Absence of pneumococcal PcsB is associated with overexpression of LysM domain-containing proteins

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The streptococcal protein required for cell separation B (PcsB) is predicted to play an important role in peptidoglycan metabolism, based on sequence motifs and altered phenotypes of gene deletion mutant cells exhibiting defects in cell separation. However, no enzymic activity has been demonstrated for PcsB so far. By generating gene deletion mutant strains in four different genetic backgrounds we could demonstrate that *pcsB* is not essential for cell survival in *Streptococcus pneumoniae*, but is essential for proper cell division. Deletion mutant cells displayed cluster formation due to aberrant cell division, reduced growth and antibiotic sensitivity that were fully reverted by transformation with a plasmid carrying *pcsB*. Immunofluorescence staining revealed that PcsB was localized to the cell poles, similarly to PBP3 and LytB, enzymes with demonstrated peptidoglycan-degrading activity required for daughter cell separation. Similarly to other studies with PcsB homologues, we could not detect peptidoglycan-lytic activity with recombinant or native pneumococcal PcsB *in vitro*. In addition to defects in septum placement and separation, the absence of PcsB induced an increased release of several proteins, such as enolase, MalX and the SP0107 LysM domain protein. Interestingly, genes encoding both LysM domain-containing proteins that are present in the pneumococcal genome (SP0107 and SP2063) and predicted to be involved in cell wall metabolism were found to be highly overexpressed (14–33-fold increase) in $\Delta pcsB$ cells in two different genetic backgrounds. Otherwise, we detected very few changes in the global gene expression profile of cells lacking PcsB. Thus our data suggest that LysM domain proteins partially compensate for the lack of PcsB function and allow the survival and slow growth of the pneumococcus.

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INTRODUCTION

The pneumococcus is one of the most important human pathogens, and defeats its host mainly by its fast multiplication and the tissue damage induced by inflammatory responses. A better understanding of the critical and essential steps in pneumococcal cell division and multiplication is important in order to develop novel strategies to reduce the high mortality associated with invasive pneumococcal infections.

As a crucial stress-bearing and shape-maintaining element, the integrity of the bacterial cell wall is essential to cell viability. Peptidoglycan, a cross-linked polymer of glycan strands and peptide chains, forms the scaffold of this structure. To maintain the protective nature of the cell

wall, bacterial cell division requires a tightly regulated and complex interplay of topological processes, biosynthetic reactions and cleavage mechanisms. In ovococcus bacteria such as *Streptococcus pneumoniae*, cell division proceeds along successive parallel planes perpendicular to the long axis of the cell. This mode of division contrasts with that of spherical coccus species such as *Staphylococcus aureus*, which divide in alternating perpendicular planes reflected by the mode of peptidoglycan synthesis in these species (for a review, see Zapun *et al.*, 2008).

In ovococcus species, peptidoglycan is synthesized at the septum and at the equatorial rings by the insertion of peptide-carrying disaccharide units into the existing murein sacculus through transglycosylation and transpeptidation, whereas spherical species have only septal peptidoglycan synthesis (for a review, see Zapun *et al.*, 2008). Cell division is completed by the action of murein hydrolases cleaving the peptidoglycan at the septum and thus releasing the adjacent daughter cells. Hydrolases are important for cell wall growth and separation during cell division, peptidoglycan recycling and turnover. These

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Abbreviations: CHAP, cysteine, histidine-dependent amidohydrolase/peptidase; C_T, cycle threshold; DAPI, 4,6-diamidino-2-phenylindole; qRT-PCR, quantitative real-time PCR; RBB, Remazol brilliant blue.

enzymes also participate in autolysis under certain growth conditions, and contribute to bacterial pathogenicity and susceptibility to antibiotics (Höltje & Tuomanen, 1991; Höltje, 1995; Vollmer *et al.*, 2008).

PcsB of *S. pneumoniae* (annotated as SP2216 in the TIGR4 genome) is a predicted murein hydrolase, and has orthologues in several streptococcal species that have been characterized to a varying degree, e.g. GbpB in *Streptococcus mutans* (Chia *et al.*, 2001a, b; Mattos-Graner *et al.*, 2001, 2006) and PcsB in *Streptococcus agalactiae* (Reinscheid *et al.*, 2001, 2003). PcsB and its orthologues consist of four major parts: a signal peptide, an N-terminal coiled-coil domain containing leucine-zipper motifs, an alanine-rich region of variable length and a C-terminal cysteine, histidine-dependent amidohydrolase/peptidase (CHAP) domain.

Orthologues of PcsB in several species have been described as immunodominant antigens during human infection and confer protection in animal models (Chia *et al.*, 2001a; Gieffing *et al.*, 2008; Smith *et al.*, 1994, 2001; McIver *et al.*, 1996; Teng *et al.*, 2003). The pneumococcal PcsB has also been shown to be a highly immunogenic protein in humans and a protective antigen in animal models in our laboratory (Gieffing *et al.*, 2008). GbpB in *S. mutans* and PcsB in *S. agalactiae* have been found to be important, but non-essential for growth. Gene deletion mutant strains display greatly reduced growth rates and altered cell morphology, with multiple and misplaced division septa within a single cell (Chia *et al.*, 2001a; Reinscheid *et al.*, 2001). Although pneumococcal PcsB has been described as being essential for *S. pneumoniae* strains D39 and R6 (Barendt *et al.*, 2009; Ng *et al.*, 2003, 2004, 2005), we reported previously that we were able to generate *pcsB* null strains in two different genetic backgrounds, and found similar phenotypic defects to those previously described for its streptococcal homologues (Gieffing *et al.*, 2008).

The CHAP domain is found in proteins from bacteria, bacteriophages, archaea and eukaryotes, and is commonly associated with domains, such as the bacterial-type SH3 and amidase domains, that have been shown to be involved in peptidoglycan hydrolysis (Bateman & Rawlings, 2003; Rigden *et al.*, 2003). The CHAP domain itself has peptidoglycan lytic activity, and conserved cysteine and histidine residues, which have been found to be required for its function, are also present and essential in pneumococcal PcsB (Ng *et al.*, 2004). Some of the CHAP-domain proteins exert robust hydrolase activity also *in vitro*, e.g. Iap from *Listeria monocytogenes* (Wuenschel *et al.*, 1993), whereas PcsB and its orthologues have failed to show murein hydrolase activity in biochemical assays (Mattos-Graner *et al.*, 2006; Reinscheid *et al.*, 2001, 2003). Similarly, analysis of the muropeptide composition in gene deletion or low-expressing strains has not revealed any differences between wild-type and mutant strains (Reinscheid *et al.*, 2003; Barendt *et al.*, 2009).

PcsB and its homologues in other streptococcal, enterococcal and lactococcal species share a similar organization

of the genome region, namely they localize in the immediate vicinity of the *mreC* and *mreD* genes, which encode the homologues of morphogenic proteins in rod-shaped bacteria (Stewart, 2005; Osborn & Rothfield, 2007), pointing towards a possible involvement of PcsB in the determination of cell morphology. The membrane-spanning essential protein MreC has been shown to link the bacterial cytoskeleton to a variety of cell wall-modifying enzymes. Additionally, MreC can form filaments that run perpendicularly to the membrane (Divakaruni *et al.*, 2007; Kyburz *et al.*, 2010). However, it has been shown that perturbations of expression of the upstream *mreCD* genes do not contribute to the cell division defects of *pcsB* mutants (Barendt *et al.*, 2009).

As the specific function of PcsB and its homologues remains unknown to date, we aimed to contribute to a better understanding of the role of pneumococcal PcsB in the course of this study. We show that PcsB is not essential for *in vitro* viability in four different genetic backgrounds, and that the phenotypic defects of *pcsB* deletion strains can be restored by extrachromosomal expression. In addition we demonstrate that PcsB localizes at the cell poles, but is excluded from the division septum. The most obvious changes in gene transcription in the *pcsB* null mutant cells are associated with highly increased expression of LysM domain-containing proteins, indicating a similar or overlapping function shared by these proteins.

METHODS

Bacterial strains and growth conditions. TIGR4 (serotype 4) and PJ1324 (serotype 6B) strains were provided by Birgitta Henriques-Normark (Swedish Institute for Infectious Disease control), EF3030 (serotype 19F) by David Briles (University of Alabama) and 4DS2341-94 (serotype 4) strain by Eddie Ades [Centers for Disease Control (CDC), USA]. $\Delta pcsB$ and $\Delta SP2063$ mutant strains were generated as described previously (Gieffing *et al.*, 2008). Mutant strains were verified first by PCR amplification of the altered genomic region using primer combinations specific for flanking and integrated fragments. Southern blot analysis confirmed integration into the expected chromosomal region, and Western blotting proved the absence of protein expression. Northern blotting was performed to confirm the absence of target gene mRNA and the presence of flanking mRNA transcripts. Bacteria were cultured in Todd-Hewitt broth supplemented with 0.5% yeast extract (THY), including the appropriate antibiotic for mutant strains at 37 °C in an atmosphere of 5% CO₂.

Complementation of the *pcsB* deletion strain. For complementation, extrachromosomal expression of PcsB from a plasmid was chosen. Briefly the complete *pcsB* gene was cloned into the multiple cloning site of pMU1328 with a fucose-inducible promoter (Chan *et al.*, 2003) via *EcoRI* and *XbaI*. The plasmid was transformed as described previously (Gieffing *et al.*, 2008), and erythromycin- and kanamycin-resistant clones were selected for further analysis. Empty plasmid alone is abbreviated as pMU, the one containing *pcsB* as pMU-PcsB throughout the text.

Antibiotic sensitivity testing. Pneumococci were grown until they reached OD₆₂₀ ~0.5. Subsequently, 2 × 10⁷ bacteria were streaked on a blood agar plate to ensure a dense bacterial lawn and after 15 min an

antibiotic test disc (Fluka and bioMérieux) was placed into the centre of each plate. Plates were incubated at 37 °C and 5 % CO₂ for 24 h, and the diameters of growth inhibition zones were measured. A mean value was calculated from three independent experiments and used to express the percentage increase in antibiotic sensitivity relative to the respective wild-type strain.

Cloning and expression of recombinant pneumococcal proteins. The following PcsB constructs were generated from TIGR4 genomic DNA: amino acids 28–392 (PcsB-FL), amino acids 28–278 (PcsB-N) and amino acids 258–392 (PcsB-CHAP) (see also Fig. 4a). FtsZ (amino acids 2–419) and PspA (SP0117) were generated in previous studies (Giefing *et al.*, 2008, 2010). All genes were cloned into the pET28b(+) vector (Novagen) and expressed with a C-terminal His-tag in *Escherichia coli* BL21 Star (Invitrogen). Proteins were bound to Ni-Sepharose beads (GE Healthcare) and eluted with 500 mM imidazole in 20 mM NaH₂PO₄, 0.5 mM NaCl, pH 7.4. Proteins were extracted from inclusion bodies that were solubilized in 8 M urea in 50 mM Tris/HCl, pH 8, and gradually dialysed against decreasing concentrations of urea-containing Tris buffer and β -mercaptoethanol. Insoluble proteins were removed by centrifugation and the protein solution was prepared in Tris buffer at 1–2 mg ml⁻¹. The purity of the proteins used for interaction studies was >90 % based on SDS-PAGE (Fig. 4b).

Generation of hyperimmune mouse and rabbit sera. C3H/HeNHsd mice (10 mice per group, female, 6–10 weeks, Harlan Winkelmann) were immunized three times at 14 day intervals subcutaneously (into the flank) with 50 μ g recombinant protein (FtsZ, PcsB-FL, PcsB-N, PcsB-CHAP, SP0107, SP0082, SP0564, SP0498, MalX or PspA) and hyperimmune sera were collected on day 35. New Zealand White rabbits (Charles River) were immunized at days 0, 28, 42 and 56 with 250 μ g PcsB-N, StkP-C or PsaA recombinant protein, and complete Freund's adjuvant (CFA)/incomplete Freund's adjuvant (IFA) as adjuvant, and terminally bled on day 70.

Electron microscopy. Wild-type, Δ pcsB and extra-chromosomally complemented mutant cells of *S. pneumoniae* strains were fixed with 2.5 % glutaraldehyde in Dulbecco's PBS (Gibco) (pH 7.4) for 2 h on ice and for 1 h at room temperature, transferred into cellulose capillary tubes (0.2 mm diameter), fixed with 2 % OsO₄ in Sorensen's phosphate buffer (0.1 M sodium phosphate, pH 7.4) for 90 min and dehydrated with increasing concentrations of ethanol. Samples were embedded in epoxy resin (Agar 100), and thin sections (60–80 nm) were cut with a Leica Ultracut S ultramicrotome, mounted on copper grids, contrasted by uranyl acetate and lead citrate, and examined at 80 kV in a JEOL JEM-1210 electron microscope. Images were acquired using a digital camera (Morada) for the wide-angle port of the transmission electron microscope and with analySIS FIVE software (Soft Image System).

Western blotting. Proteins were separated on 4–20 % gradient SDS-PAGE gels under reducing conditions and blotted to nitrocellulose membranes using a dry-blotting system (Invitrogen). Blocking and incubation with primary (1:1000 dilution) and horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000 dilution) were done in 5 % milk in PBS containing 0.1 % Tween 20 for 1 h at room temperature. Membranes were imaged with chemiluminescent substrate (Chemiglow, Alpha Innotech) and a charge-coupled device (CCD) camera (FluorChem SP, Alpha Innotech).

Immunofluorescence microscopy. Pneumococci were stained as described previously (Giefing *et al.*, 2010). Briefly, bacteria were fixed with 3 % paraformaldehyde, transferred to polylysine-coated microscope slides and permeabilized with 0.1 % Triton X-100 in PBS. After blocking, primary antibodies were added at a 1:50 dilution, followed

by incubation with anti-rabbit-Cy2 and anti-mouse-Texas Red antibodies (JacksonImmuno) and staining with 4,6-diamidino-2-phenylindole (DAPI). Slides were mounted with Eukitt (Fluka) and visualized with a Zeiss Axiovert 200M microscope with a Zeiss LSM510META confocal laser-scanning unit and an α Plan-Fluor $\times$ 100 1.45 oil (aperture, 0.11 mm) objective (Zeiss). Images were prepared with Zeiss LSM Image Browser version 4.2.0.121 and Adobe Photoshop CS4 software by adjusting the brightness and contrast of the staining.

Determination of cellular DNA content by flow cytometry. Pneumococci in different growth stages were fixed with 70 % ethanol. After washing with PBS, bacteria were incubated in staining solution (0.2 mg DNase-free RNase A ml⁻¹, 20 μ g propidium iodide ml⁻¹ and 0.1 % Triton X-100 in PBS) for 30 min at room temperature. Samples were measured with a flow cytometer (FC500, Beckman Coulter) and data were analysed using CXP software (Beckman Coulter).

In-gel peptidoglycan lysis assay. The peptidoglycan lytic activity of PcsB was tested in a zymogram assay according to Lepeuple *et al.* (1998). Total bacterial lysate and culture supernatant of TIGR4 wild-type and recombinant PcsB-FL were analysed on a 12 % polyacrylamide gel containing 0.5 % SDS-treated pneumococcal cells. Cells were prepared by heating at 100 °C for 30 min in 4 % SDS according to the method of Schubert *et al.* (2000). After electrophoresis, the 'cell-gel' was renatured in 0.1 % Triton X-100 in 50 mM MES-NaOH, pH 6.0, at 37 °C for 48 h. Gels were stained with 0.1 % methylene blue in 0.01 % KOH and destained with water. Bacteriolytic activity was visible as clear bands on a blue background.

Peptidoglycan isolation and dye-release assay. Pneumococcal cell wall was isolated as described previously (Zhou *et al.*, 1988). Briefly, TIGR4 and PJ1324 cells were killed by autoclaving, resuspended in 10 % TCA and incubated at 4 °C overnight. Insoluble material was collected, heated in 5 % TCA for 10 min and trypsinized overnight at 37 °C, followed by heat-inactivation. After extensive washing with PBS, the purified cell wall material was stored at -20 °C.

Remazol brilliant blue (RBB) derivatives were prepared by incubating insoluble peptidoglycan in 0.02 M RBB in 0.25 M NaOH for 6 h at 37 °C and 12 h at 4 °C. Dyed products were washed extensively with PBS until the supernatant was colourless (Zhou *et al.*, 1988). A 1 ml volume of peptidoglycan solution corresponded to the amount isolated from 10¹⁰ bacteria.

For dye-release assays, 100 μ g recombinant PcsB protein (full-length, N-terminal and C-terminal CHAP domain) was incubated with 900 μ l peptidoglycan solution in a total volume of 1 ml at 37 °C overnight, then the mixture was separated by centrifugation for 1 min at 10 000 g and the release of blue dye to the supernatant was measured at 595 nm.

RNA isolation. Bacteria grown in THY medium were harvested in the exponential phase (OD₆₂₀ ~0.4) and total RNA was isolated using the RNeasy Midi kit (Qiagen) according to the manufacturer's instructions, performing on-column DNase digestion using the RNase-free DNase set (Qiagen). The integrity and purity of the RNA were verified by agarose gel electrophoresis, determining OD₂₆₀:OD₂₈₀ ratios and performing RT-PCR using the Superscript III First-Strand Synthesis system (Invitrogen) and random hexamers in the standard protocol.

Analysis of changes in mRNA levels. Microarray analysis was performed at Eurogentec (Seraing, Belgium) using Cy3- and Cy5-labelled samples on an *S. pneumoniae* MicroArray [Eurogentec, 2085 ORFs of *S. pneumoniae* (TIGR4)], including data processing and

normalization (*S. pneumoniae* Hybridization Service package). Based on these data, genes that showed changes of at least twofold in mRNA expression were selected for quantitative real time-PCR (qRT-PCR).

qRT-PCR analyses were performed using the LightCycler 480 SYBR Green I Master system (Roche). The mRNA expression of the selected genes was analysed in three independent RNA isolations from TIGR4 and PJ1324 wild-type and isogenic $\Delta pcsB$ strains using gene-specific internal primers. Quantitative differences for each sample were determined using the $2^{-(\Delta C_T^{\text{sample}} - \Delta C_T^{\text{calibrator}})}$ method (Livak & Schmittgen, 2001), where C_T is the cycle threshold.

RESULTS

PcsB is not essential for *in vitro* viability of *S. pneumoniae*, and phenotypic changes can be restored by complementation of PcsB

Pneumococcal *pcsB* has been reported to be an essential gene in the R6 and D39 strains (Barendt *et al.*, 2009; Ng *et al.*, 2003, 2004, 2005). However, we were able to generate *pcsB* null mutants in the genetic background of TIGR4 (a serotype 4 strain) and a clinical isolate of a 6B strain, both capable of inducing lethal sepsis in mice (Gieffing *et al.*, 2008). During this study we generated viable $\Delta pcsB$ mutants in two additional genetic backgrounds using the transparent strain 4DS2341-94 (serotype 4) and EF3030 (serotype 19F), which induce mucosal and lung colonization, respectively (van Ginkel *et al.*, 2003). All four deletion mutant strains displayed greatly reduced *in vitro* growth rates even in nutrient-rich culture medium (Fig. 1a; Gieffing *et al.*, 2008). The mean mass doubling times increased in all mutant strains compared with the corresponding parent wild-type, and were 124 versus 62 min for TIGR4, 82 versus 56 min for 6B PJ1324, 109 versus 76 min for 4DS2341-94 and 85 versus 58 min for EF3030. We did not observe any significant difference in terms of autolysis, either in the rate or in the level to which they returned, as all strains, mutant and wild-type, declined to similar optical density values after reaching stationary phase. *In vivo* these strains can no longer cause disease in mouse models at a very high challenge dose (Gieffing *et al.*, 2008; data not shown).

Our attempts to delete PcsB in the D39 and R6 strains were not successful, suggesting that *pcsB* is indeed essential in these strains. However, based on the successful generation of deletion mutants in four unrelated strains, we concluded that PcsB is not essential for all pneumococcal strains, but many genetic backgrounds, notably recent field isolates, tolerate the absence of PcsB *in vitro*.

Since addition of recombinant PcsB to the growth medium could not restore the wild-type phenotype *in vitro* (data not shown), we complemented the $\Delta pcsB$ strain in the 6B background with plasmid pMU1328 carrying *pcsB* in order to connect the observed phenotype unambiguously to the absence of PcsB. We observed that $\Delta pcsB$ strains were hampered in competence, showing approximately 200-fold lower transformation efficiency than the wild-type strain. Nonetheless, plasmid pMU-*pcsB* complemented $\Delta pcsB$ cells

and restored the growth rate to the level of wild-type cells, while transformation with the empty plasmid was ineffective (Fig. 1b). There was a slight and not significant increase in the growth rate of wild-type cells transformed with pMU-PcsB. Importantly, the expression of PcsB at the protein level in the culture supernatant was similar in the wild-type and complemented strains (Fig. 1c).

Previously we reported an increased antibiotic sensitivity of $\Delta pcsB$ cells in the TIGR4 background (Gieffing *et al.*, 2008). Here we extended this analysis to $\Delta pcsB$ 6B cells and confirmed our previous findings obtained with the TIGR4 strain. The increased sensitivity of $\Delta pcsB$ strains relative to the corresponding parent wild-type, which was most pronounced towards antibiotics that target peptidoglycan synthesis, was almost completely eliminated by extra-chromosomal PcsB expression (Fig. 2a).

Similarly to other phenotypic changes, the severe defect in septum placement and cell separation, observed by electron microscopic analysis of gene deletion mutant cells (Gieffing *et al.*, 2008), was reversed by transformation with the pMU-*pcsB* plasmid, but not with the empty plasmid. Successfully complemented cells again displayed the typical wild-type diplococcus phenotype with regular septum formation (Fig. 2b).

Nucleoid partition is not affected in PcsB null mutants

To assess whether DNA duplication and segregation into daughter cells are affected in $\Delta pcsB$ cells, we determined the DNA content of growing cultures by propidium iodide staining combined with flow cytometric analysis (Fig. 3a). DNA staining of wild-type cells revealed two distinct populations, represented by two sharp peaks. The left peak with lower intensity corresponded to non-dividing cells, based on its abundance in stationary phase, while the right peak with higher intensity represented dividing cells with duplicated DNA content. In *pcsB* deletion mutant cells, instead of two sharp peaks, we observed one broad peak in growing cultures that extended to a higher intensity on the fluorescence scale, suggesting that several DNA duplication events occurred in the cell clusters. Immunofluorescent staining with DAPI revealed that each cell visualized by anti-PspA antibodies contained a similar amount of DNA (Fig. 3b).

The two observations together suggested that the cell separation defect in $\Delta pcsB$ cells occurred after DNA duplication.

PcsB does not lyse peptidoglycan *in vitro*

Based on the observed cluster formation and the presence of a CHAP domain, PcsB has been suggested to be involved in peptidoglycan metabolism (Ng *et al.*, 2004; Reinscheid *et al.*, 2001). Therefore, we attempted to detect the cell wall hydrolytic activity of pneumococcal PcsB in *in vitro*

Fig. 1. Wild-type growth rate is restored upon complementation of PcsB in $\Delta pcsB$ cells. (a) *In vitro* growth of 4DS2341-94 (left panel) and EF3030 (right panel) wild-type and corresponding $\Delta pcsB$ strains. (b) *In vitro* growth of PJ1324 (serotype 6B) wild-type and $\Delta pcsB$ strains transformed with either empty pMU or pMU-PcsB. (c) Western blot analysis of culture supernatants (overnight culture) of wild-type and *pcsB* mutant PJ1324 cells using PcsB-specific antibodies.

biochemical assays in a way similar to that described for the group B streptococcal PcsB homologue (Reinscheid *et al.*, 2001).

We generated recombinant proteins corresponding to the full-length PcsB, as well as its N- or C-terminal part containing the coiled-coil or CHAP domain, respectively (Fig. 4a, b). First we used an in-gel zymogram assay according to Lepeuple *et al.* (1998). SDS-treated pneumococcal cells were embedded as substrate in a polyacrylamide gel, and pneumococcal lysate, culture supernatant fractions or recombinant full-length PcsB were separated by

SDS-PAGE. After renaturation, lytic bands were detected by methylene blue staining. We observed only one band with an approximate molecular mass of 36 kDa (probably corresponding to the major autolysin LytA). However, no activity at the expected size of PcsB (43 kDa) in lysate or culture supernatant fractions or with recombinant PcsB-FL was observed (Fig. 4c). Lysate and culture supernatants of $\Delta pcsB$ strains displayed the same pattern of lytic bands as the corresponding wild-type cells (data not shown).

Next, we set up an assay according to Zhou *et al.* (1988) for the detection of the dye release from RBB-stained

Fig. 2. Complementation by extrachromosomal expression of *pcsB* restores wild-type antibiotic sensitivity and cell morphology. (a) Antibiotic sensitivity was determined by the disc-diffusion method in three independent experiments in PJ1324 $\Delta pcsB$ cells or the same cells transformed with either empty pMU or pMU-PcsB. The average increase in the diameter of the growth inhibition zone (increase in sensitivity) was expressed as a percentage relative to the wild-type. Error bars, SD. (b) Transmission electron microscopy of the $\Delta pcsB$ gene deletion strain transformed with either pMU alone or pMU-PcsB for complementation.

peptidoglycan that would be expected in the presence of peptidoglycan hydrolase activity. We incubated the recombinant PcsB-FL and its N- and C-terminal subdomains with the stained peptidoglycan substrate and measured the amount of dye release in the supernatant after incubation. No significant dye release was observed with any of the samples (data not shown).

PcsB has a polar subcellular localization and is excluded from the septum where new peptidoglycan is synthesized

To determine the subcellular localization of PcsB during cell division and growth, we performed double staining of wild-type pneumococcal cells with antibodies specific for PcsB-FL and FtsZ, the bacterial tubulin homologue that forms the division septum. We did not observe an overlap

between FtsZ and PcsB staining in any of the cells. The PcsB signal was excluded from the septal site and the divisome complex and appeared at the cell poles (Fig. 5a, b). The signal intensity was the greatest during exponential growth and was significantly reduced as cells progressed towards the stationary phase, most likely due to increased shedding of PcsB into the culture supernatant (data not shown). When *pcsB* deletion mutant cells were stained with PcsB-specific antibodies, no immunofluorescence signal was detected (Fig. 5c).

Absence of PcsB induces increased release of proteins to the culture medium

By analysing culture supernatants of $\Delta pcsB$ strains with SDS-PAGE, we detected differences in the banding pattern relative to the wild-type strain. Certain protein bands, most

Fig. 3. DNA duplication and separation are not affected in $\Delta pcsB$ mutant cells. (a) In wild-type (WT) and $\Delta pcsB$ cells, DNA was stained using propidium iodide and the intensity of the signal was measured in a flow cytometer throughout the progression of the cell cycle as monitored by OD_{620} readings. All histograms were generated by counting 10 000 events. (b) $\Delta pcsB$ mutant cells (strain 4DS2341-94) were double-stained with PspA/Cy2 and DNA/DAPI, as described in Methods, and imaged using a confocal microscope.

prominently an approximately 50 kDa protein, appeared to be more abundant in the culture supernatant of the mutant strain (Fig. 6a). This observation prompted us to further analyse culture supernatants by 2D-PAGE. Mass spectrometric analysis of the 50 kDa protein spot that was the most over-represented in the gene deletion strain supernatant revealed that it was identical to pneumococcal enolase (data not shown). When we sampled the culture supernatant of wild-type and $\Delta pcsB$ strains at multiple time points during growth and analysed them by Western blotting, we observed strong enrichment of enolase in the culture supernatant of the mutant strain that was most intense in the stationary phase (Fig. 6b). The most straightforward explanation for the appearance of enolase in the extracellular space would be the increased fragility of $\Delta pcsB$ cells. However, the selective appearance of certain protein bands did not support bacterial lysis as an explanation.

To investigate the selectivity of protein release, we compared the amounts of a selected set of pneumococcal

proteins in wild-type and $\Delta pcsB$ strains. We analysed the culture supernatant fractions of wild-type and deletion mutant pneumococcal cells from the early exponential to the late stationary growth phase by Western blotting with antibodies generated against different pneumococcal proteins available in our laboratory (Gieffing *et al.*, 2008). Out of the nine proteins analysed, four were present in increased amounts in the PcsB gene deletion strain, namely SP0107, a LysM domain protein, SP0564 (hypothetical protein), StkP (SP1732) and MalX (maltose/maltodextrin ABC transporter, SP2108) (Fig. 6c). SP0107 and MalX possess predicted signal peptides, the latter with a lipoprotein attachment motif. StkP and SP0564 are not predicted to be secreted by *in silico* analysis; however, we have reported surface localization of StkP previously (Gieffing *et al.*, 2010). Two cell wall proteins with an LPXTG anchor motif, SP0082 and SP0498 (putative endo- β -N-acetylglucosaminidase), the choline-binding cell wall protein PspA and the membrane lipoprotein PsaA were not present in increased amounts in culture supernatants

Fig. 4. Lack of lytic activity of PcsB with peptidoglycan. (a) PcsB is composed of four major domains: a signal peptide (SP), a conserved N-terminal domain of unknown function, an alanine-rich region (ALA) and a C-terminal CHAP domain. Recombinant PcsB subdomain proteins expressed in this study are displayed below the full-length PcsB: PcsB-FL (amino acids 28–392), PcsB-N (amino acids 28–278) and PcsB-CHAP (amino acids 258–392). (b) Coomassie blue-stained SDS-PAGE of purified recombinant PcsB variants used in this study. (c) Bacterial lysate (BL) and culture supernatant (CS) from the TIGR4 wild-type strain, as well as recombinant PcsB-FL, were resolved on a cell-gel containing SDS-treated TIGR4 cells and assayed for their lytic activity on the embedded material.

of *pcsB*-deleted cells relative to wild-type cells. Cytoplasmic pneumolysin was not released to the supernatant, and no difference in expression of this protein between wild-type and mutant strains was observed (data not shown).

Analysis of total lysate fractions (cell-bound proteins) for the same antigens revealed no increased expression for these proteins, except for the LysM domain-containing SP0107 (Fig. 6d and data not shown).

Absence of PcsB results in increased expression of genes encoding LysM domain proteins

Based on the above observations, we wanted to learn whether compensation mechanisms to overcome the defect of *pcsB* deletion could be observed at the transcriptional level as well. We performed microarray analysis of bacteria cultivated under the same conditions used for Western blot analysis to screen for differences in mRNA levels between wild-type and gene deletion mutant cells (data not shown). Genes with altered transcription (decreased or increased at

least twofold) were further analysed and confirmed by qRT-PCR using RNA from wild-type and $\Delta pcsB$ strains in two different genetic backgrounds (TIGR4 and PJ1324). The most striking difference was the greatly increased transcript levels for two LysM domain proteins, SP0107 and SP2063, which were >14-fold and >25-fold increased in the TIGR4 strain and the 6B isolate, respectively (based on three independent biological replicates of qRT-PCR analysis). Otherwise, there were a few genes that were affected in both strains, although to a lower extent than the LysM protein genes (Table 1). These included upregulated genes encoding the *rrg* pilus islet (SP0461–468), a phosphate transport regulatory protein (SP2088), stress proteins GrpE and 10 kDa chaperonin (SP0516, SP1907), and an ATP-dependent Clp protease (SP0338), and down-regulated genes encoding translation initiation factor IF-1 (SP0232) and transcriptional regulator GntR (SP1714), as well as three ABC transporter proteins (SP1715, SP1869 and SP1872).

These results suggest that there is a very selective change in transcription as a consequence of the lack of PcsB.

Fig. 5. PcsB is excluded from the site of septal cell wall synthesis. (a, b) Pneumococcal cells (4DS2341-94 wild-type strain) were double-stained with anti-PcsB rabbit serum/Cy2-labelled anti-rabbit IgG and anti-FtsZ mouse serum/Texas Red-labelled anti-mouse IgG. The merged picture shows the overlay of PcsB and FtsZ staining. (c) $\Delta pcsB$ mutant cells (strain 4DS2341-94) were double-stained with PcsB/Cy2 and DNA/DAPI, as described in Methods, and imaged using a confocal microscope. The merged picture shows the overlay of PcsB and DAPI staining.

DISCUSSION

PcsB in *S. pneumoniae* and in other streptococcal species has been suggested to have a critical role in cell division. There is a controversy, however, as to whether *pcsB* null mutant pneumococcal cells are viable or not. PcsB has been found to be essential in the D39 and R6 pneumococcal strains in several studies (Barendt *et al.*, 2009; Ng *et al.*, 2003, 2004, 2005); similarly, we were unable to delete *pcsB* in these genetic backgrounds (this study). However, we reported recently the successful generation of *pcsB* null mutants in two genetic backgrounds (TIGR4 and a recent serotype 6B human isolate) (Giefing *et al.*, 2008). Second site (suppressor) mutations have been implicated as an explanation for the viability of these strains (Barendt *et al.*, 2009). In this study we generated

additional pneumococcal *pcsB* mutants in two more genetic backgrounds using the 4DS2341-94 (serotype 4) and EF3030 (serotype 19F) strains (van Ginkel *et al.*, 2003). All four *pcsB* deletion mutant strains displayed the same cluster-forming phenotype, altered septum formation and retarded *in vitro* growth, also described for PcsB-underexpressing R6 strains (Ng *et al.*, 2004) and *pcsB* knockout cells generated in other streptococcal species (Chia *et al.*, 2001a; Reinscheid *et al.*, 2001). Similarly to previous reports (Barendt *et al.*, 2009), we also detected no changes in the transcript levels of the proximal MreCD proteins in our $\Delta pcsB$ mutant strains (based on microarray and Northern blot analysis; data not shown).

In order to directly relate these defects to the absence of PcsB, we complemented one of these mutants by

Fig. 6. $\Delta pcsB$ cells display enhanced protein release *in vitro*. (a) Culture supernatants (CS) of PJ1324 wild-type (WT) and $\Delta pcsB$ cells were separated by SDS-PAGE and stained with Coomassie brilliant blue. (b) Culture supernatants of TIGR4 wild-type and $\Delta pcsB$ cells at different growth stages, based on OD_{620} readings, were analysed in Western blots using enolase-specific rabbit serum. (c, d) Western blot analysis of culture supernatants from 2×10^6 cells (c) and bacterial lysates (approximately 10^8 cells) (d) of TIGR4 wild-type and $\Delta pcsB$ cells in the exponential growth phase using antibodies specific for the proteins indicated under each panel. Antisera generated against SP0498, SP0082 and SP0564 showed reactivity with unknown proteins that displayed enhanced secretion in mutant strains. The band corresponding to the respective protein is indicated by an arrow to the right of each panel.

Table 1. Changes in relative transcript amounts due to the absence of PcsB

Genes were selected for real-time PCR analysis based on microarray data. Values represent fold-changes in the $\Delta pcsB$ strains relative to the wild-type strains, shown as the mean of three independent experiments. Only genes downregulated or upregulated at least twofold in the $\Delta pcsB$ strain are indicated. ND, Not determined.

ORF	Gene product	TIGR4	6B
SP0107	LysM domain protein	14.2	26.4
SP2063	LysM domain protein	14.8	33.6
SP0461	Transcriptional regulator RlrA	2.0	11.0
SP0462	LPXTG protein RrgA	3.4	4.8
SP0463	LPXTG protein RrgB	3.8	ND
SP0464	LPXTG protein RrgC	4.3	8.3
SP0467	Sortase, SrtC	1.4	4.4
SP0468	Sortase, SrtD	2.5	5.1
SP0338	ATP-dependent Clp protease, ATP-binding	13.8	2.6
SP0516	Heat-shock protein GrpE	10.0	2.7
SP1907	Chaperonin, 10 kDa	2.8	2.6
SP2026	Alcohol dehydrogenase, iron-containing	2.3	2.2
SP2088	Phosphate transport system regulatory protein	7.3	18.9
SP2216	PcsB	−234 769	−268 817
SP0232	Translation initiation factor IF-1	−3.7	−2.1
SP1714	Transcriptional regulator, GntR family	−3.3	−3.0
SP1715	ABC transporter, ATP-binding protein	−2.5	−2.8
SP1869	Iron-compound ABC transporter, permease	−7.4	−2.7
SP1872	Iron-compound ABC transporter, iron-compound-binding	−5.9	−2.8

transformation with a plasmid carrying *pcsB* for extrachromosomal expression of the protein. Indeed, the growth rate, cell morphology and antibiotic sensitivity reverted back to wild-type. Thus we conclude that PcsB is not essential for *in vitro* viability in all pneumococcal strains, but is important for cell division and separation in *S. pneumoniae*. D39 and its R6 derivative may lack certain sets of genes needed to survive the lack of PcsB that are present in the majority of pneumococci, especially recent field isolates.

Interestingly, the absence of PcsB resulted in apparent differences in protein abundance in the culture supernatant of wild-type and *pcsB* null mutants, the most obvious increase being associated with enolase (based on 2D PAGE and MS, as well as Western blotting). Interestingly, an increased amount of enolase was also reported in *pcsB* deletion strains of *S. agalactiae* (Reinscheid *et al.*, 2003). In addition to its catalytic function in the glycolytic pathway, enolase has been found to be an important virulence factor and to be regulated by HtrA, a surface protease involved in the processing and maturation of extracellular proteins (Bergmann *et al.*, 2005; Kolberg *et al.*, 2006). $\Delta htrA$ mutants in *S. mutans* display enhanced accumulation not only of enolase but also of PcsB (Biswas & Biswas, 2005). The accumulation of certain proteins in the supernatant of $\Delta pcsB$ strains seems to be related to their anchoring mechanism: while LPXTG-anchored proteins such as SP0082 or SP0498, or choline-binding proteins such as PspA, were not enriched in the $\Delta pcsB$ strains, other proteins that are probably more loosely attached to the cell wall such as MalX and SP0107, were found at higher concentrations in the

supernatant, suggesting micro-changes in the cell wall due to the lack of PcsB.

Based on the strikingly similar morphology of PBP3 (*dacA*) and *pcsB* deletion strains we determined the localization of PcsB within the bacterial cell (Schuster *et al.*, 1990; Morlot *et al.*, 2004). By immunofluorescence studies we discovered that PcsB, similarly to PBP3, also mainly localizes at the cellular poles of dividing cells and is excluded from the septal site. In *in vitro* protein-binding assays using recombinant proteins we did not detect an interaction of PcsB with any of the cell division proteins we tested: FtsZ, FtsA, PBP1b, PBP2a, PBP2x, PBP3 or MreC (data not shown). Despite these parallels to PBP3, no changes in peptidoglycan composition were detected in *pcsB* mutants (Barendt *et al.*, 2009; Reinscheid *et al.*, 2003), while altered muropeptide composition is characteristic of PBP3 mutants (Severin *et al.*, 1992).

Interestingly, LytB, a putative endo- β -N-acetylglucosaminidase, also accumulates at the cell poles and has been shown to be important for daughter cell separation (De Las Rivas *et al.*, 2002). In contrast to LytB, addition of recombinant PcsB to the growth medium could not restore the wild-type phenotype (data not shown), as described for the cell-chaining phenotype of $\Delta lytB$ strains (De Las Rivas *et al.*, 2002). Thus the inside-to-outside path of PcsB seems to be important for its proper function.

Similarly to others' attempts to associate hydrolytic activity with PcsB in group B streptococci (Reinscheid *et al.*, 2001, 2003), we also failed to detect any peptidoglycan lytic activity

in vitro either by an in-gel assay or in solution by dye release. CHAP domains are often found in association with other domains that cleave peptidoglycan, and it has been proposed that some members of the CHAP superfamily have lost their amidase activity but still possess a substrate-binding function, e.g. the glucan-binding capacity of GbpB from *S. mutans* (Rigden *et al.*, 2003). Our data do not exclude the possibility that PcsB exerts a localized hydrolase activity *in vivo* that requires an interaction with other proteins. It is interesting in this context that DivIVA, which is important for cell polarity (reviewed in Errington *et al.*, 2003), interacts with PcsB (Fadda *et al.*, 2007). Thus PcsB might be recruited to the cell pole by interaction with DivIVA and thereby exert a localized hydrolytic function that is only active in cooperation with other proteins as soon as the cell division and cell wall synthesis machinery is moved on to the new equatorial sites.

The role of PcsB in peptidoglycan metabolism is further strengthened by our observation that LysM domain-containing proteins, SP0107 and SP2063, were highly overexpressed in $\Delta pcsB$ strains created in two different genetic backgrounds. Interestingly, these genes were also identified as the most upregulated transcripts in a PcsB-underexpressing R6 strain (Barendt *et al.*, 2009). Similarly to the earlier study, the transcriptional changes were otherwise restricted to a small set of genes. We did not observe changes in other genes of the VicRK regulon, such as *lytB*, which were described elsewhere (Barendt *et al.*, 2009; Ng *et al.* 2005). However, we also found increased expression of stress response genes, although different ones, namely chaperonin SP1907 and heat-shock protein GrpE instead of *groEL* and *groES*, confirming an overall stressed condition of the mutants. Interestingly, we found the pilus islet overexpressed in our pilated strains, possibly pointing towards a link between the *pcsB* regulatory network and pilus regulation.

To investigate the possible interplay between PcsB and the two LysM domain proteins, which are all regulated by the two-component transcriptional regulator VicR (Ng *et al.*, 2005), we attempted to generate deletion mutants of SP0107 and SP2063; however, we obtained deletion mutants (in two different backgrounds) only for the latter. $\Delta sp2063$ strains did not exhibit altered expression of PcsB or SP0107, or any growth or morphological defect observed in $\Delta pcsB$ strains (unpublished data). Vega Masigniani and co-workers recently presented evidence that deletion of SP0107 (spr0096) leads to overall thickening of the cell wall, which points to a role in peptidoglycan hydrolysis (7th International Symposium on Pneumococci and Pneumococcal Diseases, Tel Aviv, Israel, March 16 2010). Our data suggest that lack of PcsB might be partially compensated by overexpression of SP2063 and SP0107 to allow growth, although at a greatly reduced rate.

The activity of hydrolases is often specific for a certain peptidoglycan type, the presence or absence of secondary modifications, high-molecular-weight peptidoglycan or small fragments (Vollmer *et al.*, 2008). Based on our observations and data from other laboratories we suggest a model according to which PcsB is recruited by DivIVA to the future

cell pole to generate, through micro-changes in peptidoglycan, the substrate(s) for LytB, LysM domain proteins or other hydrolases that then fulfil their role in cell separation.

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