Revised mechanism of d-alanine incorporation into cell wall polymers in Gram-positive bacteria

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Teichoic acids (TAs) are important for growth, biofilm formation, adhesion and virulence of Gram-positive bacterial pathogens. The chemical structures of the TAs vary between bacteria, though they typically consist of zwitterionic polymers that are anchored to either the peptidoglycan layer as in the case of wall teichoic acid (WTA) or the cell membrane and named lipoteichoic acid (LTA). The polymers are modified with d-alanines and a lack of this decoration leads to increased susceptibility to cationic antimicrobial peptides. Four proteins, DltA–D, are essential for the incorporation of d-alanines into cell wall polymers and it has been established that DltA transfers d-alanines in the cytoplasm of the cell onto the carrier protein DltC. However, two conflicting models have been proposed for the remainder of the mechanism. Using a cellular protein localization and membrane topology analysis, we show here that DltC does not traverse the membrane and that DltD is anchored to the outside of the cell. These data are in agreement with the originally proposed model for d-alanine incorporation through a process that has been proposed to proceed via a d-alanine undecaprenyl phosphate membrane intermediate. Furthermore, we found that WTA isolated from a Staphylococcus aureus strain lacking LTA contains only a small amount of d-alanine, indicating that LTA has a role, either direct or indirect, in the efficient d-alanine incorporation into WTA in living cells.

INTRODUCTION

The bacterial cell wall is a complex and highly organized structure that allows bacteria to interact with and protects them against hostile insults encountered in the environment. In Gram-positive bacteria, multi-functional teichoic acids (TAs) are key components of the cell wall. Many Gram-positive bacteria contain two types of TAs; wall teichoic acid (WTA), which is covalently linked to the peptidoglycan layer and lipoteichoic acid (LTA), which is embedded in the membrane via a lipid anchor (Reichmann & Gründling, 2011; Xia et al., 2010a). Bacteria display diverse defects in the absence of either polymer and their combined absence is lethal to the cell (Oku et al., 2009; Schirner et al., 2009).

In Staphylococcus aureus, LTA is composed of a polyglycerolphosphate backbone chain that is linked via a glycolipid anchor to the outside of the membrane (Reichmann & Gründling, 2011). The backbone chain is polymerized on the outside of the cell by the LTA synthase enzyme LtaS using the membrane lipid phosphatidylglycerol as its substrate (Gründling & Schneewind, 2007b; Karatsa-Dodgson et al., 2010; Koch et al., 1984; Lu et al., 2009). WTA in S. aureus, on the other hand, is composed of a ribitolphosphate backbone chain that is connected through a linker unit to muramic acid residues of peptidoglycan (Brown et al., 2008; Neuhaus & Baddiley, 2003). WTA is further decorated with χ- or β-O-N-acetylglucosamine residues and the enzymes required for this modification have been recently identified as TarM and TarS (Brown et al., 2012; Xia et al., 2010b). In S. aureus and many other Gram-positive bacteria, both polymers are further decorated with d-alanine esters, which confer a positive charge on the negative polymer (Neuhaus & Baddiley, 2003). Pulse–chase experiments using [14C]-d-alanine indicated that d-alanines are first incorporated into LTA (Haas et al., 1984). Based on the observation that a decrease in radioactivity in the LTA fraction is followed by an increase in radioactivity in the WTA fraction, it has been suggested that d-alanine-LTA serves as donor for d-alanine substitutions in WTA (Haas et al., 1984).

D-alanine modification of TAs is known to play an important role in the regulation of autolytic activity and binding of Mg2+ ions within the cell wall (Fischer et al., 1981; Koprivnjak et al., 2006; Lambert et al., 1975). The absence of d-alanine esters leads to an increase in the susceptibility of bacteria to nisin, defensins and other cationic antimicrobial peptides and more rapid killing by phagocytic cells and neutrophils (Collins et al., 2002; Kristian et al., 2005; Peschel et al., 1999; Poyart et al., 2003; 2009).
Proteins required for the D-alanine incorporation into TAs are encoded in the dlt operon (Neuhaus & Baddiley, 2003; Neuhaus et al., 1996) and in S. aureus this operon consists of five genes dltXABCD (Koprivnjak et al., 2006). Based on the dlt operon in Bacillus subtilis only proteins encoded by dltABCD are thought to be essential for D-alanine incorporation (Koprivnjak et al., 2006; Perego et al., 1995). The function of DltA and DltC in this process has been established. DltA is a D-alanine-D-alanyl carrier protein ligase, which catalyses the adenylation of D-alanine and then the transfer of the activated amino acid onto the D-alanyl carrier protein DltC (Debabov et al., 1996; Fischer, 1994; Heaton & Neuhaus, 1994). The roles played by DltB and DltD are less clear. DltB is a multi-membrane-spanning protein and hydropathy profiles indicate that DltD is also anchored to the membrane via an N-terminal hydrophobic sequence. Two models for the functions of these proteins have been proposed; Fischer and colleagues proposed that D-alanylation of TAs proceeds through a lipid-linked undecaprenyl phosphate (C55-P) intermediate. In this model, it was hypothesized that DltB facilitates the transfer of D-alanines from DltC to C55-P to produce D-Ala-P-C55 and possibly the subsequent transfer of this lipid-linked intermediate across the membrane (Fig. 1a) (Perego et al., 1995). However, it should be noted that such a lipid-linked intermediate has not yet been confirmed experimentally. The final step in the D-alanylation process was proposed to be catalysed by DltD, which in this model functions on the outside of the cell (Perego et al., 1995). The second model was formulated by Neuhaus and Baddiley following experiments performed by Debabov et al. on the DltD protein from Lactobacillus rhamnosus (Debabov et al., 2000). Using purified proteins, it was shown that the rate of ligation of D-alanines from DltA to DltC increases twofold in the presence of DltD (Debabov et al., 2000). This led to the hypothesis that DltD acts in the cytoplasm of the cell as a platform to bring DltA and DltC in close proximity allowing efficient charging of DltC with D-alanines (Fig. 1b). The charged DltC protein is then thought to translocate across the membrane via a channel formed by DltB and to transfer D-alanines in a final step onto LTA (Neuhaus & Baddiley, 2003).

In this study we revisited the mechanism of D-alanine incorporation into Gram-positive cell wall polymers. Using an S. aureus strain lacking LTA, we show that D-alanine is only very inefficiently incorporated into WTA, providing experimental evidence that in living cells D-alanine-LTA is required for the efficient incorporation of D-alanine into WTA. By performing a protein localization and membrane topology analysis in S. aureus, we show that DltC remains within the cell and that DltD is targeted to the outside of the cell, which is only consistent with the model proposed by Werner Fischer and colleagues. Based on our findings, we suggest that future studies addressing the mechanism of D-alanine incorporation into LTA should be designed based on the originally proposed model.

METHODS

Bacterial stains and culture conditions. Bacterial strains used in this study are listed in Table 1 and primers in Table 2. Escherichia coli strains were grown in LB medium and S. aureus strains in tryptic soy broth (TSB) or agar (TSA). All strains were grown at 37 °C and media were supplemented when appropriate with the antibiotics or inducers as listed in Table 1.

Plasmid and strain construction. Plasmid piet-dltC-His was constructed for detection of DltC by Western blot analysis. The dltC gene from S. aureus Newman chromosomal DNA was amplified with primers 721/722, resulting in the addition of a C-terminal Histag. The PCR product was digested with AvrII/BglII and ligated with plasmid piet, which had been digested with the same enzymes. Plasmid piet-dltC-His was initially obtained in E. coli XL1 Blue.
resulting in strain ANG1482 and subsequently integrated into the lipase gene geh of *S. aureus* RN4220Δspa giving rise to strain ANG1484. For use as an empty vector control strain, *pitet* was introduced into *S. aureus* RN4220 Δspa resulting in strain ANG1729.

Plasmids *pitet-dltD-lacZ*, *pitet-dltD_{40 \text{ aa}}-lacZ*, *pitet-3 aa-lacZ* and *pitet-aurscr-lacZ* were constructed for membrane topology studies in *S. aureus*. Plasmids *pitet-dltD-lacZ* and *pitet-dltD_{40 \text{ aa}}-lacZ* were constructed by amplifying the appropriate *dltD* sequence from plasmid pUT18-dltD (ANG1314) with primers 882/883 and 882/884, respectively. Following digestion with *AvrII/Sall*, PCR products were ligated with *pitet-lacZ* (ANG286), which had been digested with the same enzymes. Plasmids *pitet-dltD-lacZ* and *pitet-dltD_{40 \text{ aa}}-lacZ* were initially obtained in *E. coli* XL1 Blue yielding strains ANG1718 and ANG1719, and subsequently transformed into *S. aureus* RN4220Δspa resulting in strains ANG1723 and ANG1724, respectively. To construct plasmid *pitet-3 aa-lacZ*, the sequence encoding the ribosome-binding site and the first three amino acids of DltD was generated by annealing the primers 887/888. The annealed primers were ligated with *pitet-lacZ*, which had been digested with *AvrII/Sall*. Plasmid *pitet-3 aa-lacZ* was initially obtained in *E. coli* XL1 Blue giving rise to strain ANG1720 and subsequently transformed into *S. aureus* RN4220Δspa yielding strain ANG1725. Plasmid *pitet-aurscr-lacZ* was constructed by amplifying the sequence encoding the signal sequence of aureolysin (*aurSc*) from *S. aureus* Newman chromosomal DNA with primers 1096/1097. The PCR product was digested with *SalI*, which had been digested with the same enzymes. Plasmid *pitet-aurscr-lacZ* was initially obtained in *E. coli* XL1 Blue yielding strain ANG1722 and

<table>
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<th>Strain</th>
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<tr>
<td><strong>E. coli</strong></td>
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<td>XL1 Blue</td>
<td>Cloning strain; Tet^R^-ANG127</td>
<td>Stratagene</td>
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<td><em>pitet-lacZ</em> in XL1 Blue; Amp^R^</td>
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<td>pOK-ltaS-T300A in XL1 Blue; Kan^R^</td>
<td>Lu <em>et al.</em> (2009)</td>
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<td><strong>S. aureus</strong></td>
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<td>SEJ1</td>
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<td>Gründling &amp; Schneewind (2007a)</td>
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<td>Wörmann <em>et al.</em> (2011)</td>
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<td>ANG1786</td>
<td>4S5; derivative of RN4220ΔspaΔabiS with mapped suppressor mutations, lacking LTA</td>
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<td><em>pitet-3 aa-lacZ</em> integrated in strain ANG314; Cam^R^</td>
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<td>ANG1727</td>
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**Table 1.** Bacterial strains used in this study

Antibiotics and inducers were used at the following concentrations: for *Escherichia coli* cultures, ampicillin (Amp^R^) 100 μg ml^-1_, kanamycin (Kan^R^) 30 μg ml^-1_; for *S. aureus* cultures, chloramphenicol (Cam^R^) 7.5 or 10 μg ml^-1_; anhydrotetracycline (Atet) at 200 ng ml^-1_.

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**Table 2. Primers used in this study**

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<td>ANG420</td>
<td>3-BglII-His6-719</td>
<td>GAAATCTTTTGTATGGTGGTGATGGACCTTTTGAAGTTGCTTGAAGTCTG</td>
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<td>ANG721</td>
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<td>ANG722</td>
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*Restriction sites in primer sequences are underlined.

subsequently transformed into *S. aureus* RN4220Δspa giving rise to strain ANG1727.

Fusions to the extracellular domain of the inactive LtaS variant eLtaST300a, with a C-terminal 6 × His-tag were used for membrane topology studies. Plasmids pietet-dltD40 aa-eltaST300a-His, pietet-dltD100 aa-eltaST300a-His, pietet-3 aa-eltaST300a-His and pietet-auratSST300a-His were generated for this purpose. Plasmids pietet-dltD40 aa-eltaST300a-His and pietet-dltD100 aa-eltaST300a-His were constructed by amplifying the sequence encoding the first 40 or 100 amino acids of DltD from pUT18-dltD (ANG1134) with primers 889/420 and 892/420, respectively. The resulting products were fused by splicing by overlap extension (SOE) PCR using primers 889/420. The final PCR products were digested with AvrII/BglII and ligated with plasmid pietet, which had been digested with the same enzymes. Plasmids pietet-dltD40 aa-eltaST300a-His and pietet-dltD100 aa-eltaST300a-His were initially transformed into *E. coli* XL1 Blue resulting in strains ANG2041 and ANG2022, and subsequently transformed into *S. aureus* RN4220ΔspaBslb yielding strains ANG2042 and ANG2026, respectively. In order to generate plasmid pietet-3 aa-eltaST300a-His, the eltaST300a sequence was amplified from pOK-ltas-T300A (ANG1103) with primers 890/420 and 892/420, respectively. The resulting products were fused by splicing by overlap extension (SOE) PCR using primers 889/420. The final PCR products were digested with AvrII/BglII and ligated with plasmid pietet, which had been digested with the same enzymes. Plasmid pietet-3 aa-eltaST300a-His was initially transformed into *E. coli* XL1 Blue yielding strain ANG1908 and subsequently transformed into *S. aureus* RN4220ΔspaBslb yielding strain ANG2024. Plasmid pietet-auratSST300a-His was constructed by amplifying the sequence encoding the signal sequence of aureolysin (aurSS) from pietet-auratSST300a-His (ANG1722) with primers 1096/1216 and the eltaST300a-His sequence from pOK-ltas-T300A (ANG1103) with primers 1217/420. The resulting products were fused by SOE PCR using primers 1096/1216. The final PCR product was digested with AvrII/BglII and ligated with plasmid pietet, which had been digested with the same enzymes. Plasmid pietet-auratSST300a-His was initially transformed into *E. coli* XL1 Blue resulting in strain ANG2021 and subsequently transformed into *S. aureus* RN4220ΔspaBslb yielding strain ANG2025. The sequences of all inserts were verified by fluorescent automatic sequencing at the MRC Clinical Sciences Centre at Imperial College London.

**Cell fractionation and Western blot analysis.** For DltC-His detection, overnight cultures of *S. aureus* were diluted 1:100 into 5 ml TSB medium with anhydrotretracycline (Ate) and grown for 4.5 h at 37 °C with shaking. For cell fractionation into cytoplasm plus membrane (cell), cell wall and supernatant, cells of a 1 ml culture were pelleted by centrifugation at 7000 g for 15 min. Nine hundred microlitre of the supernatant was precipitated with trichloroacetic acid (TCA) as previously described (Wörmann et al., 2011). The remaining supernatant was removed from the cell pellet and bacteria suspended in 1 ml osmotically stabilizing lysis buffer (50 mM Tris/HCl pH 7.5, 20 mM MgCl2, 30% raffinose and 200 µg ml−1 lysostaphin) and incubated at 37 °C for 30 min. The protoplasts were collected by centrifugation at 6000 g for 20 min and suspended in protein sample buffer, yielding the cell fraction (cytoplasm and membrane). Nine hundred microlitre of the supernatant (cell wall fraction) was TCA precipitated as described above. For detection of the eLtaST300a-His fusion proteins, the supernatant fraction was prepared as described above and for the cell fraction the bacterial pellet from 1 ml culture was suspended in 1 ml lysis buffer (100 mM Tris/HCl pH 7.5, 10 mM MgCl2, 30% raffinose and 200 µg ml−1 lysostaphin) and incubated at 37 °C for 30 min. The cell and membrane fractions were collected by centrifugation at 17000 g for 5 min and 900 µl of the supernatant was precipitated with trichloroacetic acid. For detection of the DltC-His protein, the sample buffer was 1% (v/v) SDS-PAGE gels for Western blot analysis. His-tagged proteins were detected with HRP-conjugated anti-His antibody (Sigma) at a 1:1 000 dilution. The control proteins L6 (cytoplasmic) (gift from O. Schneewind, University of Chicago, USA), Spa (membrane) (Mazmanian et al., 2000), SdrD (cell wall) (DeDent et al., 2008) and α-haemolysin (Hla) (supernatant) (Bubek Wardenburg & Schneewind, 2008) were detected with
were pelleted by centrifugation at 17 000 g and grown for 4 h at 37 °C with shaking. Cells from a 1 ml culture aliquot were collected by centrifugation at 17 000 g for 10 min and the pellet was frozen at −20 °C overnight. Samples were thawed and pellets suspended in 1 ml ABT buffer (60 mM K2HPO4, 40 mM KH2PO4, 100 mM NaCl, pH 7, 1% Triton X-100) containing 20 µg ml−1 lysostaphin and incubated at 37 °C for 30 min. Cell debris were pelleted by centrifugation at 17 000 g for 10 min and 100 µl of the supernatant was added to 20 µl 0.4 mg ml−1 4-methylumbelliferyl β-D-galactopyranoside (MUG) in a black 96-well plate. As background control 100 µl ABT was added to 20 µl MUG solution. Following incubation in the dark at room temperature for 1 h, a 20 µl sample was mixed with 180 µl ABT buffer and fluorescence readings were detected at an excitation wavelength of 336 nm and emission wavelength of 445 nm. A standard curve was generated using serial dilutions of 4-methyllumphanetidine (L-MUG) in ABT buffer at known concentration. Subsequently these values were used to determine the concentration (µM) of product in each sample and results are given as µM per OD600 of 1. The experiment was performed in triplicate and mean values and standard deviations plotted.

**WTA purification and NMR analysis.** Purification of peptidoglycan-WTA complexes was performed as previously described (Bernal et al., 2009; Kopp et al., 1996; Strandén et al., 1997) with slight modifications. Overnight cultures of *S. aureus* strain RN4220Spa were collected by centrifugation for 40 min at 13 300 g and incubated with 0.1 mm glass beads using a bead beater and the broken cells collected by centrifugation for 40 min at 13 300 g. The cell wall material was washed once with 1 M NaCl, three times with 0.5% SDS and three times with water. The material was suspended in 30 ml water and incubated for 30 min at 60 °C with gentle stirring. The cell wall material was recovered by centrifugation at 34 600 g for 20 min, washed once with water and recovered again by centrifugation. The final pellet was washed in 20 ml 0.15 mTris/HCl pH 7.0 containing 0.2 mg ml−1 trypsin and incubated at 37 °C for 18 h. The material was recovered by centrifugation and washed with 1 M Tris/HCl pH 7.0, 1 M Tris/HCl pH 7.0 containing 1 M NaCl, 1 M Tris/HCl pH 7.0 and three times with water. To hydrolyse WTA from peptidoglycan, the cell wall material was incubated in 10% TCA at 4 °C for 18 h. The peptidoglycan was removed by centrifugation at 9700 g for 45 min. WTA was precipitated with 0.1 volume of 3 M sodium acetate pH 5.2 and 3 volumes of 95% ice-cold ethanol and held overnight at −80 °C. The following day, the WTA was precipitated by centrifugation at 9700 g for 30 min and was washed five times with 95% ethanol. Following the last wash step the pellet was air-dried and the WTA was suspended in 500 µl of water and lyophilized overnight. For the NMR analysis, 6 mg of WTA was suspended and lyophilized twice in 99.96 % D2O and finally suspended in 99.99 % D2O. Spectra were acquired on a 600 MHz Bruker AVANCE III spectrometer equipped with TCI cryoprobe and processed using Bruker TopSpin 3.1 software. The experiment was performed in triplicate.

**RESULTS**

**DltC remains within the cell**

A key difference between the proposed models for D-alanine incorporation into TAs is the cellular location of the small carrier protein DltC. In the Fischer model this protein remains within the cytoplasm of the cell, while in the Neuhaus and Baddiley model the protein crosses the membrane and is at least transiently located on the outside of the cell (Fig. 1). To distinguish between these possibilities, we set out to determine the cellular location of DltC. To this end, *S. aureus* strain ANG1484 was constructed for expression of DltC with a C-terminal His-tag from the anhydratetracycline (Atet) inducible *tet* promoter (Fig. 2a). As a control, strain ANG1729 containing an empty vector was used. Both strains were grown to mid-exponential phase in the presence of Atet and subsequently cell (cytoplasm and membrane), cell wall and supernatant fractions prepared. The DltC protein was detected by Western blot using a His-tag specific antibody and detection of the cytoplasmically located ribosomal protein L6, the membrane protein SrtA, the cell wall protein SdrD and the secreted z-haemolysin (z-Hla) provided fractionation controls (Fig. 2b). DltC was only detected in the cell fraction and no signal was observed in the cell wall or supernatant fraction (Fig. 2b). These data are in better agreement with the model proposed by Werner Fischer and colleagues.

**DltD is oriented towards the outside of the cell**

DltD contains an N-terminal hydrophobic domain, which serves to anchor the protein in the membrane. According to the Neuhaus and Baddiley model, the protein is anchored in the membrane with an N terminus out/C terminus in topology, while in the Fischer model DltD has the opposite orientation (Fig. 1). To investigate the membrane topology of DltD, LacZ, fusions were constructed with the first 40 amino acids or full-length DltD and the fusion proteins were expressed in *S. aureus* from the inducible *tet* promoter by the addition of Atet (Fig. 3a). LacZ is only active within the cytoplasm of the cell and LacZ fusions with the first three amino acids of DltD or the signal sequence of aureolysin served as cytoplasmic or secreted controls, yielding as expected high or low β-galactosidase activities, respectively (Fig. 3b). Expression of the 40 amino acid or full-length DltD–LacZ fusion proteins yielded very low activity, indicating that DltD has an N terminus in/C terminus out membrane topology consistent with the Fischer model (Fig. 3b).

To verify this result, protein fusions were designed with the stably folded extracellular eLtaS domain of the LTA synthesize enzyme LtaS. It has been previously shown that when this domain is fused to a signal peptide or transmembrane domain with an N terminus in/C terminus out topology, it is cleaved by the signal peptidase and can be readily detected in the culture supernatant by Western blot (Wörmann et al., 2011). The enzymatically inactive eLtaS*T300A variant containing a C-terminal His-tag was fused to the first 40 or 100 amino acids of DltD and fusions with the first three amino acids of DltD or the aureolysin signal peptide were produced as cytoplasmic or exported controls, respectively (Fig. 4a, b). Cell and supernatant fractions were prepared from *S. aureus* strains containing...
Fig. 2. Cellular localization of DltC as assessed by Western blot. (a) Schematic representation of dltC expression construct. The gene encoding a C-terminally His-tagged version of DltC is placed under the inducible tetracycline promoter (pitet) and its expression induced by the addition of the less toxic tetracycline-derivative Atet. (b) Determination of DltC localization by Western blot analysis. S. aureus strain ANG1729 containing the empty vector pietet (v) or strain ANG1484 containing pietet-dltC-His (dltC) were grown for 4.5 h at 37 °C in the presence of Atet and samples were subsequently separated into cell (cytoplasm and membrane), cell wall and supernatant (super) fractions and analysed by Western blot using a His-tag specific antibody for the detection of DltC or antibodies specific for the ribosomal protein L6 (cytoplasmic), the sortase enzyme SrtA (membrane), the cell wall anchored protein SdrD (cell wall) and the secreted α-haemolysin Hla (supernatant). The molecular mass of protein standards is indicated on the left of each panel. The experiment was performed in triplicate and a representative blot is shown.

Fig. 3. Membrane topology of DltD as assessed by LacZ fusions and β-galactosidase activity assays. (a) Schematic representation of the different lacZ fusions. The dltD gene or the sequence encoding the first 40 aa of dltD was cloned into pietet-lacZ giving rise to dltD-lacZ fusions expressed from the Atet inducible promoter. Sequences encoding the first three amino acids of dltD (3 aa) and the signal sequence of aureolysin (aurSS) were cloned upstream of lacZ, providing cytoplasmic and secreted controls, respectively. (b) Determination of β-galactosidase activity. S. aureus strains containing pietet-3 aa-lacZ, pietet-aurSS-lacZ, pietet-dltD-lacZ (dltD) and pietet-dltD_{40 aa}-lacZ were grown for 4 h at 37 °C in the presence of 200 ng ml⁻¹ Atet and samples were prepared for β-galactosidase activity assays as described in the Methods section. The assay was performed in triplicate and the mean values and standard deviations were plotted. Activity is given as μM per OD₆₀₀ unit. t-Test analysis was performed and values which are significantly different from the positive control (3 aa) are indicated with asterisks as follows: *P<0.05, **P<0.01.

an empty vector (–) or vectors for the expression of the different fusions proteins. The fusion proteins were detected by Western blot using an anti-His-tag antibody and the fractionation technique was verified using antibodies specific for the cytoplasmically located ribosomal protein L6 or the α-haemolysin (Fig. 4c). As expected, eLtaS_{300A}·His was detected in the supernatant fraction for the aureolysin signal peptide control fusion. Proteins were also detected in the supernatant fraction for the 40 and 100 amino acid DltD fusions, while no signals were detected for samples isolated from the empty vector containing control strain or a strain expressing the cytoplasmic 3 aa-eLtaS_{300A}·His control fusion, the latter of which was detected in the cell fraction (Fig. 4c). Double bands were also observed in the cell fraction for the aurSS-eLtaS_{300A}, DltD_{40 aa} and DltD_{100 aa} fusion proteins (Fig. 4c). The less intense upper bands, which differ in size between the different fusion proteins, likely correspond to the full-length proteins. The lower protein bands, which are of similar size for all fusion proteins, are likely fusion protein fragments that have been transported to the cytoplasm.

LTA is required for efficient incorporation of d-alanine into WTA

Disruption of dltA-D in S. aureus results in a lack of d-alanine substitutions not only in LTA, but also in WTA.
proteins are involved in the transfer of D-alanine onto WTA, to retain the D-alanine modifications. The purified WTA were purified and the WTA was released in acid conditions. Wild-type *S. aureus* operon (Corrigan study) a whole genome sequence analysis was performed on these findings, it has been proposed that D-alanine-LTA is mechanism in which LTA has an indirect role in the D-alanine donor for WTA; however in this case it has been invoked that the process is enzyme-catalysed (Haas et al., 1984). While in this study we did not address whether or not the redistribution of WTA isolated from the LTA negative strain showed a drastically specific signal, yielding a D-Ala to GlcNAc ratio of 0.11 ± 0.01 (Fig. 5b and Fig S1). These results show that LTA is important for efficient D-alanylation of WTA and are consistent with a model in which D-alanine-LTA serves as major D-alanine donor for WTA. However, an alternative mechanism in which LTA has an indirect role in the D-alanylation of WTA could also take place, as discussed below.

**DISCUSSION**

In this study, we revisited the Dlt-protein-mediated D-alanine incorporation mechanism into TAs of Gram-positive bacteria. Using an *in vitro* assay system, it has been reported in a previous study that alanine can transfer without the requirement of ATP and in an enzyme- and Dlt-protein-independent manner between LTA molecules (Childs et al., 1985). In a second study, it has been proposed that D-alanine-LTA is the D-alanine donor for WTA; however in this case it has been invoked that the process is enzyme-catalysed (Haas et al., 1984). While in this study we did not address whether or not the redistribution of wild-type *S. aureus* strain (Fig. 5a and Fig S1). On the other hand, the WTA isolated from the LTA negative strain showed a drastically and statistically significant reduction in the D-alanine specific signal, yielding a D-Ala to GlcNAc ratio of 0.11 ± 0.01 (Fig. 5b and Fig S1). These results show that LTA is important for efficient D-alanylation of WTA and are consistent with a model in which D-alanine-LTA serves as major D-alanine donor for WTA. However, an alternative mechanism in which LTA has an indirect role in the D-alanylation of WTA could also take place, as discussed below. **DISCUSSION**

In this study, we revisited the Dlt-protein-mediated D-alanine incorporation mechanism into TAs of Gram-positive bacteria. Using an *in vitro* assay system, it has been reported in a previous study that alanine can transfer without the requirement of ATP and in an enzyme- and Dlt-protein-independent manner between LTA molecules (Childs et al., 1985). In a second study, it has been proposed that D-alanine-LTA is the D-alanine donor for WTA; however in this case it has been invoked that the process is enzyme-catalysed (Haas et al., 1984). While in this study we did not address whether or not the redistribution of...
of LTA rather than that, as we suggest, D-alanine LTA is the
major donor of D-alanine for WTA. This might
be explained by the fact that WTA polymers, which have been transported
to the outside of the membrane but are still linked to the
undecaprenyl phosphate membrane carrier, can, although
very inefficiently, serve as acceptor molecules for D-alanine
incorporation into TAs although the initial
steps are identical in both models. It has been well
established that the cytoplasmic D-alanine D-alanyl carrier
protein ligase DltA, which shows homology to the acetyl
coenzyme A synthases, uses ATP to activate D-alanine to
D-alanyl-AMP. In a second step, DltA then transfers
this intermediate onto the small D-alanyl carrier protein
DltC, where the D-alanine is bound through a thiol ester
bond to the phosphopantetheine prosthetic group in DltC
(Du et al., 2008; Neuhaus & Baddiley, 2003; Osman et al.,
2009; Yonus et al., 2008). DltC shows sequence and
structural homology to acyl carrier proteins (ACP), which
in bacteria function in the cytoplasm of the cell and are
involved in fatty acid and polyketide biosynthesis pathways
(Volkman et al., 2001). In this study, we show that DltC
does not cross the membrane (Fig. 2) and therefore it
is unlikely that the protein is involved in the final
d-alanylation step of LTA on the outside of the cell.
Furthermore, the results presented in this study indicate
that DltD has an N terminus in/C terminus out membrane
topology (Fig. 3). This places the functional part of the
protein on the outside of the cell and suggests that DltD
aids in the final step of the D-alanine incorporation into
LTA. Both of these findings are only consistent with the
model proposed by Werner Fischer and colleagues (Fig. 1a). According to the Fischer model, once DltC is charged
with a D-alanine, the multi-membrane-spanning protein
DltB transfers D-alanine from DltC to C55-P, resulting in
the formation of a D-alanine-P-C55 membrane intermediate.
This hypothesis is based on the proposed model for the
glycosylation process of LTA (Fischer, 1994), though this
membrane intermediate has never been experimentally
confirmed. Based on the hydropathy profile and the
TMHMM membrane topology prediction program, DltB
assembles as a ten transmembrane helix protein with both
N- and C-termini located on the outside. DltB has been
grouped among membrane-bound O-acyltransferases
(MBOAT) family proteins, a group of enzymes that
transfer organic acids onto hydroxyl groups of mem-
brane-embedded components (Hofmann, 2000). Some of
the best-characterized members of MBOAT proteins are
enzymes involved in the reacylation of lysophospholipids
(Shindou et al., 2009). This would be consistent with the
idea that DltB does not merely form a membrane channel
but also contains enzymatic activity, which will be
necessary for the formation of a membrane-linked
D-alanine intermediate. In addition, it cannot be ruled
out that DltB plays a role together with DltD in the final
cleavage and ligation of D-alanine to LTA.

The dlt operon in S. aureus encodes a fifth protein, DltX,
which is a small protein with an expected size of 5.9 kDa.
DltX has been annotated to belong to the DUF3687
superfamily of proteins and currently 185 proteins with
this domain are listed in Pfam. With two exceptions, these
proteins are encoded immediately upstream of dltA in S.
aureus strains, other Staphylococcus sp. and several other
Firmicutes including some Bacillus, Lactobacillus, Listeria,
Streptococcus and Enterococcus sp. However, additional

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**Fig. 5.** NMR analysis of WTA isolated from WT and LTA negative
*S. aureus* strains. *S. aureus* strains SEJ1 (WT) and 4S5 (LTA negative)
grown to mid-exponential phase and WTA was purified as described in the Methods section. Six milligrams of
dried WTA were suspended in 99.99% D2O and 1-D proton
spectra were acquired at 600 MHz. The experiment was performed in
triPLICATE and representative spectra are shown. The ratio of the D-
Ala to GlcNAc signal is 0.54 ± 0.08 for WTA isolated from a WT
strain and 0.11 ± 0.01 for WT isolated from the LTA negative
strain. A two-tailed unequal variance t-test gave a P-value <0.01
indicating statistically significant differences. Peaks are annotated
as previously described (Bernal et al., 2009), and the full spectra are shown in Fig. S1.

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D-alanines between TAs is enzyme-catalysed, the recent
description of an LTA negative *S. aureus* strain allowed us to
investigate the requirement of LTA for the D-alanylation of
WTA further. We show here that the D-alanine content in
WTA is drastically reduced in the absence of LTA, which is
in agreement with the hypothesis that D-alanylated LTA is
important for efficient modification of WTA (Fig. 5).
However, our data also showed that, even in the absence of
LTA, some D-alanine is still present in WTA. This might
suggest that WTA polymers, which have been transported
to the outside of the membrane but are still linked to the
undecaprenyl phosphate membrane carrier, can, although
very inefficiently, serve as acceptor molecules for D-alanine
modification by the Dlt system. This may in part reflect the
transient location of lipid-carrier anchored WTA at the
membrane-wall interface prior to incorporation into the
cell wall. An LTA negative *S. aureus* strain has usually a
severe growth defect (Gründling & Schneewind, 2007b);
however the LTA negative *S. aureus* strain used in this study
survives in the absence of LTA and grows nearly like a wild-
type strain through the acquisition of compensatory
mutations (Corrigan et al., 2011). Genome sequence analysis
confirmed that the dlt operon is intact (Corrigan et al., 2011),
which could have been an alternative explanation for the
reduced levels of D-alanine in WTA in this strain. However, it
cannot be completely ruled out that the observed reduction
of D-alanine in WTA is not due to an incorrect assembly of
the DltB or DltD proteins in the membrane caused by a lack
of LTA rather than that, as we suggest, D-alanine LTA is the
major donor of D-alanine for WTA.

Two conflicting models have been proposed for the
incorporation of D-alanine into TAs although the initial
work is needed to determine the function of DltX and establish whether or not this protein is involved in the D-alanlylation process in S. aureus or other Firmicutes.

Recently it has been shown that the lipopolysaccharide (LPS) in the Gram-negative bacterial pathogen Vibrio cholera O1 El Tor is also modified with amino acids, specifically glycine or diglycine residues (Hankins et al., 2012). The machinery used shows similarities to the D-alanine modification system of TAs in Gram-positive bacteria. AlmF, which shows homology to DltA, activates the glycine residues using ATP and ligates it to AlmE. AlmE does not show homology on the sequence level with DltC, but shows functional and likely structural homology to DltC. Once AlmE is charged with a glycine residue, it is transferred by AlmG, which contains a lysophospholipid acyltransferase (LPLAT) domain, onto LPS. Again, AlmG does not share any sequence homology with DltB, but both proteins are predicted to belong to acyltransferase enzyme families, and therefore it seems likely that as functional homologues these proteins are required for the transfer of the amino acids from the charged carrier protein to lipid-linked acceptor molecules (Hankins et al., 2012).

While additional work is needed to fully elucidate the mechanism of D-alanine incorporation into Gram-positive cell wall polymers, our cellular location and membrane topology studies on the S. aureus DltC and DltD proteins are in better agreement with the model proposed by Werner Fischer and colleagues. Therefore, we suggest that future investigations into the D-alanine incorporation mechanism should be designed with this model in mind. Our preliminary findings indicate that proteins involved in the LTA synthesis and the D-alanlylation process may physically interact within bacterial cells and it will be interesting to investigate in future studies the spatial and temporal coordination of the cell wall polymer synthesis machineries and proteins responsible for their modification.

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