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

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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 ω- 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]-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;
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 His-tag. 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, pittet was introduced into *S. aureus* RN4220Δspa resulting in strain ANG1729.

Plasmids pittet-dltD-lacZ, pittet-dltD*Δ40-aclacZ*, pittet-3 aa-lacZ, and pittet-aurs*Δ*55-lacZ were constructed for membrane topology studies in *S. aureus*. Plasmids pittet-dltD-lacZ and pittet-dltD*Δ40-aclacZ* 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/*SalI, PCR products were ligated with pittet-lacZ (ANG286), which had been digested with the same enzymes. Plasmids pittet-dltD-lacZ and pittet-dltD*Δ40-aclacZ* 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 pittet-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 pittet-lacZ, which had been digested with *AvrII/*SalI. Plasmid pittet-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 pittet-aurs*Δ*55-lacZ was constructed by amplifying the sequence encoding the signal sequence of aureolysin (aur*SS*) from *S. aureus* Newman chromosomal DNA with primers 1096/1097. The PCR product was digested with *AvrII/*SalI and ligated with plasmid pittet-lacZ, which had been digested with the same enzymes. Plasmid pittet-aurs*Δ*55-lacZ was initially obtained in *E. coli* XL1 Blue resulting in strain ANG1722 and

Table 1. Bacterial strains used in this study

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

| Strain | Relevant features | Reference |
|--------|------------------|-----------|
| *E. coli* | | |
| XL1 Blue | Cloning strain; Tet<sup>R</sup>-ANG127 | Stratagene |
| ANG284 | pittet in XL1 Blue; pCL55 containing Atet inducible promoter; Amp<sup>R</sup> | Grundling & Schneewind (2007a) |
| ANG286 | pittet-lacZ in XL1 Blue; Amp<sup>R</sup> | Lab. strain collection |
| ANG1103 | pOK-lata-T300A in XL1 Blue; Kan<sup>R</sup> | Lu et al. (2009) |
| ANG1314 | pUT18-dltD in XL1 Blue; Amp<sup>R</sup> | Lab. strain collection |
| ANG1482 | pittet-dltC-His in XL1 Blue; dltC-His under Atet inducible promoter control; Amp<sup>R</sup> | This study |
| ANG1718 | pittet-dltD-lacZ in XL1 Blue; dltD fused to lacZ under Atet inducible promoter control; Amp<sup>R</sup> | This study |
| ANG1719 | pittet-dltD<sub>Δ40</sub>-lacZ in XL1 Blue; first 40 aa of dltD fused to lacZ under Atet inducible promoter control; Amp<sup>R</sup> | This study |
| ANG1720 | pittet-3 aa-lacZ in XL1 Blue; first 3 aa of dltD fused to lacZ under Atet inducible promoter control; Amp<sup>R</sup> | This study |
| ANG1722 | pittet-aurs<sub>Δ55</sub>-lacZ in XL1 Blue; aur signal sequence fused to lacZ under Atet inducible promoter control; Amp<sup>R</sup> | This study |
| ANG1908 | pittet-3 aa-elts<sub>300A</sub>-His in XL1 Blue; first 3 aa of dltD fused to extracellular domain of ltaS<sub>300A</sub> variant and His-tag under Atet inducible promoter control; Amp<sup>R</sup> | This study |
| ANG2021 | pittet-aurs<sub>Δ55</sub>-elts<sub>300A</sub>-His in XL1 Blue; aur signal sequence fused to extracellular domain of ltaS<sub>300A</sub> variant and His-tag under Atet inducible promoter control; Amp<sup>R</sup> | This study |
| ANG2022 | pittet-dltD<sub>Δ100</sub>-aurs<sub>Δ55</sub>-elts<sub>300A</sub>-His in XL1 Blue; first 100 aa of dltD fused to extracellular domain of ltaS<sub>300A</sub> variant and His-tag under Atet inducible promoter control; Amp<sup>R</sup> | This study |
| ANG2041 | pittet-dltD<sub>Δ40</sub>-aurs<sub>Δ55</sub>-elts<sub>300A</sub>-His in XL1 Blue; first 40 aa of dltD fused to extracellular domain of ltaS<sub>300A</sub> variant and His-tag under Atet inducible promoter control; Amp<sup>R</sup> | This study |
| *S. aureus* | | |
| SEJ1 | RN4220Δspa-ANG314 | Grundling & Schneewind (2007a) |
| ANG1755 | RN4220Δspaasıb | Wörmann et al. (2011) |
| ANG1786 | 4S5; derivative of RN4220Δspaası with mapped suppressor mutations, lacking LTA | Corrigan et al. (2011) |
| ANG1484 | pittet-dltC-His integrated in strain ANG314; Cam<sup>R</sup> | This study |
| ANG1723 | pittet-dltD-lacZ integrated in strain ANG314; Cam<sup>R</sup> | This study |
| ANG1724 | pittet-dltD<sub>Δ40</sub>-lacZ integrated in strain ANG314; Cam<sup>R</sup> | This study |
| ANG1725 | pittet-3 aa-lacZ integrated in strain ANG314; Cam<sup>R</sup> | This study |
| ANG1727 | pittet-aurs<sub>Δ55</sub>-lacZ integrated in strain ANG314; Cam<sup>R</sup> | This study |
| ANG1729 | pittet integrated in strain ANG314; Cam<sup>R</sup> | This study |
| ANG2024 | pCL5S5tet-3 aa-elts<sub>300A</sub>-His integrated in strain ANG1755; Cam<sup>R</sup> | This study |
| ANG2025 | pCL5S5tet-aurs<sub>Δ55</sub>-elts<sub>300A</sub>-His integrated in strain ANG1755; Cam<sup>R</sup> | This study |
| ANG2026 | pCL5S5tet-dltD<sub>Δ100</sub>-aurs<sub>Δ55</sub>-elts<sub>300A</sub>-His integrated in strain ANG1755; Cam<sup>R</sup> | This study |
| ANG2034 | pCL5S5tet integrated in strain ANG1755; Cam<sup>R</sup> | This study |
| ANG2042 | pCL5S5tet-dltD<sub>Δ40</sub>-aurs<sub>Δ55</sub>-elts<sub>300A</sub>-His integrated in strain ANG1755; Cam<sup>R</sup> | This study |
Table 2. Primers used in this study

| Number | Primer                      | Sequence*                      |
|--------|-----------------------------|--------------------------------|
| ANG420 | 3-BglII-His6-719            | GAAATCTTTAGTGTGATGTTGATGACCTTTTTTAGAGTTTGCTTTAGGTCCTG        |
| ANG721 | 5’-AvrII-DltC               | GGAGATCTTTAGTGTGATGTTGATGACCTTTTTTAGAGTTTGCTTTAGGTCCTG        |
| ANG722 | 3’-BglII-His-tag-DltC       | GGAGATCTTTAGTGTGATGTTGATGACCTTTTTTAGAGTTTGCTTTAGGTCCTG        |
| ANG882 | 5’-AvrII-RBS-dltD for       | GGAGATCTTTAGTGTGATGTTGATGACCTTTTTTAGAGTTTGCTTTAGGTCCTG        |
| ANG883 | 5’-Sal-dltD rev             | CGAGGTTCACTTTTTAGGTTTGCTTTAGGTCCTG                          |
| ANG884 | 3’-Sal-dltD 40 aa rev       | CGAGGTTCACTTTTTAGGTTTGCTTTAGGTCCTG                          |
| ANG887 | AvrII-RBS-3 aa-SalI         | P-CTAGGAAAAAAATAAAGGAGGAAAAAATGAAATTAAAGGACCCAGACCGACACCTTTTTAGGTCCTG |
| ANG888 | AvrII-RBS-3 aa-SalI rev     | P-CTAGGAAAAAAATAAAGGAGGAAAAAATGAAATTAAAGGACCCAGACCGACACCTTTTTAGGTCCTG |
| ANG890 | dltD 40 aa-eLtaS rev        | GATAATAGCTTGAAGACTGACAGACCAGACCGACACCTTTTTAGGTCCTG          |
| ANG891 | dltD 100 aa-eLtaS rev       | GCTGTCTAGCTTGAAGACTGACAGACCAGACCGACACCTTTTTAGGTCCTG          |
| ANG892 | dltD 100 aa-eLtaS rev       | GCTGTCTAGCTTGAAGACTGACAGACCAGACCGACACCTTTTTAGGTCCTG          |
| ANG1096| 5’-AvrII-RBS-aur for        | CCCTGCTAGGAAAAAAATAAAGGAGGAAAAAATGAAATTAAAGGACCCAGACCGACACCTTTTTAGGTCCTG |
| ANG1097| 3’-Aur-salI rev             | CCCTGCTAGGAAAAAAATAAAGGAGGAAAAAATGAAATTAAAGGACCCAGACCGACACCTTTTTAGGTCCTG |
| ANG1138| AvrII-RBS-3 aa-eltaS        | CCCTGCTAGGAAAAAAATAAAGGAGGAAAAAATGAAATTAAAGGACCCAGACCGACACCTTTTTAGGTCCTG |

*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 piet-dltD_ac_eltaST300A-His, piet-dltD_ac_eltaST300A-His, piet-3 aa-eltaST300A-His and piet-aufST300A-His were generated for this purpose. Plasmids piet-dltD_ac_eltaST300A-His and piet-dltD_ac_eltaST300A-His were constructed by amplifying the sequence encoding the first 40 or 100 amino acids of DltD from pUT18-dltD (ANG1314) with primers 882/889 and 882/891 and the eLtaST300A-His sequence from pOK-IdltD (ANG1103) using primers 890/420 and 892/420, respectively. The resulting products were fused by splicing by overlap extension (SOE) PCR using primers 882/420. The final PCR products were digested with AvrII and ligated with plasmid piet, which had been digested with the same enzymes. Plasmids piet-dltD_ac_eltaST300A-His and piet-dltD_ac_eltaST300A-His were initially transformed into *E. coli* XL1 Blue resulting in strains ANG2041 and ANG2022, and subsequently transformed into *S. aureus* RN4220ΔspaΔshi yielding strains ANG2042 and ANG2026, respectively. In order to generate plasmid piet-3 aa-eltaST300A-His, the eLtaST300A sequence was amplified from pOK-IdltD (ANG1103) with primers 1138/420, resulting in the addition of the ribosome-binding site and sequences encoding the first three amino acids of DltD to the 5′ end and a 6 × His-tag to the 3′ end. This PCR product was digested with AvrII/BglII and ligated with plasmid piet, which had been digested with the same enzymes. Plasmid piet-3 aa-eltaST300A-His was initially transformed into *E. coli* XL1 Blue giving rise to strain ANG1908 and subsequently transformed into *S. aureus* RN4220ΔspaΔshi yielding strain ANG2024. Plasmid piet-aufST300A-His was constructed by amplifying the sequence encoding the signal sequence of aureolysin (aureolysin) from piet-aufST300A-LacZ (ANG1722) with primers 1096/1216 and the eltaST300A-His sequence from pOK-IdltD (ANG1103) with primers 1217/420. The resulting products were fused by SOE PCR using primers 1096/420. The final PCR product was digested with AvrII/BglII and ligated with plasmid piet, which had been digested with the same enzymes. Plasmid piet-aufST300A-LacZ-His was initially transformed into *E. coli* XL1 Blue resulting in strain ANG2021 and subsequently transformed into *S. aureus* RN4220ΔspaΔshi 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 anhydrotetracycline (Atet) 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 lysis buffer (100 mM Tris/HCl pH 7.5, 10 mM MgCl2, 30% raffinose and 200 μg ml⁻¹ lysozyme) 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 osmotically stabilizing lysis buffer (50 mM Tris/HCl pH 7.5, 20 mM MgCl2, 30% raffinose and 200 μg ml⁻¹ lysozyme) 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).

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were pelleted by centrifugation at 17 000 g and washed once with 1 M NaCl, three times with 0.5 % SDS and water before resuspension in 1 ml of ABT buffer (60 mM K2HPO4, 40 mM KH2PO4, 100 mM NaCl, pH 7.2, 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-methylumbelliferone 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.

**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 anhydrotetracycline (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 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 LtaS domain of the LTA synthase 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 eLtaS300A 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
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 antibodies specific for the cytoplasmically located ribosomal protein L6 or the α-haemolysin (Fig. 4c). As expected, eLtaST300A-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 (-) or strain ANG1484 containing pitet-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.

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 cell wall between the different fusions constructs. The experiment was performed in triplicate and a representative blot is shown. 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, eLtaST300A-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 aureolysin signal peptide control fusion. 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 cell wall.
proteins are involved in the transfer of D-alanine onto WTA, were purified and the WTA was released in acid conditions. Strain 4S5 were grown to mid-exponential phase, cell walls of S. aureus wild-type dlt operon (Corrigan et al., 1995). While this may imply that the Dlt proteins are involved in the transfer of D-alanine onto WTA, studies using [14C]-alanine have shown that when the radioactivity is lost from the LTA fraction it increases in WTA samples isolated from the wild-type strain (Fig. 5a and Fig. S1). On the other hand, the WTA isolated from the LTA negative strain showed a drastically reduced radioactivity at 1.6 p.p.m. to GlcNAc at 2.1 p.p.m. was 0.54 ± 0.08 for WTA isolated from a WT 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 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.
of LTA rather than that, as we suggest, D-alanine LTA is the major donor of D-alanine for WTA. It was previously shown that DltB or DltD proteins in the membrane caused by a lack of 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.

Fig. 5. NMR analysis of WTA isolated from WT and LTA negative S. aureus strains. S. aureus strains SEJ1 (WT) and 4SS (LTA negative) were 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 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 WTA 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.

The revised model for D-alanylation of teichoic acids 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 form 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 (ACPs), 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 hydrophathy 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 (MOBAT) family proteins, a group of enzymes that transfer organic acids onto hydroxyl groups of membrane-embedded components (Hofmann, 2000). Some of the best-characterized members of MBOAT proteins are enzymes involved in the reacylation of lyosphospholipids (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
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. AlmG, 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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REFERENCES

Bernal, P., Zloh, M. & Taylor, P. W. (2009). Disruption of -alanyl esterification of *Staphylococcus aureus* cell wall teichoic acid by the β-lactam resistance modifier (−)-epicatechin gallate. *J Antimicrob Chemother* 63, 1156–1162.

Brown, S., Zhang, Y. H. & Walker, S. (2008). A revised pathway proposed for *Staphylococcus aureus* wall teichoic acid biosynthesis based on in vitro reconstitution of the intracellular steps. *Chem Biol* 15, 12–21.

Brown, S., Xia, G., Luhachack, L. G., Campbell, J., Meredith, T. C., Chen, C., Winstel, V., Gekeler, C., Irazoqui, J. E. & other authors (2012). Methicillin resistance in *Staphylococcus aureus* requires glycosylated wall teichoic acids. *Proc Natl Acad Sci U S A* 109, 18909–18914.

Bubeck Wardenburg, J. & Schneewind, O. (2008). Vaccine protection against *Staphylococcus aureus* pneumonia. *J Exp Med* 205, 287–294.

Childs, W. C., Ill, Taron, D. J. & Neuhaus, F. C. (1985). Biosynthesis of D-alanyl-lipoteichoic acid by *Lactobacillus casei* interchain transacylation of D-alanyl ester residues. *J Bacteriol* 162, 1191–1195.

Collins, L. V., Kristian, S. A., Weidenmaier, C., Faigle, M., Van Kessel, K. P., Van Strijp, J. A., Götz, F., Neumeister, B. & Peschel, A. (2002). *Staphylococcus aureus* strains lacking D-alanine modifications of teichoic acids are highly susceptible to human neutrophil killing and are virulence attenuated in mice. *J Infect Dis* 186, 214–219.

Corrigan, R. M., Abbott, J. C., Burhenne, H., Kaefer, V. & Gründling, A. (2011). c-di-AMP is a new second messenger in *Staphylococcus aureus* with a role in controlling cell size and envelope stress. *PLoS Pathog* 7, e1002217.

Debakov, D. V., Heaton, M. P., Zhang, Q., Stewart, K. D., Lambalot, R. H. & Neuhaus, F. C. (1996). The D-alanyl carrier protein in *Lactobacillus casei*: cloning, sequencing, and expression of dltC. *J Bacteriol* 178, 3869–3876.

Debakov, D. V., Kirikhin, M. Y. & Neuhaus, F. C. (2000). Biosynthesis of lipoteichoic acid in *Lactobacillus rhamnosus* role of DltD in D-alanlylation. *J Bacteriol* 182, 2855–2864.

DeDent, A., Bae, T., Missiakas, D. M. & Schneewind, O. (2008). Signal peptides direct surface proteins to two distinct envelope locations of *Staphylococcus aureus*. *EMBO J* 27, 2656–2668.

Du, L., He, Y. & Luo, Y. (2008). Crystal structure and enantiomer selection by D-alanyl carrier protein ligase DltA from *Bacillus cereus*. *Biochemistry* 47, 11473–11480.

Fischer, W. (1994). Lipoteichoic acid and lipids in the membrane of *Staphylococcus aureus*. *Med Microbiol Immunol (Berl)* 183, 61–76.

Fischer, W., Rösel, P. & Koch, H. U. (1981). Effect of alanine ester substitution and other structural features of lipoteichoic acids on their inhibitory activity against autolysins of *Staphylococcus aureus*. *J Bacteriol* 146, 467–475.

Gross, M., Cramton, S. E., Götz, F. & Peschel, A. (2001). Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces. *Infect Immun* 69, 3423–3426.

Gründling, A. & Schneewind, O. (2007a). Genes required for glycolipid synthesis and lipoteichoic acid anchoring in *Staphylococcus aureus*. *J Bacteriol* 189, 2521–2530.

Gründling, A. & Schneewind, O. (2007b). Synthesis of glycerol phosphate lipoteichoic acid in *Staphylococcus aureus*. *Proc Natl Acad Sci U S A* 104, 8478–8483.

Haas, R., Koch, H. U. & Fischer, W. (1984). Alanyl turnover from lipoteichoic acid to teichoic acid in *Staphylococcus aureus*. *FEBS Microbiol Lett* 21, 27–31.

Hankins, J. V., Madsen, J. A., Giles, D. K., Brodbelt, J. S. & Trent, M. S. (2012). Amino acid addition to *Vibrio cholerae* LPS establishes a link between surface remodeling in Gram-positive and Gram-negative bacteria. *Proc Natl Acad Sci U S A* 109, 8722–8727.

Heaton, M. P. & Neuhaus, F. C. (1994). Role of the D-alanyl carrier protein in the biosynthesis of D-alanyl-lipoteichoic acid. *J Bacteriol* 176, 681–690.

Hofmann, K. (2000). A superfamily of membrane-bound O-acyltransferases with implications for wnt signaling. *Trends Biochem Sci* 25, 111–112.

Karatsa-Dodgson, M., Wörmann, M. E. & Gründling, A. (2010). In vitro analysis of the *Staphylococcus aureus* lipoteichoic acid synthase enzyme using fluorescently labeled lipids. *J Bacteriol* 192, 5341–5349.

Koch, H. U., Haas, R. & Fischer, W. (1994). The role of lipoteichoic acid biosynthesis in membrane lipid metabolism of growing *Staphylococcus aureus*. *Eur J Biochem* 138, 357–363.
Koch, H. U., Döker, R. & Fischer, W. (1985). Maintenance of d-alanine ester substitution of lipoteichoic acid by reesterification in Staphylococcus aureus. J Bacteriol 164, 1211–1217.

Kopp, U., Roos, M., Wecke, J. & Labischinski, H. (1996). Staphylococcal peptidoglycan interpeptide bridge biosynthesis: a novel antistaphylococcal target? Microb Drug Resist 2, 29–41.

Koprivnjak, T., Milak, V., Swanson, L., Fournier, B., Peschel, A. & Weiss, J. P. (2006). Cation-induced transcriptional regulation of the dlt operon of Staphylococcus aureus. J Bacteriol 188, 3622–3630.

Kristian, S. A., Datta, V., Weidenmaier, C., Kansal, R., Fedke, I., Peschel, A., Gallo, R. L. & Nizet, V. (2005). d-Alanylation of teichoic acids promotes group a Streptococcus antimicrobial peptide resistance, neutrophil survival, and epithelial cell invasion. J Bacteriol 187, 6719–6725.

Lambert, P. A., Hancock, I. C. & Baddiley, J. (1975). Influence of alanyl ester residues on the binding of magnesium ions to teichoic acids. Biochem J 151, 671–676.

Lu, D., Wörmann, M. E., Zhang, X., Schneewind, O., Gründer, A. & Freemont, P. S. (2009). Structure-based mechanism of lipoteichoic acid synthesis by Staphylococcus aureus LtaS. Proc Natl Acad Sci U S A 106, 1584–1589.

Mazmanian, S. K., Liu, G., Jensen, E. R., Lenoy, E. & Schneewind, O. (2000). Staphylococcus aureus sortase mutants defective in the display of surface proteins and in the pathogenesis of animal infections. Proc Natl Acad Sci U S A 97, 5510–5515.

Neuhaus, F. C. & Baddiley, J. (2003). A continuum of anionic charge: structures and functions of d-alanyl-teichoic acids in gram-positive bacteria. Microbiol Mol Biol Rev 67, 866–723.

Neuhaus, F. C., Heaton, M. P., Debabov, D. V. & Zhang, Q. (1996). The dlt operon in the biosynthesis of d-alanyl-lipoteichoic acid in Lactobacillus casei. Microb Drug Resist 2, 77–84.

Oka, Y., Kurokawa, K., Matsu, M., Yamada, S., Lee, B. L. & Sekimizu, K. (2009). Pleiotropic roles of polylipidolipidophosphate synthase of lipoteichoic acid in growth of Staphylococcus aureus cells. J Bacteriol 191, 141–151.

Osman, K. T., Du, L., He, Y. & Luo, Y. (2009). Crystal structure of Bacillus cereus d-alanyl carrier protein ligase (DltA) in complex with ATP. J Mol Biol 388, 345–355.

Pereg, M., Glaizer, P., Minutello, A., Strauch, M. A., Leopold, K. & Fischer, W. (1995). Incorporation of d-alanine into lipoteichoic acid and wall teichoic acid in Bacillus subtilis. Identification of genes and regulation. J Biol Chem 270, 15598–15606.

Peschel, A., Otto, M., Jack, R. W., Kalbacher, H., Jung, G. & Götz, F. (1999). Inactivation of the dlt operon in Staphylococcus aureus confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J Biol Chem 274, 8405–8410.

Poyart, C., Pellegrini, E., Marceau, M., Baptista, M., Jaubert, F., Lamy, M. C. & Trieu-Cuoit, P. (2003). Attenuated virulence of Streptococcus agalactiae deficient in d-alanyl-lipoteichoic acid is due to an increased susceptibility to defensins and phagocytic cells. Mol Microbiol 49, 1615–1625.

Reichmann, N. T. & Gründling, A. (2011). Location, synthesis and function of glycolipids and polyglycerolphosphate lipoteichoic acid in Gram-positive bacteria of the phylum Firmicutes. FEMS Microbiol Lett 319, 97–105.

Schirner, K., Marles-Wright, J., Lewis, R. J. & Errington, J. (2009). Distinct and essential morphogenetic functions for wall- and lipoteichoic acids in Bacillus subtilis. EMBO J 28, 830–842.

Shindou, H., Hishikawa, D., Harayama, T., Yuki, K. & Shimizu, T. (2009). Recent progress on acyl CoA:lysophospholipid acyltransferase research. J Lipid Res 50 (Suppl.), S46–S51.

Strandén, A. M., Ehler, K., Labischinski, H. & Berger-Bächi, B. (1997). Cell wall monoglycine cross-bridges and methicillin hypersusceptibility in a femAB null mutant of methicillin-resistant Staphylococcus aureus. J Bacteriol 179, 9–16.

Volkmann, B. F., Zhang, Q., Debabov, D. V., Rivera, E., Kresheck, G. C. & Neuhaus, F. C. (2001). Biosynthesis of d-alanyl-lipoteichoic acid: the tertiary structure of apo-d-alanyl carrier protein. Biochemistry 40, 7964–7972.

Walter, J., Loach, D. M., Alqumber, M., Rockel, C., Herrman, C., Pfitzenmaier, M. & Tannock, G. W. (2007). D-alanyl ester depletion of teichoic acids in Lactobacillus reuteri nasal colonization, a major risk factor in nosocomial infections. Nat Med 10, 243–245.

Wörmann, M. E., Reichmann, N. T., Malone, C. L., Horswill, A. R. & Gründling, A. (2011). Proteolytic cleavage inactivates the Staphylococcus aureus lipoteichoic acid synthase. J Bacteriol 193, 5279–5291.

Xia, G., Kohler, T. & Peschel, A. (2010a). The wall teichoic acid and lipoteichoic acid polymers of Staphylococcus aureus. Int J Med Microbiol 300, 148–154.

Xia, G., Maier, L., Sanchez-Carballo, P., Li, M., Otto, M., Holst, O. & Peschel, A. (2010b). Glycosylation of wall teichoic acid in Staphylococcus aureus by TarM. J Biol Chem 285, 13405–13415.

Yonous, H., Neumann, P., Zimmermann, S., May, J. J., Marahiel, M. A. & Stubbs, M. T. (2008). Crystal structure of DltA. Implications for the reaction mechanism of non-ribosomal peptide synthetase adenylation domains. J Biol Chem 283, 32484–32491.

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