Cellular localization of choline-utilization proteins in Streptococcus pneumoniae using novel fluorescent reporter systems

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Summary

The molecular mechanisms underlying cell growth, cell division and pathogenesis in Streptococcus pneumoniae are still not fully understood. Single-cell methodologies are potentially of great value to investigate S. pneumoniae cell biology. Here, we report the construction of novel plasmids for single and double cross-over integration of functional fusions to the gene encoding a fast folding variant of the green fluorescent protein (GFP) into the S. pneumoniae chromosome. We have also established a zinc-inducible system for the fine control of gfp-fusion gene expression and for protein depletion experiments in S. pneumoniae. Using this novel single cell toolkit, we have examined the cellular localization of the proteins involved in the essential process of choline decoration of S. pneumoniae teichoic acid. GFP fusions to LicA and LicC, enzymes involved in the activation of choline, showed a cytoplasmic distribution, as predicted from their primary sequences. A GFP fusion to the choline importer protein LicB showed clear membrane localization. GFP fusions to LicD1 and LicD2, enzymes responsible for loading of teichoic acid subunits with choline, are also membrane-associated, even though both proteins lack any obvious membrane spanning domain. These results indicate that the decoration of teichoic acid by the LicD enzymes is a membrane-associated process presumably occurring at lipid-linked teichoic acid precursors.

Introduction

Streptococcus pneumoniae (the pneumococcus) is a major pathogen causing invasive (pneumonia, meningitis, bacteraemia) and non-invasive (acute otitis media, sinusitis) diseases in young children and in elderly and/or immunocompromised adults (Kadioglu et al., 2008; Scott et al., 2008). The last decades have seen the emergence and spread of pneumococcal strains with multiple antibiotic resistance posing a serious threat to human health (Scott et al., 2008). The molecular mechanisms underlying cell growth, cell division and pathogenesis in S. pneumoniae are still not fully understood. Single-cell methodologies, including subcellular localization of proteins by fluorescence microscopy, are instrumental in studying fundamental processes in bacteria such as DNA replication, cell division, cell wall growth and development of these methodologies are potentially of great use to investigate S. pneumoniae cell biology. In particular, the use of fusions to the green fluorescence protein (GFP) has allowed the study of protein dynamics in living cells of Escherichia coli, Caulobacter crescentus and Bacillus subtilis. These studies showed that many essential proteins, like the cell division apparatus and the DNA-replication and segregation machineries, have distinct localization patterns and dynamics in the cell (Haeusser and Levin, 2008). Importantly, correct cellular localization is required for the proper functioning of these machineries and is vital for the organism. Thus, the identification and characterization of such dynamically localized proteins, for example the cell division protein FtsZ, can even aid in the successful design of novel antimicrobials (Haydon et al., 2008).

There are few reported studies on protein localization in S. pneumoniae, all of which applied the invasive technique of immunostaining (Morlot et al., 2003; Buist et al., 2006; Fadda et al., 2007; Zapun et al., 2008). Compared with the use of fluorescent proteins, immunostaining has the disadvantage that cells need to be fixed and have to undergo a damaging cell wall permeabilization treatment.
Apart from being laborious and potentially altering the localization pattern of a protein, time-lapse imaging of the dynamics of protein localization cannot be done following immunostaining. GFP fusions can overcome all of these draw backs. However, maturation of GFP requires post-translational oxidation and maximal activity is achieved at pH values around 7.0 (Tsien, 1998). It is likely that these features have hindered employment of this useful tool in microaerophiles, such as S. pneumoniae (Acebo et al., 2000).

Here we describe the design and construction of a molecular toolbox consisting of new cloning vectors that will allow stable (either single or double cross-over) integration into the S. pneumoniae genome and can be used to make both C- and N-terminal fusions to a fast folding variant of GFP. Using GFP-tagging, we have examined the cellular localization of the proteins involved in the essential pathway of choline metabolism. The amino alcohol choline is an essential growth factor for S. pneumoniae (Rane and Subbarow, 1940). Choline is taken up from the growth medium and used exclusively to decorate cell wall teichoic acid (WTA) and lipoteichoic acid (LTA) (Tomasz, 1967; Brundish and Baddiley, 1968; Briels and Tomasz, 1973; Fischer et al., 1993). The enzymes involved in choline metabolism are encoded by the lic locus (Zhang et al., 1999) which contains two transcriptional units, lic1 and lic2 (Kharat and Tomasz, 2006). lic1 encodes the putative choline-transporter LicB, two enzymes (LicA and LicC) for the activation of choline to CDP-choline (Whiting and Gillespie, 1996; Campbell and Kent, 2001; Rock et al., 2001; Kwak et al., 2002), and two enzymes (TarI and TarJ) for the formation of CDP-ribitol (Baur et al., 2009) that is required for the synthesis of the backbone of WTA and LTA. lic2 encodes the putative LicD1 and LicD2 phosphotransferases which utilize CDP-choline to load the teichoic acid precursors with choline (Zhang et al., 1999), and for the putative teichoic acid flipase TacF (Damjanovic et al., 2007; Gonzalez et al., 2008).

Here we describe successful in vivo protein localization in living S. pneumoniae cells, in which the genes encoding the fusions are present as single copies on the chromosome. Furthermore, we established a Zn$^{2+}$-inducible system for the fine control of gfp-fusion gene expression and for gene depletion experiments, providing a powerful tool for functional analysis of genes in S. pneumoniae. In addition to this, we have developed protocols to perform time-lapse microscopy on this organism. This allows us to follow the localization of proteins in single live S. pneumoniae cells with high temporal resolution through growth and division for many generations. Taken together, the tools and techniques described here have the potential to open up the field for cell and systems biology in the human pathogen S. pneumoniae.

**Results**

**Novel plasmids for GFP-tagging in S. pneumoniae**

In order to establish tools for protein localization in S. pneumoniae we examined the feasibility of protein tagging. We tested a number of fluorescent proteins including a cyan fluorescent protein (Doan et al., 2005), a yellow fluorescent protein (Veening et al., 2004), a red fluorescent protein (mCherry; Shaner et al., 2004), and two variants of GFP [GFPmut1 (Cormack et al., 1996) and GFP+ (Scholz et al., 2000)]. In most cases, fusions to GFP+ were significantly brighter than fusions to the other fluorescent proteins tested (data not shown). Therefore, we designed two novel shuttle vectors that are capable of replicating in E. coli but not in S. pneumoniae, and allow stable and single copy integration of either C- or N-terminal fusions to GFP+ into the S. pneumoniae chromosome (Fig. 1).

Plasmid pAE03 was constructed by introducing the ermB gene obtained from pJDC9 into the gfp+ containing pJWV017 vector (see Experimental procedures). ermB provides full erythromycin resistance in S. pneumoniae, even as a single copy integration (Chen and Morrison, 1988). The pAE03 vector contains three unique restriction sites, SbfI, EcoRI and Nhel upstream of the promoter-less gfp+ gene (Fig. 1A). It should be noted that SbfI shares compatible cohesive ends with PstI; EcoRI with MunI; and Nhel with AvrII, Spel, Styl and Xbal, thus expanding the list of applicable restriction enzymes. Plasmids for expression of C-terminal GFP fusions can be readily constructed by cloning the gene of interest (or a fragment of it) upstream of gfp+ into pAE03. As pAE03 cannot replicate in S. pneumoniae and does not contain any homologous sequences with the S. pneumoniae chromosome, the plasmid can only integrate at the region of homology provided by the cloned DNA via single cross-over (Fig. 2A). This will result in the integration of the fusion construct at the native locus, leaving it under the control of the native promoter (Fig. 2A), and producing a fusion protein with a C-terminal GFP+ tag. Note that in certain cases, for instance with polycistrons, it is desirable to include the native promoter upstream of gfp+ in the cloning strategy to allow for transcriptional read-through of downstream genes (see Experimental procedures).

Often it is useful to make N-terminal GFP fusions, for instance in the case of membrane proteins where the C-terminus is predicted to be outside [GFP does not fold properly outside the cell (Drew et al., 2002)] and the N-terminus inside, or when the C-terminal fusion protein is not functional. Therefore, we constructed plasmid pJWV25 that contains a Zn$^{2+}$-inducible promoter driving gfp+. We decided to make use of this novel inducible system to be able to fine-tune GFP expression (see below). Furthermore, the gfp+ upstream region carries a
strong *S. pneumoniae* ribosomal binding site and a sequence coding for the first three amino acids of the abundant HtrA protein. It was shown previously that these strong translation signals significantly improve heterologous protein production in *S. pneumoniae* (Halfmann et al., 2007). Finally, we designed the system in such a way that the 3’ end of *gfp*+ contains sequences encoding a hinge region (Arai et al., 2001) to flexibly separate the GFP+ moiety from the target protein, and a multiple cloning site at its 3’ end (Fig. 1B). The gene of interest can be cloned in frame via the SpeI and/or NotI restriction sites. It is noteworthy to mention that NotI only cuts once in the *S. pneumoniae* R6 genome and thus can be used for most fusions, and that SpeI shares compatible cohesive ends with Nhel, AvrII, StyI and XbaI. Plasmid pJWV25 also carries the flanking regions of the nonessential *S. pneumoniae bgaA* gene, facilitating a double cross-over event at this locus (Fig. 2B). It should be noted that double cross-over events of heterologous plasmid DNA is much more efficient than single cross-over events in *S. pneumoniae* (Pozzi and Guild, 1985) and, accordingly, we observed a significant increase in transformation efficiency when using pJWV25-derived plasmids than with pAE03 derivatives (data not shown).
Fine control of GFP-fusion protein expression by the addition of Zn\(^{2+}\)

Recently, Kloosterman and coworkers have identified SczA, a transcriptional regulator that specifically represses expression from the *S. pneumoniae czcD* promoter in the absence of Zn\(^{2+}\), but activates transcription from *PczcD* in the presence of Zn\(^{2+}\) (or Co\(^{2+}\) and Ni\(^{2+}\)) (Kloosterman et al., 2007). In the absence of Zn\(^{2+}\), SczA binds to a conserved DNA motif that overlaps with the −10 region of *PczcD*, thereby blocking transcription. In the presence of Zn\(^{2+}\), SczA is released from this 15 bp inverted repeat and instead binds to a different motif just upstream of the −35 region, thereby activating transcription from *PczcD* (Kloosterman et al., 2007). Because of this unique dual role of SczA as both a repressor and an activator of transcription, and as the transcriptional consequences of the addition of Zn\(^{2+}\) to growing *S. pneumoniae* are well characterized (Kloosterman et al., 2008), we hypothesized that *PczcD* would be a suitable promoter for fine-tuning of protein expression in *S. pneumoniae*.

As a proof of principle, we constructed a GFP+ fusion to the well-characterized DivIVA protein, a protein that is involved in the formation and maturation of the cell poles (Fadda et al., 2007). DivIVA localizes to the cell poles and new cell division sites in many Gram-positive bacteria and, using immunofluorescence microscopy it has been shown that *S. pneumoniae* DivIVA also follows this localization pattern (Fadda et al., 2007). The *divIVA* gene was cloned into pJWV25 and the resulting vector was integrated via double cross-over into the *S. pneumoniae* *bgaA* locus. This resulted in GFP+−DivIVA expression under the control of the Zn\(^{2+}\)-inducible *czcD* promoter.

*S. pneumoniae* cells harbouring the *PczcD-gfp−divIVA* construct were grown in C\(^{+}\)Y medium either with or without 0.15 mM ZnCl\(_2\) (see Experimental procedures). At mid-exponential phase of growth, cells were collected and examined by fluorescence microscopy. As shown in Fig. 3A, in the presence but not absence of ZnCl\(_2\), a clear GFP+−DivIVA signal was observed at the cell poles and often also at mid-cell, strikingly similar to the observed localization pattern of wild-type DivIVA as assessed by immunofluorescence microscopy (Fadda et al., 2007).

To evaluate the dynamic range of protein production using the Zn\(^{2+}\)-inducible promoter, we performed Western blot analysis of strain R6 *PczcD-gfp−divIVA* cells grown at

Fig. 2. Schematic presentation of the genomic context of strains constructed using plasmids pAE03 and pJWV25.

A. Strain R6 *licD2-gfp* was generated by a single cross-over event of plasmid pAE03-licD2 into the *S. pneumoniae* chromosome at the *licD2* locus. In this way, *licD2-gfp* is under the control of its native promoter.

B. Strain R6 *PczcD-gfp−licB* was generated by a double cross-over event of plasmid pJWV25-licB at the *bgaA* locus, thereby replacing most of the nonessential *bgaA* gene with the *PczcD-gfp−licB* construct.
Fig. 3. Cellular localization of GFP+/-DivIVA and its Zn\(^{2+}\)-dependent production.

A. Fluorescence microscopy of R6 $P_{czcD}\text{gfp}^+\text{-divIVA}$ cells grown in C+Y medium either with (top panel) or without (bottom panel) 0.15 mM ZnCl\(_2\).

B. Fine control of GFP+/-DivIVA expression using the Zn\(^{2+}\)-inducible $P_{czcD}$ promoter. Cells were grown in C+Y medium in the absence or presence of the indicated concentrations of ZnCl\(_2\) and harvested at mid-exponential growth for Western blot analysis using either anti-DivIVA (top panel) or anti-GFP (bottom panel) antibodies.
different concentrations of ZnCl₂ (Fig. 3B). In support of the fluorescence microscopy results, GFP+DivIVA was not detected by Western blotting using either polyclonal anti-GFP or polyclonal anti-DivIVA antibodies in cultures grown in the absence of Zn²⁺ (Fig. 3B). With increasing concentration of Zn²⁺ in the growth medium, the abundance of the fusion protein increased in a gradual fashion. At a concentration of approximately 0.1 mM of ZnCl₂, GFP+DivIVA reached a similar concentration compared with untagged DivIVA transcribed from its own promoter (Fig. 3B). At a Zn²⁺ concentration of 0.25 mM, GFP-DivIVA was overproduced to reach approximately two to three-fold the levels of untagged DivIVA (Fig. 3B). We noticed a decrease in the growth rate of S. pneumoniae at ZnCl₂ concentrations higher than 0.25 mM and we therefore never exceeded this concentration. Interestingly, the addition of Mn²⁺ partially overcomes these ZnCl₂-dependent growth defects (T. Kloosterman, pers. comm.), indicating that the system can be further optimized to overproduce proteins. Together, these results show that GFP+ can be successfully used to accurately visualize protein localization in S. pneumoniae. Furthermore, this novel Zn²⁺-inducible promoter system we describe offers fine control of gene expression over a wide dynamic range. Notably, for Lactococcus lactis a reciprocal expression system was developed whereby the gene of interest is repressed in the presence of Zn²⁺ (Llull and Poquet, 2004).

The use of P_czcD for gene depletion studies

A convenient way to study the functions of essential genes is to rapidly shut down the transcription of the gene of interest. A prerequisite for efficient protein depletion is tight control of transcription. As shown above, the Zn²⁺-inducible system could be efficiently used to modulate and even overexpress GFP+DivIVA (Fig. 3B). To test whether P_czcD might also be used to efficiently maintain and then shut down the expression of essential genes, we chose the essential gene licB that encodes for a membrane protein involved in the uptake of the essential amino alcohol choline. We first cloned licB under the control of P_czcD (see Experimental procedures) and introduced the construct at the bgaA locus by double crossover integration. Then a long flanking PCR mutagenesis strategy was used to replace the chromosomally encoded licB gene with an erythromycin-resistance cassette (licB::ermB; see Experimental procedures). As expected based on the essentiality of licB, we were unable to replace licB with the ermB marker gene in wild-type cells (data not shown). However, a licB replacement mutant was readily obtained in a strain ectopically harbouring a P_czcD-gfp–licB construct at the bgaA locus, but only in the presence of Zn²⁺ (Fig. 4A), suggesting that the GFP+LicB fusion is functional. This strain (R6 licB::ermB P_czcD-gfp–licB) did not grow on agar plates lacking Zn²⁺, indicating

Fig. 4. The use of P_czcD for efficient gene complementation and gene depletion studies. A. Strains R6 and R6 licB::ermB P_czcD-gfp–licB were plated on TSB-blood agar plates either with (left) or without (right) the addition of ZnCl₂ (0.25 mM).
B and C. Growth curves of strain R6 licB::ermB P_czcD-gfp–licB with (open squares) or without (filled squares) 0.15 mM ZnCl₂ in (B) C+Y medium or (C) Cden medium as described in the Experimental procedures.
that under such conditions the cellular level of LicB was not high enough to support growth. Growth experiments in liquid media (C+Y and Cden) confirmed this conclusion. R6 licB::ermB PczcD-gfp+licB ceased growth during the third successive culture without Zn\textsuperscript{2+} in the semi-synthetic C+Y medium (Fig. 4B), whereas in the chemically defined Cden medium growth slowed after 1 h and stopped 2 h after growing without Zn\textsuperscript{2+} (Fig. 4C). These results suggest that in the absence of Zn\textsuperscript{2+}, the czcD promoter is tightly repressed and thus can be efficiently used for protein depletion and gene complementation studies in S. pneumoniae.

Time-lapse microscopy reveals that choline is essential for the maintenance of S. pneumoniae cell shape and viability

Teichoic acids are the major cell wall components of Gram-positive bacteria and have a number of important physiological functions (Neuhaus and Baddiley, 2003). S. pneumoniae has a chemically unusual complex cell wall, in which both WTA and LTA carry identical repeating units containing phosphorylcholine residues (Fischer et al., 1993). Choline is an amino alcohol that is abundant in eukaryotes but is rarely found in bacteria. Interestingly, although growth of S. pneumoniae depends on the presence of exogenous choline, several choline-independent mutant strains have been obtained and characterized (Severin et al., 1997; Yother et al., 1998; Damjanovic et al., 2007). Importantly, the cell-wall-bound choline residues are absolutely required for the expression of virulence (Kharat and Tomasz, 2006; Gehre et al., 2008; 2009; Kharat et al., 2008). To examine the phenotypic consequences of S. pneumoniae growth in the absence of choline at the single cell level in real time, we performed time-lapse microscopy. Cells of the laboratory strain R6 were grown in a chemically defined medium (Cden) containing choline and subsequently spotted onto an agarose slide containing Cden with or without choline. Growth was followed by automated phase contrast time-lapse microscopy and images were acquired every 8 min for more than 5 h. As shown in Fig. 5 and Movies S1 and S2, in the absence of choline, cells quickly lost their oval cell shape and increased in cell diameter before they lysed. This time-lapse analysis confirms previous reports and highlights the essentiality of choline for growth of S. pneumoniae.

Cellular localization of the choline utilization proteins

The pneumococcal cell wall enlarges by incorporation of peptidoglycan and choline-containing WTA at a central growth zone in the oval shaped cell (Briles and Tomasz, 1970; Tomasz et al., 1971), but it remains unclear whether decoration of teichoic acid precursors with choline also occurs exclusively at the growth zone. To investigate how the proteins required for choline metabolism are localized in live S. pneumoniae we constructed gfp+ fusions to each gene in the lic locus (Fig. 6A). In the case of licA, licC, tarI, tarJ, licD1 and licD2, C-terminal gfp+ fusions were constructed by cloning the entire coding region or a 3′ gene fragment into pAE03. The resulting plasmids were integrated by single cross-over into the bgaA locus (Tables 1 and 2). At least in the case of LicD1 (with licD2 not transcribed) and LicC we could assume that the fusion proteins are functional, as they represent the only full-length copy of the essential gene. As both LicB and TacF encode hypothetical membrane proteins with a predicted N-terminus inside, N-terminal GFP fusions under the control of P\textsuperscript{czcD} were constructed using pJWV25 and the plasmids were integrated by double cross-over into the bgaA locus (Tables 1 and 2). Our depletion experiment

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Fig. 6. Genetic organization of the lic region and proposed topology of the gene products.
A. The lic1 operon contains at least two promoters and encompasses the tarl, tarj, licA, licB and licC genes. The lic2 operon consists of tacF, licD1 and licD2.
B. LicB is a predicted membrane protein and imports choline. LicA and LicC activate choline that is used by LicD1 and LicD2 to decorate the teichoic acid precursor. Tarl and TarJ enzymes produce CDP-ribitol required for repeating unit synthesis (not shown). Finally, TacF flips the choline loaded teichoic acid precursor across the cytoplasmic membrane (CM).

Table 1. Plasmids used in this study.

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>pJDC9</td>
<td>ery (ermB), lacZ</td>
<td>Chen and Morrison (1988)</td>
</tr>
<tr>
<td>pMP2</td>
<td>amp, tet, bgaA (spr0565), P_000-lacZ</td>
<td>Kloosterman et al. (2007)</td>
</tr>
<tr>
<td>pJWV017</td>
<td>spec, cat, promoter-less gfp+ (C-terminal fusions)</td>
<td>Veening et al. (2009)</td>
</tr>
<tr>
<td>pAE03</td>
<td>spec, ery, promoter-less gfp+ (C-terminal fusions)</td>
<td>This study</td>
</tr>
<tr>
<td>pAE03-tarl</td>
<td>spec, ery, *tarI-gfp+</td>
<td>This study</td>
</tr>
<tr>
<td>pAE03-Ptarl-tarJ</td>
<td>spec, ery, P_000-tarI-gfp+</td>
<td>This study</td>
</tr>
<tr>
<td>pAE03-Ptarl-licA</td>
<td>spec, ery, P_000-licA-gfp+</td>
<td>This study</td>
</tr>
<tr>
<td>pAE03-licC</td>
<td>spec, ery, licC-gfp+</td>
<td>This study</td>
</tr>
<tr>
<td>pAE03-licD1</td>
<td>spec, ery, licD1-gfp+</td>
<td>This study</td>
</tr>
<tr>
<td>pAE03-licD2</td>
<td>spec, ery, licD2-gfp+</td>
<td>This study</td>
</tr>
<tr>
<td>pJWV25</td>
<td>amp, tet, bgaA, P_000-gfp+ (N-terminal fusions)</td>
<td>This study</td>
</tr>
<tr>
<td>pJWV25-divIVA</td>
<td>amp, tet, bgaA, P_000-gfp+divIVA</td>
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</tr>
<tr>
<td>pJWV25-licB</td>
<td>amp, tet, bgaA, P_000-gfp+licB</td>
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</tr>
<tr>
<td>pJWV25-tacF</td>
<td>amp, tet, bgaA, P_000-gfp+tacF</td>
<td>This study</td>
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demonstrated that GFP+LicB from the Zn\(^{2+}\)-inducible promoter is fully functional and can support cell growth in the absence of native LicB (see above; Fig. 4).

To test whether all fusion proteins were correctly expressed and not prone to proteolytic cleavage, lysates from mid-exponentially growing cells were analysed by SDS-PAGE and Western blotting using polyclonal GFP antibodies. As shown in Fig. 7A, all fusions were expressed and TarI-GFP, TarJ-GFP, LicA-GFP, LicC-GFP, LicD1-GFP+ and LicD2-GFP+ migrated to their expected positions as judged by SDS-PAGE and did not show any significant proteolytic cleavage (Fig. 7A). GFP+LicB migrated much slower than expected, but this could be due to its multiple transmembrane spanning domains and it is not uncommon for membrane proteins to migrate differently during SDS-PAGE. Strikingly, while GFP+TacF was expressed, it migrated to about the position expected for untagged GFP+, indicating that this fusion protein has been quantitatively degraded (Fig. 7A).

Fluorescence microscopy of cells producing the GFP+ fusions demonstrated a clear cytosolic localization pattern for TarI-GFP+, TarJ-GFP+, LicA-GFP+ and LicC-GFP+ (Fig. 7B), in accordance with their primary sequences. While TacF is a predicted membrane protein, the GFP+TacF fusion demonstrated a cytosolic localization pattern (Fig. 7B), in line with the Western blot result that showed proteolytic degradation of this fusion protein (Fig. 7A). The other, unprocessed membrane protein, GFP+LicB, clearly localized to the cell membrane without having any preference for particular sites, such as the pole or new cell division sites. Strikingly, both LicD1-GFP+ and LicD2-GFP+ localized to the cell membrane (Fig. 7B), and not to the cytoplasm as predicted from their primary sequences (data not shown). Taken together, these localization data suggest that activation of choline and ribulose occurs within the cytoplasm, but that the loading of teichoic acid with choline by LicD1 and LicD2 is a membrane-associated process.

### Discussion

The ability to apply the methods of cell biology to bacteria has had a huge impact on the understanding of a range of fundamental processes. Using GFP-tagging, it has been shown that bacterial cells are highly organized. For instance, rod-shaped bacteria contain actin-like proteins that form spiral structures along the helical wall of the cell and are required for cell shape and, most bacteria, including *S. pneumoniae*, contain tubulin-like proteins that form ring-shaped structures to carry out cell division (Errington *et al.*, 2003; Thanbichler and Shapiro, 2008). GFP fusions to core components of the replication machinery have shown that replication in bacteria is also highly organized (Lemon and Grossman, 1998).

Here, we have developed tools to apply cell biology methods to the important human pathogen *S. pneumoniae* and we describe a set of vectors allowing for C- and N-terminal GFP fusions. The GFP variant used in this study, GFP+, was shown to be more than 320-fold brighter than standard GFP and contains the chromophore F64L mutation as well as mutations for enhanced folding (F99S, M153T, V163A) (Scholz *et al.*, 2000). Furthermore, the *gfp*+ gene has a bacterial codon usage and has a G+C content of 41%, both of which are similar to the respective properties of the *S. pneumoniae* genome (http://genomes.urv.es/OPTIMIZER/) (Puigbo *et al.*, 2007). The combination of optimizing amino acid substitutions and codon usage might explain why GFP+ is significantly more efficient than other fluorescent protein variants, especially under the low oxygen conditions present during microaerophilic growth of *S. pneumoniae*.

Using GFP+, we were able to determine the localization patterns of a cell division protein (DvlIVA), a membrane protein (LicB), cytosolic proteins (TarI, TarJ, LicA, LicC) and we uncovered the membrane association of proteins (LicD1 and LicD2) that, based on their primary sequences, were predicted to be cytosolic. These examples demonstrate the versatility and resolution of our
GFP tagging system and show that these vectors and methods should be generally applicable to tag proteins in pneumococci and other low-GC Gram-positive microaerophiles.

Recently, it was shown that the WTA synthetic machinery in *Bacillus subtilis* is localized in helix-like structures along the cylindrical shaped cell (Formstone et al., 2008). The helical pattern of the *B. subtilis* WTA machine is likely governed by the helical actin-like MreB cytoskeleton, which is not present in *S. pneumoniae* (Formstone et al., 2008). Using the new tools described here for cell biology in *S. pneumoniae*, we have shown that the steps of choline- and ribitol-activation occur in the cytoplasm, whereas the later step of loading of the teichoic acid precursors with choline occurs throughout the membrane and is not restricted to septal sites (Fig. 7B). Presumably, activated choline is linked to the teichoic acid precursor that is attached to the membrane by its lipid anchor, and that then diffuses to mid-cell where it is incorporated into the cell wall (Fig. 6B). Many pneumococcal teichoic acid genes remain to be identified, including most genes for precursor synthesis, for polymerization of the teichoic acid chains, and for the attachment of the chains to peptidoglycan (WTA) or glycolipid anchor (LTA). Nascent teichoic acid chains are incorporated into the cell wall at the central wall growth zone (Briles and Tomasz, 1970; A. Eberhardt et al. / H17039 © 2009 The Authors Journal compilation © 2009 Blackwell Publishing Ltd, *Molecular Microbiology*, 74, 395–408)
Tomasz et al., 1971). Thus it is likely that at least those enzymes responsible for the later steps in teichoic acid synthesis localize to the growth zone, as do the enzymes for peptidoglycan synthesis, the penicillin-binding proteins (Morlot et al., 2003; Zapun et al., 2008). The new plasmids developed in this work and their derivatives will be powerful tools for further studies on cell wall growth, cell division and pathogenesis of S. pneumoniae.

Experimental procedures

Strains and growth conditions

Streptococcus pneumoniae R6 (Ottolenghi and Hotchkiss, 1960) was grown at 30°C or 37°C in Cden medium (Tomasz, 1964) with or without choline (5 μg ml⁻¹), or in complex C+Y medium containing 1 mg ml⁻¹ yeast extract (Horne and Tomasz, 1993). Blood agar plates were made of TSBS medium containing 1.5% agar and 3% defibrinated sheep blood. Cden agar plates contained 1.5% agar. Growth on Cden agar plates was documented by replica plating on nitrocellulose filters, followed by staining with 0.1% amido black solution (45% methanol, 10% acetic acid) and destaining with water. For induction of licB::ermB PczcD-gfp in E. coli DH5α (Invitrogen) was grown in Luria broth at 37°C with aeration. Where necessary, erythromycin was added at a concentration of 1 mg ml⁻¹ and tetracycline was added at 12.5 μg ml⁻¹ for E. coli and 2.5 μg ml⁻¹ for S. pneumoniae, and growth of this strain was monitored in C+Y medium and 1:20 into 50 ml pre-warmed Cden medium with and without addition of ZnCl₂. A 6 h culture of R6 licB::ermB PczcD-gfp+ licB, growth of this strain was monitored in C+Y medium and without addition of ZnCl₂. A 6 h culture of R6 licB::ermB PczcD-gfp+ licB was grown in 50 ml C+Y at 37°C with 0.15 mM ZnCl₂. The cells were washed twice by centrifugation for 20 min at 4°C and 4000 r.p.m. and resuspension in 50 ml of cold C+Y without ZnCl₂, and then inoculated 1:20 into 50 ml pre-warmed C+Y with or without 0.15 mM ZnCl₂. The cultures were incubated at 37°C. Growth was monitored by measuring optical density at 620 nm (OD620). As soon as the culture containing ZnCl₂ reached an OD620 of 0.8 the cells of both cultures were washed as described above and inoculated 1:20 in fresh C+Y with or without 0.15 mM ZnCl₂. The depletion in Cden medium was done as described for C+Y except that the concentration of ZnCl₂ was 0.05 mM and that cells were washed and diluted only once.

Growth curves

To verify the Zn²⁺ dependency of R6 licB::ermB PczcD-gfp+ licB, growth of this strain was monitored in C+Y medium and Cden medium with and without addition of ZnCl₂. A 6 h culture of R6 licB::ermB PczcD-gfp+ licB was grown in 50 ml C+Y at 37°C with 0.15 mM ZnCl₂. The cells were washed twice by centrifugation for 20 min at 4°C and 4000 r.p.m. and resuspension in 50 ml of cold C+Y without ZnCl₂, and then inoculated 1:20 into 50 ml pre-warmed C+Y with or without 0.15 mM ZnCl₂. The cultures were incubated at 37°C. Growth was monitored by measuring optical density at 620 nm (OD620). As soon as the culture containing ZnCl₂ reached an OD620 of 0.8 the cells of both cultures were washed as described above and inoculated 1:20 in fresh C+Y with or without 0.15 mM ZnCl₂. The depletion in Cden medium was done as described for C+Y except that the concentration of ZnCl₂ was 0.05 mM and that cells were washed and diluted only once.

PCR amplification, cloning and nucleotide sequencing

Routine DNA procedures were performed essentially as described elsewhere (Sambrook, 2001). S. pneumoniae chromosomal DNA was isolated using a Promega Wizard® Genomic DNA Purification System except cells were lysed by the addition of 4% deoxycholic acid (DOC) and incubation at 37°C for 10 min. Plasmid purification was done according to the manufacturer’s protocol. The Qiagen plasmid midi kit. Restriction enzymes were purchased from Fermentas or New England Biolabs. DNA fragments were purified by using the QIAquick® PCR purification kit. For PCR amplification Phusion® high-fidelity DNA polymerase from Finnzymes was used. The T4 DNA ligase was from Fermentas. Oligonucleotides were purchased from Invitrogen or Eurogentec and are listed in Table S1. All constructs were sequence verified using custom-made oligonucleotides at GATC in Konstanz, Germany or at the Sequence facility of Dundee University, UK.

Construction of pAE03 and pJWV25

To construct plasmid pAE03 (Fig. 1A), plasmid pJDC9 (Chen and Morrison, 1988) was digested with Eco47III and EcoRI and the 2.6 kb fragment containing an erythromycin-resistance marker was inserted into the corresponding sites of pJWV017 (Veening et al., 2009) thereby replacing the chloramphenicol-resistance marker present on this plasmid.

To construct plasmid pJWV25 (Fig. 1B), a DNA fragment containing strong S. pneumoniae translation signals, gfp+ and a flexible linker region was obtained by PCR with the primers gfp-F+BglII and gfp-R+BlpI using chromosomal DNA of Bacillus subtilis strain JWV112 (Veening et al., 2009) as a template. The amplified fragment was subsequently cleaved with BglII and BlpI and inserted into the BamHI and BlpI sites of plasmid pMP2 (Kloosterman et al., 2007), resulting in plasmid pJWV25.

Cloning of the pAE03 derivatives

For cloning of pAE03-derived plasmids the complete gene of interest or its 3′-region was amplified from chromosomal DNA of strain R6 using oligonucleotides containing an SbfI, EcoRI (upstream) or NheI (downstream) restriction site. In the case of pAE03-Pwar.tarA and pAE03-Pwar.licA the promoter region of tarA was inserted in front of tarA and licA to provide transcription of the essential downstream genes. Pwar was amplified using the oligonucleotides tarA-promoter-F(SbfI) and tarA-promoter-R(BamHI). tarJ was amplified using the primers tarJ-F(BamHI) and tarJ-hingeregion-R(NheI). For amplification of licA, the primers licA-F(BamHI) and licA-hingeregion-R(NheI) were used. The DNA fragments containing the promoter region and the coding region of the gene of interest were ligated after digestion with BamHI. The products were then amplified using the terminal primers tarA-promoter-F(SbfI) and tarJ-hingeregion-R(NheI) or licA-hingeregion-R(NheI) respectively. These were then digested with SbfI and NheI and ligated to plasmid pAE03 which had been cleaved with the same restriction enzymes. Inserts and flanking regions on all plasmids constructed were verified by sequencing.

Cloning of the pJWV25 derivatives

pJWV25 and the PCR-amplified genes were digested with SpeI and NotI and purified from agarose gels using a Peqlab.
The two fragments were then ligated and transformed into *E. coli* DH5α. These pJWV25-based plasmids encode for a domain breaking linker sequence (SRGSGGEAAAKAGTS) inserted between GFP and the protein of interest to provide structural flexibility (Arai *et al*., 2001).

**Strains for the expression of GFP-fusion proteins**

For transformation of R6, 2 μl of plasmid DNA (~600 ng) were added to competent cells. Transformation was performed by 20 min of incubation at 37°C followed by a phenotypic expression period of 90 min at 37°C and overnight growth on TSB blood agar plates containing appropriate antibiotic (1 μg ml⁻¹ erythromycin or 2.5 μg ml⁻¹ tetracycline respectively). A single clone was picked and the correct integration of the pAE03 plasmids derivates was verified by PCR amplification of the erythromycin-resistance cassette *ermB* and two PCR reactions covering DNA regions from the integrated plasmid to the chromosomal DNA. pAE03-derived plasmids integrates into a single cross-over recombination event in the chromosomal DNA at the locus where the insert of the plasmid is taken from. Thereby the gene fused to *gfp* is expressed under the native promoter (Fig. 2A). pJWV25 and its derivates integrate by a double cross-over into the *bgaA* locus on the pneumococcal chromosome and the gene fused to *gfp* is under the control of the Zn²⁺-inducible promoter P*cond*, with the native locus remaining intact (Fig. 2B). Correct integration of gene fusions from pJWV25-based plasmids into the *bgaA* locus was verified by PCR reactions covering a DNA region from the integrated gene to the flanking chromosomal DNA.

**Construction of a licB deletion/complementation strain**

For the marker replacement of the *licB* gene, the upstream and downstream flanking regions of *licB* were PCR-amplified and fused to an erythromycin-resistance cassette (*ermB*) amplified by PCR from plasmid pJDC9. The three resulting PCR products were digested with BamHI, purified and ligated in a three-point ligation. The ligation mixture was directly transformed to competent *S. pneumoniae* strain R6 P*cond-gfp*−-*licB* cells and colonies were screened for correct gene replacement by PCR and Zn²⁺ dependency. To exclude any polar effects of the *ermB* marker gene, the *ermB* PCR product did not include the *ermB* promoter and terminator sequences.

**Time-lapse microscopy**

Cells were grown at 30°C in Cden with choline and inoculated onto a thin semisolid agarose matrix (low melting agarose) based on Cden, either with or without choline (5 mg ml⁻¹) and attached to a microscope slide as described (Veening *et al*., 2008). Microscope slides were incubated on a temperature-controlled automated Deltavision RT microscope (Applied Precision). Images were obtained up to 6 h with a CoolSNAP HQ (Princeton Instruments) at 100× magnification. Phase-contrast images were recorded every 8 min.

**Fluorescence microscopy**

Cells were grown at 30°C in C+Y medium without shaking but with an approximate 50% air volume to allow for proper GFP folding. Where relevant, media were supplemented with 0.15 mM ZnCl₂. For GFP, the excitation light of a 100-W Hg-vapor lamp was limited to 480–500 nm and the emission wavelengths were 509–547 nm (filters from Chroma). Typical exposure times were between 1 and 2 s. Microscopy images were deconvolved using softWoRx (Applied precision) and figures were prepared for publication using ImageJ (http://rsb.info.nih.gov/ij/).

**Verification of GFP and DivIVA expression by Western blot analysis**

Western blot analysis was performed to test for cellular production of fusion proteins. Cells were grown in C+Y at 30°C with 0.15 mM ZnCl₂. At an OD₆₀₀ of 0.5, 10 ml of cell culture was centrifuged for 20 min at 4°C and at 4000 r.p.m. The cells were resuspended in 200 μl of lysis buffer [50 mM Tris/HCl pH 8.0, 5 mM MgCl₂, 0.1 mg ml⁻¹ DNase I, 0.025 mg ml⁻¹ RNase A, 1 μl ml⁻¹ protease inhibitor (Sigma)]. The cell suspension was frozen overnight at −80°C. To 100 μl of the thawed cell suspension, 10 μl of 4% DOC was added. The sample was incubated for 5–10 min at 37°C until cells lysed and 50 μl of SDS-gel loading buffer was added. The sample was boiled for 10 min prior to loading on a 12% SDS-PAGE. After electrophoresis, the gel was transferred on a nitrocellulose membrane by Western blotting. The membrane was blocked with 0.5% casein in TBS (100 mM Tris/HCl pH 7.5, 0.9% NaCl) for 18 h at 4°C. It was then washed three times with TTBS (100 mM Tris/HCl pH 7.5, 0.9% NaCl, 0.2% Tween20) for 5 min. For detection of GFP+, the membrane was incubated with a 1:2000 dilution of anti-GFP (polyclonal from rabbit, IgG fraction, Invitrogen) in TTBS containing 0.5% casein in TBS (100 mM Tris/HCl pH 7.5, 0.9% NaCl) for 1 h at 4°C. It was then washed three times with TTBS (100 mM Tris/HCl pH 7.5, 0.9% NaCl, 0.2% Tween20) for 5 min. For detection of GFP+ the membrane was incubated with a 1:2000 dilution of anti-GFP (polyclonal from rabbit, IgG fraction, Invitrogen) in TTBS containing 0.5% casein in TBS (100 mM Tris/HCl pH 7.5, 0.9% NaCl) for 1 h at 4°C. It was then washed three times with TTBS (100 mM Tris/HCl pH 7.5, 0.9% NaCl, 0.2% Tween20) for 5 min. For detection of DivIVA and GFP-DivIVA, a purified anti-DivIVA antibody (kindly provided by Orietta Massidda) was used at a dilution of 1:10 000. The membrane was then incubated with a goat-anti-rabbit IgG-peroxidase antibody (Sigma) at a dilution of 1:10 000 in TTBS containing 0.5% casein for 1 h at room temperature, followed by three washes with TTBS (5 min each) prior to ECL detection using a kit and film from GE Healthcare.

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**References**

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Supporting information

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