

Eukaryotic-Type Serine/Threonine Protein Kinase StkP Is a Global Regulator of Gene Expression in *Streptococcus pneumoniae*^{∇†}

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Signal transduction pathways in both prokaryotes and eukaryotes utilize protein phosphorylation as a key regulatory mechanism. Recent studies have proven that eukaryotic-type serine/threonine protein kinases (Hank's type) are widespread in many bacteria, although little is known regarding the cellular processes they control. In this study, we have attempted to establish the role of a single eukaryotic-type protein kinase, StkP of *Streptococcus pneumoniae*, in bacterial survival. Our results indicate that the expression of StkP is important for the resistance of *S. pneumoniae* to various stress conditions. To investigate the impact of StkP on this phenotype, we compared the whole-genome expression profiles of the wild-type and Δ *stkP* mutant strains by microarray technology. This analysis revealed that StkP positively controls the transcription of a set of genes encoding functions involved in cell wall metabolism, pyrimidine biosynthesis, DNA repair, iron uptake, and oxidative stress response. Despite the reduced transformability of the *stkP* mutant, we found that the competence regulon was derepressed in the *stkP* mutant under conditions that normally repress natural competence development. Furthermore, the competence regulon was expressed independently of exogenous competence-stimulating peptide. In summary, our studies show that a eukaryotic-type serine/threonine protein kinase functions as a global regulator of gene expression in *S. pneumoniae*.

Signal transduction pathways are essential for the regulation of gene expression in both prokaryotes and eukaryotes. Signal transduction in prokaryotes was considered to occur primarily by histidine protein kinases that activate transcription by the phosphorylation of cognate response regulators (72). Recent studies have clearly demonstrated that this paradigm requires further investigation (reviewed in reference 13). Eukaryotic-type Ser/Thr protein kinases (STPKs) as well as Ser/Thr phosphoprotein phosphatases (STPPs) operate in many bacteria, constituting a signaling network independent of the canonical two-component system circuits. However, compared to their eukaryotic counterparts, the signals to which STPKs and STPPs respond, the mechanism of signal transduction, and their substrates remain poorly understood.

Prokaryotic STPKs have been shown to regulate various cellular functions, such as developmental processes (47, 37, 49), secondary metabolism (77), stress responses (27, 50), metabolic processes (48, 66), biofilm formation (27), and also virulence in streptococci (18, 28, 64), *Yersinia pseudotuberculosis* (21), *Pseudomonas aeruginosa* (80), and *Mycobacterium tuberculosis* (14, 79). Although these studies established the function of protein kinases, only several target substrates have been identified to date (16, 31, 42, 48, 55, 64, 66, 78). The result of posttranslational modification of a substrate on its function has been demonstrated in just a minority of cases (49, 66).

In contrast to the case with many microorganisms, *Streptococcus pneumoniae* and other streptococci contain a single gene encoding an STPK, which is genetically linked to a protein phosphatase-encoding gene (18, 28, 64). As a result, this feature makes them a more straightforward and practical model for defining the regulatory functions provided by these proteins.

We recently characterized the biochemical properties of both the protein kinase StkP and the protein phosphatase PhpP of *S. pneumoniae* and showed that the autophosphorylated form of StkP is a substrate for PhpP. These results suggest that StkP and PhpP could operate as a functional pair in vivo (55). It has been demonstrated that such functional coupling could play a role in the control of kinase activity (4). An analysis of phosphoproteome maps of both wild-type and *stkP*-null mutant strains revealed that one of the endogenous substrates of StkP is phosphoglucosamine mutase GlmM (55). GlmM is an essential enzyme involved in the biosynthesis of UDP-*N*-acetylglucosamine, a key common precursor to cell envelope components (41). The phosphorylation of GlmM is necessary for its activity in *Escherichia coli* and is achieved during an autophosphorylation process (29). On the other hand, the phosphorylation of GlmM by StkP is not essential for its enzymatic activity since the *S. pneumoniae* *stkP* mutant is viable. In addition to GlmM, we have identified the α -subunit of RNA polymerase as another substrate of StkP in in vivo-labeling experiments, although the significance of its modification is unclear (55).

It has also been shown that the mutation of *stkP* attenuates virulence in both pneumonia and bacteremia models of infection, indicating that StkP plays an important role for bacterial survival in vivo (18). A recent report by Oggioni et al. provided evidence that the expression of *stkP* was significantly increased

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in vivo in both the blood and tissues of infected mice (56). During the infection process, *S. pneumoniae* is exposed to various stress factors, including nutritional deprivation, temperature shift, pH changes, increased osmolality, and reactive oxygen species generated by host phagocytes. Due to the transmembrane topology of StkP and the presence of an extracellular sensor domain containing reiterated PASTA (penicillin binding protein and Ser/Thr protein kinase-associated domain) signature sequences (81), we hypothesized that StkP could transmit environmental cues into the cell.

Here we report the growth characteristics of an *S. pneumoniae* *stkP* mutant under different stress conditions. These results suggest that the expression of StkP can contribute to the resistance of *S. pneumoniae* to environmental stresses. Microarray analysis was used to compare the expression profile of the *stkP* mutant with that of the wild type. This analysis revealed that the *stkP* mutation is broadly pleiotropic and affects the transcription of several sets of important genes. Several functional gene categories have been identified that could account for a reduced stress response and attenuated virulence. Furthermore, we show that StkP of *S. pneumoniae* plays a role in the maintenance of low expression levels of competence genes under conditions that do not support competence development. Finally, we propose a hypothesis to describe the putative mechanism of competence deficiency in an *stkP* mutant.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. pneumoniae* strain CP1015 (44) and its isogenic derivative, CP1015 Δ *stkP* (like CP1015 but Δ *stkP*::Cm [55]), were grown at 37°C in casein tryptone (CAT) medium supplemented with 0.2% glucose and 1/30 volume 0.5 M K₂HPO₄, pH 7.5. DNA from strain CP1016 (*rif*-23) was used as donor DNA for transformation assays (45). The following antibiotics were added when necessary at the indicated concentrations: chloramphenicol, 10 µg ml⁻¹; and rifampin, 1 µg ml⁻¹.

Construction of *S. pneumoniae* *stkP* mutant. The deletion of the *stkP* gene was achieved by transforming an *S. pneumoniae* wild-type strain with a vectorless DNA fragment consisting of *stkP* downstream and upstream regions of homology and a *cat* cassette replacing the *stkP* coding region as previously described (55). Viable chloramphenicol-resistant clones arising from a double-crossover event were readily obtained after *S. pneumoniae* transformation. The resulting clones were further examined for successful allelic exchange by diagnostic PCR and Southern hybridization. The junctions between exogenous and chromosomal DNA were verified by sequencing.

In vitro stress experiments. To compare the effect of temperature stress on the wild-type and Δ *stkP* mutant strains, we used the same number of cells (4×10^4 CFU ml⁻¹) to inoculate CAT medium prewarmed to 37 and 40°C. Cultures were incubated statically under aerobic conditions at the indicated temperatures. At 1-h intervals, samples were removed and their optical densities (OD) were determined at a wavelength of 400 nm.

The sensitivity of cells to H₂O₂ was tested by exposing aliquots of cultures (10^7 CFU ml⁻¹; OD₄₀₀ = 0.4) grown in CAT medium at 37°C to 40 mM H₂O₂ for 5, 10, 15, and 20 min at 37°C. Viable cells were counted by plating them onto agar plates before and after exposure to H₂O₂, and results were expressed as percentages of survival.

Adaptability of the wild-type and isogenic CP1015 Δ *stkP* mutant to osmotic stress was evaluated by monitoring bacterial growth in CAT medium containing 400 mM NaCl. To initiate the growth experiments, bacteria grown to early exponential phase (OD₄₀₀ = 0.2) were inoculated into prewarmed CAT medium with or without NaCl. At 1-h intervals, bacterial growth was monitored by measuring the OD₄₀₀.

To study the role of StkP in acid tolerance, we used equal numbers of cells (4×10^4 CFU ml⁻¹) of both the wild-type strain and the Δ *stkP* mutant to inoculate CAT medium adjusted to pHs 6.5 and 7.5. At 1-h intervals, samples were withdrawn and the OD₄₀₀ values were determined.

RNA extraction. For an analysis of the transcriptome and quantitative real-time PCR, RNA was isolated from 50 ml of pneumococcal culture grown at 37°C

in CAT medium to an OD₄₀₀ of 0.4. Total bacterial RNA was extracted with hot acid phenol according to the method of Cheng et al. (10). After preliminary purification and precipitation with isopropanol, the crude RNA was treated with DNase I (Ambion) and purified further using RNeasy columns (QIAGEN) according to the manufacturer's protocol. The typical yield from 50 ml of cells was approximately 200 µg of purified RNA.

Reverse transcription and microarray hybridization. Commercial *S. pneumoniae* whole-genome microarray slides from Eurogentec (Belgium) were used in this study. The full genome array consists of amplicons representing segments of 2,085 open reading frames from *S. pneumoniae* reference strain TIGR4 spotted in duplicate on a glass slide.

Ten micrograms of purified RNA samples was reverse transcribed and differentially labeled with Cy3-dCTP and Cy5-dCTP (Amersham Biosciences) in the presence of random hexamer primers (Amersham Biosciences) and SuperScript II RNase H⁻ RT (Invitrogen) in a total volume of 20 µl. The reaction mixture was incubated at 42°C for 1.5 h. Dye swap labeling was performed systematically for all labeled RNA samples. Pairs of Cy3- and Cy5-labeled cDNA samples were mixed and hybridized to the array at 37°C for 15 h. After hybridization, the slides were washed in 0.2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate for 5 min, followed by a wash in 0.2× SSC for 5 min.

Data analysis. The arrays were scanned using the GeneTAC UC-4 scanner (Genomic Solutions). TIGR Spotfinder 2.2.3 (71) was used for determining the average signal intensity and the local background for each spot. The Cy3/Cy5 fluorescence ratios were log₂ transformed and normalized by LOWESS normalization in the TIGR microarray data analysis system (MIDAS) 2.17 (71). In total, six hybridizations using samples from three independent bacterial cultures and RNA extractions were performed and the average was calculated from the independent experiments. The statistical significance of the expression ratios was determined by applying the Student *t* test. Genes whose expression ratios changed by a factor of at least 2 and had *P* values of <0.01 were considered significantly regulated by StkP (95 genes). In order to verify the significance of regulation by a different statistical method, the data matrix of log₂-transformed gene expression ratios of all replicates was imported into TIGR MultiExperiment Viewer (MeV) 4.0 (71) and analyzed by a one-class significance analysis of microarrays (SAM) test (76). SAM statistics were based on all 4,096 possible permutations, and the threshold delta value ($\Delta = 0.91$) was based on delta versus the percent false discovery rate table reported by SAM to avoid any falsely significant genes. SAM identified 208 significantly regulated genes and confirmed all 97 genes identified by Student *t* test and expression ratio threshold.

Validation of array data. The significance of signals obtained from microarray hybridizations was verified by quantitative real-time PCR (qRT-PCR) (see below) using Sybr green I JumpStart *Taq* ReadyMix kit (Sigma). Six derepressed genes (spr0013, spr0857, spr1109, spr1724, spr1864, and spr2041), eight repressed genes (spr0096, spr0867, spr0934, spr1057, spr1154, spr1320, spr1684, and spr2021), and two stably expressed genes (spr0708 and spr1757) were chosen for this confirmatory study. qRT-PCRs were performed with the same RNA samples that were used for microarray analysis. For each gene, three biological replicates with three technical replicates each were used.

Competence gene expression. Cells were grown in CAT medium at 37°C until an OD₄₀₀ of 0.4 before exposure to competence-stimulating peptide (CSP) (250 ng ml⁻¹). At specified intervals (8 and 13 min) after the addition of CSP, total RNAs were extracted (see above). Sample "0" was taken before the addition of CSP. Reverse transcription was performed as described above. For the expression profile analysis of competence genes, qRT-PCR was performed (see below) on a select group of competence genes: spr0013, spr0020, spr0043, spr0857, spr1724, spr1864, spr2041, and spr2043.

qRT-PCR. qRT-PCRs were carried out in a Mx3000 LightCycler instrument (Stratagene). All primers were calculated using Primer3 services at <http://frodo.wi.mit.edu>. PCR products were approximately 100 bp in length. A master mix of the following reaction components was prepared to the following end concentrations: 6.9 µl water, 1 µl forward primer (0.5 µM), 1 µl reverse primer (0.5 µM), 0.2 µl internal Rox reference dye, and 9.9 µl Sybr green JumpStart *Taq* ReadyMix (Sigma). One microliter of cDNA (50 ng reverse-transcribed total RNA) was added as a PCR template. Amplification was performed using the following cycling protocol: 3 min of initial denaturation at 95°C, followed by a three-step profile consisting of 30 s of denaturation at 95°C, 25 s of annealing at 60°C, and 25 s of extension at 72°C for a total of 40 cycles. Each qRT-PCR was performed in triplicate using RNA from two independent isolations, and no-reverse-transcriptase controls were also included. The change in expression of each gene in the wild-type and CP1015 Δ *stkP* mutant strain was calculated according to the equation of Pfaffl (62). Values were normalized to the reference *gyrA* gene (spr1099) encoding the DNA gyrase subunit A. Melting curve analysis

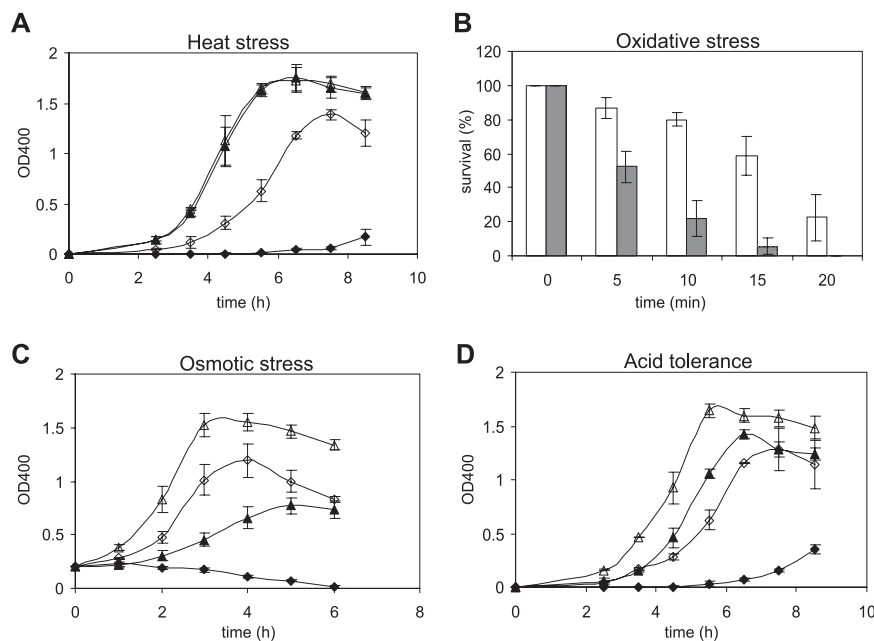


FIG. 1. In vitro stress experiments. (A) Growth curves of the wild type (triangles) and the *stkP*-deficient mutant (diamonds) at different temperatures. A total of 4×10^4 CFU ml^{-1} of each strain was used to inoculate CAT medium prewarmed at the indicated temperatures. Bacteria were grown at 37°C (open symbols) and 40°C (filled symbols). Samples were taken at 1-h intervals for measurement of the OD₄₀₀. Data are representative of three independent experiments. (B) H₂O₂ survival test of the wild type (open columns) and the *stkP*-deficient mutant (gray columns). In the assays, cells were grown in CAT medium at 37°C to an OD₄₀₀ of 0.4 and the aliquots (10^7 CFU) were used in each assay. Cells were incubated at 37°C for 5, 10, 15, and 20 min without or with 40 mM H₂O₂, and viable counts were carried out. Experiments were performed in triplicate. (C) Growth curves as measured by the ODs of the wild type (triangles) and the *stkP*-deficient mutant (diamonds) grown in CAT (open symbols) and CAT plus 400 mM NaCl (filled symbols). The growth of cultures was monitored from an initial OD₄₀₀ of 0.2. Data are representative of three independent experiments. (D) Growth curves of the wild type (triangles) and the *stkP*-deficient mutant (diamonds) at different pHs. Equal numbers of cells (4×10^4 CFU ml^{-1}) of both the wild-type strain and the Δ *stkP* mutant were used to inoculate CAT medium adjusted to pHs 6.5 (filled symbols) and 7.5 (open symbols), and bacteria were grown at 37°C. Data are representative of three independent experiments. Error bars indicate standard deviations of the means.

was also performed to evaluate PCR specificity and resulted in single primer-specific melting temperatures. For a list of the primers used, see Table S1 in the supplemental material.

Complementation experiment. An *S. pneumoniae* strain with an ectopic copy of the *pcsB* gene was constructed by transformation with a synthetic linear PCR amplicon kindly provided by M. Winkler (51). A *bgaA::[kan-t1t2-P_{tesK}]-pcsB⁺* fragment was inserted into the dispensable *bgaA* chromosomal gene. The presence of the desired mutation was verified by screening for changes in the sizes of fragments amplified from chromosomal DNA using the flanking primers.

Competence assay. The transformation was performed as described previously (35). Briefly, cells were grown in CAT medium at 37°C to OD₄₀₀ values of 0.1 and 0.4. Appropriate aliquots were withdrawn, incubated in the presence or absence of CSP (250 ng ml^{-1}), and mixed with *rif-23* donor DNA (1 μg ml^{-1}). Further incubation was carried out at 37°C for 1 h before plating in CAT agar containing 1 μg Rif ml^{-1} . Serial dilutions of transformed cultures were plated, and transformation efficiencies were calculated as the ratio of the viable counts on plates with and without rifampin. Results are expressed as the mean counts from two independent experiments.

RESULTS AND DISCUSSION

S. pneumoniae has been shown to contain a single gene coding for a eukaryotic-type protein kinase functionally coupled to its cognate protein phosphatase (55). Studies performed with virulent strains suggested that an *stkP* mutation severely affects virulence in a murine model of infection (18). The results presented in this work report two significant findings. First, we demonstrate that the *stkP* mutation confers a phenotype that is sensitive to many stress conditions, including

elevated temperature, oxidizing agents, osmotic pressure, and acidic conditions. Second, by using microarray analysis, we have identified genes belonging to several functional categories whose expressions were affected at the transcriptional level in an *stkP* mutant.

In vitro growth phenotype of the Δ *stkP* mutant. Prior to studying the effect of the *stkP* mutation on the global expression profile of *S. pneumoniae*, we first examined the growth characteristics of the *stkP*-null mutant in vitro. The *stkP* mutant strain displayed small but significant changes in growth properties under nonstress conditions. A comparison of the growth levels of the mutant and parent strains in CAT medium at 37°C showed that the mutant had a significantly reduced growth rate compared to that of the parent strain in the early exponential phase (Fig. 1A). The calculated doubling time of the mutant was increased to 55 min compared to 35 min for the parent strain. In addition, cultures of the mutant strain exhibited an increased rate of autolysis at the stationary phase of growth and did not achieve the same final optical density as the parent strain. The extended lag phase and slower doubling time in the early exponential phase and the recovery of growth with a rate approaching that of the wild type in the mid-exponential phase, which we observed in our studies, are reminiscent of those observed for protein kinase mutants in other streptococci (27, 28, 64).

We also studied the growth phenotype of the $\Delta stkP$ mutant at an elevated temperature. In contrast to the parent strain, which was unaffected at 40°C, the growth of the mutant strain was almost completely impaired (Fig. 1A). This result suggests that StkP regulates genes involved in adaptation to high temperatures.

Sensitivity of the $\Delta stkP$ mutant to oxidative stress. To determine whether the $\Delta stkP$ strain has an altered sensitivity to oxidative stress, its ability to survive H₂O₂- and superoxide (O₂⁻)-induced oxidative stress was examined and compared with that of the parental strain. Decreased survival of the $\Delta stkP$ mutant, compared to that of the wild type, was observed at 40 mM H₂O₂ (Fig. 1B). After 15 min of treatment with 40 mM hydrogen peroxide, 96% of the $\Delta stkP$ cells were killed, whereas 69% of the wild-type cells survived. Both the wild-type and mutant strains were also exposed to 50 mM paraquat over a 30-min period. In contrast to the results of experiments using hydrogen peroxide as an oxidative agent, both the wild type and the $\Delta stkP$ mutant exhibited virtually the same sensitivity to superoxide (data not shown). This sensitivity to oxidative killing might be a major contributor to the attenuated virulence of the *stkP* mutant (18), since such cells would be less likely to survive attacks by the innate host defense system.

The $\Delta stkP$ mutant strain is more susceptible to osmotic stress. To further characterize the ability of the *stkP* mutant strain to cope with stress conditions, its growth was measured under high osmolality. Growth of the wild-type strain in CAT medium containing 400 mM NaCl was impaired (Fig. 1C). The *stkP* mutant strain exhibited no discernible growth, and virtually all of the cells lysed at the end of cultivation. Hence, the *stkP* mutant has a reduced tolerance to osmotic stress.

The $\Delta stkP$ mutant strain is less tolerant to acidic pH. A comparison of the growth of the wild-type and mutant strains under mild acidic conditions (pH 6.5) showed that the *stkP* mutant is much less tolerant to an acidic pH (Fig. 1D). In contrast to that of the parent strain, which was only slightly affected, the growth of the mutant strain was significantly slower with an extended lag phase. A similar effect of Ser/Thr protein kinase deletion on acid tolerance has been observed in *Streptococcus mutans* (27). Taken together, these data indicate that the *stkP* mutation confers sensitivity to a variety of stressors, suggesting that it very likely affects numerous different cellular functions.

Global transcription profile of the $\Delta stkP$ mutant. To begin to address the genetic basis of these phenotypes, we performed an analysis of global transcription of the $\Delta stkP$ mutant compared to the wild-type parent in the exponential phase (OD₄₀₀ = 0.4) in CAT. Custom *S. pneumoniae* whole-genome microarray slides from Eurogentec (Materials and Methods) were used throughout this study. In total, six independent hybridizations using RNA samples from three separate bacterial cultures were performed for both strains. After background subtraction and normalization, the data were processed and statistically evaluated (Materials and Methods). Among 2,085 genes evaluated, changes in relative transcript amounts of at least two-fold were observed for 95 genes. Under the conditions used, more than 4% of *S. pneumoniae* genes were identified as transcriptionally affected, indicating a global regulatory function for the StkP signaling protein. Complete lists of genes with affected transcript levels are given in Tables 1 and 2. The

majority of altered genes could be clustered into several different functional categories.

As an internal control, *stkP* transcripts were not detected from the $\Delta stkP$ mutant (decrease of 22-fold) compared to the presence of the parent strain (Table 1). *stkP* is located downstream of, and overlaps with, *phpP*, a gene coding for StkP cognate phosphatase (55). The presence of a transcriptional terminator following *stkP* suggested that *stkP* is the last gene in the operon (18). Thus, the polar effect resulting from *stkP* replacement is unlikely. Furthermore, the level of transcription from the *phpP* (*spr1578*) and *orf-1576* genes, which flank *stkP*, remained unaffected in the $\Delta stkP$ mutant and its *stkP*⁺ parent, indicating a lack of polar effects on the surrounding genes.

Validation of gene expression by qRT-PCR analysis. The RT-PCR approach was chosen as an independent method to validate the microarray results. We quantified transcripts of selected genes by using the same RNA samples that were used for the microarray assays. Average data from at least two experimental replicates are shown in Table 3. In each instance, the qRT-PCR results correlated well ($R^2 = 0.9$) with those obtained from the microarrays (Fig. 2). qRT-PCR assays provided a higher dynamic range for relative gene expression than did the DNA microarrays, as indicated for *spr1057* as well as *spr0857* (*celB*), *spr1684* (*fatD*), *spr1724* (*ssbB*), and *spr1864* (*cglA*). Both microarray and qRT-PCR analysis showed that the expression levels of *recA* and *ciaH* are unaltered in the *stkP* mutant.

Functional classification of downregulated genes in $\Delta stkP$ mutant. Apart from a few exceptions, genes encoding proteins potentially involved in the regulation of cell wall biosynthesis were most strongly repressed (Table 1). *spr0096* (11-fold decrease) encodes a hypothetical protein containing a LysM domain, one of the most common protein modules in bacterial cell surface proteins. LysM occurs most often in cell wall-degrading enzymes, where it is believed to act to anchor the catalytic domains to their substrates (30). Several proteins that contain the domain, including staphylococcal immunoglobulin G binding proteins and *Escherichia coli* intimin, are involved in bacterial pathogenesis (3). *lytB* (fivefold) encodes glucosaminidase, which catalyzes the last step in cell wall division that disperses intact cells (17). It has been shown recently that *lytB* mutants exhibited a significantly reduced colonization of the nasopharynx but exhibited a behavior similar to that of the parental *lytB*⁺ strain when they were tested in a model of pneumococcus-induced sepsis (22).

A significant decrease of expression (10-fold) of *spr2021* (*pcsB*) has been shown in microarray analysis and validated further by qRT-PCR. The *pcsB* homologues of *Streptococcus mutans* (11) and *Streptococcus agalactiae* (67) have been implicated in responses to general stress, but their functions remain unknown. *pcsB* mutants in both species of streptococcus were more sensitive to low pH, high osmotic pressure, and high temperature than was their *pcsB*⁺ parent strain (67, 68). A similar increase of sensitivity to elevated temperature, high osmolality, and pH was observed in an *S. pneumoniae* R6 derivative strain expressing less than half the normal PcsB amount (M. Winkler, personal communication). Unlike the case in *S. mutans* and *S. agalactiae*, PcsB is strictly essential in *S. pneumoniae* (51). It has been demonstrated that severe depletion of PcsB led to a rapid cessation of growth. Therefore,

TABLE 1. Down-regulated genes in the *ΔstkP* mutant strain

Functional category	Gene no.	Gene product (gene)	Mean of log ₂ ratios	P value	Expression ratio ^a
Cell wall	spr0096	Hypothetical protein, LysM domain protein	-3.46	6.57E-07	-11.01
	spr0867	Endo-beta-N-acetylglucosaminidase (<i>lytB</i>)	-2.26	3.25E-11	-4.78
	spr2021	General stress protein PcsB (<i>pcsB</i>)	-3.24	5.37E-09	-9.47
Glycerol metabolism	spr1344	Glycerol uptake facilitator protein paralog (<i>glpF</i>)	-1.05	2.02E-06	-2.08
	spr1990	Glycerol-3-phosphate dehydrogenase, truncation (<i>glpD</i>)	-1.01	6.00E-04	-2.01
	spr1991	Glycerol kinase (<i>glpK</i>)	-1.37	4.82E-05	-2.58
Pyrimidine metabolism	spr0613	Orotidine-5'-decarboxylase (<i>pyrF</i>)	-1.54	8.75E-09	-2.90
	spr0614	Orotate phosphoribosyltransferase (<i>pyrE</i>)	-1.55	1.92E-10	-2.92
	spr0865	Dihydroorotate dehydrogenase electron transfer subunit (<i>pyrDII</i>)	-2.07	2.84E-08	-4.19
	spr0866	Dihydroorotate dehydrogenase (<i>pyrD</i>)	-2.38	2.49E-11	-5.21
	spr1053	Dihydroorotate (<i>pyrC</i>)	-1.50	7.59E-07	-2.83
	spr1153	Carbamoylphosphate synthase (ammonia), heavy subunit (<i>carB</i>)	-1.43	1.21E-08	-2.69
	spr1154	Carbamoylphosphate synthase (glutamine-hydrolyzing) light subunit (<i>carA</i>)	-1.27	4.64E-09	-2.41
	spr1155	Aspartate carbamoyltransferase (<i>pyrB</i>)	-1.30	6.19E-10	-2.47
	spr1156	Transcriptional attenuation of the pyrimidine operon/uracil phosphoribosyltransferase activity (<i>pyrR</i>)	-1.49	5.80E-08	-2.80
	spr1165	Uracil permease (<i>pyrP</i>)	-1.94	3.65E-09	-3.83
DNA repair	spr1054	8-oxo-dGTP nucleoside triphosphatase (<i>mutX</i>)	-1.95	4.63E-10	-3.88
	spr1055	DNA-uracil glycosylase (<i>ung</i>)	-2.03	1.48E-09	-4.08
	spr1317	O6-methylguanine-DNA methyltransferase (<i>ogt</i>)	-1.02	8.51E-11	-2.02
	spr1660	Exodeoxyribonuclease (<i>exoA</i>)	-1.20	5.16E-05	-2.30
Iron uptake	spr0934	ABC transporter substrate binding protein-iron transport	-1.16	1.30E-05	-2.23
	spr1684	ABC transporter membrane-spanning permease-ferric iron transport (<i>fatD</i>)	-1.04	1.86E-02	-2.10
	spr1685	ABC transporter membrane-spanning permease-ferric iron transport (<i>fatC</i>)	-1.51	1.15E-04	-2.85
	spr1686	ABC transporter ATP binding protein-ferric iron transport (<i>fecE</i>)	-1.67	1.93E-03	-3.17
	spr1687	ABC transporter substrate binding protein-ferric iron transport (<i>fatB</i>)	-2.23	1.43E-04	-4.70
Oxidative stress	spr1321	Conserved hypothetical protein, similar to amidotransferase, SNO family	-1.25	2.08E-03	-2.38
	spr1322	Pyridoxine biosynthesis protein (<i>pxdI</i>)	-1.10	1.22E-03	-2.14
	spr1495	Thioredoxin-linked thiol peroxidase (<i>psaD</i>)	-1.15	2.88E-03	-2.21
Other	spr0551	Branched-chain amino acid transport system carrier protein (<i>bmQ</i>)	-1.21	3.18E-05	-2.31
	spr1047	Lipoate protein ligase A (<i>lplA</i>)	-1.01	4.08E-05	-2.02
	spr1048	Dihydrolipoamide dehydrogenase (<i>acoL</i>)	-1.10	3.91E-04	-2.14
	spr1577	Eukaryotic-type serine/threonine kinase (<i>stkP</i>)	-4.46	3.66E-04	-22.04
	spr1657	ABC transporter ATP binding/membrane spanning protein	-1.01	3.37E-06	-2.01
Hypothetical genes	spr0084	Conserved hypothetical protein, rhodanese-like protein	-1.70	5.31E-06	-3.24
	spr0552	Conserved hypothetical protein, M42 glutamyl aminopeptidase homologue	-1.23	9.50E-04	-2.34
	spr0652	Conserved hypothetical protein, degV family	-2.20	4.94E-10	-4.61
	spr0709	Hypothetical protein	-1.14	6.85E-05	-2.20
	spr0755	Conserved hypothetical protein, putative lipoprotein precursor	-1.04	1.22E-04	-2.06
	spr1052	Conserved hypothetical protein, MATE efflux protein family	-1.08	6.76E-07	-2.12
	spr1056	Hypothetical protein	-3.14	3.76E-04	-8.81
	spr1057	Conserved hypothetical protein, hydrolase HAD-like family	-5.87	3.33E-10	-58.52
	spr1316	Conserved hypothetical protein, similar to arsenate reductase	-1.08	1.16E-10	-2.12
	spr1318	Hypothetical protein, putative acetyltransferase	-1.13	1.72E-08	-2.19
	spr1319	Hypothetical protein	-2.69	1.76E-06	-6.47
	spr1320	Conserved hypothetical protein, similar to hemolysin III	-3.34	1.66E-05	-10.16
	spr1436	Conserved hypothetical protein, methyltransferase-like protein	-1.57	3.01E-05	-2.98
	spr1498	Conserved hypothetical protein, putative large secreted protein	-1.03	2.33E-04	-2.05
	spr1790	Conserved hypothetical protein, related to sporulation protein, SpoIIIJ	-1.17	5.43E-08	-2.26

^a The ratio of the gene expression level in the *ΔstkP* mutant strain compared to that of the wild-type strain.

a model in which PcsB acts as a critical murein hydrolase that modulates cell wall biosynthesis has been proposed.

To test whether decreased resistance to stress conditions of the *ΔstkP* mutant resulted from low abundance of *pcsB* transcript, we constructed a merodiploid strain expressing an ectopic copy of the *pcsB* allele under the control of a fucose-inducible P_{fcSK} promoter. No significant effect on either growth characteristics or stress resistance was observed when the ectopic expression of *pcsB* was induced with fucose over a wide range of inducer concentrations (data not shown). Therefore, it seems that the increased sensitivity of the *stkP* mutant to stress conditions is multifactorial, not simply dependent on the decreased level of *pcsB* expression.

Recent studies revealed that *pcsB* expression is positively regulated by the VicRK two-component system (52). VicRK is a homologue of the essential two-component system, which is highly conserved in and specific to the low-G+C-content gram-positive bacteria, where it is often designated YycFG based on its initial discovery in *Bacillus subtilis* (20). Both the *yycF* and *yycG* genes are required for viability. Conversely, in *S. pneumoniae*, only the response regulator *vicR* gene was found to be essential (32, 74), suggesting that VicR functions independently of sensor kinase VicK, possibly participating in signal transduction with another two-component system kinase. In a recent study performed by Ng et al. (53), binding sequences for VicR were determined by band shift and footprinting experi-

TABLE 2. Up-regulated genes in the *ΔstkP* mutant strain

Functional category	Gene no.	Gene product (gene)	Mean of log ₂ ratios	P value	Expression ratio ^a	
Early competence genes	spr0020	Competence protein (<i>comW</i>)	2.84	1.80E-04	7.18	
	spr0043	Transport ATP binding protein ComA (<i>comA</i>)	3.27	2.77E-05	9.65	
	spr0044	Transport protein ComB (<i>comB</i>)	4.99	7.60E-06	31.73	
	spr2041	Response regulator (<i>comE</i>)	5.12	1.20E-05	34.76	
	spr2042	Histidine protein kinase (<i>comD</i>)	5.24	1.98E-06	37.81	
	spr2043	Competence stimulating peptide precursor (<i>comC</i>)	3.29	2.29E-04	9.76	
	spr0013/1819	Competence-specific global transcription modulator (<i>comX/comX2</i>)	4.22	1.13E-04	18.59	
Late competence genes	spr0857	Competence protein (<i>celB</i>)	1.39	7.00E-03	2.62	
	spr1144	DNA processing Smf protein (<i>smf</i>)	1.46	3.43E-03	2.75	
	spr1724	Single-stranded DNA binding protein (<i>ssbB</i>)	1.71	8.68E-05	3.27	
	spr1758	Competence induced protein A (<i>cinA</i>)	1.17	5.70E-04	2.26	
	spr1861	Competence protein (<i>cglD</i>)	1.35	4.29E-03	2.54	
	spr1863	Competence protein (<i>cglB</i>)	3.00	2.55E-03	8.02	
	spr1864	Competence protein (<i>cglA</i>)	2.92	1.19E-03	7.57	
Competence-related CSP-induced genes	Bacteriocin production	spr0472	BlpY protein (<i>blpY</i>)	1.50	3.99E-03	2.83
		spr0473	Hypothetical protein (<i>blpZ</i>)	1.24	6.54E-03	2.36
		spr0474	ABC transporter ATP binding protein (<i>pncP</i>)	1.07	3.25E-03	2.10
		spr1561	ABC transporter membrane-spanning permease-Na ⁺ export (<i>natB</i>)	1.88	2.43E-03	3.69
		spr1562	ABC transporter ATP binding protein-Na ⁺ export (<i>natA</i>)	2.10	1.09E-03	4.30
Purine metabolism	spr0021	Adenylosuccinate synthetase (<i>purA</i>)	2.11	1.54E-05	4.31	
	spr0053	Phosphoribosylaminoimidazole carboxylase, catalytic subunit (<i>purE</i>)	1.46	1.32E-03	2.75	
	spr0054	Phosphoribosyl glucinamide formyltransferase (<i>purK</i>)	1.33	3.12E-04	2.52	
	spr1109	Formate-tetrahydrofolate ligase (<i>fts</i>)	1.07	7.59E-07	2.10	
	spr1128	GMP reductase (<i>guaC</i>)	1.01	2.20E-06	2.01	
Hypothetical genes	spr0128	Hypothetical protein	1.75	3.26E-04	3.36	
	spr1407	Hypothetical protein	1.66	1.45E-04	3.16	
	spr1760	Conserved hypothetical protein	1.12	1.73E-03	2.18	
	spr1761	Conserved hypothetical protein	1.18	9.78E-04	2.27	
	spr1857	Hypothetical protein			2.67	
	spr1858	Hypothetical protein	1.72	2.79E-03	3.30	
	spr1859	Conserved hypothetical protein	2.46	9.51E-03	5.50	
Riboflavin metabolism	spr0161	Riboflavin synthase beta chain (<i>ribE</i>)	1.08	9.07E-06	2.12	
	spr0162	Riboflavin biosynthesis; GTP-cyclohydrolase II (<i>ribA</i>)	1.60	2.04E-07	3.03	
	spr0164	Riboflavin biosynthesis; a deaminase (<i>ribD</i>)	1.53	1.12E-07	2.89	
	spr1017	Macrolide efflux protein (<i>mreA</i>)	2.73	2.97E-04	6.64	
	Other	spr0059	Beta-galactosidase 3 (<i>bgaC</i>)	1.01	2.31E-03	2.01
spr0060		Phosphotransferase system sugar-specific EIIB component	1.37	6.01E-04	2.58	
spr0561		Cell wall-associated serine proteinase precursor PrtA (<i>prtA</i>)	1.16	1.15E-04	2.23	
spr1324		Thiamine biosynthesis lipoprotein (<i>apbE</i>)	1.17	8.63E-05	2.25	
spr1408		Formylmethionine deformylase (<i>def</i>)	1.88	8.62E-05	3.69	
spr1935		Dihydroxyacid dehydratase (<i>ilvD</i>)	1.73	3.32E-05	3.31	
spr1945		Choline binding protein (<i>pcpA</i>)	1.47	9.96E-05	2.77	
Hypothetical genes		spr1482	Hypothetical protein	1.22	1.56E-06	2.33
		spr1623	Hypothetical protein	1.82	3.30E-10	3.52
		spr1624	Conserved hypothetical protein	1.91	9.22E-10	3.75
	spr1625	Conserved hypothetical protein	1.75	1.60E-11	3.37	
	spr1626	Hypothetical protein	1.52	6.09E-07	2.87	
	spr1768	Conserved hypothetical protein, related to Trp repressor binding protein	1.11	8.90E-06	2.15	

^a The ratio of the gene expression level in the *ΔstkP* mutant strain compared to that of the wild-type strain.

TABLE 3. qRT-PCR confirmation of microarray data

Gene product	Gene no.	Expression ratio ^a	
		DNA microarray	Real-time PCR
Transcriptional regulator ComX1	spr0013	18.6	16.9
Hypothetical protein, LysM domain protein	spr0096	-11.0	-15.9
Sensor histidine kinase CiaH	spr0708	*	-1.6
Competence protein CelB	spr0857	2.6	22.0
Endo-beta-N-acetylglucosaminidase	spr0867	-4.8	-5.5
ABC transporter substrate binding protein-iron transport	spr0934	-2.2	-1.8
Conserved hypothetical protein, member of the hydrolase HAD-like family	spr1057	-58.5	-502.2
Formate-tetrahydrofolate ligase	spr1109	2.1	2.1
Carbamoylphosphate synthase (glutamine-hydrolyzing) light subunit	spr1154	-2.4	-3.0
Conserved hypothetical protein, similar to hemolysin III	spr1320	-10.2	-15.7
ABC transporter membrane-spanning permease-ferric iron transport	spr1684	-2.1	-10.6
Single-stranded DNA binding protein SsbB	spr1724	3.3	23.6
RecA protein	spr1757	*	1.6
Competence protein CglA	spr1864	7.6	53.7
General stress protein GSP-781	spr2021	-9.5	-7.4
Response regulator ComE	spr2041	34.7	30.1

^a The ratio of the gene expression level in the $\Delta stkP$ mutant strain compared to that of the wild-type strain indicating genes up- or down-regulated in the $\Delta stkP$ mutant strain. *, gene is not significantly regulated by StkP.

ments. VicR bound to regions upstream of *pcsB*, *pspA*, spr0096, spr1875, and spr0709. The phosphorylation of VicR to VicR-P increased the apparent strength and changed the nature of binding to these regions.

A comparison of our microarray results with those obtained by Ng et al. (52, 53) shows that spr0096, spr0709, *lytB*, and *pcsB* genes were strongly downregulated in both the *vicR* and *stkP* isogenic mutants. No changes were observed in the expression of the *vicRKY* genes in the $\Delta stkP$ mutant compared to that in the *stkP*⁺ parent. Thus, the positive regulatory effect on the expression of spr0096, spr0709, *lytB*, and *pcsB* genes can be confined to direct interaction of StkP with VicR. This finding could suggest that in addition to its cognate kinase, VicK, the activity of the response regulator VicR can be modulated by StkP. Whether StkP influences the ability of VicR to bind DNA and/or regulate downstream transcription or affect the stability of VicR is currently being investigated. Recently, Rajagopal et al. (65) brought the first experimental evidence that direct cross talk between two different signaling systems exists.

The genes encoding proteins involved in pyrimidine de novo biosynthesis comprised the largest category, containing 10

genes that were coordinately downregulated by 2.4- to 5.2-fold. In contrast to a number of different gram-positive organisms, the pyrimidine biosynthesis genes are scattered on the chromosome of *S. pneumoniae* and are organized in five putative transcriptional units: *pyrFpyrE*, *pyrDIIpyrD*, *pyrC*, *pyrP*, and *pyrRpyrBcarAcarB*. The first gene in the *pyrRpyrBcarAcarB* operon is *pyrR* encoding a regulatory protein. In *B. subtilis*, regulation of the *pyr* operon occurs through a transcriptional attenuation mechanism in which PyrR promotes transcriptional termination at three attenuation regions in the operon when uridine nucleotide levels are high (34, 75). High levels of UMP stimulate PyrR to bind to a conserved sequence and secondary structure in the mRNA in each attenuation region called the antiantiterminator or the binding loop (73). Binding of PyrR to this sequence prevents the formation of an antiterminator stem-loop and allows a downstream ρ -independent transcription terminator to form, reducing the expression of downstream genes. When UMP levels are low, the RNA binding affinity of PyrR is reduced and the more stable antiterminator stem-loop is favored, leading to transcriptional read-through and expression of the downstream genes.

By analyzing the sequences of the upstream and intergenic regions of genes comprising the PyrR regulon in *S. pneumoniae*, we identified the highly conserved sequence motif 5'-ARUCCN GNGAGGYU-3' in the *pyrRpyrBcarAcarB* mRNA leader, in the *pyrBcarA* intergenic region, and in the *pyrFpyrE* and *pyrP* leader sequences (data not shown). The secondary structures that may be formed by the RNA in these regions in *S. pneumoniae* are similar to the structures found in the RNA transcribed from the *B. subtilis pyr* operon. These findings suggest that the expression of the *pyr* genes of *S. pneumoniae* can be regulated by the same attenuator mechanism as that suggested for *B. subtilis*. The fact that all genes of the *S. pneumoniae pyr* regulon, including *pyrR*, are repressed in the *stkP* mutant could indicate that in addition to the attenuation mechanism, their expressions are regulated by an alternative mechanism. Collectively, these results suggest that the regulation of pyrimidine biosynthesis in *S. pneumoniae* involves

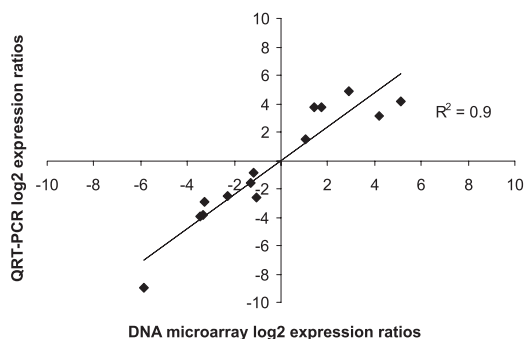


FIG. 2. Correlations between gene expression levels determined by DNA microarray and qRT-PCR. The solid line is the linear regression line; the correlation coefficient is 0.9.

multiple levels of control (i.e., transcriptional and posttranslational).

There were two- to fourfold decreases in the relative transcript amounts of several genes involved in DNA repair. Two of these, spr1055 and spr1054, coding for DNA uracil glycosylase (*ung*) and 8-oxodGTP nucleoside triphosphatase (*mutX*), respectively, are clustered with *pyrC* (see above). MutX of *S. pneumoniae* is a homologue of *E. coli* MutT, an antimutator protein (40). In addition, the expression of spr1317 (O6-methylguanine-DNA methyltransferase *ogt*) and spr1660 (exodeoxyribonuclease *exoA*) was moderately affected (twofold decrease).

Decreased expression of a gene cluster, *piuBCDA* (spr1684-spr1687), containing the components of an iron uptake ATP binding cassette (ABC) transporter (also called a *fat* or *pit1* operon) that is required for virulence (6, 7) was observed (2.8- to 4.7-fold decrease). This result was further validated by qRT-PCR, which showed even higher values for the change in expression for the first gene in the operon, spr1684 (*fatD*) (Table 3). In addition, there was a 2.2-fold reduction in spr0934 transcripts, a homologue of *piaA*. *piaA* is the first gene of a four-gene iron transport operon (Pit2) and has been identified as a ferrophore binding protein (7). Three gene clusters, *pit*, *pia*, and *piu*, each encoding four elements of an iron uptake ABC transporter, have been implicated in iron uptake by *S. pneumoniae* (6, 7).

To test the susceptibility to inhibition by streptonigrin, which can be used as an indirect indicator of intracellular iron concentration (5), we measured the zone of growth inhibition surrounding an antibiotic disk impregnated with 5 μ g of streptonigrin on blood agar plates. Since no differences in zone size were observed for mutant and wild-type strains, we concluded that both strains have similar iron transport capacities (data not shown). This result is in agreement with the previous finding that the *pia* product is likely to be the dominant *S. pneumoniae* iron transporter during both in vitro and in vivo growth (7). Thus, the attenuated virulence of the *stkP* mutant strain cannot be attributed to the defect in iron transport.

Another functional category of repressed genes comprises those that mediate resistance to oxidative stress, including spr1322 and spr1495, encoding pyridoxine biosynthesis protein Pdx1 and thioredoxin-linked thiol peroxidase PsaD. Pdx1 has been reported as a singlet oxygen resistance protein (19), and it has been suggested that PsaD is a homologue of *E. coli* thiol peroxidase, which scavenges superoxide and peroxide ions (54).

In addition to the genes with experimentally defined functions, a large group of repressed genes coding for proteins with either unknown or putative functions were observed. The alteration in relative transcription of several of these genes was considerable, suggesting that they may function in the observed phenotypic properties. A notably large decrease for the spr1057-spr1056 cluster (59- and 9-fold, respectively) encoding conserved hypothetical polypeptides was detected. spr1057, the most strongly repressed gene (as high as 500-fold by qRT-PCR) in the *stkP* mutant, encodes a homologue of the haloacid dehalogenase (HAD)-like protein superfamily, which includes a variety of enzymes with different functions. Despite the fact that it is named after a dehalogenase, the vast majority of the known catalytic activities in the HAD family involve phospho-

ryl transfer (2). The few members of the family that have a defined function are associated with membrane transport, metabolism, signal transduction, and nucleic acid repair. The *E. coli* YjiG protein, a member of the HAD superfamily, exhibits a high phosphatase activity towards 5'-dTMP, 5'-dUMP, and 5'-UMP and might be involved in the pyrimidine substrate cycles (63). Further work is necessary to elucidate the function of the HAD protein in *S. pneumoniae*. Nevertheless, the extent and strength of its repression in the *stkP* mutant suggest that its expression can be completely inhibited. Indeed, a proteomic analysis of the wild-type and *stkP* strains demonstrated that mutant cells are depleted of Spr1057 protein (data not shown).

Among the other repressed hypothetical genes, we observed a substantial reduction (10-fold by microarrays and 15-fold by qRT-PCR) in the expression of the spr1320 gene encoding a putative second pneumococcal hemolysin that is distinct from pneumolysin. Spr1320 is a conserved protein in all streptococci as well as additional bacterial species and is similar to hemolysin III. It has been described as a lysin responsible for a beta-hemolytic effect in pneumococci (9); however, no data are available regarding its potential role in pathogenesis.

Inactivation of *stkP* leads to overexpression of competence genes. The analysis of the induced genes in the *stkP* mutant showed that the majority of upregulated genes belong to the competence regulon (Table 2). This finding was surprising as it has been previously demonstrated that an *stkP* mutation effectively abolishes natural competence development (18). Furthermore, the *S. pneumoniae* strains were grown in CAT medium, in which the wild-type strain CP1015 fails to develop spontaneous competence. We selected these culture conditions to uncouple the regulatory effects of StkP on growth and physiology from those on competence. All the ComE-dependent genes (early *com* genes) described previously as key components of the competence regulatory cascade, including *comCDE*, *comAB*, *comX*, and *comW* (26, 33, 35, 36, 58), were strongly upregulated in the Δ *stkP* mutant (Table 2). These genes encode functions absolutely required for competence induction. A comparison of the induction ratios of the core early competence genes, including *comCDE*, *comAB*, *comX*, and *comW*, with those reported in previous reports (15, 61) suggested that the competence regulatory circuit is fully induced. Although there were differences in the amplitude of induction between particular surveys, these differences could be attributed to the distinct microarrays, variations in medium composition, and in the strains used.

In contrast, the levels of induction of σ^x -dependent genes (late competence genes) required for the process of DNA uptake, processing, and recombination were generally 10- to 20-fold lower than those reported previously (15, 61). In addition, several competence genes had transcript levels below the cutoff value of twofold, suggesting that their expression levels were not affected in the *stkP* mutant. These genes include *celA*, *coiA*, *dalA*, *cclA*, *recA*, *cglC*, *cflA*, and *cflB*. It has been experimentally demonstrated that these genes encode proteins whose functions are indispensable in efficient DNA binding, uptake, and recombination (8, 38, 46, 59, 61). Among these, the absence of *recA* induction was confirmed by qRT-PCR (Table 3). Previous studies have demonstrated that the *recA* gene is induced at competence (38, 57), and de novo synthesis of RecA molecules is absolutely required for full recombina-

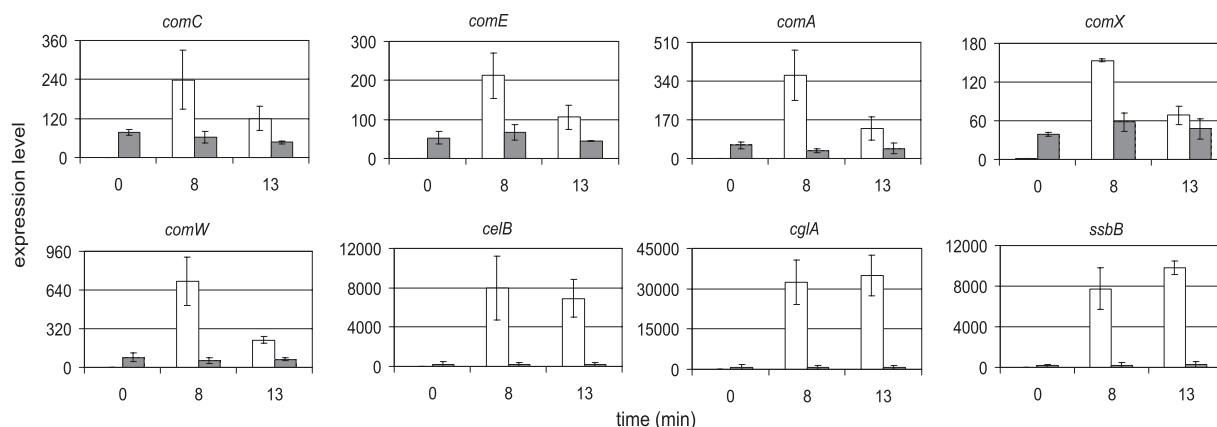


FIG. 3. Competence genes expression determined by qRT-PCR. Relative levels of mRNA of selected competence genes before (time zero) and 8 min and 13 min after the addition of CSP to wild-type (open columns) and *stkP* mutant bacteria (gray columns). mRNA levels in both wild-type and mutant are expressed relative to uninduced wild-type levels. Data are representative of two separate experiments. The data shown are means and standard deviations (error bars) of at least three technical replicates.

tion proficiency during transformation (46, 61). *recA* is the second gene in a *cinA-recA-dinF-lytA* late operon and, in competent cells, is induced from the competence-inducible promoter located in front of *cinA* (*Pcin*). In noncompetent cells, *recA* is expressed from its own σ^A -type promoter (46). The competence-specific induction of *recA* (that is, expression from *Pcin*) accounts for 95% of the yield of transformants. Thus, we conclude that low basal level expression of *recA* and possibly other DNA uptake and processing genes could be a major factor contributing to the reduced rate of transformation in the *stkP* mutant strain.

Responsiveness of competence genes to CSP induction in the wild-type and Δ *stkP* strains. The results from our global transcription analysis showed that the “core” early competence genes are induced in the *stkP* mutant during mid-exponential growth. In order to address whether an observed increase of expression of selected competence genes in the *stkP* mutant corresponds to the levels of the fully induced *com* regulon, we quantified the mRNA levels in the CSP-induced and uninduced cultures of both wild-type and mutant strains. *comC*, *comE*, *comA*, *comX*, and *comW* were selected as representatives of early genes. Cultures of both strains were grown in parallel to an OD_{400} of 0.4 (the same conditions as cultures used for microarray analysis) and sampled immediately before and after the addition of CSP (250 ng ml^{-1}) for the purification of RNA (see Materials and Methods). This dose of CSP was sufficient to induce full competence in the wild-type strain. To analyze the kinetics of the CSP response, samples were taken 8 and 13 min after CSP addition. As expected, in the wild-type strain, a low basal signal increase was observed for representatives of early genes (*comC*, *comE*, *comA*, *comX*, and *comW*) tested upon CSP treatment, reaching the maximum level at 8 min and then declining by 13 min (Fig. 3). This rapid response and sharp decline in mRNA levels are typical of what was previously reported for competence genes (1, 60, 69). In contrast, the level of the corresponding transcripts in the mutant strain, although significantly elevated, remained unaffected by the addition of CSP (Fig. 3). Transcription levels of the in-

duced genes in the wild-type strain reached values two- to eightfold higher than those measured in the *stkP* mutant.

Representatives of late competence genes, including *cglA*, *celB*, and *ssbB*, were analyzed in the same experimental setup. A large increase in transcript levels for these genes was observed upon CSP treatment in the wild-type cells at 8 min, reaching the expected maximum level at 13 min (Fig. 3). As before, the kinetics of late gene induction were in good agreement with those stated in previous reports (1, 15, 60, 61, 69). As observed with the early genes, the transcript levels of late competence genes were elevated and remained unaffected by CSP treatment in the *stkP* mutant. The induction amplitude of the late genes represented by *celB*, *cglA*, and *ssbB* showed a much higher increase (20- to 25-fold) (Fig. 3) relative to “induced” levels in the *stkP* background, suggesting that this increase of expression is likely not sufficient to derepress competence development. This result suggests that the reduced transformation efficiency in the *stkP* mutant is caused by weak induction of DNA uptake and processing genes. On the other hand, the elevated level of the competence regulon in the *stkP* mutant is sufficient for the increase of transformation frequency by a factor of 10^3 compared to that of the wild-type strain under conditions that repress competence development.

Competence development. To exclude the possibility that the observed failure of *stkP* strain cells to respond to exogenous CSP may result from an occurrence of a transient postcompetence period, when cells remain refractory to CSP (43), we performed the analysis of transcript levels as well as transformation assays at a lower optical density ($OD_{400} = 0.1$). This density was selected because the refractory phase persists for about 1 to 1.5 generations after spontaneous as well as CSP-induced development of competence (12, 43). The cell counts at given optical densities corresponded to approximately 1.5 generations. The transcript level of the sampled genes was essentially the same as that in the cultures at a higher cell density, suggesting that the absence of a CSP response in the mutant strain is not a consequence of a refractory period (data not shown). Competence assays were performed with cultures

TABLE 4. Transformation efficiencies of *S. pneumoniae* wild-type and Δ *stkP* mutant strains in the medium that did not allow natural competence development and after CSP treatment^a

OD ₄₀₀	<i>S. pneumoniae</i> strain	CSP induction ^b	Total count (CFU ml ⁻¹ [10 ⁷])	Transformants (CFU ml ⁻¹ [10 ⁴])	Transformation efficiency (%)
0.1	CP1015	No	4.85 ± 0.52	<0.003	<6.22E-05 ± 0.71E-05
		Yes	4.95 ± 0.54	102.00 ± 26.23	2.04 ± 0.32
	CP1015 Δ <i>stkP</i>	No	4.20 ± 0.38	4.50 ± 0.53	0.11 ± 0.01
		Yes	3.80 ± 0.68	9.25 ± 0.82	0.25 ± 0.04
0.4	CP1015	No	14.53 ± 0.64	<0.003	<2.07E-05 ± 0.09E-05
		Yes	11.58 ± 0.73	264.10 ± 17.15	2.29 ± 0.28
	CP1015 Δ <i>stkP</i>	No	10.00 ± 0.37	7.33 ± 0.49	0.07 ± 0.01
		Yes	11.91 ± 0.66	2.27 ± 0.38	0.02 ± 0.0

^a Transformation efficiencies were calculated as the ratio of the viable counts on plates with and without rifampin (see Materials and Methods). Results are expressed as the mean counts (\pm standard deviations) from three independent experiments.

^b Competence induction with CSP at 250 ng ml⁻¹.

of the wild-type and *stkP* mutant strains. Competence development was measured in cultures both induced by the addition of exogenous CSP and uninduced and grown in CAT medium, a broth which prevents Cp1015 from developing spontaneous competence. As expected, basal transformation efficiency was observed in the wild-type cultures in conditions that repressed competence development ($<10^{-5}\%$) (Table 4). In the isogenic *stkP* mutant strain, the yield of transformants attained a transformation efficiency \sim 1,000-fold higher than that of the wild type. The addition of CSP to the wild-type culture resulted in a 10^5 -fold increase in the transformation frequency (it reached 2.3%), whereas no change was observed in the *stkP* mutant strain. This result demonstrates that the transformation efficiency in the mutant strain is not affected by the addition of exogenous CSP. The transformation efficiencies of CSP-treated and untreated cells were similar to those obtained at an OD₄₀₀ of 0.4 (Table 4). Thus, it seems plausible that the competence regulon is permanently induced; however, the degree of induction does not reach sufficient levels for full activation of the competence cascade. Thus, the results of the transformation assays are consistent with those of qRT-PCR and microarray analysis.

Our finding that the basal-level expression of competence genes is increased and transformability is reduced is reminiscent of the phenotype reported for a particular *S. pneumoniae* mutant (24). This mutation, which is localized in the transcription terminator of the tRNA^{Arg} gene located immediately upstream of *comCDE*, was suggested to destabilize the terminator and to allow transcriptional read-through of *comCDE* (39). Contrary to expectation, an increased basal level expression of *comCDE* impeded both spontaneous and CSP-induced competence in *S. pneumoniae*. However, reduced competence and response to CSP can be bypassed either through the addition of synthetic CSP or by increasing CSP export capacity, e.g., overexpression of *comAB* (24). In contrast, the transformability of the *stkP* strain is unaffected by the addition of exogenous synthetic CSP. Evidently, the mechanism leading to an increased expression of competence genes in the *stkP* mutant is different, although its basis is at present unclear.

In summary, our microarray analysis of the transcription profile of a *stkP* mutant revealed that the posttranslational modification of an as-yet-unidentified target(s) affects the expression of many functionally different genes in *S. pneumoniae*.

Nevertheless, considering the fact that the contribution of *stkP* to virulence varies with the different *S. pneumoniae* strains and the site of infection (18), it is very likely that the impact on the repertoire of genes regulated will be strain dependent. It is becoming increasingly clear that genes encoding signaling proteins make different contributions to gene expression depending on the genetic background of the parental strain (25).

The broad pleiotropic effect of the *stkP* mutation is noteworthy. One possible explanation is that some phenotypic properties of the mutant strain can be ascribed to the deregulation of an essential GlnM enzyme (55), resulting in so-far-unidentified, although envisaged, alterations in the cell wall biosynthesis. These perturbations could be reflected in some features of the mutant strain likely involving susceptibility to stress conditions. Apart from this possibility, StkP could assume a direct role, interacting with transcription machinery. However, as StkP lacks canonical DNA binding domains and as a result is unlikely to directly regulate transcription, the input signal must be transmitted through an effector molecule. Considering that this effect is exerted to many different gene categories involved in diverse cellular processes, the existence of a common factor mediating the transfer of signal to transcription machinery seems plausible. We hypothesize that the α -subunit of RNA polymerase is a candidate for such an effector. Our studies revealed that RpoA is a substrate of StkP either in vivo (55) or in vitro (unpublished results). Its covalent modification, particularly at the C-terminal domain (23), can contribute to the altered efficiency of transcription initiation by changing its affinity for promoters or transcription factors (70). The identification of the target amino acids that are phosphorylated will provide the grounds for further investigation.

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