Characterization of pneumococcal Ser/Thr protein phosphatase phpP mutant and identification of a novel PhpP substrate, putative RNA binding protein Jag

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Abstract

Background: Reversible protein phosphorylation catalyzed by protein kinases and phosphatases is the primary mechanism for signal transduction in all living organisms. Streptococcus pneumoniae encodes a single Ser/Thr protein kinase, StkP, which plays a role in virulence, stress resistance and the regulation of cell wall synthesis and cell division. However, the role of its cognate phosphatase, PhpP, is not well defined.

Results: Here, we report the successful construction of a ΔphpP mutant in the unencapsulated S. pneumoniae Rx1 strain and the characterization of its phenotype. We demonstrate that PhpP negatively controls the level of protein phosphorylation in S. pneumoniae both by direct dephosphorylation of target proteins and by dephosphorylation of its cognate kinase, StkP. Catalytic inactivation or absence of PhpP resulted in the hyperphosphorylation of StkP substrates and specific phenotypic changes, including sensitivity to environmental stresses and competence deficiency. The morphology of the ΔphpP cells resembled the StkP overexpression phenotype and conversely, overexpression of PhpP resulted in cell elongation mimicking the stkP null phenotype. Proteomic analysis of the phpP knock-out strain permitted identification of a novel StkP/PhpP substrate, Spr1851, a putative RNA-binding protein homologous to Jag. Here, we show that pneumococcal Jag is phosphorylated on Thr89. Inactivation of jag confers a phenotype similar to the phpP mutant strain.

Conclusions: Our results suggest that PhpP and StkP cooperatively regulate cell division of S. pneumoniae and phosphorylate putative RNA binding protein Jag.

Keywords: Signal transduction, Protein phosphatase, Protein kinase, Cell division, Streptococcus, Phosphorylation, Jag

Background

Signal transduction via protein phosphorylation is one of the basic mechanisms that modulate numerous cellular processes in both prokaryotes and eukaryotes. Signal transduction in prokaryotes has been considered to occur primarily by two-component systems consisting of a histidine protein kinase and its cognate response regulator [1]. However, studies published in the last two decades have clearly demonstrated that this paradigm requires modification. Eukaryotic-type Ser/Thr protein kinases (ESTKs) as well as Ser/Thr phosphatases (ESTPs) operate in various bacterial species in parallel or overlapping signaling networks and regulate various cellular functions [2]. A distinct group of ESTKs which regulate cell cycle and cell division in many Gram-positive bacteria are conserved transmembrane proteins with a cytoplasmic kinase domain and repeated PASTA (penicillin-binding protein and Ser/Thr kinase-associated) domains in their extracellular region [2–6].

ESTKs are often co-expressed with their cognate phosphatases which are necessary for regulation of ESTK activity and quenching of signaling cascades; however, their physiological function in bacteria is still poorly understood.

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understood. The ESTPs associated with PASTA-possessing ESTKs are Mg\(^{2+}\) or Mn\(^{2+}\)-dependent enzymes of the PPM family of Ser/Thr phosphatases, which share homology with the eukaryotic PP2C phosphatase [7]. Unlike ESTKs, only a few cognate ESTPs have been studied in detail, in part because several of them have been reported to be essential [8–10]. However, other detailed studies have demonstrated that knock-out mutants of phosphatase genes are viable and that ESTPs play a role in virulence, cell wall metabolism and cell segregation [11–16].

*S. pneumoniae* encodes a single PASTA-containing ESTK named StkP and a co-transcribed phosphatase, PhpP [8, 17]. Unlike PhpP, StkP has been extensively studied in past years, and its pleiotropic function in the regulation of different cellular processes has been described. StkP is a virulence determinant that is important for lung infection and bloodstream invasion in vivo and regulates pilus expression and bacterial adherence in vitro [8, 18]. StkP is essential for the resistance of *S. pneumoniae* to various stress conditions and competence development. Microarray analysis has revealed that StkP affects the transcription of a set of genes involved in cell wall metabolism, pyrimidine biosynthesis, DNA repair, iron uptake and oxidative stress response [8, 19]. StkP localizes to the division sites and plays important role in the regulation of cell division [20–22]. Cells with stkP mutations demonstrated disrupted cell wall synthesis and displayed elongated morphologies with multiple, often uncondensed, cell division septa, which suggest that StkP coordinates cell wall synthesis with cell division and thus helps pneumococcus to achieve its characteristic ovoid shape. Consistent with its role in cell division, StkP was found to phosphorylate several proteins involved in cell wall synthesis and cell division. The cell division proteins DivIVA [21, 23], LocZ (named also MapZ) [23–25] and the phosphoglucomutase mutase GlnM [17] are phosphorylated by StkP in vitro and in vivo. The cell division proteins FtsZ [22] and FtsA [20] and the cell wall biosynthesis enzyme MurC [26] are substrates of StkP in vitro; however, their phosphorylation by StkP in vivo has not been confirmed.

StkP is dephosphorylated by the cognate phosphatase PhpP, which is a PP2C-type Mn\(^{2+}\)-dependent enzyme. The PhpP catalytic domain contains 11 conserved signature motifs [27], and mutations of the highly conserved residues D192 and D231, which have been implicated in metal binding, completely abolish PhpP activity in vitro [17]. GFP-PhpP fusion protein is localized in the cytoplasm; however, the protein is often enriched in the midcell. The localization of PhpP to cell division sites depends on the presence of active StkP, indicating that both enzymes form a signaling couple in vivo [20]. Previously, *phpP* was reported to be essential for the viability of the unencapsulated Rx1 and R800 strains [10, 21]. According to global analysis performed by Thanassi et al. [28], both *phpP* and *stkP* genes were found to be essential; however, in the other global studies, *phpP* was not recognized as an essential gene [29, 30]. A recent report generated nonpolar markerless *phpP* knock-out mutants in two encapsulated pathogenic strains, *S. pneumoniae* D39 and 6A, indicating that PhpP is dispensable for pneumococcal survival [11]. Characterization of these mutants demonstrated the strain-specific role of PhpP in cell wall biosynthesis, adherence and biofilm formation. The StkP/PhpP signaling couple has been demonstrated to regulate the two-component system HK06/RR06, which modulates the expression of a major pneumococcal adhesin, CbpA [11].

In the present study, we show that the unencapsulated Rx1 *phpP* knock-out strain is viable. The morphology of both, the unencapsulated *phpP* null mutant and the *phpP* overexpression strain, clearly demonstrated that PhpP participates in the regulation of cell division and has an opposite regulatory effect to that of StkP. Our data suggest that PhpP modulates the level of protein phosphorylation in vivo both, through direct dephosphorylation of target proteins and dephosphorylation of its cognate kinase, StkP, resulting in coordination of cell wall synthesis and division in *S. pneumoniae*. Proteomic analysis of the ∆*phpP* strain revealed a novel StkP/PhpP substrate, Spr1851, a putative RNA-binding protein homologous to Jag protein of *B. subtilis* [31].

**Results and discussion**

**PhpP is not essential for pneumococcal survival and catalyzes dephosphorylation of StkP and its substrates**

Although *phpP* was reported to be essential in an *stkP*′ genetic background [10], a nonpolar markerless *phpP* knock-out was generated in two encapsulated *S. pneumoniae* strains using the Janus cassette-based two-step negative selection strategy [11]. We attempted to use the same strategy to knock out *phpP* in the unencapsulated Rx1 strain as described in the Methods. We obtained viable ∆*phpP* transformants and characterized them further (see below). To exclude the possibility that the ∆*phpP* strain might carry an unlinked extragenic suppressor of the potentially lethal effect of loss of PhpP, the dose-response pattern for ∆*phpP* versus a wild type (WT) backcross was determined [32, 33]. Transformation by a single marker in pneumococcus displays a linear dependence on the dose of donor DNA (slope of regression curve equal to 1), whereas less efficient co-transformation by two markers follows a quadratic dependence on donor DNA dose (slope of regression curve equal to 2). Transfer of the ∆*phpP* mutation followed first order kinetics, and, therefore, viability of the ∆*phpP* strain does not depend on an extragenic suppressor
mutation (Fig. 1a). In addition, the sequence of the neighboring genes spr1579 and stkP was verified for the absence of mutation by DNA sequencing. The contradictory results reporting the essentiality [10, 21, 28] and non-essentiality [11, 29, 30] of the phpP gene may result from the different methods used for gene inactivation or from the genetic variability of the pneumococcal strains used. As reviewed in Massidda et al. [34], the genome of S. pneumoniae is very dynamic, and the number of genes found to be conditionally essential is dependent on the genetic background or the presence of capsule.

Using specific anti-PhpP (α-PhpP) and anti-StkP (α-StkP) antibodies, we confirmed that PhpP was deleted from the genome of the ΔphpP strain (Sp113), while the expression level of StkP was similar in both the ΔphpP and wild type strain (Fig. 1b). To evaluate the level of protein phosphorylation, we performed immunodetection with an anti-phospho-threonine (α-pThr) antibody. Thr phosphorylation in S. pneumoniae is largely dependent on StkP, and the majority of its substrates are membrane or membrane-associated proteins [23]. As previously reported, no Thr phosphorylated proteins were detected in the ΔstkP mutant (Sp10) (Fig. 1b). Immunodetection of phosphoproteins in the ΔphpP membrane fraction revealed a pattern similar to the StkP-dependent phosphoproteome [23]; however, we observed an increase in signal intensity corresponding to 192 ± 58.4 % of the wild type, indicating hyperphosphorylation of StkP substrates, including StkP itself (Fig. 1b). These data indicate that PhpP negatively regulates phosphorylation of StkP and its substrates.

To verify that the observed phosphorylation profile was the result of the phpP deletion, we constructed two complementation strains. First, we reverted the ΔphpP mutation back to the wild type genotype by transforming the complementation strains. First, we reverted the Δα mutation with an anti-phospho-threonine (α-pThr) antibody. Thr phosphorylation of StkP and its substrates.

The phpP knock-out strain is sensitive to elevated temperature and oxidative stress

The stkP null mutant has an altered growth rate, and it is sensitive to environmental stresses [19], which highlights the importance of StkP in the resistance of pneumococcus to hostile environmental conditions in the host. The growth rate of the phpP knock-out strain was reduced in TSB medium (38 min doubling time) compared to the wild type strain (31 min doubling time). In addition, the mutant strain had a significantly prolonged lag phase and reached a lower final optical density (Fig. 2a), similar to the ΔstkP mutant strain. Further we examined the growth of the ΔphpP mutant in response to heat stress, osmotic stress and pH variation, as well as its viability after exposure to H2O2. The ΔphpP, ΔstkP and the wild type strains were inoculated in liquid medium and cultivated as described in detail in the Methods. Our experiments showed that unlike StkP, PhpP did not significantly affect the sensitivity to osmotic stress induced by high salt concentration or the tolerance to acidic or alkaline pH (data not shown). However, PhpP was important for normal growth at elevated temperatures: the doubling time and final density achieved were significantly affected when the ΔphpP strain was grown at 40 °C (Fig. 2b). In addition, we tested the resistance of the mutant strain to oxidative damage. When exposed to varying concentrations of H2O2, the ΔphpP strain, similar to the ΔstkP strain, displayed a lower survival rate than the wild type strain, indicating increased sensitivity to oxidative stress (Fig. 2c). In summary, the phenotype of the unencapsulated Rx1 derived ΔphpP mutant differs from the encapsulated strain 6A which displayed retarded growth under all stress conditions tested but also differs from the strain D39 which was affected only in the high-salt stress
These results suggest that genetic background significantly affects the demonstration of phpP mutation although we cannot exclude the role of polysacharide capsule itself. Considering that the Rx1 derived stkP mutant strain was sensitive to elevated temperature, acidic pH, osmotic and oxidative stress [19], we did not confirm the opposite effect of PhpP. Our data suggest that unbalanced activity of both, PhpP and StkP, is critical for bacterial physiology, and the adaptive response to environmental stress is not cooperatively regulated by the PhpP/StkP signaling couple.
Fig. 2 (See legend on next page.)
The ΔphpP strain displays decreased competence for genetic transformation

Competence for genetic transformation is a powerful mechanism for generating genetic diversity and acquiring antibiotic resistance. Natural competence in *S. pneumoniae* is a transient event regulated by a quorum-sensing system and occurs via a peptide pheromone signal (e.g., competence-stimulating peptide (CSP)). Previous studies demonstrated the importance of StkP for competence development [10, 19]. Here, we tested the capability of the ΔphpP mutant strain to develop natural and induced competence in conditions optimal for competence development [36]. Induced competence was defined as the transformation efficiency in response to the addition of synthetic CSP. Similar to the ΔstkP strain, the phpP null mutant strain weakly developed induced competence, and the transformation efficiency was low compared to wild type (Fig. 2d). Natural competence was defined as the transformation efficiency in the absence of added CSP and was monitored during the growth. As shown in Fig. 2e, that wild type strain developed natural competence during the early exponential phase of growth (OD$_{600}$ 0.08–0.16), which is observed as a peak in viable transformants obtained by transformation with control DNA. On the other hand, two low peaks of competence, one during the exponential phase and the second upon the entry into the stationary phase, were detected during growth of the phpP knock-out strain (Fig. 2f). However, the transformation efficiency was about fivefold lower than that observed for the wild type, and therefore, the strain is competence deficient, similar to the ΔstkP strain (Fig. 2g). In the ΔstkP strain the reduced transformation efficiency may be the result of a weak induction of DNA uptake and processing genes [19]. However, the molecular mechanism responsible for the transformation deficiency in the ΔstkP mutant remains unclear. To date, none of the proteins that play a direct role in competence development have been identified as a substrate of StkP. Our results suggest that StkP and PhpP do not function as antagonists in the control of competence regulation. Therefore, we cannot exclude that competence deficiency of both mutants is an indirect consequence of the pleiotropic effects of phpP and stkP mutations on pneumococcal physiology.
Fig. 3 PhpP is involved in regulation of cell division in S. pneumoniae. a Morphology and cell length analysis of WT strain (Sp1), reverted strain WTα (Sp222) and ΔphpP (Sp113) or ΔstkP (Sp10) strains grown in C + Y medium to mid-exponential phase. b Cell length depends on expression of PhpP in complementation strain ΔphpPPzn- (Sp120). Micrographs of complementation strain cultivated in the presence of 0, 0.2, 0.25 and 0.3 mM ZnSO4 in C + Y medium. Cell length in panel A and B is expressed as a median value ± MAD (n = 300). Bar 5 μm. c Cell length analysis. Cell length parameters measured with MicrobeTracker software were analyzed and plotted in box-and-whiskers graph. Mann-Whitney U test: * cell length of mutant strain is significantly different from that of the WT strain P < 0.0001. 300 cells were scored per sample. d Scanning electron microscopy of WT strain (Sp1), ΔstkP strain (Sp10) and ΔphpP strain (Sp113) cultivated in TSB medium. Magnifications are the same for all panels. Bar 0.5 μm. a-d: Representative data for three independent experiments are shown.
associated with the loss of *phpP* (data not shown) which were observed in the encapsulated Δ*phpP* strain [11].

Analysis of 600 cells showed that 24.2% of the Δ*phpP* cells formed chains longer than 4 cells in contrast with 2.5% of chaining cells in the wild type. However, we did not observe aggregation and abnormally long chains which were detected in the encapsulated mutant strains [11]. Regulation of chain length in streptococci depends on wall-associated autolytic activity. Therefore we tested the expression of genes encoding the peptidoglycan hydrolases *pcsB*, *lytA* and *lytB*, which may affect cell separation, using qRT-PCR, but we did not detect any differences in transcript levels in the Δ*phpP* and wild type strain (data not shown); thus, the reason for the increased chain formation remains unknown.

To characterize the role of PhpP in cell division in more detail, we investigated the localization of nascent PG synthesis sites in live Δ*phpP* cells (Sp113) stained with fluorescently labeled vancomycin (Van-FL), a marker of nascent peptidoglycan synthesis (PG) (Fig. 4). Labeling was observed predominantly at current and future cell division sites in the mutant cells, a pattern similar to that observed in the wild type cells. However, 4.5% of mutant cells (58/1300) showed disturbed Van-FL labeling (Fig. 4) indicating that minority of cells display perturbed cell wall synthesis. When we induced overexpression of PhpP in complementation strain Sp120 (Δ*phpP* P_{Zn}-*phpP*) by the addition of 0.3 mM ZnSO_{4}, we observed significant elongation of cells, and Van-FL staining revealed that the cells often contained multiple unconstricted division septa, which is a distinct feature of stkP-depleted cells (Fig. 4). Further we investigated localization of cell division proteins LocZ/MapZ, FtsA and DivIVA but we did not find significant differences between mutant and wild type cells (data not shown).

Our data clearly show that PhpP plays an opposing role to StkP in regulation of cell division which was not recognized in the previous study by Agarwal et al. [11]. We hypothesize that the morphological differences between Δ*phpP* mutants derived from different strains may be largely caused by the presence or absence of capsule. However, the cell division defect caused by the depletion of *phpP* is less severe than the abnormalities observed either in the absence of StkP or in the presence of the excess of PhpP suggesting that hyperphosphorylation of StkP substrates is better tolerated than the absence of phosphorylation.

**Conserved residues D192 and D231 are essential for PhpP activity in vivo**

PhpP contains two conserved aspartate residues, D192 and D231, which are directly involved in metal ions binding and are essential for the activity of eukaryotic PP2C phosphatases [37]. Previously, we reported that substitution of D192 and D231 for alanine abolished PhpP activity in vitro [17]. Here, we investigated the importance of D192 and D231 for enzymatic activity and localization of PhpP in vivo. We constructed strains expressing PhpP mutant alleles D192A and D231A fused to GFP under an inducible P_{Zn} promoter in the Δ*phpP* genetic background. Strains expressing GFP-PhpP-WT (Sp140), the D192A allele (Sp292) and the D231A allele (Sp293) were cultivated in C + Y medium with or without zinc, and PhpP expression and the phosphorylation pattern were detected using specific antibodies. Expression of PhpP-WT and PhpP-D192A was similar; however, the expression of PhpP-D231A was lower as indicated by immunodetection with the α-GFP antibody.
(Fig. 5a). Immunodetection with the α-pThr antibody showed that increasing the expression of the wild type allele resulted in a decrease in overall phosphorylation intensity. On the other hand, increased expression of PhpP-D192A or D231A upon addition of zinc did not affect the phosphorylation intensity in strains Sp292 and Sp293, respectively, indicating that both alleles are catalytically inactive. Phase contrast microscopy revealed that the morphology of strain Sp140 expressing GFP-PhpP-WT changed depending on zinc concentration, and cell length increased (Fig. 5b). On the other hand, the morphology of strains expressing the mutant alleles of PhpP did not change (Fig. 5b) indicating inability to complement the mutant phenotype. Cell size analysis confirmed these observations (Fig. 5c). PhpP-WT expression in the Sp140 strain led to increases in cell length up to 2.11 ± 0.36 μm when 0.3 mM ZnSO₄ was added to the medium, while cell length remained unchanged in strains Sp292 (PhpP-D192A) (1.49 ± 0.2 μm) and Sp293 (PhpP-D231A) (1.43 ± 0.23 μm).

Previously, we demonstrated that the protein phosphatase PhpP is localized in the cytoplasm, but it is significantly enriched at the midcell during the early exponential phase of growth, and this localization depends on the presence of active StkP [20]. To determine localization of catalytically inactive GFP-PhpP, we cultivated strains Sp140, Sp292, and Sp293 in medium supplemented with 0.2 mM ZnSO₄ until the early exponential phase (OD₆₀₀ 0.2) and examined live cells using fluorescence microscopy. PhpP-WT was clearly associated with the cell division septum in 23% (176/766) of the cells and showed cytoplasmic distribution in 77% of cells (Fig. 5d). PhpP-D192A was enriched at the midcell in 19% (152/800) of the cells; however, the GFP signal in these cells was more diffuse (Fig. 5d). To quantify the difference, we measured the fluorescence intensity profiles in the cells at the first stage of cell division (predivisional cells). We confirmed different distributions of the GFP-PhpP-WT and GFP-PhpP-D192A signals along the cell axis, which indicates that PhpP-D192A is more abundant in the cytoplasm (Fig. 5d). Interestingly, PhpP-D231A was localized exclusively in the cytoplasm (Fig. 5d). These data suggest that mutant alleles of PhpP not only lose the catalytic activity but also lose the ability to co-localize with cell division apparatus.

**Jag protein (Spr1851) is a previously unknown substrate of StkP and PhpP**

The elevated level of Thr phosphorylation in the phpP null mutant helped us to detect phosphorylated membrane proteins previously unrecognized in wild type lysates. Because the phosphorylated proteins designated P35 and P40 migrate close to phosphorylated DivIVA, we generated strain ΔphpPΔdivIVA (designated Sp169), which would enable appropriate separation of the new substrates. To identify these StkP substrates we extracted proteins from the membrane fraction using trifluorethanol (TFE) [38] and separated them using two-dimensional (2D) SDS-PAGE as described in the Methods. The protein spot corresponding to P40 was successfully resolved, and its phosphorylation was confirmed by immunoblotting (Fig. 6a). The protein spot was excised, digested by trypsin and identified using MALDI-TOF mass spectrometry as Spr1851, a homolog of Jag/SpoIIIJ-associated protein from *B. subtilis*. We named the product of the spr1851 gene JagSpn (Fig. 6b). JagSpn contains an N-terminal Jag_N domain and KH domain followed by an R3H domain at the C-terminus (Conserved Domain Database (CDD) [39]) (Fig. 6b). The Jag_N domain located at the N-terminus of bacterial Jag proteins is a conserved stretch of 50 amino acids without a defined function (CDD). The KH domain is a single-stranded nucleic acid-binding domain that mediates RNA target recognition in proteins that regulate gene expression in eukaryotes and prokaryotes (reviewed in [40]). The R3H motif is present in proteins from a diverse range of organisms that includes Eubacteria, green plants, fungi, and various groups of metazoans, and it is predicted to bind ssDNA or ssRNA in a sequence-specific manner. Jag homologues are conserved in bacteria, especially in *Firmicutes*, and their domain architecture suggests that they bind RNA; however, their function is unknown.

To verify the phosphorylation of JagSpn we constructed a jag null mutant named Sp295 using the Janus cassette strategy described in the Methods. We detected phosphorylated proteins in whole cell lysates of the wild type, Δjag and ΔstkP strains and compared them with strain ΔphpPΔdivIVA to better distinguish different phosphoprotein bands. Immunoblotting showed that a phosphoprotein corresponding to 40 kDa (P40) is present in the wild type and ΔphpPΔdivIVA strain but absent in the Δjag strain, which indicates that Spr1851/Jag corresponds to StkP substrate P40 (Fig. 6c).

To verify further phosphorylation of Jag we prepared the complementation strain Sp304 expressing Jag with a Flag-tag at the C-terminus (Jag-Flag) under the inducible PZn promoter in the Δjag genetic background. Figure 6d shows that addition of zinc induced expression of Jag-Flag in strain Sp304 (Δjag PZn-Flag), and immunodetection with the α-pThr antibody confirmed that the protein is phosphorylated. A second, faster migrating band of Jag was detected with the α-Flag antibody when ZnSO₄ was added at concentrations of 0.25 and 0.3 mM. This protein band interacted weakly with the α-pThr antibody, which indicated that this form of Jag is also phosphorylated.

**Jag is phosphorylated on Thr89**

Spr1851/Jag was previously found to be phosphorylated on Thr89 in a global study published by Sun et al. [41]. Thr89
Fig. 5 D192 and D231 are essential for PhpP catalytic activity in vivo. a Phosphorylation pattern after induction of expression of GFP-PhpP-WT (Sp140), GFP-PhpP-D192A (Sp292) and GFP-PhpP-D231A (Sp293) in ΔphpP genetic background. Total cell lysates from cultures grown in C + Y medium in the presence or absence of ZnSO₄ were separated by SDS-PAGE and immunoblotted with α-pThr antibody to document protein phosphorylation. α-GFP antibody was used to show expression level of GFP-PhpP and immunodetection of RpoA was used as a loading control. Position of StkP and its substrates is indicated by arrows. b Morphology of strains expressing GFP-PhpP-WT (Sp140), GFP-PhpP-D192A (Sp292) and GFP-PhpP-D231A (Sp293) in ΔphpP genetic background. Pneumococcal strains were cultivated in C + Y medium supplemented with ZnSO₄. Phase contrast images show cell morphology in the presence of 0.2 and 0.3 mM ZnSO₄ and median cell lengths ± MAD (n = 300) corresponding to each image are shown below. Bar, 5 μm. c Cell length analysis. Cell length parameters were analyzed and plotted in box-and-whiskers graph. Mann-Whitney U test: * cell length in the presence of inducer is significantly different from uninduced conditions (0 mM ZnSO₄) P < 0.0001. 300 cells were scored per sample. d Localization of PhpP. Strains expressing GFP-PhpP-WT (Sp140), GFP-PhpP-D192A (Sp292) and GFP-PhpP-D231A (Sp293) were cultivated in C + Y medium supplemented with 0.2 mM ZnSO₄. GFP signal and overlay of phase contrast and GFP signal are shown. Enrichment of PhpP at midcell of cells at first stage of cell division (predivisional cells) is indicated by full arrow; cells showing cytoplasmic localization of PhpP are indicated by open arrow. Bar, 1 μm. Predivisional cells (n = 20) showing either midcell enrichment of PhpP (WT and D192A) or cytoplasmic localization (D231A) were selected to quantify distribution of GFP-PhpP along the cell axis. Fluorescence intensity (arbitrary units) versus cell length is plotted in corresponding graphs (error bars show SD).
is located in a region that does not show significant homology to any conserved domain; however, Thr89 is conserved in many streptococcal species (KEGG, Kyoto Encyclopedia of Genes and Genomes). To verify phosphorylation of Thr89, we mutagenized this residue to unphosphorylatable alanine and constructed strain Sp302 expressing the phosphoablative allele \( \text{jag-Flag-T89A} \) under the \( \text{PZn} \) promoter in the \( \Delta \text{jag} \) genetic background. Upon induction of expression, we detected production of Jag-Flag-T89A migrating faster than the major form of Jag-Flag-WT. This suggests that alteration of mobility is related to the unphosphorylated state (Fig. 6d). Immunodetection with the \( \alpha\)-pThr antibody showed a significant decrease in phosphorylation; however, the signal was not completely lost, and Jag-T89A reacted weakly with the \( \alpha\)-pThr antibody when expressed at higher levels (0.25–0.3 mM ZnSO\(_4\)) (Fig. 6d, lanes 11, 12). These results confirm that Thr89 is indeed a phosphoacceptor residue in vivo; however, another still unidentified secondary phosphorylation site is present in Jag.

**Jag is dephosphorylated by PhpP**

To demonstrate that PhpP directly dephosphorylates Jag, we isolated phosphorylated Jag-Flag from cell lysates of strain Sp304 via affinity chromatography and performed in vitro dephosphorylation reactions as described in the

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**Fig. 6** Spr1851/Jag is a new substrate of StkP and PhpP. a Identification of Jag. Membrane fraction isolated and extracted by TFE/chloroform from strain Sp169 (\( \Delta \text{phpP} \Delta \text{DivIVA} \)) was resolved on 2D SDS-PAGE, immunoblotted with \( \alpha\)-pThr antibody and matched with parallel gel stained with Coomassie Blue. Spot corresponding to P40 was excised, analyzed by MALDI-MS and identified as Spr1851. b Schematic structure of Spr1851/Jag. Jag_N: conserved domain found at the N-terminus of Jag proteins; T89: phosphorylated threonine 89; KH: single-stranded RNA binding domain; R3H: putative single-stranded nucleic acid binding domain. c Deletion of jag. Whole cell lysates of WT (Sp1), \( \Delta \text{jag} \) (Sp295), \( \Delta \text{phpP} \Delta \text{DivIVA} \) (Sp169) and \( \Delta \text{stkP} \) (Sp10) were separated by SDS-PAGE and immunoblotted with \( \alpha\)-pThr antibody to detect protein phosphorylation. Immunodetection of \( \alpha\)-subunit of RNA polymerase (\( \alpha\)-RpoA) was used as a loading control. Arrows indicate position of StkP and its substrates. d Phosphorylation of T89. Total protein lysates (30 \( \mu \)g) of WT (Sp1), \( \Delta \text{jag} \) (Sp295), complementation strain \( \Delta \text{jag} \ P_{2\Delta}\text{jag-Flag} \) (Sp304) and \( \Delta \text{jag} \ P_{2\Delta}\text{jag-Flag-T89A} \) (Sp302) were separated by SDS-PAGE and immunoblotted with \( \alpha\)-pThr antibody to detect protein phosphorylation. Expression of Jag-Flag was monitored by immunodetection with anti-Flag antibody (\( \alpha\)-Flag) and immunodetection of RpoA (\( \alpha\)-RpoA) was used as a loading control. Arrows indicate position of proteins. e Dephosphorylation of Jag. Purified Jag-Flag was incubated with His-PhpP in vitro and reaction was stopped at given time. Samples were subjected to SDS-PAGE and immunoblotted with \( \alpha\)-pThr antibody to visualize dephosphorylation in time. PhpP-His was detected with \( \alpha\)-PhpP antibody and Jag-Flag was detected with \( \alpha\)-Flag antibody. To exclude the spontaneous decay, phosphorylated form of Jag-Flag was incubated for 90 min in phosphatase reaction buffer without addition of His-PhpP. Arrows indicate position of two differentially phosphorylated forms of Jag.
Methods. Phosphorylation of Jag was monitored by immunodetection with the α-pThr antibody, and the results showed that the loss of the phosphorylation signal was time dependent (Fig. 6e). This experiment confirmed that PhpP directly dephosphorylates Jag. Two different forms of Jag were detected upon incubation with PhpP, correlating with our finding that Jag most likely contains more than one phosphorylated residue (Fig. 6d).

Characterization of the Δjag phenotype

To obtain insight into Jag function in pneumococcus, we characterized the phenotype of a Δjag mutant. The Δjag mutant showed retarded growth in TSB medium, with a longer doubling time (32 min) than the wild type (29 min). The mutant had a significantly longer lag phase and reached stationary phase at a lower optical density (Fig. 7a). Phase contrast microscopy indicated that mutant cells are smaller, a phenotype reminiscent of the ΔphpP mutant. Cell size analysis confirmed that the median cell length \((1.33 \pm 0.19 \mu m)\) and cell width \((0.59 \pm 0.01 \mu m)\) of the mutant were significantly smaller than the median cell length and width of the wild type \((1.57 \pm 0.2 \mu m \text{ and } 0.67 \pm 0.09 \mu m)\) \((P < 0.0001; \text{ Mann-Whitney rank sum test})\). Scanning electron microscopy further supported these data; however, no significant abnormalities in cell shape and morphology were observed (Fig. 7b).

We also determined cell size of the complementation strain Sp304 (Δjag PZn-jag-flag) upon addition of increasing zinc concentrations. Induced expression of Jag-WT resulted in complementation characterized by increasing cell length (Fig. 7c). This rescue of the phenotype confirmed the relationship between inactivation of jag and decreased cell dimensions. The cells reached wild type cell length at a concentration of approximately 0.25 mM ZnSO_4 \((1.67 \pm 0.25 \mu m)\). The cell length increased further upon addition of 0.3 mM ZnSO_4 until it reached 1.76 ± 0.26 μm, suggesting that overexpression of Jag led to significant cell elongation. These data suggest that Jag plays a role in pneumococcal cell division and helps to maintain proper cell shape.

The jag homologue in B. subtilis forms a bicistronic operon with the spoIII gene [31], which corresponds to pneumococcal spr1852 encoding the YidC1/Oxa1 membrane protein insertase. This gene cluster is widely conserved (KEGG). YidC homologues are required for the insertion and/or proper folding of integral membrane proteins [reviewed in [42]]. Most Gram-positive bacteria encode two YidC paralogues, YidC1 and YidC2, which correspond to Spr1852 and Spr1790, respectively, in S. pneumoniae. The role of YidC homologues in S. pneumoniae has not been described; however, in S. mutans, disruption of YidC2 results in a loss of genetic competence, decreased membrane-associated ATPase activity and stress sensitivity. Loss of YidC1 has less severe defects, with little observable effect on growth or stress sensitivity [43]. Although the two insertases have different physiological functions, both of them contribute to biofilm formation and cariogenicity in rats [43].

The Jag association with the membrane and likely co-transcription with yidC1 suggest that both proteins might be functionally linked. It is tempting to speculate
that JagSpn plays an indirect role in targeting of the integral membrane proteins. Given the Δjag phenotype and its phosphorylation by StkP, which regulates cell division in pneumococcus, JagSpn might specifically affect targeting of cell division proteins. YidC homologues are involved in cell division processes in different bacteria. The well-studied YidC1/SpoIIIJ in B. subtilis is required for sporulation [31]. Interestingly, the cell division proteins FtsQ and FtsEX have been found to be substrates of YidC in E. coli and Shigella, respectively [44, 45]. A recent report also showed that YidC assists in the biogenesis of penicillin-binding proteins (PBP) in E. coli, and in the absence of YidC, two critical PBPs, PBP2 and PBP3, are not correctly folded, and their substrate-binding capacity is reduced, although the total amount of protein in the membrane is not affected [46].

Conclusions

Streptococcus pneumoniae has a characteristic ovoid shape, which is most likely achieved by the concerted action of two peptidoglycan biosynthetic machineries: peripheral and septal [47, 48]. We previously proposed a model in which Ser/Thr protein kinase StkP coordinates cell wall synthesis and cell division in S. pneumoniae [20]. Here, we demonstrate that the cognate Ser/Thr protein phosphatase PhpP is not essential as published previously [10, 21, 28] and plays an opposing role in cell division to that of StkP. Overexpression of PhpP, which leads to dephosphorylation of StkP substrates, mimics the stkP null phenotype and the dividing cells are elongated and contain multiple unconstricted cell division septa. In the absence of phpP we observe enhanced autophosphorylation of StkP and hyperphosphorylation of StkP substrates. We show that PhpP regulates not only activity of StkP but dephosphorylates directly StkP substrates. The morphology of ΔphpP cells resembles StkP overexpression, and the cells do not achieve the size of the wild type, most likely due to insufficient elongation of cells or premature constriction of the Z-ring. We hypothesize that PhpP and StkP co-ordinately regulate the shift from peripheral to septal cell wall synthesis through phosphorylation of several substrates, including cell division proteins. In contrast, we did not confirm a straightforward regulatory impact of PhpP on the other functions of StkP. Characterization of the phpP null mutant revealed that like the stkP null mutant, it is more sensitive to elevated temperature, oxidative stress and that both mutant strains have reduced competence for genetic transformation. These results suggest that unbalanced activity of each of these enzymes is critical for bacterial physiology. We cannot exclude the possibility that PhpP may also have broader substrate specificity and may dephosphorylate phosphoproteins other than StkP substrates. Detection of proteins specifically phosphorylated in the ΔphpP strain allowed us to identify new substrate modified by StkP/PhpP couple. The product of gene spr1851 called JagSpn is a putative RNA binding protein phosphorylated on Thr89. Jag proteins are widely conserved in bacteria and their role is unknown. Phenotype of the Δjag mutant suggests that JagSpn is involved in cell division and maintaining proper cell shape of S. pneumoniae.

Methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. E. coli DH5α used as a general purpose cloning host and E. coli BL21 used for protein expression were cultured in Luria–Bertani (LB) broth at 37 °C. The wild type S. pneumoniae strain Rx1 and its corresponding mutants were grown statically at 37 °C in Brain–heart Infusion (BHI) medium, Tryptone Soya Broth (TSB) medium, semi-synthetic C medium supplemented with 0.1 % yeast extract (C + Y) [49] or in Casein Tryptone (CAT) medium supplemented with 0.2 % glucose and 1/30 volume 0.5 M K₂HPO₄, pH 7.5 [49]. DNA from the strain CP1016 (rif-23) was used as the donor DNA for the competence assays [50]. The following antibiotics were added when necessary at the indicated concentrations (in μg ml⁻¹): rifampin (Rif), 1; kanamycin (Kan), 200; streptomycin (Sm), 500; tetracycline (Tet), 2.5; erythromycin (Erm), 1 (for S. pneumoniae); ampicillin (Amp), 100; kanamycin (Kan), 50; erythromycin (Erm), 100 (for E. coli).

Plasmid construction

Plasmids used in this study are listed in Table 1 and oligonucleotides are listed in Additional file 1: Table S1. To construct plasmid pZn-PhpP, phpP was amplified with primers JG19 and JG20 using WT chromosomal DNA as a template. The PZn promoter was amplified with primers LN123 and AU79. The final PCR product was used as a template in a fusion PCR with primers LN123 and JG21 from a template plasmid pJWV25 [35]. Both PCR products were used as a template in a fusion PCR with primers LN123 and JG20. The final PCR product PZn-phpP was cloned into the KpnI and NotI restriction sites of the plasmid pJVW25. Plasmid pZn-jag-flag was constructed as follows: jag gene was amplified with primer pairs AU77 and AU79 (containing Flag sequence and NotI restriction site) using wild type chromosomal DNA as a template. The PZn promoter was amplified with primers LN123 and AU76 from a template plasmid pJVW25. Both PCR products were used as a template in a fusion PCR with primers LN123 and AU76. The final PCR product was cloned into the EcoRI and NotI restriction sites of the plasmid pJVW25. To generate pZn-flag-locZ, the PZn promoter was amplified with primers LN123 and NS1 from a template pJVW25 and locZ was amplified with
Table 1  Bacterial strains and plasmids used in this study

| Strain/plasmid | Genotype or description | Source |
|----------------|-------------------------|--------|
| **Strains**    |                         |        |
| S. pneumoniae  |                         |        |
| Sp1 (Rx1)      | unencapsulated, wild-type, str1, hexA | [54]   |
| Sp10           | Cm, stkP::cm             | [17]   |
| Sp26           | Cm, divIVA::cm           | [55]   |
| Sp57           | locZ::lox72              | [25]   |
| Sp100          | Kan, phpP::kan rpsL      | This work |
| Sp113          | ΔphpP                    | This work |
| Sp120          | Tet, ΔphpP bgaA::P20::phpP | This work |
| Sp140          | Tet, ΔphpP bgaA::P20::gfp-phpP | This work |
| Sp161          | Kan, ΔphpP divIVA::kan rpsL | This work |
| Sp169          | ΔphpP ΔdivIVA             | This work |
| Sp174          | Erm, ΔdivIVA pMU-P96-divIVA-flag | This work |
| Sp188          | Erm, ΔlocZ pMU-P96-flag-locZ | This work |
| Sp220          | Kan, phpP::kan rpsL (reverted from Sp113) | This work |
| Sp222          | wild-type (reverted from Sp113) | This work |
| Sp292          | Tet, ΔphpP bgaA::P20::gfp-phpP-D192A | This work |
| Sp293          | Tet, ΔphpP bgaA::P20::gfp-phpP-D231A | This work |
| Sp295          | Δjag                     | This work |
| Sp302          | Tet, Δjag bgaA::P20-jag-flag-T89A | This work |
| Sp304          | Tet, Δjag bgaA::P20-jag-flag | This work |
| E. coli        |                         |        |
| DH5α           | F- lacZΔM15 Δ(lacZYA-argF) U169 recA1 endA1 hsdR17 (k-, mK+) phoA supE44 λ- thi-l gyrA96 relA1 | Invitrogen |
| BL21           | F- ompT gal [dcm] [λ AraD31] hsdS2 (R- mB-) (DE3) | Novagen |
| **Plasmids**   |                         |        |
| pJWV25         | Amp, tet, bgaA, P20::gfp-+ | [35]   |
| pJWV25-phpP    | Amp, tet, bgaA, P20::phpP | This work |
| pZn-flag-locZ  | Amp, tet, bgaA, P20::flag-locZ | This work |
| pZn-locZ       | Amp, tet, bgaA, P20::locZ   | This work |
| pZn-gfp-phpP-P20 | Amp, tet, bgaA, P20::gfp-phpP-P20 | This work |
| pZn-gfp-phpP-P231 | Amp, tet, bgaA, P20::gfp-phpP-D231A | This work |
| pZn-jag-flag-T89A | Amp, tet, bgaA, P20::jag-flag-T89A | This work |
| pZn-jag-flag   | Amp, tet, bgaA, P20::jag-flag | This work |
| pEXphpP-D231A  | Kan, phpP::D231A         | [17]   |
| pMU1328        | Erm, empty vector        | [51]   |
| pMU-P96-divIVA-flag | Erm, P96-divIVA-flag | This work |
| pMU-P96-locZ   | Erm, P96-locZ             | This work |
| Janus cassette | Kan, kan-rpsL+           | [52]   |

Amp ampicillin resistance marker, cm chloramphenicol resistance marker, kan kanamycin resistance marker, tet tetracycline resistance marker, erm erythromycin resistance marker
primers NS2 (containing Flag sequence) and LN155 using wild type chromosomal DNA as a template. Both PCR products were used as a template in a fusion PCR with primers LN123 and LN155 and the final PCR product was digested and cloned into the EcoRI and NotI restriction sites of the plasmid pJWV25. To construct plasmid pMU-P96.flag-loCZ, the P96 promoter was amplified with primer LN231 and LN215 from a template plasmid pMU1328 [51]. The flag-loCZ fragment was amplified with primers NS3 and NS4 using pZn-flag-loCZ as a template. Both PCR products were used as a template in a fusion PCR with primers LN231 and NS4. The resulting PCR fragment was cloned into EcoRI and Sall sites of pMU1328 vector. To generate plasmid pMU-P96-divIVA-flag, the P96 promoter was amplified with primers LN214 and LN215 from a template pMU1328. The divIVA gene was amplified with primers LN218 and LN229 (containing Flag sequence) using wild type chromosomal DNA as a template. Both PCR fragments were fused in a fusion PCR with primer pair LN214/LN229 and the acquired PCR fragment was inserted into BamHI and Sall sites of pMU1328. All constructs were verified by DNA sequencing.

Site directed mutagenesis
To introduce specific mutations in the phpP and jag genes we used the QuickChange mutagenesis kit (Strategene) according to manufacturer’s instructions. T89A mutation was introduced into plasmid pZn-jag-flag using primer pair AU80/AU81 to generate plasmid pZn-jag-flag-T89A. D192A mutation was introduced into pJWV25-phpP plasmid using primer pair AU69/AU70 to generate plasmid pZn-gfp-phpP-D192A. Plasmid pZn-gfp-phpP-D231A was constructed as follows: gene phpP-D231A was amplified by PCR using plasmid pEXphpP-D231A as a template and primers AU67 and AU68 containing Spel and NotI restriction site, respectively. PCR product was cloned into pJWV25 generating pZn-gfp-phpP-D231A. All constructs were verified by DNA sequencing.

Construction of pneumococcal strains
pJWV25 derived strains expressing proteins under control of PZn promoter were prepared by transformation of S. pneumoniae competent cells with the corresponding pJWV25 derived plasmids previously linearized by digestion with PvuI. Tetracycline resistant transformants were obtained by a double-crossover recombination event between the chromosomal bgA gene of the parental strain and bgA regions located on the plasmids as described previously [35]. Following plasmids were used for construction of corresponding strains: pZn-PhpP: Sp120; pJWV25-phpP: Sp140; pZn-gfp-phpP-D192A: Sp292; pZn-gfp-phpP-D231A: Sp293; pZn-jag-flag: Sp304; pZn-jag-flag-T89A: Sp302. Strain Sp174 was prepared by transformation of strain Sp26 (ΔdivIVA) with plasmid pMU-P96-divIVA-flag. Strain Sp188 was prepared by transformation of strain Sp57 (ΔlocZ) with pMU-P96-flag-loCZ.

Strain Sp113 (ΔphpP) was constructed as described by Agarwal et al. [11], using a Janus cassette (kana- mycin resistance gene followed by the recessive rpsL gene)-based two-step negative selection strategy [52]. In the first step 1100 bp and 1037 bp fragments corresponding to the upstream and downstream flanking regions of the phpP gene were amplified from the wild type chromosomal DNA with JG24/JG25 and JG26/JG27 primer pairs, respectively. The Janus cassette (1333 bp) amplified by JG28/JG29 primers from the Janus cassette DNA fragment was attached to the phpP flanking regions by fusion PCR using primers JG24 and JG27. The resulting PCR fragment was used for the transformation of the S. pneumoniae strain Rx1, and KanR/SmS transformants (Sp100, phpP::kan rpsL) were selected. The PCR fragments, consisting of the upstream and downstream flanking region of the phpP gene, were amplified by JG24/JG31 and JG27/ JG30 primer pairs, respectively, and fused by overlap extension using primers JG24/JG27. The resulting fragment was transformed into the strain Sp100 to gain Sp113 (SmR/KanS). Reverted strain Sp222 (WTΔ) was constructed as follows: in the first step, ΔphpP strain (Sp113) was transformed by PCR fragment consisting of upstream and downstream flanking regions of the phpP gene fused with Janus cassette, as described in the previous section and SmS/KanR transformants (strain Sp220, phpP::kan rpsL) were selected. The PCR fragments, consisting of the upstream and downstream flanking region of the phpP gene, were amplified by JG24/JG68 and JG27/JG67 primer pairs, respectively, and fused by overlap extension with phpP gene (amplified by primers JG65 and JG66) using the primers JG24/JG27. The resulting fragment was transformed into the strain Sp220 to obtain reverted strain Sp222 (SmR/KanS).

Strain Sp295 (Δjag) was constructed using a Janus cassette strategy [52]. In the first step, upstream and downstream flanking regions of the jag gene were amplified from the wild type chromosomal DNA with AU57/AU58 and AU59/AU60 primer pairs, respectively. The Janus cassette (1333 bp) amplified by JG28/JG29 primers from the Janus cassette DNA fragment was attached to the jag gene flanking regions by fusion PCR using primers AU57 and AU60. The resulting PCR fragment was used for the transformation of the S. pneumoniae strain Rx1, and SmS/KanR transformants (jag::kan rpsL)
were selected. The PCR fragments, consisting of the upstream and downstream flanking region of the jag gene, were amplified by AU57/AU74 and AU60/AU75 primer pairs, respectively, and fused by overlap extension using the primers AU57/AU60. The resulting fragment was transformed into the jag::kan rpsL strain and Sm^R/Kan^R transformants were selected (strain Sp295).

Strain Sp169 (ΔphpP ΔdivIVA) was constructed as follows: the upstream and downstream flanking regions of the divIVA gene were amplified from the wild type chromosomal DNA with JG57/JG58 and JG59/JG60 primer pairs, respectively. Both flanking regions were attached to the amplified Janus cassette (see above) by fusion PCR using primers JG57 and JG60. The resulting PCR fragment was used for the transformation of the ΔphpP strain (Sp113), and Sm^R/Kan^R transformants were selected to obtain Sp161 (ΔphpP divIVA::kan rpsL). To construct deletion of divIVA gene without selectable marker, the PCR fragments, consisting of the upstream and downstream flanking region of the divIVA gene, were amplified by JG57/JG62 and JG60/JG61 primer pairs, respectively, and fused by overlap extension using the primers JG57/JG60. Resulting fragment was transformed into Sp161 strain to yield Sm^R/Kan^R strain named Sp169.

Western blot analysis and immunodetection

Cells were grown in C + Y medium with or without the addition of an appropriate concentration of ZnSO_4 to an OD_600 of 0.4, harvested and resuspended in 1 ml of precooled lysis buffer containing 25 mM Tris (pH 7.5), 100 mM NaCl, Benzonase (Merck), and protease inhibitors (Roche). Cells were disintegrated using glass beads in a FastPrep homogenizer (ThermoScientific). Cell debris was pelleted by a centrifugation at 5,000 × g. The total cell lysate was further fractionated by centrifugation at 100,000 × g for 1 h at 4 °C and the cytoplasmic and membrane fractions were obtained. The protein concentration was determined using a bicinchoninic acid (BCA) protein estimation kit (Pierce). An aliquot of 30 μg of each protein fractions or total cell lysate was diluted 1:20 in BHI supplemented with 0.2% BSA (Bovine Serum Albumin) and 1 mM CaCl_2 and pH 4000 Da. For protein identification the peptide mass fingerprints were analyzed by MALDI-TOF mass spectrometry (BrukerDaltonics) in a mass range of 700–4000 Da. For protein identification the peptide mass spectra were searched against SwissProt or NCBI (National Center for Biotechnology Information) bacterial database database using an in-house Mascot search engine. The identity of protein candidates was confirmed using MS/MS analysis.

Competence assays

*S. pneumoniae* was induced to competence using competence stimulating peptide (CSP) as described previously [36], with minor modifications. Briefly, an exponential culture of cells cultivated in BHI medium was diluted 1:20 in BHI supplemented with 0.2% BSA (Bovine Serum Albumin) and 1 mM CaCl_2 and pH
adjusted to 7.8. The recipient strain was activated by the addition of CSP (250 ng ml\(^{-1}\)) and incubated for 10 min at room temperature. The \(rif-23\) donor DNA (1 \(\mu\)g ml\(^{-1}\)) was then added and DNA uptake was obtained by 20 min incubation at room temperature. The mixture was then diluted 1:10 in BHI medium and incubated at 37 °C for 2 h. 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. To generate natural competence profiles of the wild type and mutant strains, method according to Echenique et al. [36] was used with several modifications. Briefly, stocks of bacteria grown in BHI medium to an OD\(_{600}\) of 0.5 were diluted 100-fold in the same medium supplemented with 0.2 % BSA and 1 mM CaCl\(_2\) and pH adjusted to 7.8 and grown at 37 °C. Samples were withdrawn in 15-min intervals, diluted 10-fold into BHI medium containing \(rif-23\) donor DNA, and incubated for 30 min at 30 °C. Further incubation was carried out at 37 °C for 90 min before plating serial dilutions with and without rifampin. Transformation efficiencies were calculated as the ratio of the viable counts on plates with and without rifampin.

**Growth and environmental stress tolerance**

To generate the growth curves pneumococcal strains were inoculated (6.8 \(\times\) 10\(^5\) CFU ml\(^{-1}\)) in TSB medium, cultivated statically and the growth was monitored every 30 min by measuring OD\(_{600}\) for period of 7 to 8 h. The tolerance of pneumococcal strains to environmental stress was examined in a manner similar to that described previously [19]. To investigate heat stress resistance cultures were inoculated into TSB medium prewarmed to 37 and 40 °C. The acid tolerance of all strains was monitored by measuring the growth curve in TSB medium adjusted to pHs 6.5 and 7.5. The alkaline tolerance was monitored at pH 8.0. To test the tolerance to osmotic stress, bacteria were first grown to early exponential phase (OD\(_{600}\) 0.2) and then inoculated into prewarmed TSB medium with or without 400 mM NaCl. The sensitivity of cells to H\(_2\)O\(_2\) was tested by exposing exponential cultures (OD\(_{600}\) 0.4) grown in CAT medium at 37 °C to 10 mM and 20 mM H\(_2\)O\(_2\) for 15 min. Viable cell counts were determined by plating serial dilutions of cultures onto agar plates before and after exposure to H\(_2\)O\(_2\). The results were expressed as percentages of survival.

**Protein purification and dephosphorylation assay**

Recombinant His-PhpP was purified as described previously [17]. To purify Flag-tagged proteins form *S. pneumoniae* the strains Sp174 (ΔdivIVA pMU-P96-divIVA-flag), Sp188 (ΔlocZ pMU-P96-flag-locZ) and Sp304 (Δjag bga:: P\(_{\text{Zn}}\)jag-flag) were grown statically at 37 °C in C + Y medium supplemented with 0.25 mM ZnSO\(_4\). Total cell lysates were prepared as described above and Flag-tagged proteins were purified by affinity chromatography using ANTI-Flag M2 Affinity Gel (Sigma-Aldrich) according to the manufacturer’s instructions. Dephosphorylation assay was performed basically as described previously [17]. Briefly, Flag fusion-proteins of interest (Flag-LocZ, DivIVA-Flag or Jag-Flag) bounded on the M2 affinity gel were mixed with 55 μl of reaction buffer and 4 μg of purified His-PhpP and incubated at 37 °C. Phosphatase reaction was terminated by the addition of 5× SDS-PAGE sample buffer at different time intervals (0–90 min). Samples were boiled, subjected to SDS-PAGE and immunoblotted as described above.

**Electron microscopy**

Samples for electron microscopy were prepared as described elsewhere [25] except the dried samples were sputter coated with 3 nm of platinum in a Q150T ES sputter coater (Quorum Technologies Ltd.). The final samples were examined in a FEI Nova NanoSem 450 scanning electron microscope (FEI Czech Republic s.r.o.) at 5 kV using Circular Backscatter Detector and back-scattered electrons.

**Fluorescence microscopy**

Fluorescence microscopy was performed basically as described before [20]. Cells were grown statically at 37 °C in C + Y medium, and the expression of the GFP fusion proteins was induced by adding desired concentration of ZnSO\(_4\). To stain the unfixed cells with fluorescently labelled vancomycin (VanFL) (Molecular Probes) the pneumococcal cultures were grown to OD\(_{600}\) 0.2 in C + Y medium, and the samples were labelled with 0.1 μg ml\(^{-1}\) of Van-FL/vancomycin (50:50) mixture for 5 min at 37 °C before examination. A quantity of 2 μl of the culture was spotted onto a microscope slide and covered with a 1 % PBS agarose slab. The samples were observed using an Olympus CellR IX 81 microscope equipped with an Olympus FV2T Digital B/W Fireware Camera and 100× oil immersion objective (N.A. 1.3) (phase contrast). The images were modified for publication using CellR Version 2.0 software, ImageJ (http://rsb.info.nih.gov/ij/) and CorelDRAW X7 (Corel Corporation). Fluorescence intensity line scans were acquired using ImageJ and plotted as a function of cell length measured with MicrobeTracker Suite [53].

**Cell size analysis**

The phase-contrast images were analyzed using automated MicrobeTracker software [53] and cell size parameters were evaluated by the Mann-Whitney rank sum test and plotted using GraphPad Prism 3.0. \(P < 0.0001\) was considered as statistically significant. Cell
size throughout the text is indicated as the median cell size ± median absolute deviation (MAD).

Additional file

Additional file 1: Table S1. Oligonucleotides used in this study.

(ODC15 15 kb)

Abbreviations

2D SDS-PAGE: Two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis; CDD: Conserved domain database; CSP: Competence-stimulating peptide; ESTK: Eukaryotic-type serine/threonine protein kinases; ESTP: Eukaryotic-type serine/threonine phosphatases; GFP: Green fluorescent protein; Jag: N-terminal domain; Jag\_N: N-terminal domain; Jag\_s: pneumococcal Jag; KEGG: Kyoto encyclopedia of genes and genomes; KH: Ribonuclease protein K homology domain; MAD: Median absolute deviation; MALDI-TOF: Matrix-assisted laser desorption/ionization- time of flight mass spectrometry; P35: Protein 35 kDa; P40: Protein 40 kDa; PASTA: Penicillin-binding protein; PBP: Penicillin-binding protein; PG: Peptidoglycan; P2PC: Protein phosphate 2C, PPM: Protein phosphatases Mg2+Mn2+ dependent; pThr: phospho-threonine; PZn+: Zinc-inducible promoter; qRT-PCR: quantitative real-time polymerase chain reaction; R3H: Arginine-x-x-Histidine domain; SD: Standard deviation; TFE: Trifluorethanol; VanFL: Fluorescently labeled vancomycin; WT: Wild type

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Availability of data and materials

The data sets supporting the results of this article are included within the article and its Additional file 1.

Authors’ contributions

LD, JG and AU designed the study. LD and PB supervised the project. JG and PB wrote the manuscript. All authors discussed the results and implications and commented on the manuscript at all stages. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interest.

Consent for publication

Not applicable.

Ethics approval and consent to participate

This study did not involve human subjects, human material, human data, animals or plants.

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