SUPPLEMENTAL MATERIALS AND METHODS

Construction of plasmids. To construct plasmid pZn-His6-LocZ, *his6-locZ* was amplified with primers LN140 and LN155 using pETPHos-LocZ as a template. The P_{czcD} promoter was amplified with primers LN123 and LN139 from pJWV25 plasmid DNA. The two amplicons were then used as a template in a fusion PCR with primers LN123 and LN155. The PCR product was digested with EcoRI and NotI and ligated into corresponding sites of pJWV25. To construct plasmid pZn-GFP-LocZ, *locZ* was amplified using primers LN156 and LN157 and Rx1 chromosomal DNA as a template; the PCR product was cleaved with SpeI and NotI and cloned in corresponding sites of pJWV25. Plasmid pCDFDuet_locZ/stkP was constructed as follows: The cytoplasmic kinase domain of StkP (1-270) was amplified by PCR using Rx1 chromosomal DNA as a template and specific primers containing an NdeI or BamHI restriction site (Nterm-StkP-NdeI and Cterm-StkP-BamHI). The 810-bp amplified product was then digested and subcloned into NdeI-BglII pCDFDuet plasmid (Novagen), generating pCDFDuet_stkP. Then, the *locZ* gene was amplified using chromosomal DNA as a template and specific primers containing either a BglII or a PstI restriction site (Nterm-LocZ-BglII and Cterm-LocZ-PstI). This 1395-bp amplified product was digested by BglII and PstI and subcloned into pCDFDuet_stkP plasmid, thus generating pCDFDuet_locZ/stkP. All constructs were verified by DNA sequencing.

Mutagenesis of T67, T78 and S80 in pETPHos-LocZ and pZn-His6-LocZ. To introduce phosphoablative mutations in the *locZ* gene, we used the QuickChange mutagenesis kit (Stratagene), according to manufacturer´s instructions. The following combinations of primers were used: LN126 and LN127 for the T67A mutation; LN117 and LN118 for the

T78A mutation; LN149 and LN150 for the S80A mutation; LN210 and LN211 for the T67E mutation; LN208 and LN209 for the T78E mutation.

Construction of *locZ* deletion mutant in Rx1 and D39 genetic background. Δ*locZ* in Rx1 genetic background (Sp57) and in D39 genetic background (Sp267) were constructed by using a Cheshire cassette as a described previously (1).Up- and downstream fragments of *locZ* were produced by PCR using respectively the primer pairs PB014/PB015 and PB012/PB013. Chromosomal DNA was used as a template to amplify both flanking regions. Fragments were cut with MluI and NcoI. The cheshire fragment was amplified with primers DAM868 and DAM869 (1) and cut with MluI and NcoI. All three amplicons were then ligated into a linear DNA fragment and amplified with primers PB012 and PB015. The product was used for transformation of Sp1 (Rx1) and Sp4 (D39) competent cells, respectively. Erythromycin-resistant transformants were obtained by a double-crossover recombination event. Subsequently, a *locZ* deletion strain harbouring *lox72* in place of *locZ* (strains Sp57 and Sp267) was obtained (1). The fidelity of Cheshire excision was checked by DNA sequencing.

Construction of Δ*locZ S. pneumoniae* R6 strain using Janus cassette and construction of strains expressing *locZ* in its native locus

To employ a Janus cassette (2) for gene replacement through negative selection, a *rpsL* gene with up- and downstream flanking sequences was amplified with primers JG74 and JG75 using Rx1 (Sp1) chromosomal DNA as a template and the amplicon was transformed into R6 strain (Sp208) to obtain streptomycin resistant strain Sp225. Strain Sp225 was used as a parent strain for replacement of *locZ* by the Janus cassette via double cross-over. Janus cassette was amplified using primers JG28 and JG29. Upstream and downstream *locZ* regions (1000bp) were amplified using primer pairs KB1/KB2 and KB3/KB4, respectively, and R6

chromosomal DNA as a template. Fusion PCR reaction was performed using the three isolated PCR fragments and primers KB1 and KB4. Strain Sp225 was transformed with the product of the fusion PCR and resulting Kan resistant strain was named Sp227. To construct the *locZ* deletion mutant without a selectable marker (strain Sp239), we first amplified the *locZ* upstream flanking region (1000 bp) with primers KB1 and KB13 and *locZ* downstream flanking region (1000 bp) with primers KB4 and KB14. The corresponding PCR products were fused using primers KB1 and KB4 and the resulting fragment was transformed into strain Sp227 to obtain Sp239. Strain Sp227 was used for Janus cassette replacement by N-terminal fusion of *locZ* with *rfp* (*mKate2*) (Sp228) or *gfp* (Sp229). *rfp* was amplified with primers KB7 and KB8 using chromosomal DNA of strain HK95 (3). *locZ* was amplified with primers KB9 and KB10 and fused in PCR reaction with *rfp* with primers KB7 and KB10. Flanking sequences up- and downstream of *locZ* were amplified with primer pairs KB1/KB5 and KB4/KB6, respectively. The resulting PCR products were fused with *rfp-locZ* PCR product and the amplicon was transformed into strain Sp227 to obtain strain Sp228. *gfp-locZ* fusion was amplified using primers KB10 and KB11 and PZn-GFP-LocZ as a template. Flanking sequences up- and downstream of *locZ* were amplified with primer pairs KB1/KB5 and KB4/KB6, respectively, and were fused with *gfp-locZ*. The resulting fusion product was transformed into strain Sp227 to obtain strain Sp229. Strains expressing phosphoablative (Sp234) or phosphomimetic (Sp235) allele of *locZ* were prepared as follows: *locZ* was amplified with primers KB10 and KB12 using PZn-His6-LocZ-T67A/T78A or pZn-His6-LocZ-T67E/T78E as a template. Flanking up- and downstream regions were amplified with primers KB1/KB5 and KB4/KB6, respectively. The resulting PCR products were fused with modified *locZ* amplicons and then transformed into strain Sp227 giving rise to Sp234 and Sp235.

Constructions of strains expressing CFP-FtsZ. Strains, expressing CFP-FtsZ (Sp240, Sp242, Sp243) from plasmid pBCSMH036 (4) under control constitutive *sigA* promoter, were obtained by transforming the parent strains Sp208, Sp228 and Sp239 with plasmid pBCSMH036. Tranformants were selected using Tet (1 μg.ml^{-1}) as a marker.

Construction of pJWV25 derived strains expressing proteins under control of P_{czcD} promoter. pJWV25 strains were prepared by transformation of *S. pneumoniae* competent cells with the corresponding pJWV25 derived plasmids linearized by digestion with PvuI. Tetracycline resistant clones (2.5 μg.ml^{-1}) were obtained by a double-crossover recombination event between the chromosomal *bgaA* gene of parental strain and *bgaA* regions located on plasmids as described previously (5). Plasmid pZn-GFP-LocZ together with pDELstkP (6) were used for construction of strain Sp58. Plasmid pJWV25-GFP-StkP was used for construction of strains Sp246, Sp248 and Sp249. pJWV25-GFP-FtsA was used to generate strains Sp254, Sp256 and Sp257. pJWV25-DivIVA-GFP was used to generate Sp250 and Sp253. Plasmid pZn-His6-LocZ was used for construction of strains Sp60.

Expression and purification of recombinant proteins and *in vitro* protein phosphorylation. His6-LocZ, corresponding mutated proteins and the kinase domain of StkP were expressed and purified by affinity chromatography using a histidine tag as described previously (6). The recombinant protein substrate (0.2 μg) was incubated with the purified StkP kinase domain (StkP-KD) (0.2 μg) in a reaction buffer containing 25 mM Tris·HCl (pH 7.5), 25 mM NaCl, 5 mM $MnCl_2$, 10 μM ATP and 1 μCi γ[^{32}P]ATP. The reaction was started by the addition of ATP and terminated after 15 min of incubation in 37 °C by the addition of 5× SDS sample buffer. Samples were separated by SDS-PAGE, and the Coomassie blue

stained gel was exposed to a sensitive screen and scanned with Molecular Imager FX (Bio-Rad).

Identification of phosphorylated sites in LocZ. The duet strategy was used to generate hyper-phosphorylated LocZ as described previously (7). His-tagged LocZ was co-expressed with kinase domain of StkP in *E. coli* BL21(DE3)Star transformed with plasmid pCDFDuet_locZ/stkP and purified as reported previously (8). Purified His-tagged hyper-phosphorylated LocZ was subjected to mass spectrometric analysis after tryptic digestion. A sequence coverage of 98% that included all Ser and Thr residues was obtained. Spectra were analyzed with the paragon algorithm from the ProteinPilot® 2.0 database-searching software (Applied Biosystems) using the phosphorylation emphasis criterion against a homemade database that included the sequences of LocZ.

SDS-PAGE and immunodetection. Total cell lysates was prepared as a previously described (3). Briefly, cells were grown in TSB or C+Y medium (10 ml) supplemented with the appropriate $ZnCl_2$ concentrations (0.45 mM for TSB and 0.15 mM for C+Y) and $MnCl_2$ to a final concentration 0.1 mM. Cultures were grown until an OD_{600} of 0.4 and then 1ml of culture was harvested by centrifugation at 9000 *g* for 5 min. For lysis, the pellet was resuspended in 20 µl of SEDS lysis buffer (SDS 0.02 %; EDTA 15 mM; deoxycholate 0.01 %; NaCl 150 mM) and incubated for 5 min at 37°C. Lysates were diluted in 20 µl 2x SDS loading buffer and boiled for 10 min. After separation by SDS/PAGE, proteins were transferred to a PVDF membrane. Phosphorylated proteins were detected using anti-phosphothreonine polyclonal antibody (Cell Signalling). Proteins LocZ, StkP, DivIVA, FtsA were detected using custom-made anti-LocZ (this work), anti-StkP (9), anti-DivIVA (10) and anti-FtsA polyclonal rabbit sera (11). His-tagged or GFP fusion proteins were detected with

polyclonal anti-His (Sigma, A7058) or monoclonal anti-GFP antibodies (Santa Cruz Biotechnology, A1111).

RNA extraction and cDNA synthesis

For an analysis of quantitative real-time PCR (qRT-PCR), total RNA was isolated from 50 ml of pneumococcal culture grown at 37°C in TSB medium to an OD_{400} of 0.4. Total bacterial RNA was extracted with hot acid phenol according to the method of Cheng, *et al.* (12). After preliminary purification and precipitation with isopropanol, RNA sediment was washed with 1 ml of 70% ethanol (wt/vol). The crude total RNA was solubilized in water. The total RNA was treated with DNase I (Qiagen) and further purified using the RNeasy Mini Kit (Qiagen), according to the manufacturer's protocol. Pure RNA was analyzed on 1.5% agarose gel and stored at -80°C.

Superscript III (Invitrogen) was used for the first strand cDNA synthesis, which was performed according to the manufacturer's protocol. Briefly, 5 μg of purified total RNA samples were used in a first-strand reaction in the presence of random hexamer primers (Invitrogen) and RNase inhibitor RNasin (Promega) in the total volume of 20 μl. The reaction mixture was incubated at 42°C for 2 hours.

qRT-PCR

qRT-PCR analyses were performed using the iQ5 real-time PCR detection system (BioRad). A master mix with the following reaction components was prepared: 0.5 μM forward primer, 0.5 μM reverse primer, 10 nM fluorescein as an internal reference dye, 20 ng cDNA as a PCR template and 1x concentrated SYBR® Green JumpStart™ Taq ReadyMix (Sigma). The total volume of reaction was 20 μl. All primers were designed by using Primer3 software (http://frodo.wi.mit.edu/primer3/) and Primer select (Lasergene). The primers used (AU7-

AU16) are listed Table S3. The following cycling protocol was used for amplification: 2 min of initial denaturation at 94°C, followed by a three-step profile consisting of 15 s of denaturation at 94°C, 25 s of annealing at 56°C, and 25 s of extension at 72°C for a total of 45 cycles. Three biological replicates with three technical replicates each were used for each gene. Changes in the gene expression between the wild-type strain and each mutant strain were estimated by the $\Delta\Delta C_T$ method according to Pfaffl[75] (10). The *gyrA* gene (*spr1099*) encoding the DNA gyrase subunit A of *S. pneumoniae* was used as a reference gene for data normalization (13).

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