# Characterization of a eukaryotic type serine/threonine protein kinase and protein phosphatase of *Streptococcus pneumoniae* and identification of kinase substrates

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Searching the genome sequence of *Streptococcus pneumoniae* revealed the presence of a single Ser/Thr protein kinase gene *stkP* linked to protein phosphatase *phpP*. Biochemical studies performed with recombinant StkP suggest that this protein is a functional eukaryotic-type Ser/Thr protein kinase. *In vitro* kinase assays and Western blots of *S. pneumoniae* subcellular fractions revealed that StkP is a membrane protein. PhpP is a soluble protein with manganese-dependent phosphatase activity *in vitro* against a synthetic substrate RRA(pT)VA. Mutations in the invariant aspartate residues implicated in the metal binding completely abolished PhpP activity. Autophosphorylated form of StkP was shown to be a substrate for PhpP. These results suggest that StkP and PhpP could operate as a functional pair *in vivo*. Analysis of phosphoproteome maps of both wild-type and *stkP* null mutant strains labeled *in vivo* and subsequent phosphoprotein identification by peptide mass fingerprinting revealed two possible substrates for StkP. The evidence is presented that StkP can phosphorylate *in vitro* phosphoglucosamine mutase GlmM which catalyzes the first step in the biosynthetic pathway leading to the formation of UDP-*N*-acetylglucosamine, an essential common precursor to cell envelope components.

In recent years, analysis of bacterial genomes revealed the widespread presence of eukaryotic-type Ser/Thr protein kinase as well as protein phosphatase genes in many bacteria. In several cases the genes encoding both enzymes are genetically linked and it has been demonstrated that the respective gene products play antagonistic roles in regulation [1–3]. Although bacterial homologues of eukaryotic-type enzymes have been identified and biochemically characterized, their functions are not well understood because of the lack of information on their endogenous targets and activating signals.

Ser/Thr protein kinases (STPK) are represented by multigene families in *Streptomyces*, *Mycobacterium*, *Myxococcus*, and *Cyanobacteria* [4–7]. These bacterial groups display complex life cycle including multistage cellular differentiation and the presence of multiple protein kinase genes seems to be associated with this behavior. However, the redundancy of STPKs in these microorganisms is a major hindrance in the study of their physiological function. It was recently demonstrated that AfsR, *Streptomyces coelicolor* transcriptional activator, could be phosphorylated by several endogenous protein kinases suggesting substrate

interchangeability at least *in vitro* [8]. In addition, the phenotypes of many of the single knockouts are relatively weak and the function of particular protein kinase cannot be clearly assigned. *Streptococcus pneumoniae*, with its one pair of protein kinase and phosphatase, provides a good model to study the role of serine–threonine phosphorylation in prokaryotes. Recently, it has been demonstrated that the disruption of *stkP* gene resulted in repression of genetic transformability and virulence of *S. pneumoniae*, suggesting an important role for StkP in the regulation of various cellular processes [9]. There are only a few examples of such significant impact of an inactivation of single STPK on the phenotype affecting physiological functions [3,10,11].

A few target substrates for bacterial STPKs have been identified so far. Most of them were identified due to the presence of their genes in the close vicinity of cognate protein kinase genes [12–14]. Another approach which could make the identification of substrates of prokaryotic STPKs possible is a comparative analysis of phosphoproteome maps of both wild-type and corresponding mutant strains. Surprisingly, this approach has not been widely used. On the other hand, in the only article reporting a comprehensive analysis of bacterial phosphoproteome no phosphoproteins with evident regulatory functions were detected [15]. In this work, we show that recombinant StkP is a functional protein kinase with Ser/Thr specificity. We also show that its cognate protein phosphatase, PhpP, dephosphorylates specifically autophosphorylated StkP and that its activity is strictly dependent on the presence of manganese ions. In order to find out the substrate(s) of protein kinase StkP, we prepared deletion of the corresponding gene in *S. pneumoniae* by PCR ligation mutagenesis and allelic exchange. Cultures of the wild-type as well as *stkP* null mutant strains were labeled *in vivo* with [$^{33}$P]orthophosphate and soluble proteins were separated by two-dimensional gel electrophoresis. Mass spectrometry analysis identified six phosphorylated proteins. Besides the phosphoproteins which are present in both the wild-type and mutant strains two likely substrates of StkP were absent in mutant strain. We bring evidence that phosphoglucosamine mutase GlmM, one of the putative protein kinase targets identified, undergoes direct phosphorylation by StkP. This is the first example of an endogenous protein substrate modified by a serine/threonine kinase in *S. pneumoniae*.

In addition, this is the first report in which analysis of two-dimensional phosphoproteome maps of both the wild-type and STPK loss-of-function mutant led to identification of protein kinase target in prokaryotes.

## Results and Discussion

### StkP is a protein Ser/Thr kinase capable of autophosphorylation

To characterize a putative protein kinase StkP, the *stkP* gene and its truncated form containing kinase domain were cloned in pET28b and expressed as His-tagged proteins in *E. coli* BL21(DE3). To rule out the possibility that the proteins synthesized in *E. coli* could be phosphorylated by an endogenous protein kinase activity rather than by an autophosphorylation process, the essential lysine residue of catalytic subdomain was replaced by arginine. A *stkP* gene with a Lys-to-Arg change (pEXstkP-K42R) was also expressed in *E. coli*. Total cellular extracts were analyzed for autophosphorylation activity in *in vitro* kinase assay. After incubation, the products were separated by SDS/PAGE and phosphorylated proteins were identified by autoradiography. Both full-length and truncated forms of StkP were detected as phosphorylated products migrating with an expected mobility (Fig. 1A, lanes 2 and 3, respectively). However, about 50% decrease of $^{32}$P incorporation into truncated form of StkP was observed by comparing bands intensity. Therefore, it can be concluded that the truncated form of StkP has altered kinetic parameters. Phosphoamino acid analysis of $^{32}$P-labeled StkP showed that full-length protein was phosphorylated by its intrinsic activity predominantly at the threonine residue and weakly at the serine residue (Fig. 1C). Replacement of an essential lysine residue in subdomain II involved in phosphotransfer reaction resulted in a dramatic reduction of phosphorylation, although the mutated protein showed residual 13% activity (Fig. 1B, lane 2). A similar feature was observed when *Pseudomonas aeruginosa* protein kinase PpkA was mutated [16]. Probably, in some particular cases, this mutation is insufficient to explain the complete loss of activity and an extensive mutational analysis of other residues involved in phosphotransfer reaction is needed.

As oligohistidine-tagged StkP was not capable of binding to metal affinity column, a GST-chimeric protein was also engineered and expressed in *E. coli*. Soluble fusion enzyme was purified by affinity chromatography, and GST-tag was cleaved with factor Xa as described in Experimental procedures. The purified protein was analyzed for its cation requirements in a standard kinase assay with variable divalent cation concentrations (Fig. 1D). $Mn^{2+}$ cation was much more effective as a cofactor than $Mg^{2+}$. Maximal activation was induced in the range of 0.5–1 mM, while concentrations between 5 and 10 mM were required for $Mg^{2+}$.
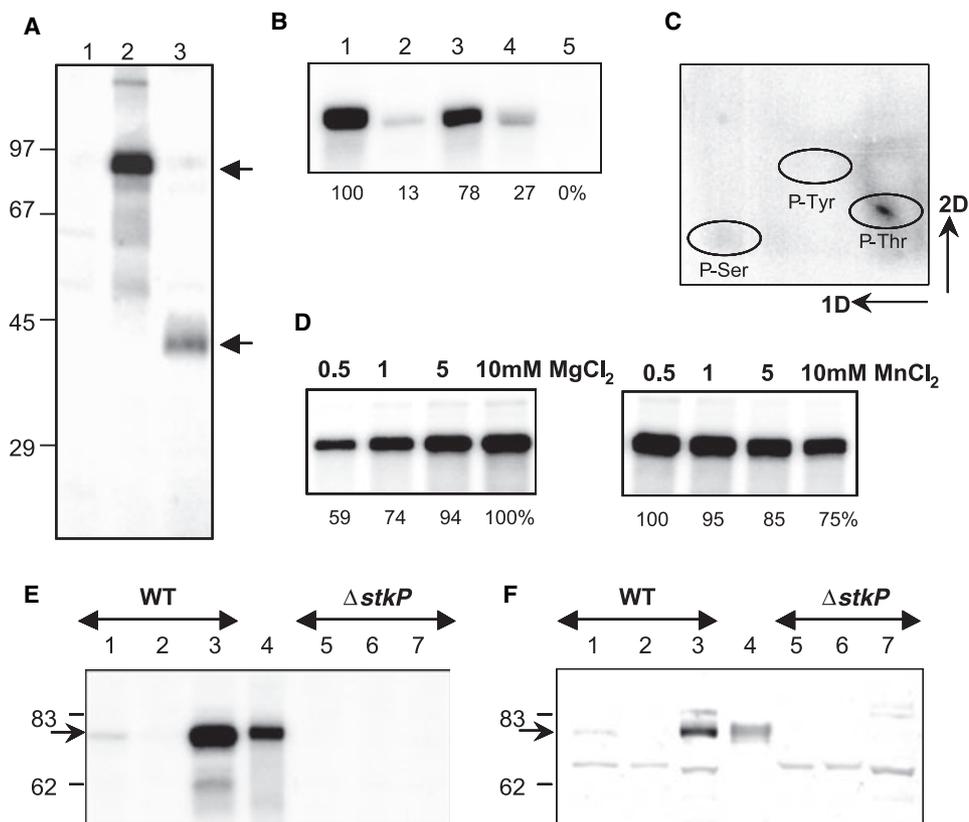
**Fig. 1.** Biochemical properties of StkP and its cellular localization in *S. pneumoniae*. (A) *In vitro* phosphorylation of His-tagged StkP (lane 2) and its truncated form StkP-T (lane 3) in *E. coli* cell-free lysates. Cell-free lysate of *E. coli* bearing empty vector pET28b was used as a control (lane 1). Arrows indicate the phosphorylated forms of StkP (72.4 kDa) and StkP-T (30.1 kDa). Molecular mass standards are indicated on the left side. (B) Effect of kinase inhibitors and essential lysine substitution on StkP activity. Autophosphorylation of purified StkP in the presence of 1 mM $MnCl_2$ (lane 1) was estimated as a basal level activity (100 %) and compared with the activity of mutated enzyme StkPK42R (lane 2) and StkP in the presence 0.1 mM, 1 mM and 10 mM of staurosporine (lanes 3, 4, and 5, respectively), a protein kinase inhibitor. Relative kinase activities are indicated in percents (bottom). (C) 2D analysis of phosphorylated amino acids. The acid-stable phosphoamino acids from $^{32}P$-labeled StkP were separated by electrophoresis in the first dimension (1D) followed by ascending chromatography in the second dimension (2-D). (P-Tyr) phosphotyrosine, (P-Thr) phosphothreonine, (P-Ser) phosphoserine. (D) Effect of cations on StkP activity *in vitro*. *In vitro* phosphorylation reaction was carried out using purified recombinant StkP in a reaction buffer supplied with 0.5 mM, 1 mM, 5 mM, 10 mM $MnCl_2$ or $MgCl_2$. Relative kinase activities are indicated in percents (bottom). (E) and (F) Subcellular localization of StkP in a wild type strain *S. pneumoniae* (WT) and in a *stkP* null mutant strain (Δ*stkP*). (E) *In vitro* phosphorylation of total cell free extract (lanes 1 and 5), cytosolic fraction (lanes 2 and 6) and membrane fraction (lanes 3 and 7) of *S. pneumoniae* strains. Purified recombinant StkP was used as a control (lane 4). (F) Immunodetection with specific polyclonal antiserum raised against recombinant StkP in a total cell-free extract (lanes 1 and 5), cytosolic fraction (lanes 2 and 6) and membrane fraction (3 and 7) of *S. pneumoniae* strains. Purified recombinant StkP was used as a control (lane 4). Arrows indicate bands corresponding to StkP. Molecular mass standards are indicated on the left. Relative kinase activities in percents were determined as the intensity of phosphorylated band evaluated with AIDA 2.11.

StkP was active over the wide range of pH from 3 to 9 (not shown). The effect of staurosporine, a potent protein kinase inhibitor was also examined. Pre-incubation of inhibitor with StkP inhibited its kinase activity in a dose-dependent manner (Fig. 1B, lanes 3–5).

## Subcellular localization of StkP in *S. pneumoniae*

The hydropathy profile of StkP revealed the presence of a unique hydrophobic domain, consisting of an 18-residue apolar stretch, suggesting that it could correspond to a transmembrane region anchoring StkP to the membrane. *In vitro* kinase assays and immunodetection were used to localize StkP in fractionated cell-free lysates of the wild-type *S. pneumoniae* and *stkP* deletion mutant strains (Fig. 1E). In the wild type a phosphorylated protein of the molecular mass corresponding to that of purified StkP was detected in either crude extract or membrane fraction (Fig. 1E, lanes 1 and 3). This phosphoprotein was missing in the

subcellular fractions of Δ*stkP* strain (lanes 5–7). Immunodetection with polyclonal antiserum confirmed these results (Fig. 1F). These results clearly showed that native pneumococcal StkP is capable of auto-phosphorylation *in vitro* and it is indeed a membrane protein as was predicted from amino acid sequence.

## PhpP is PP2C-type protein phosphatase

To characterize a putative protein phosphatase PhpP, the *phpP* gene was cloned in pET28b and expressed in *E. coli* BL21 (DE3). Mutant alleles were prepared where the essential aspartate residues in the 8th and 11th conserved motifs were replaced by alanine. Aspartate residues corresponding to D192 and D231 of PhpP are directly involved in metal ions binding and are known to be essential for the activity of eukaryotic PP2C phosphatases [17]. *phpPD192A* and *phpPD231A* alleles were cloned in pET28b plasmid and expressed in *E. coli*. All PhpP proteins fused with His-tag were purified by an affinity chromatography. The phosphatase activity of the purified PhpP was measured using a ser-ine/threonine phosphatase assay system (Promega). Figure 2A shows that PhpP has the significant protein phosphatase activity on phosphopeptide RRA(pT)VA only in the presence of $Mn^{2+}$ but not of other divalent cations, such as $Mg^{2+}$ or $Ca^{2+}$ (not shown). The optimal $Mn^{2+}$ concentration was found to be 10 mM. The preference for $Mn^{2+}$ over $Mg^{2+}$ is similar to that of the Stp1 phosphatase of *P. aeruginosa* [18] and Pph1 phosphatase of *M. xanthus* [19], rather than the mam-malian PP2C protein phosphatases, which prefer $Mg^{2+}$ [20]. Inhibitors such as NaF inhibited the PhpP activity at 50 mM concentration. Okadaic acid, a potent inhib-itor of PP2A and PP2B family of phosphatases [21], did not inhibit PhpP, which is one of the unique char-acteristics of the PP2C family of phosphatases (Fig. 2B). Thus, PhpP is indeed a PP2C phosphatase. In addition, Ala missense mutations of either of the two invariant aspartate residues in the subdomain VIII and XI, which are implicated in the metal binding, completely abolished PhpP activity. Neither PhpP (D192A) nor PhpP (D231A) was active against phos-phopeptide substrate confirming their involvement in PhpP function. This is the first direct evidence that the conserved aspartate residues are necessary for bacterial PP2C phosphatase activity.

## StkP and PhpP are functionally coupled

Sequence analysis revealed a four-nucleotide overlap between *phpP* and *stkP*; it is therefore suggested that these two genes might be tightly coregulated at the
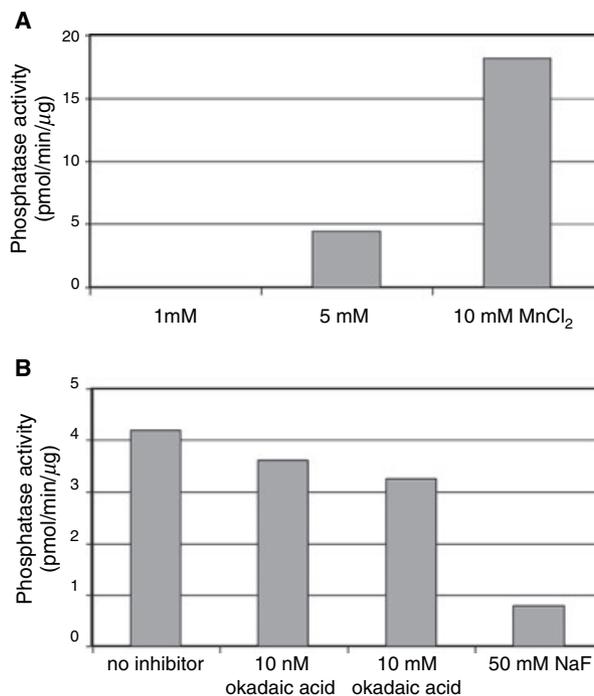


**Fig. 2.** Biochemical properties of PhpP. Phosphatase activity was determined as a concentration of free phosphate released from phosphorylated peptide RRA(pT)VA due to the catalytic activity of purified HIS-tagged PhpP and is expressed in $pmol \cdot min^{-1} \cdot \mu g^{-1}$ on the *y*-axis. See Experimental procedures for details of the assay. (A) Effect of $MnCl_2$ concentration on PhpP activity. (B) Effect of phosphatase inhibitors on PhpP activity.

transcriptional level. To test this hypothesis we per-formed RT-PCR analysis on RNA isolated from dif-ferent cultures of the wild-type bacteria using various combinations of primers (Table 1). As shown in Fig. 3A, the fragments of the expected lengths were generated by RT-PCR in RNA samples from bacteria growing in CAT medium and at different stages in growth from early exponential to stationary phase. Based on the results of RT-PCR analysis, we conclu-ded that *phpP* and *stkP* genes are transcribed as a single mRNA molecule. Because both genes are gen-etically linked their functional coupling seemed very likely. To test this hypothesis, we examined dephos-phorylation of autophosphorylated StkP by PhpP. The purified protein kinase was first incubated under optimal conditions for autophosphorylation with $[^{32}P]ATP[\gamma P]$. The radiolabeled enzyme was then mixed with purified PhpP. The results presented in Fig. 3B clearly indicate that in these conditions, StkP was extensively dephosphorylated by PhpP. These data provide evidence that PhpP can use StkP as an endo-genous substrate and support the concept that enzy-matic activity of both enzymes operate as a functional

**Table 1.** List of primers used in this study.

| Primer | Sequence (restriction site underlined) | Restriction site | Purpose |
|---|---|---|---|
| STKP-F | 5′-AGGATGCCATATGATCCAAATCGGCAA-3′ | *Nde*I | stkP expression |
| STKP-R | 5′-TTGATTATGAATTCGCTTTTAAGGAGTAGC-3′ | *Eco*RI | stkP expression |
| STKP-RT | 5′-GTAGGACAGAATTCAAGACAAGTCTACATACA-3′ | *Eco*RI | stkP expression |
| SMUT | 5′-TCCTCAGTACTCTCCACTGCCACT-3′ | *Sca*I | stkP mutagenesis |
| STKP-FNco | 5′-GGATGCACCATGGTCCAAATCGGC-3′ | *Nco*I | stkP expression |
| PHPP-F | 5′-GGACTGACATATGGAAATTTCATTA-3′ | *Nde*I | phpP expression |
| PHPP-R | 5′-CTTGCGAATTCGGATCATTCTGCATCC-3′ | *Eco*RI | phpP expression |
| PMUT1 | 5′-CTCGATAGTGCCGGCTTGACC-3′ | *Nae*I | phpP mutagenesis |
| PMUT2 | 5′-GCAGGAGGCCTAGCCAACATT-3′ | *Stu*I | phpP mutagenesis |
| PGM-F | 5′-GAACTGACATATGGGTAAATATTTTGGG-3′ | *Nde*I | glmM expression |
| PGM-R | 5′-CCGCTCGAGTTAGTCAATCCCAATTTCAGC-3′ | *Xho*I | glmM expression |
| UFKFP | 5′-CGCGAATTCCGCAAGATATCGGATTAGGAA-3′ | *Eco*RI | stkP deletion |
| UFKRP | 5′-CGCGGATCCCTTGCCGATTTGGATCATTC-3′ | *Bam*HI | stkP deletion |
| DFKFP | 5′-GCTCTAGAATCTACAAACCTAAAACAAC-3′ | *Xba*I | stkP deletion |
| DFKRP | 5′-TGCCCGCGGTCATAATATCACGGACCGCAT-3′ | *Sac*II | stkP deletion |
| CAT1 | 5′-CGCGGATCCGAAAATTTGTTTGATTTTTAA-3′ | *Bam*HI | stkP replacement |
| CAT2 | 5′-GCTCTAGAAAGTACAGTCGGCATTAT-3′ | *Xba*I | stkP replacement |
| PRTI | 5′-CAATTGACCAGCCTTGAGCA-3′ | | phpP RT-PCR |
| PRT-F | 5′-ATAGCACCTGCACTATCGTCT-3′ | | phpP RT-PCR |
| PRT-R | 5′-CGCTCGTCAACTGATGGTATT-3′ | | phpP RT-PCR |
| SX | 5′-GAACAATTCCTCGAGTATGG-3′ | | stkP RT-PCR |
| KRT-F | 5′-CGGCAAGATTTTTGCCGGAC-3′ | | stkP RT-PCR |
| KRT-R | 5′-GCGCATAGCCAAGAGAATTTG-3′ | | stkP RT-PCR |
| Cp | 5′-GCAGGTTTAGACCAACATTA-3′ | | *phpP-stkP* RT-PCR |

couple. Similar genetic linkage of Ser/Thr protein kinase and phosphatase genes is found in many bacteria. However, the functional coupling of these enzymes was demonstrated only in few cases [1,3,18].

## Analysis of phosphoproteome maps revealed differences between the wild-type and ΔstkP strains

The Coomassie blue-stained master gel of proteins between p*I* 4–7 contains approximately 470 protein spots. After metabolic labeling and subsequent 2-DE, at least 23 protein spots could be reproducibly detected (Fig. 4). Ten identical phosphoprotein spots were detected on both wild-type and mutant phosphoprotein maps. Further analysis revealed that five phosphoprotein spots were absent on the mutant map in comparison to the wild-type two-dimensional pattern. On the contrary, eight additional spots were assigned to the mutant map. Out of all the detected phosphoprotein spots, six of them were well separated and in the quantities sufficient for MALDI-TOF-MS identification. Four phosphorylated proteins were identified being present in the wild-type as well as mutant strains (Fig. 4, spots P3-6, and Table 2). Phosphoglycerate kinase and fructose-1,6-bisphosphate aldolase are glycolytic enzymes, and phosphodeoxyribomutase is involved in a pentose phosphate pathway. The presence of phosphorylated forms of these metabolic enzymes which are probably phospho-enzyme intermediates has already been described in *Corynebacterium glutamicum* [15]. Thus far, their presence in both the wild-type and mutant strains is not surprising and did not result from StkP activity. The fourth identified phosphoprotein which was identified in both the wild-type and mutant strains is S1 ribosomal protein involved in RNA binding. Phosphorylation of this protein on serine residue was described in *E. coli* [22] and *C. glutamicum* [15]. The significance of its modification and nature of modifying enzyme remains unclear.

One of the phosphoproteins which is absent in mutant strain was identified as α-subunit of RNA-polymerase (RNAP). Transcriptional activator proteins in bacteria often operate by interaction with the C-terminal domain of the α-subunit of RNAP [23]. The possibility that this interaction might be affected by covalent modification of RNAP is intriguing. However, it is not clear at the moment if observed phosphorylation of S1 protein and α-subunit of RNAP are important for their interaction. The interaction of RNAP and S1 protein has already been described in *E. coli* and resulted in significant stimulation of the RNAP transcriptional activity from a number of promoters *in vitro* [24].
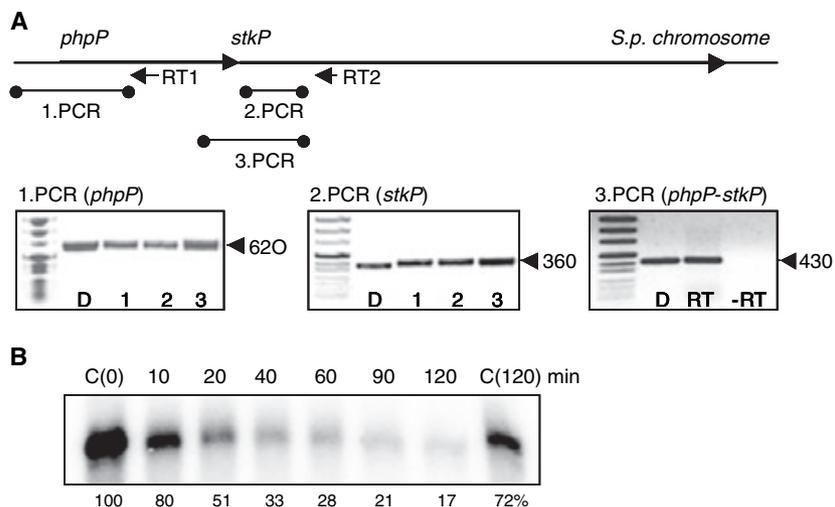
**Fig. 3.** Transcriptional and functional coupling of StkP and PhpP. (A) RT-PCR analysis of stkP and phpP expression. Total RNA was extracted from cells grown in CTM medium and harvested in precompetent (1), competent (2) and postcompetent (3) state. Control PCR was performed using genomic DNA as template (D). RT-PCR was performed as described in Materials and methods with following primers: PRTI (RT1), PRT-F and PRT-R (1.PCR) for RT-PCR of phpP; SX (RT2), KRT-F and KRT-R (2.PCR) for RT-PCR of stkP. The transcriptional coupling of phpP-stkP was tested on total RNA (RT) isolated from postcompetent cells using primers SX (RT2), Cp and KRT-R (3.PCR) for RT-PCR. Control PCR was performed using genomic DNA as template (D) and total RNA without prior reverse transcription (-RT). Arrows and numbers indicate the position and size (bp) of specific amplification product. DNA ladder from above: 1116, 883, 692, 501, 404, 331, 242, 190, 147, 110 bp. (B) Dephosphorylation of autophosphorylated StkP by PhpP. Phosphorylated StkP was incubated with PhpP in phosphatase buffer containing $Mn^{2+}$ as described in the Experimental procedures. Aliquots of the reaction were removed at various time intervals (0–120 min) and the reaction products were analyzed on SDS/PAGE. C(0): autophosphorylated StkP at 0 min in phosphatase reaction buffer; C(120): autophosphorylated StkP at 120 min in phosphatase reaction buffer.

The second putative substrate of StkP kinase determined is the phosphoglucosamine mutase (GlmM). This enzyme catalyzes the interconversion of glucosamine-6-phosphate (GlcN-6-P) and GlcN-1-P isomers, the first step in the biosynthetic pathway leading to the formation of UDP-*N*-acetylglucosamine, an essential common precursor to cell envelope components such as peptidoglycan, lipopolysaccharides, and teichoic acids. In *E. coli*, the phosphoglucosamine mutase is synthesized in an inactive, dephosphorylated form [25]. To be active, this enzyme must be phosphorylated. Two different modes for this initial phosphorylation have been proposed [26]. First, a kinase-dependent phosphorylation with a nucleoside triphosphate as phosphoryl group donor, or second, a phosphorylation by GlcN-1,6-diP, the reaction intermediate. The initial phosphorylation of purified *E. coli* phosphoglucosamine mutase is achieved *in vitro* during an auto-phosphorylation process [27]. To remain in an active phosphorylated form the GlmM enzyme requires the sugar diphosphate as a cofactor [28]. However, it is not clear yet, how this enzyme is activated *in vivo*. Our data suggest that in *S. pneumoniae* phosphorylation of the phosphoglucosamine mutase could be achieved by Ser/Thr protein kinase StkP.

## GlmM is a substrate for *in vitro* phosphorylation by StkP

To verify the results of *in vivo* phosphoproteome analysis and to demonstrate that GlmM is indeed a substrate of StkP, recombinant phosphoglucosamine mutase was expressed and purified. The ability of StkP to phosphorylate GlmM was examined via *in vitro* phosphorylation assay. Purified GlmM was added to the reaction mixture containing purified autophosphorylated GST-StkP fusion protein. The reaction products were separated by SDS/PAGE and labeled proteins were identified by autoradiography. As shown in Fig. 5 (lane 3), StkP could *trans*-phosphorylate GlmM, whereas GlmM alone was unable to incorporate γ-$^{32}$P (Fig. 5, lane 2), thus confirming that GlmM was a substrate of StkP and possessed no autophosphorylating activity.

In conclusion, the findings reported here show that eukaryotic type serine/threonine protein kinase StkP and its cognate protein phosphatase PhpP of the Gram-positive pathogen, *S. pneumoniae*, are indeed functional enzymes *in vitro*. Differential phosphoproteome analysis performed on the wild-type and stkP null mutant led to the identification of two target substrates *in vivo*. Whereas the relevance of *in vivo*
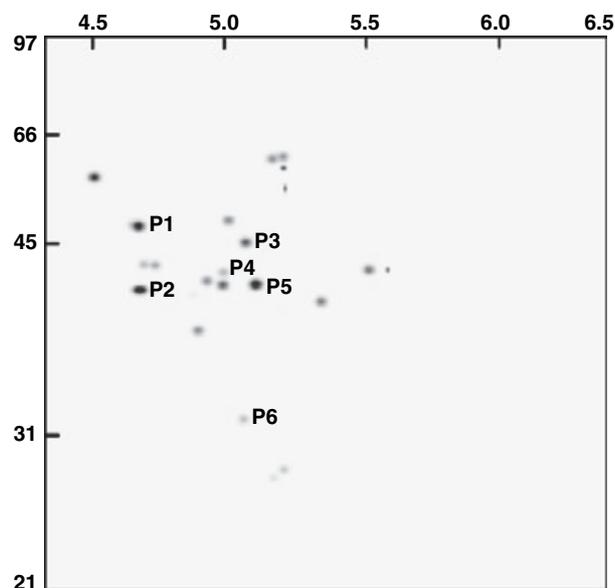
**Fig. 4.** Image of the 2D gel electrophoresis of phosphoproteins identified in both the wild type and mutant strains. Radioactive phosphoproteins were detected by scanning of Fuji imaging plates after exposition of dried gels for 10 days. Scanned images were processed with PDQUEST gel analysis software and merged together. The positions of the proteins identified in this study are indicated on the right side of the spots. Molecular mass markers are indicated on the left and p*I* values at the top of the panel.
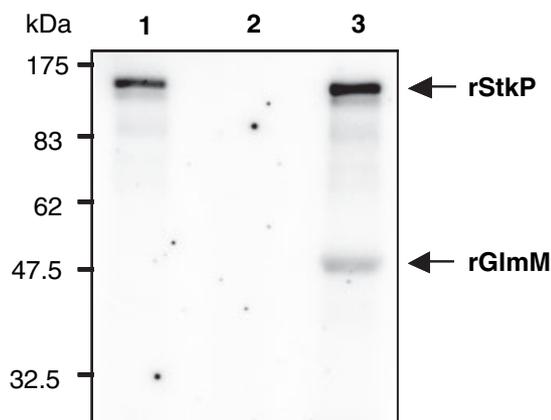


**Fig. 5.** *In vitro* phosphorylation of recombinant phosphoglucosamine mutase GlmM by protein kinase StkP. Phosphorylation reactions were performed in the standard kinase reaction mixture. The following proteins were incubated with [$^{32}$P]ATP[γP]: 100 ng of recombinant StkP (rStkP) for 30 min (lane 1); 100 ng of recombinant GlmM (rGlmM) for 30 min (lane 2); 100 ng of rStkP was autophosphorylated for 10 min, and then 100 ng of rGlmM was added to the reaction mixture and incubated for further 20 min (lane 3). Phosphorylation reactions of rGlmM were performed in the presence of 5 mM CoCl$_2$. Proteins were separated by SDS/PAGE, and radioactive bands revealed by autoradiography. Positions and molecular mass (kDa) of protein standards are indicated on the left. The arrows indicate the position of phosphorylated rStkP and rGlmM.

modification of α-subunit of RNA polymerase remains to be determined, the phosphorylation of GlmM, at least in *E. coli*, has a pivotal role for its activity. Therefore, phosphorylation of GlmM by protein kinase StkP in *S. pneumoniae* could be a factor regulating the activation of GlmM and consequently the flow of metabolites in the cell wall biosynthetic pathways. This hypothesis is supported by the fact that the cultures of stkP null mutant tend towards premature cell lysis suggesting the cell wall defects. In addition, this mutant also shows an attenuated virulence in lung infection and bloodstream invasion [9]. Both observed phenomena could suggest that the structure and composition of the cell envelope are affected in stkP null mutant.

The nature of an external factor activating StkP signaling pathway remains unknown. It is tempting to speculate that this environmental signal could be related to the cell wall stress. The experiments verifying this hypothesis are being carried out.

# Experimental procedures

## Bacterial strains and growth conditions

Culture of *S. pneumoniae* Cp 1015 [29] was grown in casein tryptone medium (CAT) [30]. Cultures of *E. coli* were routinely propagated in Luria broth. Antibiotics were added when necessary at the following concentrations: *E. coli*

**Table 2.** Identification of phosphoproteins by peptide fingerprinting. Phosphoproteins of *S. pneumoniae* detected by *in vivo* labeling and identified by mass spectrometry analysis. The spot numbers correspond to those given in Fig. 4.

| Spot number | Protein name | Number of peptides | Coverage (%) | Mass (kDa) | p*I* | Database number | Function/reaction |
|---|---|---|---|---|---|---|---|
| P1 | Phosphoglucosamine mutase | 7 | 20 | 48.1 | 4.6 | spr 1417 | Cell wall biosynthesis |
| P2 | RNA polymerase alpha subunit | 17 | 57 | 34.3 | 4.6 | spr 0215 | Transcription |
| P3 | Ribosomal protein S1 | 20 | 45 | 43.9 | 5.1 | spr 0764 | Proteosynthesis |
| P4 | Phosphoglycerate kinase | 14 | 47 | 41.9 | 4.9 | spr 0441 | Glycolysis |
| P5 | Phosphodeoxyribomutase | 15 | 46 | 47.0 | 5.2 | spr 0732 | Pentose metabolism |
| P6 | Fructose-1,6-bisphosphate aldolase | 10 | 39 | 31.5 | 5.0 | spr 0530 | Glycolysis |

hosts: ampicillin, 100 mg·L$^{-1}$; kanamycin, 50 mg·L$^{-1}$; and rifampicin, 400 mg·L$^{-1}$; *S. pneumoniae* strains: chloramphenicol, 10 mg·L$^{-1}$. *E. coli* XL1-Blue (Stratagene, La Jolla, CA, USA) was used as the recipient strain in most DNA manipulations. *E. coli* BL21(DE3) (Novagen, San Diego, CA, USA) was used as a host for the protein overexpression.

## DNA manipulations and plasmid constructions

DNA manipulations in *E. coli* were conducted as described by Sambrook *et al.* [31]. Plasmids pET28b and pET42b (Novagen) were used for the expression of *stkP* and *phpP* genes (accession no. AF285441.1). pBluescript II SK$^+$/KS$^+$ vectors (Stratagene) were used for cloning, subcloning and sequencing experiments. Plasmid pEVP3 [32] was used as the source of *cat* gene. Chromosomal DNA of *S. pneumoniae* Cp 1015 was used as a template for PCR amplifications.

To construct plasmids expressing oligohistidine-tagged full-length *stkP* gene as well as its truncated form containing N-terminal kinase domain, the *stkP* gene was amplified with primer STKP-F and reverse primers STKP-R and STKP-RT, yielding 1980 bp and 825 bp products, respectively. Both amplicons were inserted into vector pET28b, giving plasmids pEXstkP and pEXstkP-T, respectively. To create a substitution of arginine for the essential lysine residue in subdomain II of *stkP*, megaprimer PCR-based mutagenesis was used [33]. The megaprimer was generated using the mutagenic antisense primer SMUT (which introduced the K42R mutation and a silent *Sca*I site) and forward primer STKP-F. A product of 145 bp was used in the second PCR with reverse primer STKP-R yielding a 1980 bp final product. The full-length mutated *stkP* gene was ligated into pET28b vector to create pEXstkP-K42R.

To construct plasmid expressing *stkP* gene fused to glutathione *S*-transferase the full-length gene (1980 bp) was amplified with primers STKP-FNco and STKP-R and inserted into pET42b vector to obtain pEXGST-stkP.

To construct plasmids expressing *phpP* with an oligohistidine tag a 741 bp fragment was amplified using oligonucleotides PHPP-F and PHPP-R. The amplified fragment was ligated into pET28b giving pEXphpP.

The *phpP* mutations were created by megaprimer PCR-based mutagenesis using the mutagenic forward primers PMUT1 (which introduced the D192A mutation and a silent *Nae*I site) and PMUT2 (which introduced the D231A mutation and a silent *Stu*I site) and reverse primer PHPP-R in the first round of PCR. The generated fragments (190 and 75 bp, respectively) with the mutations were used as the primers for the second round of PCR with PHPP-F. The final fragments were inserted into pET28b vector. The expression plasmids, containing the full-length *phpP* gene with point mutations were named pEXphpP-D192A and pEXphpP-D231A.

To construct plasmid expressing *glmM* gene (accession number AE008512.1) with an oligohistidine tag a 1350 bp fragment was amplified using oligonucleotides PGM-F and PGM-R. The amplified fragment was ligated into pET28b giving pEXglmM.

All DNA fragments obtained by PCR amplification were sequenced with the use of universal primers and synthetic oligonucleotides based on the generated sequence.

## Expression and purification of recombinant proteins

*E. coli* BL21(DE3) strains harboring plasmids with fusion proteins were cultivated at 30° C until $D_{600}$ reached 0.6. Overproduction of recombinant proteins was initiated by addition of isopropyl thio-β-D-galactoside to a final concentration of 2 mM. Rifampicin (400 μg·mL$^{-1}$) was then added, and the cultures were incubated for a further 3 h. Induced soluble proteins were purified by either TALON$^{TM}$ metal affinity resin (Clontech, Heidelberg, Germany) or GST· Bind$^{TM}$ Resin (Novagen) affinity chromatography according to the manufacturer's instructions. Purified proteins were dialysed against a buffer containing 50 mM Tris/HCl (pH 7.5), 100 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol and 10% (v/v) glycerol. Purified StkP was used to raise rabbit polyclonal antibodies against StkP.

## *In vitro* protein phosphorylation

In standard protein kinase assay reaction mixture contained 100 ng of purified StkP in 20 μL kinase buffer (25 mM Tris/HCl (pH 7.5), 25 mM NaCl, 1 mM dithiothreitol, 0.1 mM EDTA, 5 mM MgCl$_2$, 40 μM ATP and 37 kBq of 10 μmol·L$^{-1}$ [$^{32}$P]ATP[γP]). The reaction was started by the addition of ATP and terminated after 10 min of incubation at room temperature by adding of 5× SDS sample buffer and analyzed by SDS/PAGE. After staining and drying the gels were scanned with a Fuji BAS 5000 PhosphorImager (Raytest, Straubenhardt, Germany) and evaluated with the AIDA 2.11 program. Phosphorylation of recombinant phosphoglucosamine mutase by autophosphorylated StkP was performed by adding 100 ng of purified GlmM and CoCl$_2$ (5 mM final concentration) to kinase reaction mixture and incubating for further 20 min. Phosphoamino acids from phosphorylated StkP were liberated by acid hydrolysis [34] and separated by two-dimensional electrophoresis as described in [35]. Labeled phosphoamino acids were detected by PhosphorImager.

Dephosphorylation of autophosphorylated StkP by PhpP. *In vitro* kinase assay was performed with 2 μg of purified StkP in a total volume of 20 μL. After 15 min fraction of the reaction volume containing 200 ng of StkP (2 μL) was transferred to reaction mixture containing phosphatase reaction buffer [50 mM Tris, pH 7.5, 0.2 mM EDTA, 0.02% (w/v) 2-mercaptoethanol, 5 mM MnCl$_2$] and 500 ng

of purified PhpP in a final volume of 20 µL. Phosphatase reaction was terminated by the addition of SDS/PAGE sample buffer at different time intervals. Samples were loaded on SDS/PAGE and dried gel was exposed, scanned and phosphorylation intensity was evaluated with AIDA 2.11.

## Protein phosphatase assay

Protein phosphatase activity was measured using a serine/threonine phosphatase assay system (Promega, Mannheim, Germany) according to the manufacturer's protocol. In a standard assay, 5 µg of purified PhpP reacted with 100 µM phosphopeptide (RRA(pT)VA) in PP2C buffer [50 mM imidazole, pH 7.2, 0.2 mM EDTA, 0.02% (v/v) 2-mercaptoethanol, and variable concentrations of divalent cations] for 30 min at 37° C. Reactions were stopped by adding a molybdate dye/additive mixture. The amount of free phosphate generated in the reactions was determined by the absorbance of the resulting molybdate–malachite green–phosphate complex at 600 nm.

## Construction of *S. pneumoniae* StkP mutant

Deletion of the *stkP* gene was achieved by transforming *S. pneumoniae* wild-type strain with vectorless DNA fragment consisting of *stkP* downstream and upstream regions of homology and *cat* cassette replacing the *stkP* coding region, similarly as described in [36]. Briefly, upstream flanking region (800 bp) was amplified with primers UFKFP and UFKRP, downstream flanking region (820 bp) with primers DFKFP and DFKRP, while primers CAT1 and CAT2 were used to amplify the terminatorless *cat* gene from plasmid pEVP3. The final construct was prepared by subsequent directional cloning of the fragments

**Table 3.** List of strains and plasmids used in this study.

| Strain or plasmid | Genotype or description | Phenotype[a] | Source or reference |
|---|---|---|---|
| Strain | | | |
| *E. coli* | | | |
| XL1-blue | F'::Tn10 proA+B+ lacIq ?(lacZ)M15/recA1 endA1 gyrA96 (Nalr) thi hsdR17 (rk– mk+) supE44 relA1 lac | | Stratagene |
| BL21(DE3) | F– ompT gal [dcm][lon] hsdSB (rB– mB–) (DE3) | | Novagen |
| *S. pneumoniae* | | | |
| Cp1015 | Rx derivate, str1; hexA | SmR | [16] |
| Cp1015ΔstkP | Cp1015, but stkP::cat, allelic exchange mutant | CmR | This work |
| Plasmid | | | |
| pET28b | | KmR | Novagen |
| pET42b | | KmR | Novagen |
| pBluescript II SK+/KS+ | | ApR | Stratagene |
| pEVP3 | | | [19] |
| pEXstkP | 1.98-kb NdeI/EcoRI amplicon (primers STKP-F and STKP-R) containing stkP gene inserted into pET28b | KmR | This work |
| pEXstkP-T | 0.825-kb NdeI/EcoRI amplicon (primers STKP-F and STKP-RT) containing fragment (kinase domain) of stkP gene inserted into pET28b | KmR | This work |
| pEXstkP-K42R | 1.98-kb NdeI/EcoRI amplicon (primers STKP-F, SMUT and STKP-RT (see methods)) containing stkPK42R gene inserted into pET28b | KmR | This work |
| pEXGST-stkP | 1.98-kb NcoI/EcoRI amplicon (primers STKP-FNco and STKP-R) containing stkP gene inserted into pET28b | KmR | This work |
| pEXphpP | 0.74-kb NdeI/EcoRI amplicon (primers PHPP-F and PHPP-R) containing phpPgene inserted into pET28b | KmR | This work |
| pEXphpP-D192A | 0.74-kb NdeI/EcoRI amplicon (primers PHPP-F, PMUT1 and PHPP-R (see methods)) containing phpP-D192A gene inserted into pET28b | KmR | This work |
| pEXphpP-D231A | 0.74-kb NdeI/EcoRI amplicon [primers PHPP-F, PMUT2 and PHPP-R (see methods)] containing phpP-D231A gene inserted into pET28b | KmR | This work |
| pDELstkP | 3.5-kb EcoRI/SacII fragment containing stkP flanking regions with inserted cat cassette (see methods) | ApR, KmR | This work |
| pEXglmM | 1.35-kb NdeI/XhoI amplicon (primers PGM-F and PGM-R) containing glmM gene inserted into pET28b | KmR | This work |

[a] SmR, resistant to streptomycin; CmR, resistant to chloramphenicol; KmR, resistant to kanamycin; ApR, resistant to ampicillin.

into Bluescript vector (5' region-*cat* gene-3' region) using restriction sites included in the primers. The resulting chloramphenicol-resistant clones arising from double crossover event were examined for successful allelic exchange (replacement of almost all *stkP* genes with the *cat*-cassette) by diagnostic PCR and Southern hybridization. The junctions between exogenous and chromosomal DNA in allelic exchange mutant Cp1015ΔstkP were verified by sequencing.

## RNA analysis

Total RNA was extracted from *S. pneumoniae* cultures with hot phenol method according to [37]. For RT-PCR assays the isolated RNA was treated with DNA-free[TM] (Ambion, Huntingdon, UK) to remove the contaminating DNA. cDNA synthesis was performed by using AMV reverse transcriptase (Promega) in a total 20 μL reaction volume containing 40 U RNASe Out (GibcoBRL, Gaithersburg, MD, USA) according to the manufacturer's protocol. By using various primer combinations (Fig. 3; Table 3) PCR was carried out for 30 cycles at standard conditions. The amplified products were analyzed by agarose gel electrophoresis.

## *In vivo* radio-labeling and protein sample preparation

*S. pneumoniae* cells were labeled with [33P]phosphoric acid (specific activity 148 TBq·mmol$^{-1}$; MP Biomedicals, Heidelberg, Germany). Exponentially growing cells were harvested and resuspended in 1/20 volume of prewarmed low-phosphate complex medium CAT. After adding 10 MBq [33P]phosphoric acid, cells were incubated for 45 min, harvested and resuspended in 100 μL of water containing protease inhibitor cocktail (Sigma, St Louis, MO, USA) and Benzonase (Merck, Darmstadt, Germany). Four hundred microliters of cold acetone was added and proteins were precipitated at −20° C overnight. Incorporated radioactivity was quantitated by scintillation counting using a Wallac scintillation counter 1409 DSA (Turku, Finland).

## Two-dimensional gel electrophoresis and mass spectrometry analysis

For isoelectric focusing 18 cm precast Immobiline Dry Strip (IPG) strips pI 4–7 and the MultiPhor II; (Amersham Biosciences, Uppsala, Sweden) were used. 250 000 dpm (100–200 μg of protein) were focused for 71 000 Vh. In the second dimension proteins were separated on vertical 12.5% SDS polyacrylamide gels (Investigator 2-D System; Genomic Solutions, Huntingdon, UK). After electrophoresis the gels were air dried, exposed to imaging plates (FujiFilm, Tokyo, Japan) and scanned with BAS 5000. The resulting autoradiographs were aligned with the corresponding images of the

Coomassie-stained gels using PDQUEST gel analysis software. Selected protein spots were in-gel digested with trypsin and fragment masses were measured on a BIFLEX mass spectrometer (Bruker-Franzen, xxxx, Germany). MS data obtained were matched through NCBI database using the search program PROFOUND (http://prowl.rockefeller.edu/profound_bin/WebProFound.exe).

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