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Protein Serine/Threonine Kinase StkP Positively Controls Virulence and Competence in *Streptococcus pneumoniae*

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In the *Streptococcus pneumoniae* genome, *stkP*, encoding a membrane-associated serine/threonine kinase, is not redundant (L. Novakova, S. Romao, J. Echenique, P. Branny, and M.-C. Trombe, unpublished results). The data presented here demonstrate that StkP belongs to the signaling network involved in competence triggering in vitro and lung infection and bloodstream invasion in vivo. In competence, functional StkP is required for activation of *comCDE* upstream of the autoregulated ring orchestrated by the competence-stimulating peptide. This is the first description of positive regulation of *comCDE* transcription in balance with its repression by CiaRH.

The human pathogen *Streptococcus pneumoniae* is highly versatile. Its response to environment determines the bacterium's virulence and transformability as shown by mutational studies targeting metabolic (1, 16), regulatory (4, 11), transport (5, 19), and signaling (6, 8) functions. The factors so far identified which influence the fate of the bacterium include mainly the concentrations of divalent cations (5, 19, 20) and H^+ and dioxygen (6). Signaling involves two-component signal-transducing systems (6, 8, 13), an oxidase (7), and a global regulator (4). In the present work, we provide evidence for the role of the serine/threonine kinase StkP in competence signaling and in experimental virulence. StkP is membrane associated, carries the PASTA signature (22), and has been shown to avoid LytA-dependent autolysis induced by growth at alkaline pH and by low concentrations of cell wall-directed antibiotics. It is proposed that virulence expression and competence development represent population responses to cell wall stress induced in specific growth conditions.

The rough *S. pneumoniae* RX derivatives (19) carrying the *lytA* mutation (17) and the smooth serotype 2 (2) strain D39 and serotype 6 strain 23477 (1) were used for competence tests and virulence studies, respectively. Bacterial growth and storage were as previously described (12, 17, 19). An insertion mutation in *stkP* was obtained in vitro with the pBluescript derivative (15) plasmid pPHK29 carrying a 2.96-kbp EcoRI/SalI amplimer containing *stkP* from the RX chromosome. The 1.3-kbp BamHI fragment from pPJ1 (14) containing the *aphA3* cassette was inserted into a BglII site of *stkP* to give the mutagenic plasmid pPKB3. The mutated *stkP*::*aphA3* allele was introduced by genetic transformation into the relevant genetic backgrounds, and strains carrying the allelic exchange were selected on kanamycin (50 mg/liter) plates as described previously (6). Recombinant clones were verified by PCR, and it has been verified that the *stkP*::*aphA3* mutation did not affect bacterial growth of the different strains in vitro and also that the insertion mutation was very unlikely to impact the expression of downstream genes in the region. Indeed, with the software described in reference 9, it is predicted that genes SP1731 and SP1732 following the *stkP* stop codon show no relationship with *stkP*. Moreover, in the 118-bp intergenic region between *stkP* and SP1731, we found a stem-loop with free energy of −6.7 kcal/mol. This DNA structure shows the features of a putative transcription terminator for *stkP*.

In order to assess the importance of StkP in the virulence of the pneumococcus, mice were intranasally challenged with strains D39 and 23477 and their corresponding *stkP*::*aphA3* derivatives. Figure 1A illustrates the fate of both D39 and 23477 strains after intranasal challenge of the mice, notably with regards to recovery of CFU from lungs and blood at 48 h postinfection. Data presented in Fig. 1B and C clearly indicate that the *stkP*::*aphA3* mutation significantly reduces the virulence of both strains, as shown by analysis of variance giving a P value of <0.05. The numbers of CFU of each of the two *stkP*::*aphA3* mutant strains were significantly lower than those of the corresponding wild-type strains, with a striking elimination of the 23477 *stkP*::*aphA3* bacteria from the lungs and the blood at 48 and 24 h postinfection, respectively. This strong effect of the *stkP* mutation on bloodstream invasion incited us to evaluate the role of StkP specifically during growth in the bloodstream. Results presented in Fig. 2 indeed show a requirement for StkP for optimal growth in blood after intravenous injection ($P < 0.05$); however, comparison of the numbers obtained in both models for each couple of strains indicates that StkP increased invasion efficiency by 2 orders of magnitude in the intravenous challenge and by 4 to 6 orders of magnitude in the intranasal challenge. This suggests an important role for the protein specifically in successful access into the

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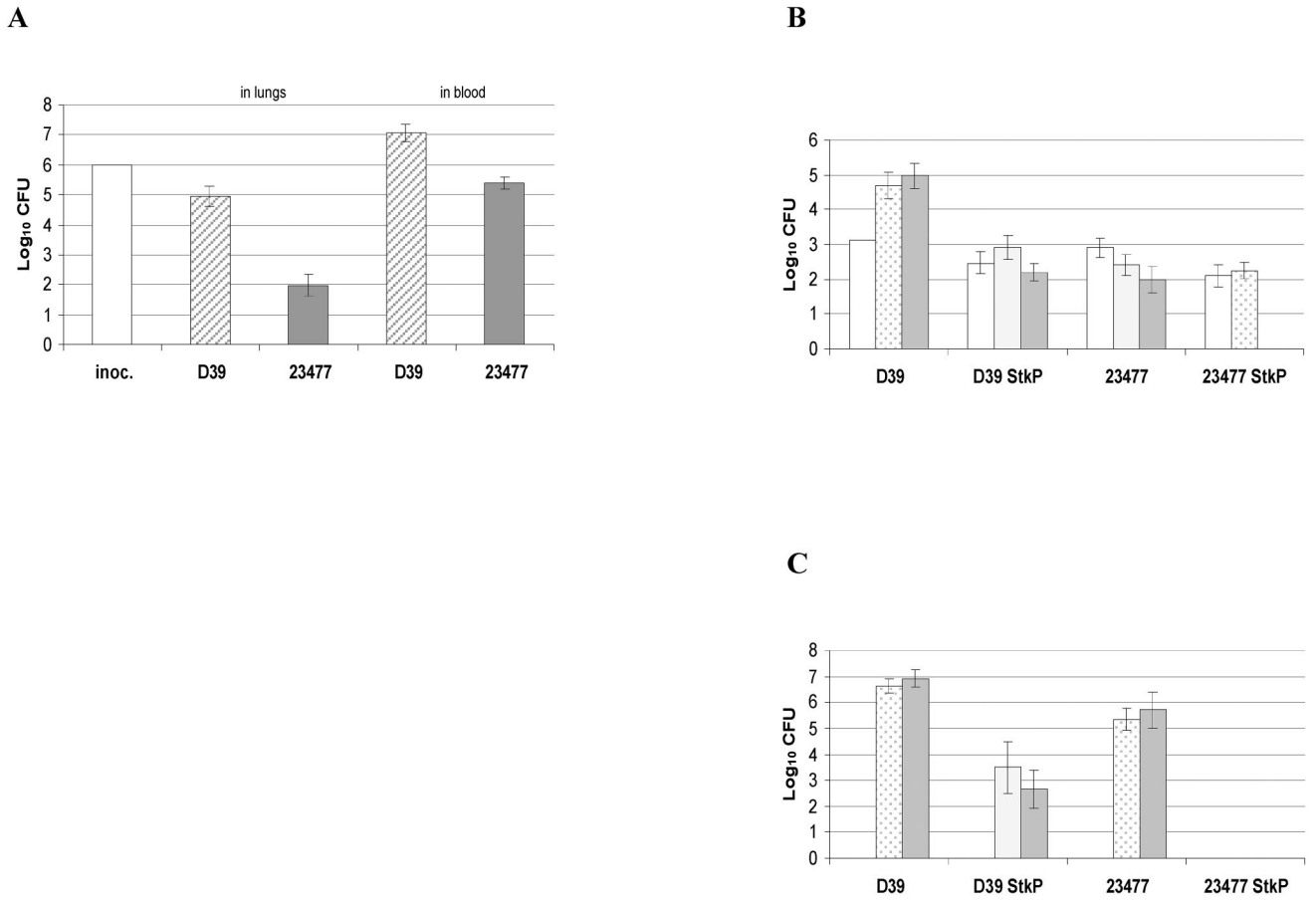


FIG. 1. Positive impact of StkP on growth in lungs and bloodstream invasion. (A) Bacteria from the wild-type strains D39 and 23477 were used for intranasal infection of female MF1 outbred mice as described in reference 12. At 48 h, CFU were recovered from the lungs and blood as described in reference 12. inoc., inoculum. (B) CFU recovery from lungs after intranasal challenge with the wild-type strains and their *stkP*::*aphA3* mutant derivatives. Infection was as described for panel A. CFU were recovered from lungs at 15 min (white bars), 24 h (dotted bars), and 48 h (grey bars). (C) CFU recovery from blood. The conditions of infection were as described for panel A, and CFU recovery was at 24 h (dotted bars) and 48 h (grey bars). Five mice were used at each time point, and suspensions of 10^6 CFU in 50 μl of phosphate-buffered saline were used for infection. The results are expressed as numbers of CFU recovered per milliliter of blood (C) and per lung (B), and the standard error of the mean (SEM) is given.

bloodstream in addition to the requirement for StkP for optimal growth in blood.

The role of StkP in the development of competence during growth has been assessed using the RX derivative, strain Cp9090 carrying the *stkP*::*aphA3* mutation in the *lytA* genetic background, in order to avoid autolysis at alkaline pH (Novakova et al., unpublished). Expression of the central competence operon *comCDE* and subsequent expression of bacterial transformability have been investigated by measuring the cellular levels of *comCDE* transcripts and transformant recovery in cultures. Results of Northern blotting of *comCDE* mRNA and the yields of transformants in cultures correlated and revealed central and positive control of *comCDE* expression by StkP (Fig. 3A). The level of *comCDE* mRNA in aerobic cultures is modulated by negative control due to CiaRH, as shown by uniform expression in strains defective for CiaRH compared to that in the wild type (6). Results presented in Fig. 3B show that the *ciaR*::*spc* mutation restored transformability of bacteria carrying the *stkP*::*aphA3* mutation, and this finding was correlated with the cellular level of *comCDE* transcripts (data not shown). Measurements of transformant yields for strains Cp9090 (*stkP*::*aphA3*), Cp1890 (*ciaR*::*spc*), and Cp9890 (*stkP*::*aphA3 ciaR*::*spc*) clearly indicate that the *ciaR*::*spc* mutation induced only partial reversion of the competence defect due to the *stkP*::*aphA3* mutation (Fig. 1 and B2). These results

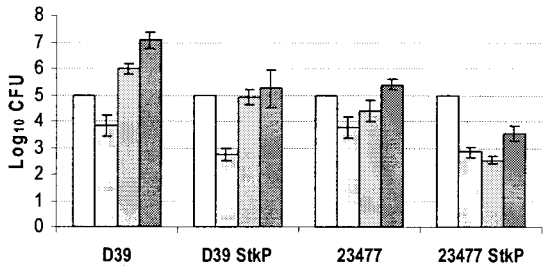
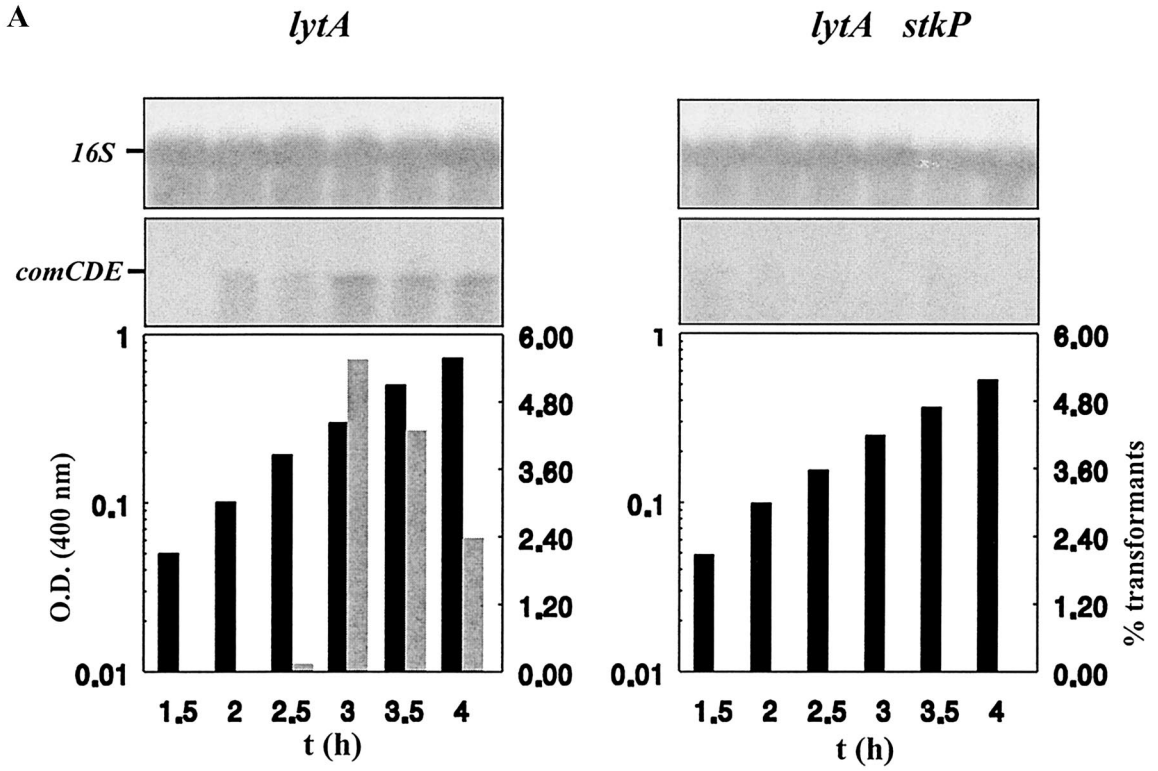


FIG. 2. StkP and bloodstream invasion. Suspensions of 10^5 bacteria in 50 μl of phosphate-buffered saline were injected intravenously into a tail vein, and growth in blood was determined by counting CFU at 15 min (white bars), 2 h (dotted bars), 24 h (grey bars), and 48 h (dark grey bars). Five mice were used at each time point. Data are expressed as numbers of viable CFU per milliliter of blood, and the SEM is given.



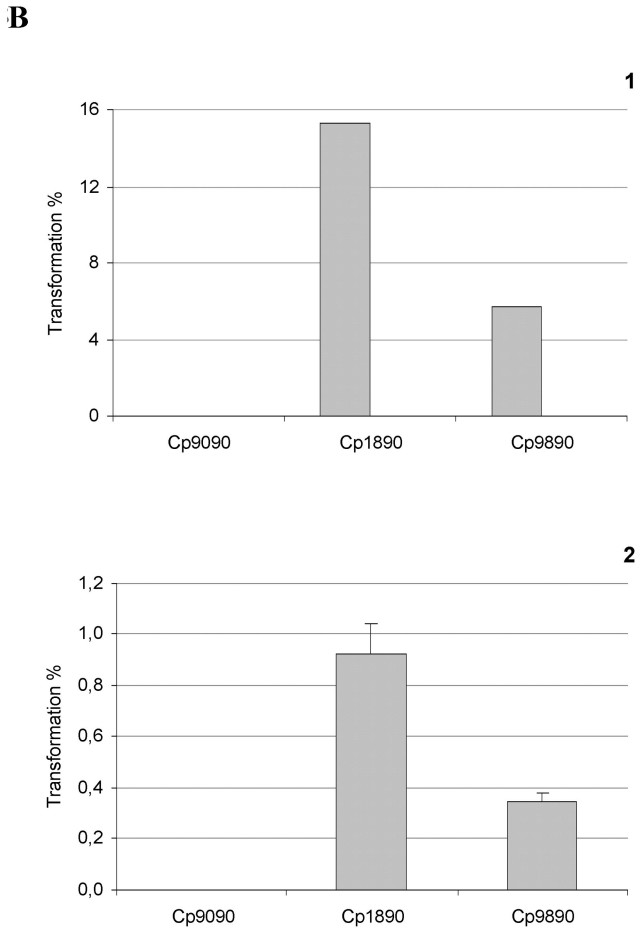


FIG. 3. Positive control of *comCDE* mRNA cellular levels by StkP and impact on transformability in the wild-type and *ciaR*::*spc* genetic backgrounds. (A) Strains Cp1095 (*lytA stkP*$^{+}$) and Cp9090 (*lytA stkP*) were grown aerobically in static flasks, and samples were withdrawn at 30-min intervals and analyzed for biomass increase (black bars), transformability (grey bars), and cellular levels of *comCDE* mRNA. Methods and material were as described in reference 8. The level of specific mRNA in cultures was evaluated by Northern blotting of total cellular RNA with the appropriate probes, and 16S rRNA was used as an internal qualitative and quantitative control. The columns representing growth, measured by comparing optical densities at 400 nm [O.D. (400 nm)], and transformability as the percentage of transformants (transformed by *rifR* chromosomal DNA) recovered relative to the total population are aligned with the Northern blot signals corresponding to the same culture. The experiment was repeated with independent cultures to test reproducibility. t, time. (B) The *ciaR*::*spc* mutation partially restores transformability of the *stkP*::*aphA3* mutant strain. Both in situ transformation tests (4) (B1) and liquid transformation tests (8) (B2) were performed with strains Cp9090 (*stkp*::*aphA3*), Cp1890 (*ciaR*::*spc*), and Cp9890 (*ciaR*::*spc stkP*::*aphA3*). In panel B1, the data are expressed as the mean of results from two independent experiments, the results of which differed by less than 10%. In panel B2, the data are expressed as the mean of results from three independent experiments and the SEM is given.

provide credence to the idea of an antagonistic role of CiaRH and StkP in *comCDE* regulation and demonstrate for the first time positive control of *comCDE* expression by StkP. In the absence of StkP and CiaRH, residual competence in strain Cp1890 may either correspond to basal *comCDE* expression or reveal additional inputs upstream of the competence-stimulating peptide autoregulated ring.

Taken together, the results provided demonstrate that in *S. pneumoniae*, virulence and genetic transformability are positively controlled by the membrane-associated serine/threonine kinase StkP. StkP positively impacts activation of the central

competence operon *comCDE* in balance with CiaRH repression, determining the developmental profile of competence expression in strain RX. In experimental virulence, StkP is crucial for lung infection and bloodstream invasion by both virulent strains D39 and 23477.

Bacterial serine/threonine kinases have been implicated in the control of virulence, notably in *Yersinia* and pseudomonads, although the inputs and outputs of serine/threonine kinase signaling are not well described (10–21; Novakova et al., unpublished). The recent identification of PASTA motifs in penicillin binding proteins and serine/threonine kinases from gram-positive bacteria led researchers to propose a role for these PASTA motifs in the interaction between these proteins and the cell wall (22). Indeed, in *S. pneumoniae* the protein is membrane associated and is required to bypass LytA-dependent autolysis triggered by growth at alkaline pH and by the cell wall-directed inhibitors penicillin G and vancomycin (Novakova et al., unpublished). Now we have shown additional phenotypes related to StkP deficiency. On the one hand, StkP is required for the expression of the central competence operon *comCDE*. Interestingly, competence development occurs during exponential growth in alkaline medium, conditions requiring StkP to avoid bacterial autolysis. It is possible that pH stress constitutes the trigger for *comCDE* expression, culminating in competence development. On the other hand, StkP deficiency dramatically reduced virulence of strains D39 and 23477. Protection against autolysis may account for the positive role of StkP in bacterial virulence. It must be noticed, however, that a LytA loss-of-function mutation attenuates virulence (3). Alternatively, StkP may constitute a checkpoint determining the autolysis rate and liberation of appropriate levels of inflammatory molecules, allowing successful infection by a given population.

To conclude, we propose a pivotal role for StkP in pneumococcal virulence and in horizontal genetic transfer in addition to its role in the response to pH and to low concentrations of cell wall-directed antibiotics. This membrane-associated protein belongs to the signaling network allowing the pneumococcus to sense and respond to its environment both in vivo and in vitro. StkP is required to overcome low concentrations of cell wall-directed inhibitors, suggesting involvement of StkP in the response to cell wall stress (Novakova et al., unpublished). Although other kinds of StkP-mediated signaling have not been excluded, it is likely that cell wall defects induced in specific growth conditions play a central role in the fate of *S. pneumoniae* in vitro and in vivo.

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