence analysis (lanes T, G, C, and A) and primer extension reactions (lane 1 with 30 μg *S. pneumoniae* RNA and lane 2 without added RNA) were performed using a specific primer for the 5′ end of the *vicR* gene. RNA was isolated from exponentially growing *S. pneumoniae* R6 cells. Only one band was observed, as indicated by the arrow. (B) Autoradiography of a Northern blot hybridization experiment. RNA was isolated from *S. pneumoniae* R6 and SOBvicR cells. The probe used in the hybridization covered the complete *vicR* gene and was labeled with [α-^{32}P]CTP by in vitro transcription with the Lig'nscribe kit (Ambion).

ylated than VanR under the same conditions. This cross-phosphorylation between a known heterologous histidine kinase and the putative response regulator demonstrated that VicR can be a phospho-acceptor protein.

Growth behavior of *vic* operon mutant strains. Attempts to disrupt the *vicR* gene were carried out as previously described (26, 40), but it was not clear whether this apparently lethal effect was due to *vicR* inactivation or to polar effects of the disruption on *vicK* or *vicX*. Therefore, several mutant strains were constructed in order to assign phenotypes to specific genes within the *vic* operon. *vicK and vicX* were successively inactivated by insertion-duplication (8). For this purpose *S.*

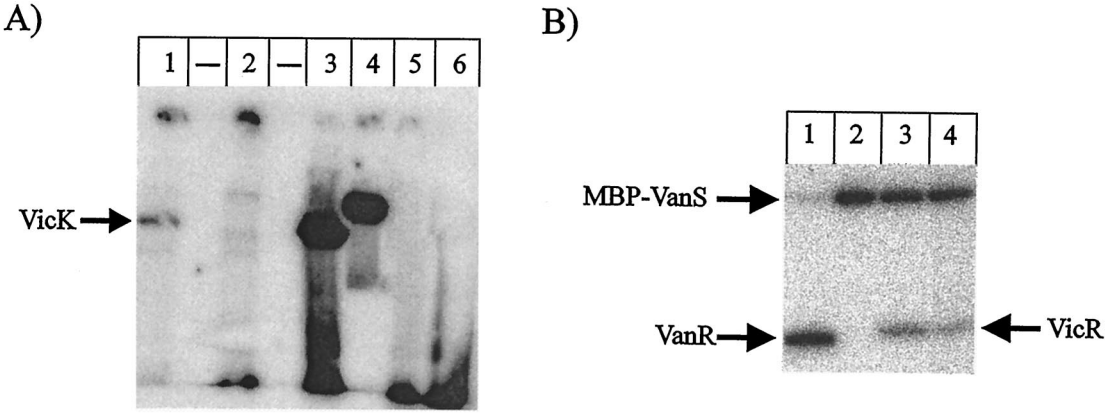

FIG. 4. Autophosphorylation and phosphotransfer activities. (A) Vesicle preparations (80 μg) were incubated for 7 min at 30°C with [γ-^{32}P]ATP, and then proteins were separated on 10-to-20% Tris-glycine precast Bio-Rad polyacrylamide gels. Lane 1, *S. pneumoniae* membrane vesicles from *vic* operon-overproducing strain SOBvicR; lane 2, *S. pneumoniae* membrane vesicles from strain vicKKO; lane 3, VicK overexpressed as an integral membrane protein in *E. coli* expression system QIAexpress; lane 4, 18 μg of purified *S. pneumoniae* HK08 histidine kinase as an MBP; lane 5, heat-treated *E. coli* overexpressed and autophosphorylated VicK; lane 6, *E. coli* expression system host strain M15[pREP4] harboring pDSNde without any insert. The strong band visible in lanes 1 and 3 corresponds to autophosphorylated VicK. (B) Autoradiography of a denaturing gel, run to analyze a phosphotransfer experiment. VanS was used as an MBP fusion protein (MBP-VanS). MBP-VanS (18.8 μg) was applied in the experiments shown. Lane 1, phosphotransfer for 5 min from MBP-VanS to VanR (3.7 μg); lane 2, control experiment with [γ-^{32}P]ATP autophosphorylated MBP-VanS (with no response regulator added to the reaction mixture). VicR phosphorylation was performed with 18.8 μg of MBP-VanS and decreasing amount of purified VicR protein in lanes 3 (10 μg) and 4 (0.1 μg), respectively. Protein separation was performed on a 10-to-20% Novex precast Tricine gel.

pneumoniae was transformed with plasmids carrying a partial copy of the target gene of the histidine kinase (pKOHK2) and the third *vic* open reading frame (pKOOX2), respectively (Table 2). These findings demonstrate that of the genes in the *vic* operon only the response regulator gene *vicR* is essential for in vitro survival. Other mutant strains, expressing the entire operon or portions of it, in approximately 20-fold excess were constructed (Table 1 and Fig. 1). Overexpression was confirmed using Western blot analysis with a polyclonal antibody against VicR (data not shown). The *vicK* disrupted strain (vicKKO) grew at about 30% of the rate of its parent (R6). Other *vic* operon mutant strains (promoter-driven knockouts or vicXKO) did not show significantly altered growth rates (Fig. 1).

Competence in *vic* mutant strains. Natural transformation is affected, directly or indirectly, by two-component systems (2, 18). Therefore, we monitored the relative ease of transformation of *vic* mutant strains and R6 with replicative plasmid pGK13-KAN (Fig. 1). Most striking was the 400-fold reduction of transformation frequency observed when *vicK* was inactivated or truncated at its C terminus (vicKst). In order to allow for an artificial effect on transformation due to slow growth phenotype, assays were performed at several time points. Thus, in addition to the standard transformation time window, assays were performed with prolonged incubation in the presence of competence-stimulating peptide (20, 25, 30, and 45 min). Even with these prolonged incubation times, the transformability of the kinase mutants was significantly less than that of the parent strain. A 10-fold difference in transformation frequency was also observed with a mutant strain in which the downstream genes were constitutively activated.

Virulence of *vic* mutant strains. *vic* null and overexpression constructs were also used to transform two clinical isolates of serotype 3 and 22 (Table 1). Mutant strains were injected intraperitoneally into groups of five mice. A 1.5-fold increase in mouse survival time could be reproducibly measured for *S. pneumoniae* mutant strain SOBvicK, indicating decreased virulence (Table 3). The remaining *vic* operon mutant strains did not show significant changes in virulence behavior in this mouse model.

DISCUSSION

Organization of the *vic* operon in different gram-positive bacteria. The *vic* operon comprises three genes: VicR, a response regulator of the OmpR family; VicK, its cognate histidine kinase; and VicX, a putative protein sharing 55% identity to the predicted product (YycJ) of an open reading frame in the *B. subtilis* genome. Similar organization was found in homologous systems in other gram-positive bacteria (Fig. 2). The organization is simplest in *S. pneumoniae* and *S. pyogenes* (only these three genes), whereas in *B. subtilis*, *Enterococcus faecalis*, and *S. aureus* there are two (*B. subtilis*) or three (*E. faecium* and *S. aureus*) more genes of unknown function. In *S. pneumoniae* the response regulator is essential, while the response regulator and the histidine kinase are both essential in *B. subtilis* and *S. aureus* (13, 26, 40). The only other two-component signal transduction system that is known to be essential is the CtrA system of *C. crescentus* (21).

Unusual features of the *vic* operon proteins. VicK probably responds to intracellular stimuli rather than extracellular environmental signals. In contrast to other histidine kinases, like VanS or ComD, VicK and its closest homologues in *Lactococcus lactis* and *S. pyogenes* appear to contain only one transmembrane domain, close to the N terminus. With the cognate response regulator being a cytosolic component, it is very likely that VicK is oriented towards the cytoplasm. Another indication for such a role comes from the N-terminal PAS/PAC core motif of VicK, since bacterial proteins containing PAS domains have been reported to sense oxygen or cytoplasmic redox potentials (43). PAS/PAC domains are involved in protein-protein interactions in response to oxygen or the physiological redox balance of NAD to NADH and FAD to FADH (5, 34). The prototype of such a PAS domain is the photoactive yellow protein from *Ectothiorhodospira halophila* (16, 32). Homologous domains have been observed in a relatively small number of prokaryotic histidine kinases so far, namely, in Aer, FixL, NifL, KinC, and NtrY systems (45). While the cytosolic domains of most histidine kinases fused to MBP have autophosphorylation activity (43), in vitro activity has been more difficult to demonstrate for histidine kinases containing PAS domains. In vitro autophosphorylation has been shown for an N-terminally truncated form of FixL devoid of its PAS and transmembrane domains (17, 29). However, in the case of VicK, autophosphorylation could be achieved only for the full-length protein incorporated into membrane vesicles. Other truncated VicK constructs, for example, those devoid of its PAS and/or transmembrane domains, did not show this activity. This may reflect the importance of the PAS core domain for full functionality of VicK. Tight control over the activation status of VicR may be deduced from the lack of phosphotransfer from the autophosphorylated VicK to VicR. This is probably because the phosphorylated form of VicR has limited stability in the presence of the histidine kinase.

Since cross-phosphorylation of VicR from VanS can occur, one can envisage that other kinases or even acetyl-phosphate helps to suppress some effects of a *vicK* kinase disruption, which may also explain how a knockout of the histidine kinase VicK is possible.

VicX, present in *vic* operon-containing bacteria. The function of the third open reading frame of *vicX* still remains to be precisely described. VicX has high homology (55% identity) to a *B. subtilis* open reading frame of unknown function. Close homologues sharing more than 50% identity were also identified in *S. pyogenes*, *S. aureus*, and *E. faecalis*. An analysis using a BLAST search against the Prodom database revealed a domain within VicX showing strong homology (P_Score = 0.002623) (Table 2) with the zinc binding motif HxHxD of a β-lactamase (accession number, P10425). An inverted repeat sequence followed by a poly(U) downstream of *vicX* may serve as a rho-independent transcriptional terminator. The first evidence for its possible role may be deduced from the constitutive expression of *vicX* in strain vicKKOP, where *vicK* simultaneously was inactivated and the transformation deficiency expected to be caused by *vicK* inactivation could be partially relieved. As *vicX* appears to possess a Zn^{2+} binding motif and the transformability of *S. pneumoniae* is known to be dependent on Zn^{2+} (12), VicX may be involved in this effect. However, the Zn^{2+} binding motif identified in *S. pneumoniae* VicX

TABLE 3. Phenotypic characteristics of mutant strains used in this study[a]

Strain	Growth rate (% of R6)	Maximum OD_{600}[b]	Log no. of colonies	Relative in vivo virulence
R6	100	0.75	2.90	1.0
vicKKO	30	0.46	0.48	1.4
vicKst	50	0.66	0.60	1.8
vicKKOP	90	0.53	2.07	0.9
vicXKO	60	0.57	1.88	0.8
SOBvicR	90	0.65	3.60	1.8
SOBvicK	90	0.60	2.71	2.5
SOBvicX	90	0.59	2.83	1.0

[a] This table indicates the phenotypic characteristics of the different mutant strains, as far as growth rate, competence, and in vivo virulence characteristics of the SOBvicK mutant are concerned. The growth rate was determined with respect to *S. pneumoniae* R6. Transformability is shown as the logarithmic number of transformants obtained. Experiments were repeated three times in triplicate, and the number of transformants was averaged. The in vivo virulence data are the calculated mean survival times of the mutant strains relative to that for a wild-type infection. Results of three in vivo virulence experiments were averaged.
[b] OD_{600}, optical density at 600 nm.

is also present in the VicX homologues of other organisms not known to be affected by Zn^{2+} in the same way. Thus, the potential Zn^{2+} binding properties of this protein most likely fulfill a more general purpose in influencing one or several enzymes that require Zn^{2+} as a cofactor. With over 100 such enzymes currently known, a wide variety of potential interaction partners can be envisaged.

Decreased virulence due to imbalance in *vic* expression. Effects on virulence after mutating the *vic* operon were observed when the expression of the two *vic* signaling components were perturbed in strain SOBvicK. Overexpressing the sensor and its downstream gene *vicX* resulted in a reproducible decrease in virulence. The remaining *vic* mutant strains did not show altered virulence in our mouse model, which is designed for detecting virulence genes responsible for maintaining an established infection.

Does the *vic* TCRS integrate several pathways? The role of the *vic* operon remains uncertain despite the observation of homologues of the *vic* TCRS in most gram-positive bacteria. The most-striking phenotype described so far implicates the homologous system of *B. subtilis* in cell division (13), and the wide distribution of *vic* systems suggests a key role in controlling growth rather than a species-specific signaling function. The severe growth impairment and significant reduction in transformability of VicK kinase mutants of *S. pneumoniae* strengthen this hypothesis by indicating an important role for this TCRS for growth.

On the basis of the data acquired from the virulence tests, it appears that the Vic proteins maintain a finely tuned balance between signals, rather than constituting a simple on-off switch. Further evidence for an integrative checkpoint comes from the demonstration of cross-phosphorylation of the response regulator. If this occurs in the living cell, it would be possible for several signals to be processed via VicR. On the basis of these observations, the unique features of the *vic* TCRS and its importance for growth in a number of gram-positive bacteria should help to guide the elucidation of a key regulatory circuit for the growth of gram-positive bacteria.

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REFERENCES

1. **Aaberge, I. S., J. Eng, G. Lermark, and M. Lovik.** 1995. Virulence of *Streptococcus pneumoniae* in mice: a standardized method for preparation and frozen storage of the experimental bacterial inoculum. Microb. Pathog. **18:** 141–152.
2. **Alloing, G., B. Martin, C. Granadel, and J. P. Claverys.** 1998. Development of competence in *Streptococcus pneumoniae*: pheromone autoinduction and control of quorum sensing by the oligopeptide permease. Mol. Microbiol. **29:**75–83.
3. **AlonsoDeVasco, E., A. F. Verheul, J. Verhoef, and H. Snippe.** 1995. *Streptococcus pneumoniae*: virulence factors, pathogenesis and vaccines. Microbiol. Rev. **59:**591–603.
4. **Apfel, C. M., B. Takacs, M. Fountoulakis, M. Stieger, and W. Keck.** 1998. Genomics approach to identification of bacterial undecaprenyl pyrophosphate synthetase: cloning expression and characterization of the essential *uppS* gene. J. Bacteriol. **181:**483–492.
5. **Aravind, L., and C. P. Ponting.** 1997. The GAF domain: an evolutionary link between diverse phototransducing proteins. Trends Biochem. Sci. **22:**458–459.
6. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl.** 1997. Current protocols in molecular biology. Wiley Interscience, New York, N.Y.
7. **Avery, O. T., C. M. MacLeod, and M. McCarty.** 1995. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III. Mol. Med. **1:**344–365.
8. **Chen, J. D., and D. A. Morrison.** 1988. Construction and properties of a new insertion vector, pJDC9, that is protected by transcriptional terminators and useful for cloning of DNA from *Streptococcus pneumoniae*. Gene **64:**155–164.
9. **Claverys, J. P., M. Prudhomme, I. Mortier-Barriere, and B. Martin.** 2000. Adaptation to the environment: *Streptococcus pneumoniae* a paradigm for recombination-mediated genetic plasticity? Mol. Microbiol. **35:**251–259.
10. **del Solar, G. H., J. Perez Martin, and M. Espinosa.** 1990. Plasmid pLS1-encoded RepA protein regulates transcription from repAB promoter by binding to a DNA sequence containing a 13-base pair symmetric element. J. Biol. Chem. **265:**12569–12575.
11. **de Saizeau, A., U. Certa, J. Warrington, C. Gray, W. Keck, and J. Mous.** 1998. Bacterial transcript imaging by hybridization of total RNA to oligonucleotide arrays. Nat. Biotechnol. **16:**45–48.
12. **Dintilhac, A., G. Alloing, C. Granadel, and J. P. Claverys.** 1997. Competence and virulence of *Streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ANC metal permease. Mol. Microbiol. **25:**727–739.
13. **Fabret, C., V. A. Feher, and A. Hoch.** 1999. Two-component signal transduction in *Bacillus subtilis*: how one organism sees its world. J. Bacteriol. **181:** 1975–1983.
14. **Fabret, C., and J. A. Hoch.** 1998. A two-component signal transduction system essential for growth of *Bacillus subtilis*: implications for anti-infective therapy. J. Bacteriol. **180:**6375–6383.
15. **Garcia Vescovi, E., F. C. Soncini, and E. A. Groisman.** 1996. Mg^{2+} as an extra-cellular signal: environmental regulation of *Salmonella* virulence. Cell **84:**165–174.
16. **Genick, U. K., G. E. Borgstahl, K. Ng, Z. Ren, C. Pradervand, P. M. Burke, V. Srajer, T. Y. Teng, W. Schildkamp, E. E. McRee, K. Moffat, and E. D. Getzoff.** 1997. Structure of a protein photocycle intermediate by millisecond time-resolved crystallography. Science **275:**1471–1475.
17. **Gilles Gonyaley, M. A., G. S. Ditta, and D. R. Helinski.** 1991. A haemoprotein with kinase activity encoded by the oxygen sensor of *Rhizobium meliloti*. Nature **350**:170–172.
18. **Guenzi, E., A. M. Gasc, M. A. Sicard, and R. Hakenbeck.** 1994. A two-component signal-transducing system is involved in competence and penicillin susceptibility in laboratory mutants of *Streptococcus pneumoniae*. Mol. Microbiol. **12**:505–515.
19. **Gunn, J. S., and S. I. Miller.** 1996. Pho-PhoQ activates transcription of *pmrAB*, encoding a two-component regulatory system involved in *Salmonella typhimurium* antimicrobial peptide resistance. J. Bacteriol. **178**:6857–6864.
20. **Havarstein, L. S., G. Coomaraswamy, and D. A. Morrison.** 1995. An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*. Proc. Natl. Acad. Sci. USA **92**:11140–11144.
21. **Hecht, G. B., T. Lane, N. Otha, J. M. Sommer, and A. Newton.** 1995. An essential single domain response regulator required for normal cell division and differentiation in Caulobacter crescentus. EMBO J. **14**:3915–3924.
22. **Hoch, J. A., and T. J. Silhavy.** 1995. Two-component signal transduction. American Society for Microbiology, Washington, D.C.

23. **Hofmann, K., and W. Stoffel.** 1993. A database of membrane spanning protein segments. Biol. Chem. Hoppe Seyler **347:**166.
24. **Jenal, U.** 2000. Signal transduction mechanisms in *Caulobacter crescentus* development and cell cycle control. FEMS Microbiol. Rev. **24**:177–191.
25. **Klein, P., M. Kanehisa, and C. DeLisi.** 1985. The detection and classification of membrane-spanning proteins. Biochim. Biophys. Acta **815**:468–476.
26. **Lange, R., C. Wagner, A. de Saizeau, N. Flint, J. Molnos, M. Stieger, P. Caspers, M. Kamber, W. Keck, and K. E. Amrein.** 1999. Domain organization and molecular characterization of 13 two-component systems identified by genome sequencing of *Streptococcus pneumoniae*. Gene **237:**223–234.
27. **Lewin, B.** 1994. Genes V. Oxford University Press, Oxford, United Kingdom.
28. **Miller, S. I., A. M. Kukral, and J. J. Mekalanos.** 1989. A two-component regulatory system (*phoP phoQ*) controls *Salmonella typhimurium* virulence. Proc. Natl. Acad. Sci. USA **86:**5054–5058.
29. **Monson, E. K., M. Weinstein, G. S. Ditta, and D. R. Helinski.** 1992. The FixL protein of *Rhizobium meliloti* can be separated into a heme-binding oxygen-sensing domain and a functional C-terminal domain. Proc. Natl. Acad. Sci. USA **89**:4280–4284.
30. **Mortier Barriere, I. A., A. de Saizeau, J. P. Claverys, and B. Martin.** 1998. Competence-specific induction of recA is required for full recombination proficiency during transformation in *Streptococcus pneumoniae*. Mol. Microbiol. **27**:159–170.
31. **Parkinson, J. S.** 1993. Signal transduction schemes of bacteria. Cell **73**:857–871.
32. **Pellequer, J. L., K. A. Wager-Smith, S. A. Kay, and E. D. Getzoff.** 1998. Photoactive yellow protein: a structural prototype for the three-dimensional fold of the PAS domain superfamily. Proc. Natl. Acad. Sci. USA **95**:5884–5890.
33. **Pestova, E. V., L. S. Havarstein, and D. A. Morrison.** 1996. Regulation of competence for genetic transformation in *Streptococcus pneumoniae* by an auto-induced peptide pheromone and a two-component regulatory system. Mol. Microbiol. **21**:853–862.
34. **Ponting, C. P., and L. Avarind.** 1997. PAS: a multifunctional domain family comes to light. Curr. Biol. **7:**R674–R677.
35. **Sabelnikov, A. G., B. Greenberg, and S. A Lacks.** 1995. An extended −10 promoter alone directs transcription of the DpnII of *Streptococcus pneumoniae*. J. Mol. Biol. **250**:144–155.
36. **Sambrook, J., E. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
37. **Schönfeld, H. J., B. Pöschel, and F. Müller.** 1998. Quasi-elastic light scattering and analytical ultracentrifugation are indispensable tools for the purification and characterization of recombinant proteins. Biochem. Soc. Trans. **26**:753–758.
38. **Schultz, J., F. Milpetz, P. Bork, and C. P. Ponting.** 1998. SMART, a simple modular architecture research tool: identification of signaling domains. Proc. Natl. Acad. Sci. USA **95**:5857–5864.
39. **Stüber, D., H. Matile, and G. Garotta.** 1990. System for high-level production in Escherichia coli and rapid purification of recombinant proteins, p. 121–152. *In* I. Lefkovitis and B. Pernis (ed.) Application to epitope mapping, preparation of antibodies and structure-function analysis, vol. IV. Academic Press, Orlando, Fla.
40. **Throup, J. P., K. K. Koretke, A. P. Bryant, K. A. Ingraham, A. F. Chalker, Y. Ge, A. Marra, N. G. Wallis, J. R. Brown, D. J. Holmes, M. Rosenberg, and M. K. Burnham.** 2000. A genomic analysis of two-component signal transduction in *Streptococcus pneumoniae*. Mol. Microbiol. **35**:566–576.
41. **Wright, G. D., T. R. Holman, and C. T. Walsh.** 1993. Purification and characterization of VanR and the cytosolic domain of VanS: a two-component regulatory system for vancomycin resistance in *Enterococcus faecium* BM4147. Biochemistry **32**:5057–5063.
42. **Wu, J., N. Ohta, and A. Newton.** 1998. An essential multi-component signal transduction pathway required for cell cycle regulation in *Caulobacter*. Proc. Natl. Acad. Sci. USA **95**:1443–1448.
43. **Zhulin, I. B., B. L. Taylor, and R. Dixon.** 1997. PAS domain S-boxes in Archea, bacteria and sensors for oxygen and redox. Trends Biochem. Sci. **22**:331–333.

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