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Pneumococcal cell biology in a new light

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Chapter 5

Phosphorylated StkP induces the CiaR/CiaH two-component system in *Streptococcus pneumoniae*: indications for a feedback system driving pneumococcal cell-cycle progression

Beilharz and Veening, this thesis

Abstract

Streptococcus pneumoniae **is an oval-shaped Gram-positive human pathogen. This oval shape is most likely obtained by the alternation of peripheral cell wall elongation and septal cell wall synthesis. We recently showed that the eukaryotic-type serine/threonine kinase StkP localizes to the division site and plays an important role in regulating this cell elongation-division cycle. How StkP exactly does this remains largely elusive. To examine the physiological response of cells to the active form of StkP, StkP~P, we constructed a strain that allows induction of StkP in absence of its cognate phosphatase PhpP. This leads to the accumulation of StkP~P due to the lack of dephosphorylation by PhpP. Cells producing StkP~P are significantly shorter than wild-type cells, indicating that phosphorylation by StkP induces cell division. Interestingly, Western blotting demonstrated an increased turnover of StkP under hyperphosphorylation conditions, suggesting the specific induction of a protease. DNA-microarrays showed upregulation of the CiaR/CiaH two-component system under StkP~P conditions. As part of this regulon, a gene coding for the extracellular serine protease HtrA was significantly upregulated. Immunofluorescence microscopy experiments showed that HtrA and StkP colocalize at midcell. Together, our results suggest that accumulation of StkP~P leads to an activation of CiaR/H. Our data, however, cannot confirm proteolysis of StkP by HtrA, but it is tempting to speculate that a finely controlled protease-based feedback-system is in place to control cell-cycle progression in *S. pneumoniae*.**

Chapter 5

Introduction

Streptococcus pneumoniae (pneumococcus) is a Gram-positive oval-shaped pathogenic bacterium that causes severe diseases such as otitis media, pneumonia, meningitis and sepsis[3]. It is estimated that annually more than 1.6 million people die as a result of an infection with *S. pneumoniae*[158]. To obtain its characteristic ovoid shape the alternation of two processes might be crucial: the elongation of the peripheral cell wall and septal cell wall synthesis[53]. The synthesis of septal and peripheral peptidoglycan requires complex protein machineries, which are still not completely described and understood. Nevertheless, the interplay of these proteins forms the basis of a tight regulation. Recently, it was shown that the eukaryotic-type serine/threonine kinase StkP is strongly involved in the regulation of cell division[43,102,104,115]. StkP is part of the family of ultra-conserved eukaryotic-type serine/threonine kinases (STKs) of Gram-positive bacteria that often show an important role in regulating cell growth and division[101,115,175]. They all consist of an intracellular Hanks-type kinase domain and extracellular PASTA (Penicillin-binding protein And Serine/Threonine-Associated) domains. StkP colocalizes with and recruits PBP2x[47], a PBP required for septal cell wall synthesis[44,49]. StkP localizes to cell septa by sensing unlinked peptidoglycan via its extracellular PASTA domains[43,101,115]. By this, StkP gets activated, autophosphorylates and phosphorylates its target proteins. Among the target proteins, that are phosphorylated by StkP, are important players for cell division and cell wall synthesis, such as GlmM, DivIVA and putatively FtsA and FtsZ[104,205]. StkP acts together with the phosphatase PhpP in a functional pair and the two proteins show antagonistic activities. In this context, it was shown that PhpP dephosphorylates StkP and also its substrates[95]. Importantly, mutants in *stkP* show an elongated phenotype indicating that StkP and PhpP are involved in the control of cell division[43,102,104,115].

Several studies have addressed the global transcriptional response of cells lacking *stkP*. The mutant phenotypes indicated that StkP might be involved in virulence and competence[94], cell division regulation[43,102,104,115] and resistance to several forms of stress[107].

The two-component system CiaRH was reported to affect different processes that are important for cell integrity such as bacteriocin production[121], competence development, autolysis[119,206] and host colonization[123]. CiaR, the response regulator, controls at least 15 promoters[124], of which five regulate small non-coding RNAs. Among these 15 promoters, one regulates the *htrA-parB* operon. HtrA is an extracytoplasmic serine protease/chaperone that is part of the HtrA/DegP protein-family which was shown to play a role in native

protein turnover and recycling as well as in protein activation[207] and protein quality control[208]. DegP, first described for *Escherichia coli*, degrades misfolded proteins in the periplasm[209]. The *htrA* gene is not essential for the pneumococcus but absence of HtrA makes it more sensitive to oxygen and heat stress[122] and *htrA* mutants show decreased colonization of the nasopharynx[123]. Previously, it was shown that HtrA localizes to new division sites in exponentially growing *S. pneumoniae* cells[133]. The septal localization of HtrA begs the question whether it is also involved in cell division control under stress conditions. How HtrA localizes to the division sites is not fully understood, but it was shown that the anionic lipid content may influence its localization[133]. Few substrates of HtrA are known, including the signalling peptide that induces competence, CSP[129]. Furthermore, it was shown that pneumocin MN is either directly or indirectly degraded by HtrA[121]. Interestingly, inhibition of penicillin binding proteins (PBPs), which are all localized at cell septa, induces upregulation of *ciaRH* and thus *htrA*[120].

Here, we show that an accumulation of StkP~P, the active form of StkP, results in significantly shorter cells. An increased turnover of StkP was observed by Western blot analysis upon accumulation of StkP~P. Transcriptome analysis revealed that in response to StkP~P overproduction, the CiaR/H two-component system is upregulated, including *parB-htrA*. By immunofluorescence microscopy we show that StkP colocalizes with HtrA at new cell division sites. Although, we have no evidence at this moment, it is tempting to speculate that either HtrA or another protease degrades StkP-P to finely control cell-cycle progression via CiaR/H in *S. pneumoniae*.

Results

Overproduction of StkP in the absence of the PhpP phosphatase results in premature cell division and increased StkP turnover

To investigate how StkP controls the cell elongation-division cycle, we constructed a strain that allowed for fine-tuned expression of StkP in its kinase-active form. To do so, we first replaced the *phpP-stkP* locus with a chloramphenicol resistance marker. Next, we introduced a fully functional N-terminal GFP fusion to StkP[43] under the control of a zinc-inducible promoter P_{Zn} at the ectopic *bgaA* locus resulting in strain KB1-20 (D39, *phpP-stkP::cm;* P_{Zn}-*gfp-stkP*). Induction of GFP-StkP expression with Zn^{2+} in this strain thus leads to increased amounts of phosphorylated StkP (StkP~P) since its cognate phosphatase, PhpP, is absent. To examine the effects of StkP~P expression on cellular morphology, strain KB1-

20 was grown with and without addition of 0.1 mM $ZnCl_2$ and cells were collected for microscopy analysis. Strikingly, cells were significantly shorter when StkP~P was induced, indicating that cell division was accelerated in these cells (Fig. 1B).

Western blot analysis of strains D39 (wild-type), KB1-15 (D39*;* P_{Zn}-*gfp-stkP*), and KB1-20 (D39, *phpP-stkP::cm;* P_{Zn}-*gfp-stkP*), that were grown with and without induction by 0.15 mM $ZnCl_2$, was performed. Intriguingly, the levels of GFP-StkP were reduced in a *phpP-stkP* mutant background as compared to the *stkP* background, although the same concentration of inducer was applied and the total protein content was the same (Fig. 1C). These reduced protein levels of GFP-StkP in the absence of *phpP* suggests that StkP~P is specifically degraded by a yet unknown protease.

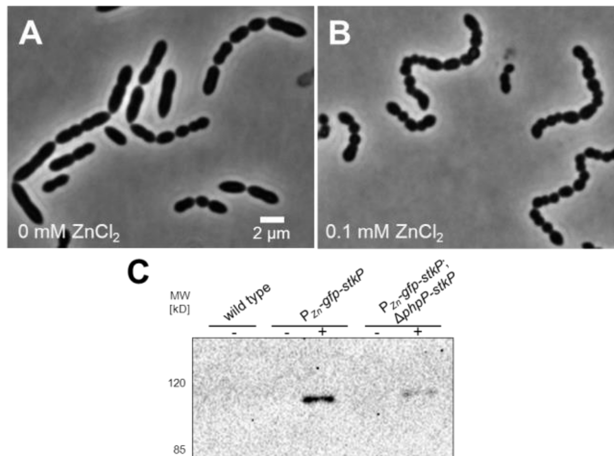

Fig. 1 Hyperphosphorylation has an effect on cell division and StkP turnover. Phase contrast micrographs of strain D39; Δ*phpP-stkP*, *bgaA::*P_{Zn}-*gfp-stkP* (A) without and (B) with addition of 0.1 mM $ZnCl_2$. The scale bar is 2 µm. (C) Quantity and degradation of GFP-StkP was immunodetected in wild type and hyperphosphorylation mutants using anti-GFP antibodies on total cell extract. Expression of GFP-StkP was induced with 0.1 mM $ZnCl_2$ (+).

Accumulation of StkP~P leads to upregulation of the *ciaRH* regulon

To investigate the global effects of accumulation of StkP~P, we performed a DNA microarray experiment comparing strain KB1-20 (D39, *stkP-phpP::cm;* P_{Zn}-*gfp-stkP*) with reference strain KB2-23 (D39, *stkP-phpP::cm*). Cells were grown in C+Y medium at 37°C and induced mid-exponentially (OD_{600} of 0.1) with 0.15 mM $ZnCl_2$. After 60 min of induction, cells were collected for RNA isolation, cDNA synthesis, labeling and hybridization to DNA microarrays containing all ORFs of *S. pneumoniae* D39 (see Materials and Methods). DNA microarray analysis showed differential gene expression of 143 genes, for which the expression was changed more than 2.5-fold. Regulatory pathway analysis showed that the *ciaRH* regulon was significantly upregulated upon StkP~P overproduction. The CiaR/H two-

component system was reported to be involved in a wide range of stress responses to maintain homeostasis of the cell[119,120]. Most interestingly, the gene encoding the serine protease HtrA, which belongs to the CiaRH-regulon[120,144], was found to be upregulated with a fold change of 3.7 (*p*-value 2.4E-15). The *htrA* gene is part of an operon with the chromosome segregation gene *parB* (Yuzenkova et al, submitted), which was also significantly upregulated (5.3 fold; *p*-value 3.8E-15).

Table 1 Summary of transcriptome comparison of D39; Δ*phpP-stkP* and D39; Δ*phpP-stkP*, P_{Zn}-*gfp-stkP* after induction with 0.15 mM $ZnCl_2$. Selection of top hits.

Gene name	Function	*p*-value	Fold change
ciaR (SPD_0701)	DNA-binding response regulator CiaR	1.45E-14	3.81
ciaH (SPD_0702)	sensor histidine kinase CiaH	1.40E-14	3.51
htrA (SPD_0268)	extracellular serine protease	2.44E-15	3.68
parB (SPD_0669)	Spo0J protein	3.77E-15	5.28
manM (SPD_0264)	PTS system mannose	1.47E-11	-3.07
manL (SPD_0264)	PTS system mannose	5.11E-15	3.01
stkP (SPD_1524)	Eukaryotic-type serine/threonine kinase	2.59E-12	2.57

HtrA and StkP colocalize at division sites

Since we found the protease *htrA* to be upregulated when StkP~P was overexpressed (Table 1), while the total StkP protein level was decreased under these conditions (Fig. 1C), this raises the question whether HtrA is responsible for StkP~P turnover. As a first step to address this question, we examined whether StkP and HtrA colocalize in single cells. Although it was previously reported that HtrA[133] and StkP[43,102,115] localize at sites of cell division, it is unclear whether they colocalize at the same stage during the pneumococcal cell cycle. To investigate localization of HtrA and StkP in one strain, we constructed strain KB3-03 (D39 *rpsL-*; *htrA*-FFF, P_{Zn}-*gfp-stkP; cps::rpsL+-kan*) for expression of GFP-StkP and Flag-tagged HtrA. To visualize HtrA, we used a fully functional C-terminally triply FLAG-tagged HtrA construct[133] in combination with immunofluorescence microscopy using an anti-Flag antibody and a red-fluorescently labeled secondary antibody. Localization of StkP in the same strain was visualized by induction with 0.1 mM $ZnCl_2$ of the functional GFP-StkP fusion. As shown in Fig. 2, HtrA-Flag3 and GFP-StkP indeed colocalize at midcell.

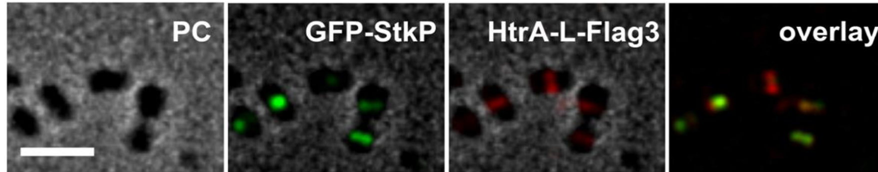

Fig. 2 Colocalization of HtrA and StkP to midcell. Immunofluorescence microscopy (IFM) of strain KB3-03 (D39 *rpsL-*; *htrA*-FFF, P_{Zn}-*gfp-stkP; cps::rpsL+-kan*). Cells were grown exponentially and harvested after induction of 40 minutes with 0.1 mM $ZnCl_2$. IFM was performed using anti-FLAG antibody and AlexaFluor®-555 (anti-IgG, rabbit). Phase contrast (PC) picture shows cell outline, localization of GFP-StkP in the GFP channel, localization of HtrA-$_L$FLAG^3 in the mCherry channel and an overlay of fluorescence channels. The scale bar is 2 µm.

Discussion

Cell division in bacteria is a complex process that involves tightly regulated activity of multiple proteins that are involved in that process. It was recently shown that the eukaryotic-type Ser/Thr-protein kinase StkP and its antagonistic protease PhpP are involved in controlling cell division in a yet unknown manner[43,115]. StkP senses the state of a cell via recognition of unlinked peptidoglycan at new division sites, which induces dimerization and autophosphorylation, resulting in StkP~P. StkP further phosphorylates proteins that are involved in cell division and StkP~P itself gets dephosphorylated by PhpP[95]. It was reported that cells lacking *stkP* are still viable but show an elongated phenotype and higher sensitivity to various types of stress, whereas *phpP* is reported to be essential for viability and thus hyperphosphorylation is lethal[112]. We therefore assume that the obtained *phpP* mutants accumulated suppressor mutantions and are thereby viable. Here we demonstrate a system with which we are able to induce hyperphosphorylation in cells lacking *phpP*. Accumulation of StkP~P results in heterogeneous cell shape and numerous shortened cells can be observed (Fig. 1B), which indicates that cell division is induced. In order to ensure proper propagation of cell division, the activity of StkP must be controlled. Interestingly, we observed an increased turnover of StkP under such conditions (Fig. 1C). What exactly is causing the increased turnover of StkP is not clear and one possible explanation is a direct proteolytic activity by a protease. It was reported before that proteases can postranscriptionally control protein abundance by proteolysis of target proteins[121]. In order to identify a potential protease that degrades StkP, we performed a DNA microarray experiment to see global changes in gene expression under hyperphosphorylated conditions. This condition is mimicked using a strain that is lacking *phpP-stkP*, with overexpression of a functional ectopic *gfp-stkP*. Results of the DNA microarray revealed activation of the two-component system CiaRH and genes of its regulon. In order to respond to environmental changes or external stresses, cells often use signaling pathways

that utilize the principle of phosphorylation and dephosphorylation. The most abundant ones in prokaryotes are the so-called two-component systems. The CiaR/H two-component system is involved in a wide range of aspects of stress response and homeostasis in *S. pneumoniae*. This system is important for colonization of the nasopharynx[123] and has an effect on competence[94], lysis[120,210] and bacteriocin production[121]. It has been shown that mutations in *ciaH* lead to a higher susceptibility to β-lactam antibiotics, which indicates that important genes involved in cell wall metabolism may be affected[117]. Further, it was shown that *ciaH* positively regulates the serine protease HtrA[122].

CiaRH is upregulated among the late competence genes and has been postulated to be important to exit the competent state[119]. As it was recently shown, HtrA degrades signal peptide CSP that induces competence of the cell[129] and it can thus be seen as a negative regulator of competence. On the other hand, *stkP* mutants show an upregulation of *comCDE*[107] but no responsiveness to CSP. Taking these results together, an antagonistic role of StkP and CiaRH in the regulation of *comCDE* expression is suggested[94]. Furthermore, it has been shown that HtrA has a regulatory role in bacteriocin activity, most likely due to posttranslational regulation[121].

HtrA is a molecular chaperone and protease and is assumed to be involved in protein quality control under normal and stress conditions. Stresses, e.g. heat stress, can induce misfolding of proteins. HtrA might thus be a protective force induced by cellular stress to refold or proteolyse damaged proteins[211]. Also, HtrA was recently shown to degrade GFP fusions to PBP2x, which contains PASTA domains[49]. It was previously reported that HtrA localizes to new division sites[133], the site of PG synthesis, in vicinity to cell division proteins such as FtsZ[102], FtsA[43], high molecular weight PBPs (PBP2x, PBP1a)[18], and DivIVA[187], that also localize to midcell. We showed by coimmunofluorescence microscopy that HtrA and StkP localize at cell septa in a similar manner (Fig. 2). It was reported that an inhibition of PG synthesis induces upregulation of *ciaRH* and *htrA* as part of the CiaRH regulon[120]. Accumulation of StkP~P led to a morphological change of cells, which indicates perturbed PG synthesis since cells appeared shorter and more heterogeneous (Fig. 1B). Under these conditions the CiaRH regulon was upregulated. This might imply a regulatory role of HtrA in cell division, by regulating activity and interplay of cell division proteins under cell wall stress. More research is required to test this hypothesis.

Materials and Methods

Growth condition. *S. pneumoniae* D39 strains were grown in C+Y medium[156] at 37°C. Blood agar plates were prepared with Columbia agar containing 3% defibrinated sheep blood (Johnny Rottier, Kloosterzade, The Netherlands). For induction of cells harbouring the inducible zinc-promoter P_{Zn} 0.1 mM $ZnCl_2$ was added to liquid culture. Transformation of D39 or Rx1 cells was performed as described previously[156]. Strains are listed in table 2.

Recombinant DNA techniques and oligonucleotides. DNA isolation, restriction, ligation, gel electropheresis and transformation of *E. coli* were performed as described[155]. For isolation of chromosomal DNA of *S. pneumoniae* the Promega Wizard Genomic DNA purification kit was used. Enzymes were purchased from Fermentas, New England Biolabs, Roche, and used as described by manufacturer. For PCR amplification Phusion high-fidelity DNA polymerase from Finnzymes was used.

Strain construction. *Strain KB3-03.* To obtain unencapsulated strain DRm (*rpsL-K56T*, Str^R) (Sorg RA, unpublished) was transformed with amplicon Δ*cps2E::*[*p-kan*^{R} *rpsL*+] of strain IU3286[212] using primers SR261 and SR262. Both *htrA* amplicons were obtained with primers P368 and P369[133]; *htrA*-L-FFF from strain IU4468 and Δ*htrA::*[*p-kan*^{R} *rpsL*+] from strain K110. All amplicons were kindly provided by Malcolm Winkler´s lab. The strain was transformed with pJWV25-*st*[43].

DNA microarray analysis. DNA microarrays were used to test the effect of StkP activity in the absence of dephosphorylation. The gene expression in strain KB1-20 (P_{Zn}-*stkP*, *phpP-stkP::cm*) was compared to that in strain KB1-23 (*phpP-stkP::cm*), where each was induced with 0.1 mM $ZnCl_2$. Two biological replicates were used. Cells were grown in 50 ml to an OD_{600} of 0.1 and then induced for 30 minutes before harvesting them by centrifugation (10 min, 10000 rpm, 4°C). Pellets were immediately frozen in liquid nitrogen and stored at -80°C. For RNA isolation the Roche RNA isolation kit was used as described by the manufacturer's manual. For cDNA synthesis and Cy3/Cy5 labeling of 0.15 ng/ml of total RNA the Cyscribe Post-Labelling kit (Amersham biosciences) was used. Hybridization of labeled cDNA was performed for 16 h at 45°C on superamine glass slides. Slides were scanned using a Genepix 4200AL laser scanner. For data analysis ArrayPro 4.5 (Media Cybernetics, Silver Spring) was used. Data were normalized using microPrep by means of a LOWESS normalization algorithm. Cyber-T was used for statistical analysis of the expression.

Western blot and immunodetection. Cells were grown in 4 ml C+Y to an OD_{600} of 0.1 and induced for 1h with $ZnCl_2$ to a final concentration of 0.1 mM. Cells were harvested by centrifugation of 10 min at 8000 rpm and 4°C. Pellets were resuspended in 100 µl SEDS lysis buffer (0.02% SDS, 15mM EDTA, 0.01% DOC, 150 mM sodium chloride) and incubated for 5 minutes at room temperature. Then, 100 µl 2-fold concentrated loading buffer was added to the lysates and boiled for 5 min before loading on a 12% SDS-gel. After separation of proteins by electrophoresis, proteins were blotted on a PVDF nitrocellulose membrane. For immunodetection of GFP-tagged proteins, a polyclonal anti-GFP antibody (Invitrogen) in a concentration 1:1000 and secondary HRP-labeled rabbit anti-IgG antibody was used.

Immunofluorescence microscopy. For immunofluorescence, cells were harvested at mid-exponential phase and fixed and treated as described by Morlot et al[18]. Cells were then transferred to poly-Lysin-coated glass slides. Slides were washed twice with PBS, air-dried, dipped in methanol at -20°C for 10 min and allowed to dry. After rehydratation with PBS, the slides were blocked for 1h at room temperature with 2% (w/v) bovine serum albumin, 0.2% Triton X-100 (v/v) in PBS (BSA-PBST), and for 1h with appropriate dilutions of anti-FLAG antibody in BSA-PBST. Slides were washed five times with PBS and incubated for 30 min with a 1:150 dilution of Alexa Fluor® 555 anti-rabbit IgG (Life technologies) in BSA-PBST.

Microcopy. Cultures of *S. pneumoniae* were grown and prepared as described. Images were taken with an Olympus IX71 Microscope (Personal DV, Applied Precision; assembled by Imsol, Preston, UK) using a CoolSNAP HQ2 camera (Princeton Instruments, Trenton, USA) with a 100× phase-contrast objective.

Table 2 Strain list

Strains and plasmids	Relevant properties	Source or reference
E. coli DH5α	F-, *araD*139, Δ(*ara-leu*)7696, Δ(*lac*)X74, *galU*, *galK*, *hsdR2*, *mcrA*, *mcrB1*, *rspL*	Laboratory stock
S. pneumoniae		
D39	encapsulated strain	[1]
KB1-20	D39, tet, *bga*::P_{Zn}-*gfp-stkP; phpP-stkP::cm*	[43]
KB1-15	D39, tet, *bga*::P_{Zn}-*gfpstkP*	[43]
KB2-23	D39, *phpP-stkP::cm*	[43]

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