Two-enzyme systems for glycolipid and polyglycerolphosphate lipoteichoic acid synthesis in Listeria monocytogenes

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Summary
Lipoteichoic acid (LTA) is an important cell wall polymer in Gram-positive bacteria and often consists of a polyglycerolphosphate backbone chain that is linked to the membrane by a glycolipid. In Listeria monocytogenes this glycolipid is Gal-Glc-DAG or Gal-Ptd-6Glc-DAG. Using a bioinformatics approach, we have identified L. monocytogenes genes predicted to be involved in glycolipid (lmo2555 and lmo2554) and LTA backbone (lmo0644 and lmo0927) synthesis. LTA and glycolipid analysis of wild-type and mutant strains confirmed the function of Lmo2555 and Lmo2554 as glycosyltransferases required for the formation of Glc-DAG and Gal-Glc-DAG. Deletion of a third gene, lmo2553, located in the same operon resulted in the production of LTA with an altered structure. Lmo0927 and lmo0644 encode proteins with high similarity to the staphylococcal LTA synthase LtaS, which is responsible for polyglycerolphosphate backbone synthesis. We show that both proteins are involved in LTA synthesis. Our data support a model whereby Lmo0644 acts as an LTA primase LtaP and transfers the initial glycerolphosphate onto the glycolipid anchor, and Lmo0927 functions as LTA synthase LtaS, which extends the glycerolphosphate backbone chain. Inactivation of LtaS leads to severe growth and cell division defects, underscoring the pivotal role of LTA in this Gram-positive pathogen.

Introduction
The cell wall envelope of Gram-positive bacteria has been an area of active research for decades. By studying its assembly not only essential functions for bacterial growth and physiology but also important aspects of host pathogen interactions have been uncovered, and studies on the Gram-positive cell wall envelope have gained increased attention in the field of bacterial pathogens. A typical Gram-positive envelope is composed of peptidoglycan, proteins, often capsular polysaccharides and secondary wall polymers, which include wall teichoic acid (WTA), a polymer covalently linked to peptidoglycan, and lipoteichoic acid (LTA), a polymer tethered by a lipid anchor to the bacterial membrane (Fischer, 1988; Navarre and Schneewind, 1999). The structure of LTA varies between organisms (Fischer, 1988; Weidenmaier and Peschel, 2008); one of the best characterized structure is a polymer with an un-branched 1-3-linked glycerolphosphate chain attached to a membrane glycolipid as for instance found in Bacillus subtilis, Staphylococcus aureus, Enterococcus faecalis, Group A and B Streptococcus and Listeria monocytogenes (Fischer, 1990). Glycerolphosphate subunits can be substituted with glycosyl residues and/or D-alanine esters, which significantly contribute to cationic peptide resistance in Gram-positive bacteria (Fischer, 1990; Peschel et al., 1999). In L. monocytogenes, the polyglycerolphosphate LTA backbone is substituted with both D-alanines and α-galactosyl residues and linked to the bacterial membrane via glycolipids Gal(α1-2)Glc(α1-3)-diacylglycerol (Gal-Glc-DAG) or Gal(α1-2)Ptd-6Glc(α1-3)DAG (Gal-Ptd-6Glc-DAG), in which the glucose moiety is lipidated at position 6 with a phosphatidyl (Ptd) group (Hether and Jackson, 1983; Uchikawa et al., 1986; Fischer et al., 1990) (Fig. 1). Despite this thorough chemical analysis, the exact function of LTA is not known.

The recent identification of enzymes responsible for glycolipid and LTA backbone synthesis allowed a phenotypic characterization of strains that are deficient in LTA synthesis or produce LTA of an altered structure. The enzyme responsible for polyglycerolphosphate backbone chain formation has been discovered recently in S. aureus and named LtaS for LTA synthase (Gründling and Schneewind, 2007a). The same and two subsequent studies on S. aureus and B. subtilis revealed that LTA is important for normal growth and observed morphological alterations indicate a crucial role of LTA in the cell division
Chemical structure of L. monocytogenes LTA

L. monocytogenes LTA is a linear polyglycerolphosphate polymer attached to the membrane by the glycolipid Gal-Glc-DAG. The free hydroxyl group of the glycerolphosphate units (X1) can be esterified with D-alanine (D-Ala) or glycosylated with galactose (Gal) and the glucose moiety of Gal-Glc-DAG can be lipidated at position 6 with a hydroxyl group of the glycerolphosphate units (X1) can be esterified attached to the membrane by the glycolipid Gal-Glc-DAG. The free LTA, has been investigated and it was found that this modification is important for bacterial adhesion to eukaryotic cells and virulence of L. monocytogenes in the mouse model of infection (Abachin et al., 2002). In addition, it has been reported that the L. monocytogenes internalin B protein (InlB), a non-covalently attached cell surface protein required for entry into various host cells, binds to LTA and is retained at the bacterial surface in this manner (Braun et al., 1997; 1998; Jonquieres et al., 1999). Thus, LTA directly and indirectly has important roles in bacterial physiology and virulence.

Here, we used a bioinformatics approach to identify L. monocytogenes genes required for glycolipid and LTA polyglycerolphosphate backbone synthesis. Using a combination of molecular biology and mass spectrometry approaches to characterize glycolipids and LTA synthesized in wild-type and mutant strains, we show that the previously uncharacterized L. monocytogenes genes lmo2555 and lmo2554 encode glycolipid synthesis enzymes, and renamed them LafA and LafB for LTA anchor formation proteins A and B. Two proteins, Lmo0927 and Lmo0644, with similarity to the S. aureus LTA synthase LtaS are involved in LTA backbone synthesis but they have clearly distinct enzymatic functions within the cell. Inactivation of Lmo0927 leads to the absence of LTA on the bacterial surface, a severe growth defect at elevated temperatures and morphological changes underscoring the importance of LTA for cellular functions in the Gram-positive pathogen L. monocytogenes.

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Identification of potential glycolipid and LTA synthesis gene in L. monocytogenes

To begin to understand the function(s) of LTA in the Gram-positive pathogen L. monocytogenes, we used a bioinformatics approach to identify putative glycolipid and LTA synthesis enzymes in this organism. Since the LTA glycolipid anchor in L. monocytogenes consists of Gal-Glc-DAG (Hether and Jackson, 1983; Uchikawa et al., 1986; Fischer et al., 1990) presumably two distinct glycosyltransferases are required for its synthesis similar to that observed for E. faecalis and S. agalactiae. In E. faecalis and S. agalactiae the glycosyltransferases responsible for the addition of the terminal glucose moiety have been identified as IagA (Gbs0682 in strain NEM316 and Bgs0683 in strain V583) for biofilm-associated glycolipid synthesis and IagA (Gbs0682 in strain NEM316) for invasion-associated gene, respectively, to denote observed phenotypes and defects of deletion strains (Doran et al., 2005; Theilacker et al., 2009). It should be noted that phenotypes observed in strains mutated in glycolipid synthesis genes may not necessarily be due to the lack of these membrane lipids as LTA structure and production are also affected in their absence (Fedtke et al., 2007; Gründling and Schnewind, 2007b).

Little is known about LTA and glycolipid synthesis in the Gram-positive pathogen L. monocytogenes. Only the function of Dlt proteins, which incorporate D-alanines into LTA, has been investigated and it was found that this modification is important for bacterial adhesion to eukaryotic cells and virulence of L. monocytogenes in the mouse model of infection (Abachin et al., 2002). In addition, it has been reported that the L. monocytogenes internalin B protein (InlB), a non-covalently attached cell surface protein required for entry into various host cells, binds to LTA and is retained at the bacterial surface in this manner (Braun et al., 1997; 1998; Jonquieres et al., 1999). Thus, LTA directly and indirectly has important roles in bacterial physiology and virulence.

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LTA production in wild-type and mutant L. monocytogenes strains. A. Operon structure of L. monocytogenes genes involved in glycolipid and LTA formation with direction of transcription indicated by the arrows and predicted terminators shown by hairpin loops. B–E. Western blot detection of cell wall-associated LTA in wild-type, deletion and complementation strains: (B) 10403S (WT), 10403S\(\Delta lmo2553\) (\(\Delta 2553\)), 10403S\(\Delta lmo2554\) (\(\Delta 2554\)) and 10403S\(\Delta lmo2555\) (\(\Delta 2555\)); (C) 10403S (WT), 10403S\(\Delta lmo0644\) (\(\Delta 0644\)) and 10403S\(\Delta lmo0927\) (\(\Delta 0927\)); (D) 10403S pHPL3 (WT), 10403S\(\Delta lmo2553\) pHPL3-\(lmo2553\) (\(\Delta 2553\) compl.), 10403S\(\Delta lmo2554\) pHPL3 (\(\Delta 2554\)) and 10403S\(\Delta lmo0927\) pHPL3 (\(\Delta 0927\) compl.); (E) 10403S pHPL3 (WT), 10403S\(\Delta lmo0644\) pHPL3-\(lmo0644\) (\(\Delta 0644\) compl.), 10403S\(\Delta lmo0644\) pPL3 (\(\Delta 0644\)) and 10403S\(\Delta lmo0927\) pPL3 (\(\Delta 0927\) compl.) and 10403S\(\Delta lmo0927\) pPL3 (\(\Delta 0927\)). Positions of protein standards (in kDa) are shown on the left.

BLAST searches (Altschul et al., 1997) against the L. monocytogenes EGD-e genome (Glaser et al., 2001). This identified the L. monocytogenes proteins Lmo2554 (E-value of 6e-20 to lAgA) and Lmo2555 (E-value of 2e-76 to Gbs0683) as the closest homologues. Using the respective E. faecalis proteins in BLAST searches the same two L. monocytogenes proteins were identified with similar E-values of 2e-22 and 3e-83. Taken together, this suggests that Lmo2555 and Lmo02554 could encode UDP-glucose- and UDP-galactose-specific glycosyltransferases responsible for Glc-DAG and Gal-Glc-DAG synthesis respectively. The coding sequences of Lmo2555 and Lmo2554 overlap by eight bases and the operon is likely to contain a third gene, Lmo2553, predicted to encode an integral membrane protein (Fig. 2A). The S. aureus enzyme LtaS, which is responsible for LTA polyglycerolphosphate backbone synthesis, was recently identified (Gründling and Schneewind, 2007a). Two proteins with high degree of similarity to the staphylococcal LtaS enzyme, Lmo0927 (E-value 0.0) and Lmo0644 (E-value 1e-58), are encoded in Listeria genomes (Fig. 2A).

To study the requirement of Lmo2555, Lmo2554, Lmo2553, Lmo0927 and Lmo0644 for glycolipid production, LTA synthesis and bacterial physiology, these genes were inactivated in the L. monocytogenes 1/2a strain 10403S. Unmarked in-frame deletions were created by allelic exchange and all gene deletions were confirmed by PCR. With the exception of strain 10403S\(\Delta lmo0927\), which only grew well at 30°C (discussed below), all deletion strains had similar doubling times as compared with the parental 10403S strain (data not shown).

**Inactivation of predicted L. monocytogenes glycolipid and LTA synthases affects LTA production and structure**

Initially we set out to assess if Lmo2555, Lmo2554, Lmo2553, Lmo0927 and Lmo0644 contribute to LTA synthesis in L. monocytogenes by analysing LTA production in wild-type and deletion strains. In the case of S. aureus, inactivation of enzymes involved in glycolipid and LTA backbone synthesis leads to structural changes in LTA, which can be readily visualized by Western blot analysis using a polyglycerolphosphate-specific LTA antibody (Gründling and Schneewind, 2007b). Wild-type L. monocytogenes 10403S and deletion strains were grown overnight at 37°C with exception of strain 10403S\(\Delta lmo0927\), which was cultivated for 2 days at 37°C, and samples were prepared for Western blot analysis of cell wall associated LTA as described under **Experimental procedures**. Inactivation of Lmo2554 and Lmo2555 led to a drastic reduction in the total amount of LTA produced, while deletion of Lmo2553 led to the production of LTA with retarded mobility (Fig. 2B). A similar mobility shift was observed upon inactivation of Lmo0644, while no LTA-specific signal could be detected for strain 10403S\(\Delta lmo0927\) (Fig. 2C). In addition, the amount of released LTA was analysed by the same Western blot method, but only minimal amounts could be detected in the culture supernatant of Lmo2555, Lmo2554 and Lmo2553 deletion strains (data not shown). To confirm that observed phenotypes were solely due to deletion of the respective gene, complementation vectors were constructed and introduced into appropriate deletion strains. Genes Lmo0644, Lmo0927 and Lmo2555 were cloned under their native promoter into the L. monocytogenes single-site integration vector pPL3, while Lmo2553 and Lmo2554 were cloned into vector pHPL3 under control of the hyper-spac promoter. As shown in Fig. 2D and E, the observed alterations in LTA production could be comple-
mented, confirming that differences in LTA synthesis are due to inactivation of the respective gene. In summary, these results indicate that all L. monocytogenes proteins identified by our bioinformatics approach are indeed involved in LTA synthesis. The complete absence of LTA in strain 10403SΔlmo0927 suggests that Lmo0927 is responsible for the synthesis of the polyglycerolphosphate backbone chain, while Lmo0644 seems to have an accessory function.

Lmo2555 and Lmo2554 are glycosyltransferases responsible for Glc-DAG and Gal-Glc-DAG production

Alterations in glycolipid synthesis will affect LTA structure and production (Gründling and Schneewind, 2007b). To correlate observed alterations in LTA production with changes in glycolipid formation in the different L. monocytogenes deletion strains, production of these membrane lipids was further analysed. Previous studies have identified the following glycolipids in membranes of Listeria spp.: Glc-DAG, Gal-Glc-DAG and glycolipids with the proposed structure of GroP-Gal-Glc-DAG (Gal-Glc-DAG with one glycerolphosphate subunit GroP) and the D-alanine-esterified derivative D-Ala-GroP-Gal-Glc-DAG (Fischer and Leopold, 1999). Bioinformatic analysis suggested that proteins encoded in the lmo2555–lmo2553 operon are directly involved in glycolipid formation. For glycolipid analysis, wild-type, lmo2553, lmo2554 and lmo2555 deletion strains were grown overnight at 30°C, total membrane lipids isolated and separated by thin-layer chromatography (TLC). TLCs were developed with α-naphthol and sulphuric acid to visualize sugar-containing lipids. Four major glycolipid bands were detected in wild-type L. monocytogenes cells (Fig. 3A) and as shown in Fig. 3A, lmo2555–lmo2553 deletion strains showed differences in the pattern of glycolipids as compared with the wild-type strain. Using matrix-assisted laser desorption/ionization (MALDI) mass spectrometry, we were able to provide structural information for glycolipids within three main bands labelled top, middle and bottom in Fig. 3. Obtained masses were consistent with expected masses for sodium adducts of Glc-DAG (753.6 m/z – top band; Fig. 4A), Gal-Glc-DAG (915.7 m/z – middle band; Fig. 4C) and GroP-Gal-Glc-DAG (1069.5 m/z – bottom band; Fig. 4D) with C17 and C15 fatty acid side-chains. Calculated absolute masses for glycolipids and experimentally observed masses are summarized in Table 1. The predominant masses for lipids with C17 and C15 fatty acid chains are consistent with previous findings that anteiso-branched C17 and C15 fatty acid at C1 and C2 positions, respectively, are the most abundant fatty acids in Listeria lipids (Kosaric and Carroll, 1971; Fischer and Leopold, 1999). In addition, we also observed masses, which are consistent with glycolipids with C15/C15 or C17/C17 fatty acid chains or disodium adducts (replacement of H+ with Na+) (Table 1). Only small amounts of Glc-DAG accumulated in wild-type cells (Fig. 3A) and as seen in Fig. 4A the observed mass signal
of 753.6 m/z for this lipid was weak as compared with background signals but the signal was specific (background peaks were separated from each other by 44 mass units). Deletion of \textit{lmo2553}, predicted to encode an integral membrane protein, resulted in small but reproducible changes in the glycolipid profile with reduction of a glycolipid of unknown structure, indicated by an asterisk in Fig. 3A. Deletion of \textit{lmo2555} led to a complete absence of glycolipids and deletion of \textit{lmo2554} led to the accumulation of Glc-DAG (Fig. 3A). Glycolipid structure as well as presence and absence of Gal-Glc-DAG (middle band) and GroP-Gal-Glc-DAG (bottom band) was confirmed by MALDI mass spectrometry (Fig. 4B and E–J, Table 1). We had difficulties detecting a signal above background for the top glycolipid band. Only in strain 10403S \textit{Dlmo2554}, in which this lipid accumulated to significant levels, a clear m/z signal of 753.5 was obtained as expected for a lipid with the structure Glc-DAG (Fig. 4B). These results are consistent with a function of \textit{lmo2555} and \textit{lmo2554} as glycosyltransferase responsible for the formation of Glc-DAG and Gal-Glc-DAG respectively. The exact function of \textit{lmo2553} remains unknown; nevertheless we propose to rename proteins encoded in the \textit{lmo2555–lmo2553} operon, LafA (\textit{lmo2555}), LafB (\textit{lmo2554}) and LafC (\textit{lmo2553}) for LTA anchor formation proteins A to C. 

**Lmo0644 functions as an LTA primase**

Based on the accumulation of glycolipids with a single glycerolphosphate in some Gram-positive bacteria such as GroP-Gal-Glc-DAG in \textit{L. monocytogenes}, a two-enzyme system for LTA synthesis has been proposed, whereby an LTA primase initiates LTA synthesis by the transfer of the first glycerolphosphate subunit onto the glycolipid and a second enzyme, an LTA synthase, extends the chain to form the polyglycerolphosphate backbone (Fischer, 1990). Lmo0927 is likely to be the LTA synthase, as in its absence no LTA could be detected on the bacterial surface (Fig. 2C). In the presence of an LTA primase, one would expect that even in the absence of the LTA synthase (Lmo0927) production of GroP-Gal-Glc-DAG should occur. To test if Lmo0644 could serve as LTA primase, we determined the glycolipid profile of \textit{lmo0644} and \textit{lmo0927} deletion strains. In the absence of Lmo0644
no GroP-Gal-Glc-DAG (bottom band) could be detected, while in the absence of Lmo0927 GroP-Gal-Glc-DAG (bottom band) was produced and accumulated to higher levels as compared with a wild-type strain (Fig. 3B). MALDI mass spectrometry analysis confirmed the structure and presence or absence of Gal-Glc-DAG and GroP-Gal-Glc-DAG in respective deletion strains (Fig. 4K–N; Table 1). The m/z signal of 1069.8, expected for a sodium adduct of GroP-Gal-Glc-DAG with a calculated mass of 1069.6, was only observed in the presence of Lmo0644. In addition, upon deletion of lmo0927, which resulted in the accumulation of the bottom glycolipid band, an additional mass signal of 1040.8 was observed, which is consistent with the expected mass of 1040.6 for D-Ala-GroP-Gal-Glc-DAG, a D-Ala esterified derivative of GroP-Gal-Glc-DAG. These results are consistent with a model whereby Lmo0644 serves as LTA primase and Lmo0927 functions as LTA synthase and we propose to rename Lmo0644, LtaP, for LTA primase and Lmo0927, LtaS, for LTA synthase.

LtaP (Lmo0644) and LtaS (Lmo0927) are processed in L. monocytogenes

In S. aureus, LtaS is efficiently processed and the extra-cellular enzymatic domain can be found in the culture supernatant as well as in the cell wall fraction (Lu et al., 2009). To test if L. monocytogenes LtaP (Lmo0644) and LtaS (Lmo0927) are processed similarly, localization and cleavage of these proteins was analysed. To this end, plasmids pPL3-lmo644His6 and pPL3-lmo927His6 were constructed for expression of C-terminally His-tagged LtaP and LtaS proteins under their native promoter and integrated into the chromosome of strain 10403S. We also introduce as controls the empty vector pPL3 and pPL3-ltaSSA His6, which encodes a C-terminally tagged version of the S. aureus LtaS protein under the control of its native promoter. Resulting strains 10403S pPL3, 10403S pPL3-lmo644His6, 10403S pPL3-lmo927His6 and 10403S pPL3-ltaSSA His6 were grown overnight at 37°C and cell-associated and supernatant protein samples were prepared for Western blot analysis as described in the Experimental procedure section and tagged proteins were detected with a His-tag specific antibody. As can be seen in Fig. 5A, L. monocytogenes LtaP and LtaS, and the S. aureus LtaSSA protein could be detected in culture supernatant, indicating that all proteins were cleaved in L. monocytogenes. Processed forms of the L. monocytogenes LTA synthase (Lmo927) and the S. aureus control protein were also detected in the cell wall-associated fraction (Fig. 5B). We were not able to detect any full-length proteins in L. monocytogenes, while our previously published results have shown that small amounts of full-length LtaSSA protein can be detected in S. aureus using an identical sample preparation method (Lu et al., 2009). Taken together, these results show that both proteins, LtaP

### Table 1. Summary of TLC and MALDI-TOF data of glycolipids produced by wild-type and mutant L. monocytogenes strains.

| Lipid (fatty acid chain length) | WT | Δ2553 | Δ2554 | Δ2555 | Δ0644 | Δ0927 |
|--------------------------------|----|-------|-------|-------|-------|-------|
| Top band Glycerol-DAG (C17, C15) | X | X | X | Not observed | X | X |
| C41H78Na1O10 | 753.5 | 753.5 | |
| Glucose-DAG (C15, C15) | 725.4 | |
| C39H74Na1O10 | |
| Middle band Galactose-DAG (C17, C15) | X | X | Not observed | Not observed | X | X |
| C47H88Na1O15 | 915.6 | 915.6 | 915.6 | 915.7 |
| Glucose-DAG (C15, C15) | 887.6 | 887.6 | 887.6 | 887.6 |
| C45H84Na1O15 | |
| Glucose-DAG (C17, C17) | 943.6 | 943.6 | 943.6 | 943.7 |
| C49H92Na1O15 | |
| Bottom band Glucose-Phosphoryl-Galactose-DAG (C17, C15) | X | X | Not observed | Not observed | Not observed | X |
| C50H95Na1O20P1 | 1069.5 | 1069.5 | |
| Glucose-Phosphoryl-Galactose-DAG (C17, C17) | 1091.5 | 1091.5 | |
| C50H94Na2O20P1 | |
| Glucose-Phosphoryl-Galactose-DAG (C17, C15) | Not observed | Not observed | |
| C53H100Na1O21P1 | |
| Glucose-Phosphoryl-Galactose-DAG (C17, C15) | | |
| C53H99Na1O20P1 | |
| Glucose-Phosphoryl-Galactose-DAG (C17, C15) | | |
| C53H98Na1O21P1 | |

Presence or absence of glycolipids in different L. monocytogenes strains shown on top is denoted with ‘X’ when present and ‘Not observed’ when absent. Abbreviations for glycolipids in top, middle and bottom bands as indicated in Fig. 3 are shown in the left column with fatty acid chain length given in parenthesis along with molecular formula and calculated absolute mass of sodium adducts or disodium adducts (minus one proton).
and LtaS, are processed in *L. monocytogenes* and suggest that simply a difference in enzyme processing cannot explain the difference in enzyme function between an LTA primase and an LTA synthase.

**Inactivation of the LTA synthase LtaS (Lmo0927) severely affects growth and morphology of *L. monocytogenes***

LtaS is required for growth of *S. aureus* under standard laboratory growth conditions (tryptic soya broth medium at 37°C) (Gründling and Schneewind, 2007a). An LtaS deletion strain is viable at 30°C when grown in the present of at least 1% NaCl and at 37°C when bacteria are cultured in the presence of 7.5% NaCl or 40% sucrose (Oku et al., 2009). However, *S. aureus* cells have severe morphological defects in the absence of LTA (Gründling and Schneewind, 2007a; Oku et al., 2009). *L. monocytogenes* is apparently able to grow in the absence of LTA since we obtained a strain with a clean deletion in *lmo0927* (see Fig. 2C). However, during the strain construction, we observed that an *lmo0927* deletion strain could only be obtained when bacteria were plated and maintained at 30°C (and not at 37°C) during the final step of the allelic exchange procedure. A more detail growth analysis revealed that strain 10403SΔ*lmo0927* had already at 30°C a reduced colony size (data not shown) and reduced growth rate as compared with a wild-type strain (Fig. 6A and B). Such membrane bubbles were never observed in 10403SΔ*lmo0927*, regardless of the growth temperature (Fig. 7C–H). The complete absence of membranous material at division sites in the *lmo0927* deletion strain was more frequent at the non-permissive growth temperature and 8.5 h after the temperature shift actual cell lysis was observed in several cells (Fig. 7E and F). In the few instances where a clear division site was observed in strain 10403SΔ*lmo0927*, these septa looked abnormal (Fig. 7H). In summary, these data show that LTA plays a crucial role in the cell division process in another Gram-positive pathogen, the rod-shaped bacterium *L. monocytogenes*.

**Discussion**

In this study, we have identified and characterized *L. monocytogenes* proteins required for glycolipid anchor and LTA backbone synthesis and a summary model for their function is shown in Fig. 8.
Based on the structural relation of glycolipids with a single glycerolphosphate subunit (GroP-glycolipids) and their parallels in occurrence with LTA, it has been suggested that GroP-glycolipids are intermediates in the LTA synthesis pathway (Fischer et al., 1978; 1990; Fischer, 1981). This led to the proposal that LTA is synthesized by two enzymes: an LTA primase, which produces the GroP-glycolipid intermediate and an LTA synthase, which extends the polyglycerolphosphate backbone chain on this intermediate (Fischer et al., 1978; 1990; Fischer, 1981).

**Fig. 6.** Growth and morphology of wild-type 10403S and 10403SΔimo0927 L. monocytogenes strains.

A. Bacterial growth curves. Overnight cultures of wild-type 10403S (WT) and 10403SΔimo0927 (Δ0927) strains were diluted into fresh BHI medium and cultures incubated at 30°C or 37°C. OD600 values were determined at timed intervals and plotted.

B–D. Phase-contrast microscopy images of (B) 10403S (WT) and (C and D) 10403SΔimo0927 (Δ0927) strains grown for 8.5 h at the indicated temperature.

**Fig. 7.** Transmission electron microscopy (TEM) images of wild-type 10403S and 10403SΔimo0927 L. monocytogenes strains. Overnight cultures of wild-type 10403S (WT) and 10403SΔimo0927 (Δ0927) strains were back-diluted and grown for the indicated time at 30°C or 37°C. Bacteria were fixed and prepared for TEM as described under Experimental procedures and representative images are shown: WT grown for 3.5 h at (A) 30°C and (B) 37°C; Δ0927 grown for 3.5 h at (C) 30°C and (D) 37°C; (E–H) Δ0927 grown for 8.5 h at 37°C. Images were taken at (A–D) 49 000×; (E) 30 000×; (F) 68 000×; (G and H) 98 000× magnification and scale bars are shown.
This and alternative models for polyglycerolphosphate chain synthesis and extension have been proposed and recently reviewed by Rahman et al. (2009). Here, we provide evidence that *L. monocytogenes* uses a two-enzyme system for LTA synthesis and show for the first time distinct enzymatic functions for two LtaS paralogues within the cell. Based on the results presented in Figs 2–4, we propose that LtaP (Lmo0644) acts as an LTA primase and produces GroP-glycolipids and LtaS (Lmo0927) functions as LTA synthase and generates the polyglycerolphosphate backbone. However, even in the absence of LtaP (*lmo0644* mutant), the *L. monocytogenes* LtaS enzyme can produce a polyglycerolphosphate polymer (Fig. 2C) and we speculate that LtaS can initiate LTA synthesis on both glycolipids and in the absence of this class of lipids directly on DAG or PG. This notion is supported by the observed LTA profile of an *ltaP/lafA* (*lmo0644/lmo2555*) double mutant (Fig. S1A). In the absence of both, glycolipids (*lmo2555* mutant) and the LTA primase (*lmo0644* mutant), LTA is produced albeit at reduced levels compared with a strain, which only lacks the LTA primase. This indicates that in the absence of LtaP, LtaS can use glycolipids as an anchor and in the absence of both, LtaP and glycolipids, DAG or PG is used. (It should be noted that the observed LTA alterations in the double mutant could be complemented to the levels of the individual single mutants by introduction of the respective complementation vector pPL3- *lmo0644* or pPL3- *lmo2555*; Fig. S1A).

In contrast to *L. monocytogenes*, *S. aureus* apparently synthesizes LTA with a single enzyme and depletion of LtaS does not lead to an accumulation of GroP-glycolipids (M. Wörmann, unpubl. results). The biological significance why some bacteria use a one-enzyme and other bacteria use a multi-enzyme system for LTA synthesis is not clear at this point, especially considering that an *L. monocytogenes* LtaP mutant still synthesizes LTA and does not show a growth defect in broth culture. While it is not clear why different Gram-positive bacteria use one or multiple enzymes for LTA synthesis, in general there seems to be a correlation between the number of genome-encoded LtaS-like proteins and bacterial shape; coccoid *Staphylococcus* spp., *Streptococcus* spp. (with exception of *S. pneumoniae*, which does not produce a polyglycerolphosphate-type LTA and does not encode an LtaS-like protein) and *Lactococcus lactis* strains encode one LtaS protein; ellipsoid-shaped *E. faecalis* strains, rod-shaped *Listeria* spp. and with a few exceptions rod-shaped *Lactobacillus* spp. encode two proteins; and the majority of *Bacillus* spp., rod-shaped bacteria with a more complex developmental cycle, encode multiple LtaS-like proteins. *B. subtilis* contains four LtaS paralogues (Grün-dling and Schneewind, 2007a) and based on published results, it was suggested that the four *B. subtilis* LtaS paralogues have partially different functions and individual or a combination of these proteins is particular important for proper septum formation during cell division (YfIE) or the sporulation process (YfIE and YfgS) (Schirner et al., 2009).

Of the five deletion strains constructed in this study, only deletion of LtaS, which caused a complete absence of LTA (Fig. 2C), had a marked effect on bacterial growth.

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characterized glycosyltransferases alMGS and alDGS present in both LafA (amino acids 293–301) and LafB (Campbell et al., 2003). In the absence of Gal-Glc-DAG, a profound reduction in the overall amount of LTA has been reported for a ypfP mutant in the SA113 strain background (Fedtke et al., 2007), which is what we observed for lafA or lafB mutant L. monocytogenes strains. The simplest explanation for this reduction in LTA production is that enzyme(s), which are subsequently needed for the formation of the polyglycerolphosphate polymer, cannot efficiently initiate LTA synthesis in the absence of glycolipids. We suggest that in L. monocytogenes in the absence of glycolipids, neither LtaP nor LtaS can efficiently initiate polyglycerolphosphate LTA backbone synthesis. Therefore, LtaP and LtaS could either have a specific recognition site for glycolipids or alternatively special constraints (the OH-group used for backbone extension will be further removed from the membrane surface in glycolipids as compared with other lipids such as DAG or PG) could dictate which lipid can be used to initiate LTA synthesis. Structural information on the extracellular S. aureus LtaS or B. subtilis YfIE domains did not provide any information on potential glycolipid binding sites (Lu et al., 2009; Schirner et al., 2009) and these sites might be embedded within the membrane portion of the protein, for which no structural information is available. For S. aureus strains, differences in LtaS protein sequence could explain observed phenotypic differences in ypfP mutants. However, the LtaS sequence does not vary between sequenced strains, with exception of strain RF122, which has one amino acid change. If not differences in LtaS protein sequence, differences in expression, post-translational modifications or differences in yet to be identified proteins involved in LTA synthesis could explain observed strain differences.

A third gene, lafC (lmo2553), predicted to code for an integral membrane protein with eight transmembrane helices is part of the laf operon. We show that LafC plays an accessory function in glycolipid and LTA synthesis as inactivation of LafC results in minor changes in the glycolipid profile (Fig. 3A) and production of LTA with a retarded mobility similar to that observed in an ltaP mutant (Fig. 2B and C). Since glycolipids Glc-DAG and Gal-Glc-DAG are produced in a lafC mutant, this protein probably acts downstream of LafA and LafB (see model in Fig. 8).

During this study, we also created an ltaP/lafC (lmo0644/lmo2553) double mutant and respective control strains for complementation analysis. LTA analysis revealed that the strain lacking both, the LTA primase LtaP and LtaC, produced only small amounts of LTA, comparable to that of the lafA (lmo2555) mutant, which is unable to synthesize glycolipids (Fig. S1B). This result indicates that either LafC or LtaP is required presumably to modify the glycolipid in such a manner that it can be used by LtaS as the LTA anchor. In several Listeria spp., LTA is anchored to the membrane not only by Gal-Glc-DAG but also by a derivative in which the glucose moiety is lipitated at position 6 with a Ptd group (Fig. 1) (Uchikawa et al., 1986) and LafC.
could be involved in its synthesis. Such a modification may physically alter the presentation of the anchor thus affecting the ability of LtaS to synthesize LTA. However, additional work is needed to determine the exact molecular function of LfIC and to establish the requirement of glycolipids and LTA during infection. Also, determining the precise function of LTA during the cell division process warrants further investigation.

**Experimental procedures**

**Bacterial strains, plasmids and growth conditions**

Bacterial strains and plasmids used in this study are listed in Table 2. *L. monocytogenes* mutants were constructed in strain 10403S, which is a streptomycin resistant isolate of the serotype 1/2a strain 10403 (Bishop and Hinrichs, 1987). *L. monocytogenes* strains were grown in brain heart infusion (BHI) medium at 30°C or 37°C as indicated and *Escherichia coli* strains were grown in Luria–Bertani (LB) medium at 37°C. When appropriate, the growth medium was supplemented with antibiotics as listed in Table 2.

**Plasmid and strain construction**

Pfu polymerase (Stratagene) was used for PCR amplification of DNA fragments subsequently used for cloning. Restriction enzymes were purchased from New England Biolabs and used according to the manufacturer’s instructions. All plasmids were constructed initially in *E. coli* strains XL1-Blue or CLG190. Allelic exchange plasmids for the construction of strains 10403S*,lmo0644*, 10403S*,lmo0927*, 10403S*,lmo2553*, 10403S*,lmo2554* and 10403S*,lmo2555* containing an in-frame deletion in the respective gene were constructed by the two-step PCR SOE method (Horton et al., 1989) using primers listed in Table 3. Briefly, primers 5-KpnI-LMO0644, 5-int-LMO0644, 3-BamHI-LMO0644 and 3-int-LMO0644-10403S (for deletion of *lmo0644*); 5-KpnI-LMO0927, 5-int-LMO0927, 3-BamHI-LMO0927 and 3-int-LMO0927 (for deletion of *lmo0927*); 5-KpnI-LMO2553, 5-int-LMO2553, 3-BamHI-LMO2553 and 3-int-LMO2553 (for deletion of *lmo2553*); 5-KpnI-LMO2554, 5-int-LMO2554, 3-BamHI-LMO2554 and 3-int-LMO2554 (for deletion of *lmo2554*); 5-KpnI-LMO2555, 5-int-LMO2555, 3-BamHI-LMO2555 and 3-int-LMO2555 (for deletion of *lmo2555*) were used to amplify and fuse approximately 950 bp upstream and downstream regions of relevant genes using 10403S chromosomal DNA in PCR reactions. The resulting PCR products were digested with the restriction enzymes KpnI and BamHI and ligated with the allelic exchange vector pKSV7 (Smith and Youngman, 1992) that had been digested with the same restriction enzymes, resulting in plasmids pKSV7*,lmo0644*, pKSV7*,lmo0927*, pKSV7*,lmo2553*, pKSV7*,lmo2554* and pKSV7*,lmo2555*. These plasmids were introduced by electroporation (Park and Stewart, 1990) into strain 10403S and a previously described allelic exchange method (Camilli et al., 1993) was used to create strains 10403S*,lmo0644*, 10403S*,lmo0927*, 10403S*,lmo2553*, 10403S*,lmo2554* and 10403S*,lmo2555*. Plasmids pKSV7*,lmo2553* and pKSV7*,lmo2555* and strains 10403S*,lmo0644* were used to create double mutants 10403S*,lmo0644*/2553 and 10403S*,lmo0644*/2555 respectively. Deletions were confirmed by PCR using primer pairs listed in Table 3, which bind outside the region of homology used for allelic exchange. For complementation analysis, genes *lmo0644*, *lmo0927* and *lmo2555* were cloned with their native promoter into the *L. monocytogenes* single-site integration vector pPL3 (Gründling et al., 2004), while genes *lmo2553* and *lmo2554* were cloned under the control of the hyper-spac promoter into the integration vector pHPL3 (Gründling et al., 2004). Briefly, primers 5-BamHI-LMO0644_pPL3 and 3-KpnI-LMO0644_pPL3 (for complementation of *lmo0644*), 5-Sall-LMO0927_pPL3 and 3-KpnI-LMO0927_pPL3 (for complementation of *lmo0927*), 5-BamHI-LMO2553_pPL3HSPAC and 3-KpnI-LMO2553_pPL3HSPAC (for complementation of *lmo2553*), 5-BamHI-LMO2554_pPL3HSPAC and 3-KpnI-LMO2554_pPL3HSPAC (for complementation of *lmo2554*) and 5-BamHI-LMO2555_pPL3 and 3-KpnI-LMO2555_pPL3 (for complementation of *lmo2555*) were used to amplify relevant fragments from chromosomal DNA of strain 10403S. The resulting PCR products were digested with the restriction enzymes KpnI and BamHI or KpnI and SalI, which were digested with the same restriction enzymes, resulting in plasmids pPL3-,lmo0644*, pPL3-,lmo0927*, pPL3-,lmo2553*, pPL3-,lmo2554* and pPL3-,lmo2555*. Plasmids pPL3-,lmo0644*, pPL3-,lmo0927*, pPL3-,lmo0927*, pPL3-,lmo0927*, pPL3-,lmo0927* were electroproporated into the relevant deletion strains resulting in complementation strains listed in Table 2. Plasmid pPL3-,lmo0927* was introduced into strain 10403S*,lmo0927* by conjugation (Lauer et al., 2002) resulting in strain 10403S*,lmo0927* pPL3-,lmo0927*. As controls, empty pPL3 and pHPL3 vectors were introduced into wild-type 10403S and relevant deletion strains by electroporation or conjugation for strain 10403S*,lmo0927*. For expression of C-terminally His-tagged Lmo0644 and Lmo0927 proteins under their native promoter control, plasmids pPL3-,lmo0644*His6* and pPL3-,lmo0927*His6* were constructed. The C-terminal His6 tag was introduced by PCR using primer pair 5-BamHI-LMO0644_pPL3/3-Sall-LMO0644-C-His and 5-PstI-LMO0927-wt/P-3-Sall-LMO0927-C-His and 10403S chromosomal DNA. The resulting PCR products were cut with BamHI and Sall (for *lmo0644*His6) or PstI and Sall (for *lmo0927*His6) and inserted into vector pPL3 cut with the same enzymes. As control, the *S. aureus* *ltaS* gene, annotated as *SAV0719* in the MU50 genome, was also cloned as C-terminal His6 fusion under its native promoter into the *L. monocytogenes* vector pPL3. Plasmid pPL3-,ltaS*,His6* was constructed by amplifying *ltaS* from *S. aureus* RN4220 chromosomal DNA using primer pair 5-BamHI-P+S SAV0719 and 3-KpnI-His6-719 and the PCR product was cut and cloned as BamHI and KpnI fragment into vector pCL55 (Lee et al., 1991), resulting in plasmid pCL55-,ltaS*,His6*. The *BamHI*KpnI fragment was subsequently excised and cloned into pPL3, resulting in plasmid pPL3-,ltaS*,His6*. Plasmids for expression of His-tag protein fusions were initially recovered in *E. coli* strain XLI-Blue and subsequently introduced by electroporation into the *L. monocytogenes* strain 10403S, resulting in strains 10403S pPL3-,lmo0644*His6* and 10403S pPL3-,lmo0927*His6*, 10403S pPL3-,ltaS*,His6*. The DNA sequences of all inserts were verified by automated fluores-
Table 2. Bacterial strains used in this study.

| Strain                  | Relevant features                                                                 | Reference                      |
|-------------------------|-----------------------------------------------------------------------------------|--------------------------------|
| **Escherichia coli strains** |                                                                                  |                                |
| XL1 Blue                | Cloning strain, TetR – ANG127                                                     | Stratagene                     |
| CLG190                 | Cloning strain, TetR – ANG1141                                                    | D. Boyd                        |
| SM10                   | *E. coli* strain used for conjugations; KanR – ANG618                             | Simon et al. (1983)            |
| ANG124                 | JM109 pKS7; allelic exchange vector; AmpR                                         | Smith and Youngman (1992)      |
| ANG243                 | XL1-Blue with *S. aureus* integration vector pCL5                                  | Lee et al. (1991)              |
| ANG583                 | XL1-Blue pCL55-ItaSSA His6; *S. aureus* LtaS (SAV0719) with C-terminal His6 tag; AmpR | This study                     |
| ANG1378                | CLG190 pKS7; *lmo0644*                                                             | This study                     |
| ANG1379                | XL1 Blue pKS7; *lmo0927*                                                           | This study                     |
| ANG1382                | XL1 Blue pKS7; *lmo2553*                                                           | This study                     |
| ANG1384                | XL1 Blue pKS7; *lmo2554*                                                           | This study                     |
| ANG1385                | XL1 Blue pKS7; *lmo2555*                                                           | This study                     |
| DH-E898                | XL1 Blue pPL3; *L. monocytogenes* integration vector; CamR – ANG127               | Gründling et al. (2004)        |
| DH-E899                | XL1 Blue pPL3; *L. monocytogenes* integration vector with hyper-spac promoter; CamR – ANG1277 | Gründling et al. (2004)        |
| AJW1392                | XL1 Blue pPL3-*lmo0644*; *lmo0644* under native promoter control; CamR             | This study                     |
| AJW1393                | XL1 Blue pPL3-*lmo0927*; *lmo0927* under native promoter control; CamR             | This study                     |
| AJW1396                | XL1 Blue pPL3-*lmo2553*; *lmo2553* under hyper-spac promoter control; CamR        | This study                     |
| AJW1397                | XL1 Blue pPL3-*lmo2554*; *lmo2554* under hyper-spac promoter control; CamR        | This study                     |
| AJW1398                | XL1 Blue pPL3-*lmo2555*; *lmo2555* under native promoter control; CamR             | This study                     |
| ANG1399                | XL1 Blue pPL3-*lmo0644*His6; *lmo0644* with C-terminal His-tag under native promoter control; CamR | This study                     |
| ANG1401                | XL1 Blue pPL3-*lmo0927*His6; *lmo0927* with C-terminal His-tag under native promoter control; CamR | This study                     |
| ANG1406                | XL1 Blue pPL3-ItaSSA His6; LtaSSA with C-terminal His-tag under native promoter control; CamR | This study                     |
| ANG1456                | SM10 pPL3; *E. coli* conjugation strain donor for plasmid pPL3; KanR, CamR        | This study                     |
| ANG1459                | SM10 pPL3-*lmo0927*; *E. coli* conjugation strain donor for plasmid pPL3-*lmo0927*; KanR, CamR | This study                     |
| **Listeria monocytogenes strains** |                                                                                  |                                |
| 10403S                  | StrepR – ANG1263                                                                  | Bishop and Hinrichs (1987)     |
| AJW1385                | 10403S-*lmo0644*; StrepR                                                          | This study                     |
| ANG1386                | 10403S-*lmo0927*; StrepR                                                          | This study                     |
| AJW1389                | 10403S-*lmo2553*; StrepR                                                          | This study                     |
| AJW1390                | 10403S-*lmo2554*; StrepR                                                          | This study                     |
| AJW1391                | 10403S-*lmo2555*; StrepR                                                          | This study                     |
| ANG1411                | 10403S-*lmo0927* pPL3; StrepR, CamR                                               | This study                     |
| ANG1412                | 10403S-*lmo0927* pPL3-*lmo0927; *lmo0927* complementation strain; StrepR, CamR     | This study                     |
| AJW1413                | 10403S pPL3; StrepR, CamR                                                          | This study                     |
| AJW1414                | 10403S pHPL3; StrepR, CamR                                                         | This study                     |
| AJW1415                | 10403S-*lmo0644* pPL3-*lmo0644; *lmo0644* complementation strain; StrepR, CamR     | This study                     |
| AJW1416                | 10403S-*lmo0644* pPL3; StrepR, CamR                                               | This study                     |
| AJW1417                | 10403S-*lmo2553* pHPL3-*lmo2553; *lmo2553* complementation strain; StrepR, CamR     | This study                     |
| AJW1418                | 10403S-*lmo2553* pHPL3; StrepR, CamR                                              | This study                     |
| AJW1419                | 10403S-*lmo2554* pHPL3-*lmo2554; *lmo2554* complementation strain; StrepR, CamR     | This study                     |
| AJW1420                | 10403S-*lmo2554* pHPL3; StrepR, CamR                                              | This study                     |
| AJW1421                | 10403S-*lmo2555* pHPL3-*lmo2555; *lmo2555* complementation strain; StrepR, CamR     | This study                     |
| AJW1422                | 10403S-*lmo2555* pPL3; StrepR, CamR                                               | This study                     |
| AJW1423                | 10403S pPL3-*lmo0644*His6; StrepR, CamR                                           | This study                     |
| AJW1424                | 10403S pPL3-*lmo0927*His6; StrepR, CamR                                           | This study                     |
| AJW1425                | 10403S pPL3-ItaSSA His6; StrepR, CamR                                            | This study                     |
| AJW1466                | 10403S-*lmo0644*-*lmo2555*; StrepR                                                | This study                     |
| AJW1497                | 10403S-*lmo0644*-*lmo2555* pPL3; StrepR, CamR                                      | This study                     |
| AJW1498                | 10403S-*lmo0644*-*lmo2555* pPL3-*lmo0644; *lmo0644* complementation strain; StrepR, CamR | This study                     |
| AJW1499                | 10403S-*lmo0644*-*lmo2555* pPL3-*lmo2555; *lmo2555* complementation strain; StrepR, CamR | This study                     |
| AJW1501                | 10403S-*lmo0644*-*lmo2555*; StrepR                                                 | This study                     |
| AJW1502                | 10403S-*lmo0644*-*lmo2553* pPL3; StrepR, CamR                                     | This study                     |
| AJW1503                | 10403S-*lmo0644*-*lmo2553* pPL3-*lmo0644; *lmo0644* complementation strain; StrepR, CamR | This study                     |
| AJW1504                | 10403S-*lmo0644*-*lmo2553* pHPL3-*lmo2553; *lmo2553* complementation strain; StrepR, CamR | This study                     |
| **Other strains**      |                                                                                  |                                |
| RN4220                 | Transformable *S. aureus* laboratory strain – ANG113                              | Kreiswirth et al. (1983)       |

Antibiotics were used at the following concentrations: for *E. coli* cultures: ampicillin (AmpR) 100 μg ml⁻¹; kanamycin (KanR) 30 μg ml⁻¹; tetracycline (TetR) 10 μg ml⁻¹; for *L. monocytogenes* cultures: chloramphenicol (CamR) 7.5 or 10 μg ml⁻¹; streptomycin 200 μg ml⁻¹ (StrepR) for the conjugation experiment.
Genomics Core Laboratory, Imperial College London.

LTA and protein detection by Western blot

Lipoteichoic acid and protein detection by Western blot was undertaken essentially as previously described (Gründling and Schneewind, 2007b). In brief, for sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and Western blot analysis of cell-associated LTA and His-tagged proteins, 1 ml of overnight culture was mixed with 0.5 ml of 0.1 mm glass beads and lysed by vortexing for 45 min in the cold. Glass beads were sedimented by centrifugation at 200 g for 1 min, and 0.5 ml of the resultant supernatant transferred to a fresh tube. Bacterial debris and LTA were sedimented by centrifugation at 17 000 g for 15 min and suspended in protein sample buffer containing 2% SDS normalized for OD_{600}; that is, samples from a culture with an OD_{600} of 2 were suspended in 50 ml of sample buffer. Samples were boiled for 20 min, centrifuged at 17 000 g for 5 min and 10 ml of samples loaded onto SDS-PAA gels. To determine the amount of LTA shed into the culture medium, 10 ml of supernatant was mixed with 0.5 ml of 2× sample buffer and loaded onto SDS-PAA gels.

Table 3. Primers used in this study.

| Number | Name                  | Sequence                                                                 |
|--------|-----------------------|--------------------------------------------------------------------------|
| ANG383 | 5-KpnI-LMO0644        | GGGGTACCCTAGGAGAAACGGCATCAAANCTAATAGCAAGAAAGAAGAAAGAATACAGTG           |
| ANG384 | 5-int-LMO0644         | CTTTCTACCTCCTCTCTCCTCTATTACTTTCAATACATCACTACATGTTGTCAC               |
| ANG637 | 3-int-LMO0644–10403S  | GGGGTACCCTAGGAGAAACGGCATCAAANCTAATAGCAAGAAAGAAGAATACAGTG           |
| ANG386 | 3-BamHI-LMO0644       | GGGGTACCCTAGGAGAAACGGCATCAAANCTAATAGCAAGAAAGAAGAATACAGTG           |
| ANG377 | 5-KpnI-LMO0927        | CGGGGATCCCCGGTATCTGCGCACAAGTTGATTTTTCGATTCCATCACCGTTCCCTCAT          |
| ANG378 | 3-BamHI-LMO0927       | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG379 | 3-BamHI-LMO0927       | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG544 | 5-KpnI-LMO2553        | CGGGGATCCCCGGTATCTGCGCACAAGTTGATTTTTCGATTCCATCACCGTTCCCTCAT          |
| ANG545 | 5-int-LMO2553         | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG546 | 3-int-LMO2553         | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG547 | 3-BamHI-LMO2553       | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG551 | 5-KpnI-LMO2554        | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG552 | 5-int-LMO2554         | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG553 | 3-int-LMO2554         | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG554 | 3-BamHI-LMO2554       | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG555 | 5-KpnI-LMO2555        | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG556 | 3-int-LMO2555         | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG561 | 3-BamHI-LMO2555       | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG562 | 3-KpnI-LMO0644_pPL3   | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG563 | 5-Sall-LMO0927_pPL3   | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG564 | 3-KpnI-LMO0927_pPL3   | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG565 | 5-BamHI-LMO2553_pPL3  | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG566 | 3-KpnI-LMO2553_pPL3   | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG567 | 3-Sall-LMO0644-C-His  | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG674 | 5-PstI-LMO0927-withP  | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG675 | 3-Sall-LMO0927-C-His  | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG086 | 5-BamHI + P SAV0719   | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |
| ANG419 | 3-KpnI-His6-719       | AAAATCTGAAAGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAATGAAAT |

Restriction sites in primer sequences are underlined and shown in bold.

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500 µl of culture was first centrifuged at 17 000 g for 5 min to pellet bacteria. Culture supernatant (100 µl) was removed, mixed with 100 µl of 2x protein sample buffer, boiled for 30 min and insoluble material removed by centrifugation at 17 000 g for 5 min. Supernatant samples were normalized based on OD$_{600}$ of 2, in that 10 µl of a culture of OD$_{600}$ of 2 was loaded. To determine if the His-tagged proteins were shed into the supernatant, 1.4 ml of culture was centrifuged at 17 000 g for 10 min to pellet the bacteria. One millilitre of the supernatant was transferred to a new tube, mixed with 100 µl of 100% trichloroacetic acid (TCA), vortexed, incubated on ice for 1 h and centrifuged for 10 min at 17 000 g. The supernatant was aspirated and the TCA precipitated pellet was washed twice with 1 ml of ice-cold acetone. Between wash steps, samples were incubated on ice for 1 h and debris collected by centrifugation as described above. After the final centrifugation step, pellets were air dried and suspended in 2x protein sample buffer normalized for OD$_{600}$, that is, samples from a culture with an OD$_{600}$ of 2 were suspended in 100 µl of sample buffer. The samples were boiled for 30 min and 10 µl analysed by Western blot. LTA samples were routinely loaded onto 15% SDS-PAA gels and probed with polyglycerolphosphate-specific LTA antibody (Clone 55 from Hycult biotechnology) and HRP-conjugated anti-mouse IgG (Cell Signalling Technologies, USA) used at 1:2000 and 1:10 000 dilutions respectively. His-tagged protein samples were routinely loaded onto 10% SDS-PAA gels and probed with HRP-conjugated His-tag-specific antibody (Sigma) used at a 1:10 000 dilution and Western blots were developed by enhanced chemiluminesce (ECL). Western blots were performed with at least three independently grown cultures in at least two independent experiments and representative images are shown.

**Growth curves and determination of colony-forming units**

Wild-type and mutant *L. monocytogenes* cultures were grown overnight at 30°C in 4 ml of BHI medium. Next day, cultures were diluted to a starting OD$_{600}$ of 0.07 into 25 ml of BHI medium, incubated with shaking at 30°C or 37°C and OD$_{600}$ values determined at timed intervals. Growth curves were performed in duplicate and representative graphs are shown. To determine the number of colony-forming units (cfu) per ml of culture normalized for OD$_{600}$ of 2, the optical density of overnight cultures grown at 30°C was determined, cultures normalized based on OD$_{600}$ readings and a dilution series prepared in phosphate-buffered saline (PBS) pH 7.4. Fifty microlitres of appropriate dilutions were plated in duplicate onto BHI plates and plates incubated at 30°C or 37°C. Colonies were enumerated after 24 or 48 h growth for wild-type and 10403S::imo927 *L. monocytogenes* strains, respectively, and average values plus standard deviations for three independently grown cultures are given.

**Wide-field and transmission electron microscopy**

For wide-field microscopy, strains 10403S and 10403S::imo927 were grown overnight at 30°C in 4 ml of BHI medium. Next day, cultures were diluted 1:100 or 1:50 (for strain 10403S::imo927 for subsequent growth at 37°C) into 25 ml of fresh medium and grown either at 30°C or 37°C. Eight and a half hours after back dilution, culture aliquots were removed, washed once with 1 ml of PBS pH 7.4 buffer and viewed under a 100x objective on a Nikon Eclipse E600 microscope and images taken with a Nikon DXM1200 digital camera. Experiments were performed with at least three independently grown cultures in at least two independent experiments and representative images are shown.

Samples for TEM were prepared similar as described in Thomaides *et al.* (2001). Briefly, *L. monocytogenes* strains were grown overnight at 30°C, back-diluted between 1:100 and 1:25 into 200 ml of BHI medium and grown for 3.5 or 8.5 h at 30°C or 37°C as indicated in the text. Bacteria from an equivalent of a 100 ml culture with an OD$_{600}$ of 0.5 were collected by centrifugation for 10 min at 8000 g, washed twice with 10 ml of 0.2 M sodium cacodylate buffer pH 7.1 and finally suspended in 2 ml of 0.2 M sodium cacodylate buffer pH 7.1 containing 4% glutaraldehyde. Bacteria were fixed for 4 h at 4°C without shaking and subsequently collected by centrifugation for 5 min at 10 000 g. Bacteria were suspended in 1 ml of 0.2 M sodium cacodylate buffer pH 7.1, transferred to 35 mm round tissue culture dishes, overlaid with 1 ml of 0.2 M sodium cacodylate buffer pH 7.1 containing 4% glutaraldehyde and incubated for 1 h at room temperature. After this second fixation step, bacterial layers were washed six times with 2 ml of 0.2 M sodium cacodylate buffer pH 7.1 and processed for electron microscopy as previously described (Thomaides *et al.*, 2001). Images were taken on an FEI Tecnai GZ transmission electron microscope at the Henry Wellcome Trust Imaging Centre, St Mary’s Campus, Imperial College London.

**Membrane lipid extraction and detection of glycolipids by TLC**

For *L. monocytogenes* membrane lipid extraction and glycolipid analysis, bacteria from 200 ml of cultures grown for 20–24 h at 30°C were collected by centrifugation for 10 min at 8000 g. Bacteria were washed once with 10–20 ml of ice-cold 0.1 M sodium citrate buffer pH 4.7, suspended in 3 ml of 0.1 M sodium citrate buffer pH 4.7 and dispensed into three 2 ml Fast Prep tubes containing 0.1 mm glass beads (~0.5 ml). Bacteria were lysed and lipids extracted as previously described using a modified Bligh-Dyer method (Kates, 1972; Gründling and Schneewind, 2007b). Dried lipids were suspend either in chloroform or in a 1:1 chloroform : methanol (Macherey-Nagel), lipids separated using a chloroform : methanol : H$_2$O (65:25:4) solvent system and glycolipids visualized by spraying plates with 0.5% α-naphthol in 50% methanol and then with 95% H$_2$SO$_4$ (Gründling and Schneewind, 2007b; Kates, 1972). Experiments were performed with at least three independently grown cultures in at least two independent experiments and representative images are shown.

**Lipid analysis by MALDI mass spectrometry**

For MALDI analysis to determine and confirm the structure of different glycolipids, a total of 4 mg of lipids were spotted...
(4 × 20 μl) and separated by TLC as described above and different lipids were further purified after scraping the silica gel from appropriate areas. Areas containing glycolipids were determined by developing one lane run in parallel with α-naphthol and H2SO4 and lipids were extracted from the silica gel with chloroform/methanol as previously described (Gründling and Schneewind, 2007b). Dried lipids were suspended in 10 μl of 0.5 M 2,5-dihydroxybenzoic acid (DHB) MALDI matrix dissolved in 1:1 methanol: chloroform or diluted 1:10 using 0.5 M DHB matrix and 1 μl spotted. Spotted MALDI plates were run on a MALDI micro MX™ machine (Waters, UK) available at the Proteomics Facility at Imperial College London. Using an automated program, 10 spectra were recorded for each spot in the reflector positive ion mode. As calibration standard, 25–50 pmols of bradykinin peptide was spotted directly onto MALDI plates or diluted 1:10 using 0.5 M DHB matrix and 1 μl spotted. The Proteomics Facility at Imperial College London. Using an automated program, 10 spectra were recorded for each spot in the reflector positive ion mode. As calibration standard, 25–50 pmols of bradykinin peptide standard (Sigma) with an absolute mass of 757.3997 (M-H+) was spotted in α-cyano-4-hydroxycinnamic acid (CHCA) matrix, which was suspended at 10 mg ml⁻¹ in 70% acetonitrile 0.1% TFA. Mass signals for lipids were manually corrected for observed mass difference of the internal peptide standard. Representative data from two independent experiments are shown.

Acknowledgements

We would like to thank Dr Judit Nagy for her technical assistance with the MALDI mass spectrometry instrument, Michael S. Hollinshead for his excellent assistance with the electron microscopy and Dr Christiana van Ooj and Dr Rebecca Corrigan for valuable discussions and critical reading of the manuscript. This work was supported by the Medical Research council grant G0701212 and the Wellcome Trust grant WT084483 to A.G.

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