Editor’s Choice

Localization and expression of the *Bacillus subtilis* DL-endopeptidase LytF are influenced by mutations in LTA synthases and glycolipid anchor synthetic enzymes

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*Bacillus subtilis* LytF plays a principal role in cell separation through its localization at the septa and poles on the vegetative cell surface. In this study, we found that a mutation in a major lipoteichoic acid (LTA) synthase gene – *ltaS* – results in a considerable reduction in the σD-dependent transcription of *lytF*. The *lytF* transcription was also reduced in mutants that affected glycolipid anchor biosynthesis. Immunofluorescence microscopy revealed that both the numbers of cells expressing LytF and the LytF foci in these mutants were decreased. In addition, the transcriptional activity of *lytF* was almost abolished in the double (*ltaS yfnI*), triple (*ltaS yfnI yqgS*), and quadruple (*ltaS yfnI yqgS yvgJ*) mutants during vegetative growth. Cell separation defects in these mutants were partially restored with artificial expression of LytF. Interestingly, when *lytF* transcription was induced in the *ltaS* single or multiple mutants, LytF was localized not only at the septum, but also along the sidewall. The amounts of LytF bound to cell wall in the single (*ltaS*) and double (*ltaS yfnI*) mutants gradually increased as compared with that in the WT strain, and those in the triple (*ltaS yfnI yqgS*) and quadruple mutants were almost similar to that in the double mutant. Moreover, reduction of the *lytF* transcription and chained cell morphology in the *ltaS* mutant were completely restored with artificial induction of the *yqgS* gene. These results strongly suggest that LTA influences the temporal, σD-dependent transcription of *lytF* and is an additional inhibitory component to the vegetative cell separation enzyme LytF.

INTRODUCTION

The cell wall (CW) of Gram-positive bacteria is composed of mesh-like peptidoglycan (PG) and teichoic acids. Teichoic acids are either covalently attached to PGs (wall teichoic acid, WTA) or anchored to membrane lipids (lipoteichoic acid, LTA) (*Bacillus subtilis* 168 mainly produces major and minor WTAs, as well as LTA during vegetative growth (Foster & Popham, 2001; Lazarevic et al., 2002). In this organism, major WTA and LTA polymers consist of a similar main chain structure, comprising poly glycerol-phosphate (GroP) repeating units (Foster & Popham, 2001; Neuhaus & Baddiley, 2003). WTAs are tethered to the PG through a linkage unit consisting of two GroP residues linked to N-acetylmannosaminyl-N-acetylglucosaminyl phosphate, whereas LTA polymers are linked through a glycolipid anchor – diglucosyl-diacylglycerol (Glc2-DAG) – on the cytoplasmic membrane (Foster & Popham, 2001; Gründling & Schneewind, 2007b; Neuhaus & Baddiley, 2003).

Three supplementary tables, two supplementary figures, supplementary Methods and references are available with the online Supplementary Material.

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**Abbreviations:** chrDNA, chromosomal DNA; CW, cell wall; DIC, differential interference contrast; ECF, extracytoplasmic function; Glc2-DAG, diglucosyl-diacylglycerol; GroP, glycerol-phosphate; IFM, immunofluorescence microscopy; LTA, lipoteichoic acid; PG, peptidoglycan; WTA, wall teichoic acid.

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This synthesis is catalysed by the UgpP glycosyltransferase (Gründling & Schneewind, 2007b; Jorasch et al., 1998). Two glucose moieties are transferred from UDP-glucose, derived from glucose-1-phosphate by the UTP: α-glucose-1-phosphate uridylyltransferase GtaB, which is also involved in the glucose modification pathway of WTA (Gründling & Schneewind, 2007b; Soldo et al., 1993). It is thought that the glycolipid anchor is translocated to the outer surface of the membrane by LtaA in Staphylococcus aureus (Gründling & Schneewind, 2007b) and an unknown flippase in B. subtilis (Reichmann & Gründling, 2011).

In contrast to anchor formation, the biosynthetic pathway of the GroP polymer was not characterized until recently. However, it has been reported that S. aureus LtaS (LtaS_A) is the key LTA synthase responsible for polymerization of the GroP main chain (Gründling & Schneewind, 2007a). LTA synthesis is required for growth and cell division in S. aureus (Gründling & Schneewind, 2007a; Oku et al., 2009), and B. subtilis ltaS restored LTA synthesis and growth in LtaS_A-depleted S. aureus cells (Gründling & Schneewind, 2007a). In B. subtilis, four ltaS orthologues, ltaS (originally yfIE), yfIN, yggS, and yvgJ, are conserved in the genome (Schirner et al., 2009; Wörmann et al., 2011). LtaS, YfIN, and YggS are LTA synthases that play a role in the polymerization of the main chain, while YvgJ is an LTA primase that transfers the initial GroP subunit onto the glycolipid anchor to produce GroP-Glc_C2-DAG (Wörmann et al., 2011). Among them, B. subtilis LtaS appears to play the principal role in LTA synthesis housekeeping because its absence affects cell division during vegetative growth (Schirner et al., 2009). By contrast, YfIN functions under stress conditions and synthesizes longer LTA polymers than those synthesized by LtaS (Gründling & Schneewind, 2007a; Wörmann et al., 2011). Finally, YggS and YvgJ are required for LTA synthesis during sporulation. The loss of LtaS activity affects cell division, cell morphogenesis, and divalent cation homeostasis in B. subtilis (Schirner et al., 2009). A quadruple mutant in the ltaS homologue genes abolishes LTA modification, displays slower growth, has an unknown flippase in S. aureus, B. subtilis ltaS homologue genes (originally yfIE), yfIN, yggS, and yvgJ, are conserved in the genome (Schirner et al., 2009; Wörmann et al., 2011). LtaS, YfIN, and YggS are LTA synthases that play a role in the polymerization of the main chain, while YvgJ is an LTA primase that transfers the initial GroP subunit onto the glycolipid anchor to produce GroP-Glc_C2-DAG (Wörmann et al., 2011). Among them, B. subtilis LtaS appears to play the principal role in LTA synthesis housekeeping because its absence affects cell division during vegetative growth (Schirner et al., 2009). By contrast, YfIN functions under stress conditions and synthesizes longer LTA polymers than those synthesized by LtaS (Gründling & Schneewind, 2007a; Wörmann et al., 2011). Finally, YggS and YvgJ are required for LTA synthesis during sporulation. The loss of LtaS activity affects cell division, cell morphogenesis, and divalent cation homeostasis in B. subtilis (Schirner et al., 2009). A quadruple mutant in the ltaS homologue genes abolishes LTA modification, displays slower growth, has an unknown flippase in S. aureus, B. subtilis ltaS homologue genes (originally yfIE), yfIN, yggS, and yvgJ, are conserved in the genome (Schirner et al., 2009; Wörmann et al., 2011). LtaS, YfIN, and YggS are LTA synthases that play a role in the polymerization of the main chain, while YvgJ is an LTA primase that transfers the initial GroP subunit onto the glycolipid anchor to produce GroP-Glc_C2-DAG (Wörmann et al., 2011). Among them, B. subtilis LtaS appears to play the principal role in LTA synthesis housekeeping because its absence affects cell division during vegetative growth (Schirner et al., 2009). By contrast, YfIN functions under stress conditions and synthesizes longer LTA polymers than those synthesized by LtaS (Gründling & Schneewind, 2007a; Wörmann et al., 2011). Finally, YggS and YvgJ are required for LTA synthesis during sporulation. The loss of LtaS activity affects cell division, cell morphogenesis, and divalent cation homeostasis in B. subtilis (Schirner et al., 2009). A quadruple mutant in the ltaS homologue genes abolishes LTA modification, displays slower growth, has an aberrant twisted morphology (Schirner et al., 2009), and shows decreased adsorption of rare earth elements (Moriwaki et al., 2013; Moriwaki & Yamamoto, 2013).

Cryo-electron microscopy of frozen-hydrated sections has indicated that Gram-positive bacteria such as B. subtilis and S. aureus also have a periplasmic space, termed the inner wall zone, between the plasma membrane and thick CW (Matias & Beveridge, 2005; Matias & Beveridge, 2006; Zuber et al., 2006) and that LTA is a major component of the B. subtilis periplasm (Matias & Beveridge, 2008). Moreover, subcellular localization analysis of UgpP fused to GFP showed that the fusion protein was predominantly localized at the septa and that proper localization was abolished in the pgcA and gtaB mutants (Weart et al., 2007). Importantly, UgpP is a metabolic sensor that localizes to the septum in a nutrient-dependent manner and inhibits assembly of the cell division protein FtsZ to control cell size (Weart et al., 2007). In addition, two LTA synthases, LtaS and YggS, when fused to GFP, are predominantly localized at division sites in both vegetative and sporulating cells (Schirner et al., 2009). These observations strongly suggest that both glycolipid anchor synthesis and main chain polymerization of LTA occur at the site of cell division. Moreover, both ugpP and ltaS mutations affect the process of cell division (Schirner et al., 2009; Weart et al., 2007).

During vegetative growth, B. subtilis produces various cell surface proteins, including a CW-associated protein (WapA), CW-bound proteases (WprA and Epr), several CW hydrolases (CwlO, CwlS, LytC, LytD, LytE, and LytF), a modifier of major autolysin (LytB), and an inhibitor of cell separation enzymes (IseA) (Antelmann et al., 2002; Foster & Popham, 2001; Fukushima et al., 2006; Smith et al., 2000; Yamamoto et al., 2003, 2008a). Among these, LytF is the major vegetative cell separation enzyme and has D₃-endopeptidase activity that cleaves the linkage of N-D-glutamyl-meso-diaminopimelic acid in the peptide moieties of the B. subtilis PG. The lytF gene is transcribed by an alternative sigma factor σ⁰ during the late exponential growth phase (Margot et al., 1999; Ohnishi et al., 1999). During the separation of the two daughter cells at the final stage of cell division, LytF specifically localizes to the cell separation sites (Yamamoto et al., 2003). In contrast, LytF localized to the septum and sidewall of the WTA-depleted cells, suggesting that WTA predominantly regulates the sidewall localization of LytF (Yamamoto et al., 2008b). This indicated that WTA along the sidewall is necessary for the septum localization of LytF.

A similar study on the localization control of an autolysin by WTA has been reported in S. aureus (Schlag et al., 2010). In this organism, cell separation largely depends on the bifunctional autolysin Atl, which has both amidase and glucosaminidase activities (Baba & Schneewind, 1998; Oshida et al., 1995; Yamada et al., 1996). Atl is targeted to the cell separation site to produce daughter cells in the WT strain (Baba & Schneewind, 1998; Yamada et al., 1996), whereas in a tagO mutant strain, which lacks WTA in the CW layer, the amidase domain is evenly distributed on the cell surface (Schlag et al., 2010). These observations suggest that WTA controls the septal localization of the major cell separation enzymes LytF in B. subtilis and Atl in S. aureus. On the other hand, Steen et al. (2003) have speculated that LTA might be an inhibitory component for the major autolysin AcmA in Lactococcus lactis. However, because they used chemically treated cells and CWs, it is unclear whether AcmA localization is prevented by LTA and/or WTA. In this report, we demonstrate that LTA influences both temporal expression and proper localization of the major vegetative cell separation enzyme LytF in B. subtilis.

METHODS

Bacterial strains and culture conditions. The strains of B. subtilis and Escherichia coli used in this study are listed in Table S1, available
in the online Supplementary Material. *B. subtilis* 168 and WEC, a double mutant strain of *wprA* and *epr* without any antibiotic resistance genes (Yamamoto et al., 2008b), were used as the parent strains throughout this study. The *B. subtilis* strains were grown in Luria–Bertani (LB) medium (Sambrook et al., 1989) at 37 °C unless otherwise noted. When necessary, chloramphenicol, kanamycin, spectinomycin, tetracycline, and erythromycin were added to final concentrations of 5, 50, 10, and 0.3 μg ml⁻¹ respectively. To culture the conditional mutants of the *ltaS* homologues and D-endorpeptidase *lytF*, IPTG was added to the culture medium. *E. coli* strains were cultured in LB medium at 37 °C, and when necessary, ampicillin was added to a final concentration of 100 μg ml⁻¹. *E. coli* JM109 and C600 cells were used for plasmid construction and multimeric plasmid DNA preparation, respectively. DNA manipulation and *E. coli* transformation were performed using standard methods (Sambrook et al., 1989). *B. subtilis* transformation was also carried out based on the conventional transformation method (Anagnostopoulos & Spizizen, 1961).

**Strain construction.** The strains, plasmids and primers used in this study are listed in Tables S1, S2, and S3, respectively. After digestion with restriction enzymes, all DNA fragments were fractionated using agarose gel electrophoresis and purified with a QiAquick gel extraction kit (Qiagen) according to the manufacturer’s instructions. The nucleotide sequences of all inserts amplified by PCR were confirmed by sequencing.

To obtain the *ltaS* null mutants with several markers, upstream (516 bp) and downstream (526 bp) regions of the *ltaS* gene were amplified with two sets of primers, yflEf-Bf and yflEf-PEr for upstream and yflEb-Ef and yflEb-MHr for downstream, respectively, and using 168 chrDNA as the template. The two amplified fragments were digested with *EcoRI* and ligated. The ligation mixture was used as the template for the second round of PCR with the primers yflEf-Bf and yflEf-MHr. After digestion with *BamHI* and HindIII, the resulting 1.0 kb DNA fragment was cloned into the corresponding sites in *pBluescript* II SK + to obtain pBAYlfE. Erythromycin, kanamycin and spectinomycin markers derived from pDG646, pDG783 and pDG1726, respectively, were cloned into pBAYlfE to generate pBAYlfEm, pBAYlfEspl and pBAYlfEsp. These plasmids were used for the *B. subtilis* transformation to obtain KY1210 (*ltaS::erm*, KY1210 (*ltaS::kan*), and KY1212 (*ltaS::spc*), respectively.

For construction of the IPTG-inducible conditional mutants of *yflD*, *yflG*, and *yflJ*, each 5'-flanking region, including their Shine–Dalgarno sequence, was amplified with 168 chrDNA as the template and the primer sets YFlnp-Ef and YFlnp-Br for *yflD*, YQGSp-Ef and YQGSp-Br for *yflG*, and YVGLp-Ef and YVGLp-Br for *yflJ*, respectively. All of the amplified fragments (approx. 0.3 kb) were cloned between the *BglII* and *SphI* sites of the *pSpac* vector (Yamamoto et al., 2003), respectively. To construct a *B. subtilis* transformation to create pKY2093 (*pSpac-lacZ*).

To construct an IPTG-inducible *lytF-6×flag* fusion gene at the *amyE* locus, *lytF-6×flag* was amplified with the primers PflyF-ZF and PryF-Smr and using 168 chrDNA as the template. The amplified fragment was digested with *BglII* and *SpeI* and then ligated into the corresponding sites of pOIDAFB (Fukushima et al., 2003). The ligation mixture was used for the transformation of *B. subtilis* WEC to obtain KY2101 (*pSpac-lytF-6×flag*).

**β-galactosidase assay.** After cultivating the cells at 37 °C, samples were withdrawn at various times to assay β-galactosidase activity. Measurement and calculation of β-galactosidase activity (expressed in the units mg-protein⁻¹) were performed as described by Shimotus & Henner (1986). One unit of β-galactosidase activity was defined as the amount of enzyme necessary to release 1 nmol of 2-nitrophenol from ONPG in 1 min at 28 °C. The assay was performed at least three times independently.

**Immunofluorescence microscopy (IFM).** Sample preparation for IFM was performed as described previously (Yamamoto et al., 2003, 2008b) with minor modifications, as follows. An anti-FLAG M2 monoclonal antibody (Sigma) was used as the primary antibody and a sheep anti-mouse IgG Cy3 conjugated antibody (Sigma) was used as the secondary antibody at 1:400 dilutions. For CW staining, wheatgerm agglutinin-Alexa Fluor 488 conjugate (WGA-AF488) was used as described previously (Yamamoto et al., 2003). Lysozyme treatment just before spotting onto a poly-l-lysine-coated microscope slide (Yamamoto et al., 2003) was omitted to avoid exfoliation of the LytF-6×FLAG protein. Fluorescence microscopy was performed as described previously (Yamamoto et al., 2008b) with an AxioImager M1 microscope, a Plan-APochromat Fluorite differential interference objective (magnification, 63 × ; numerical aperture, 1.4), and standard filter sets for visualizing rhodamine (for Cy3). The exposure times were 0.04 s for differential interference contrast microscopy and 0.1 s (gain 2) for Cy3. Cells were photographed with a charge-coupled device camera (AxioCam MRm; Carl Zeiss) with Axiosvision software (version 4.6; Carl Zeiss). The three-dimensional deconvolution utility of the Axiosvision software was used for z-axis imaging. All images were processed with Axiosvision and Adobe Photoshop software.

**RESULTS**

**Expression and localization of LytF in mutants of the LTA synthesis pathway**

Previous reports revealed that mutation of *ltaS*, which encodes a principal vegetative LTA synthase, affects cell division, cell morphogenesis, and divergent cation homeostasis. Indeed, a quadruple mutation in the *ltaS* homologue genes results in aberrant filamentous clumps (Schirner et al., 2009; Wörmann et al., 2011). Therefore, we hypothesized that the *ltaS* mutation affects not only cell division, but also cell separation. In order to examine this,
we performed a transcriptional analysis of the \textit{lytF} gene, which encodes a major Dl-endopeptidase responsible for vegetative cell separation and is transcribed by an alternative sigma factor \( \sigma^D \) (Ohnishi \textit{et al.}, 1999). As shown in Fig. 1(a), \( \sigma^D \)-dependent \textit{lytF} transcription was considerably reduced in the \textit{ltaS} mutant during the vegetative growth phase. Supporting this observation, \textit{LytF-6 \times FLAG} was decreased in the CW fraction (Fig. 2j). Moreover, analysis of the subcellular localization indicated that \textit{LytF} only weakly localizes at the septum and poles, or not at all, in the \textit{ltaS}-deficient cells (Fig. 2b). The number of foci per

![Fig. 1. Effect of mutants involved in the biosynthesis or modification pathways of teichoic acids on \textit{lytF} transcription. Cultures were inoculated at a starting OD\textsubscript{600} of 0.001 unless otherwise noted. Growth (OD\textsubscript{600}) and \( \beta \)-galactosidase activity (units (mg protein\textsuperscript{-1})) are shown by dotted and solid lines, respectively. The activity in three different cultures was assayed as described in Methods. Each experiment was repeated three times and the values obtained did not vary significantly. (a) \( \beta \)-galactosidase activity of \textit{P\textsubscript{galdehydrolacZ}} in the \textit{ltaS} homologue mutants. YK2093 (○: WT), YK2105 (□: \textit{ltaS}), YK2097 (●: \textit{yfnI yggS yvgJ}), YK2111 (△: \textit{ltaS yfnI}), YK2112 (×: \textit{ltaS yfnI yggS}), and YK2110 (●: \textit{ltaS yfnI yggS yvgJ}). In the case of multiple mutants, cultures were inoculated at a starting optical density at OD\textsubscript{600} of 0.002 (YK2111 and YK2112) or 0.003 (YK2110) because they grew slowly. (b) \( \beta \)-galactosidase activity of \textit{P\textsubscript{glycocollipid}} in glycolipid anchor biosynthesis in the anionic polymer modification. YK2093 (○: WT), YK2123 (■: \textit{ugtP}), YK2121 (●: \textit{gtaB}), YK2120 (●: \textit{pgcA}), YK2186 (△: \textit{dltA}) and YK2122 (×: \textit{tagE}).]

Next, we examined \textit{lytF} expression in mutants of genes involved in the glycolipid anchor biosynthesis pathway. \textit{UgtP} is responsible for the synthesis of the \( \text{GlC}_{2}\text{-DAG} \) glycolipid anchor of LTA (Gründling \& Schneewind, 2007b; Jorasch \textit{et al.}, 1998; Kiriykin \textit{et al.}, 2001; Weart \textit{et al.}, 2007). In the \textit{ugtP} mutant, \textit{lytF} transcription and the amount of \textit{LytF-6 \times FLAG} on the cell surface were slightly reduced during vegetative growth (Fig. 1b, 3g). In addition, IFM revealed that the \textit{LytF-6 \times FLAG} foci were lower in frequency compared with the WT strain (Fig. 3a, b). In addition to the \textit{ugtP} mutant, a distinct reduction in \textit{LytF} expression was observed in the \textit{gtaB} and \textit{pgcA} mutants (Fig. 1b, 3g). \textit{PgcA} and \textit{GtaB} are responsible for supplying the substrates, glucose-1-phosphate and UDP-glucose, respectively, in the biosynthetic pathway of the glycolipid anchor (Gründling \& Schneewind, 2007b; Lazarevic \textit{et al.}, 2005; Weart \textit{et al.}, 2007). The \textit{LytF} foci at the septum were also weak or missing in both mutants (Fig. 3c, d).

It is thought that the \( \text{d}-\text{alanyl ester} \) content of LTA changes as a function of the pH of the growth medium. Loss of \( \text{d}-\text{alanylation} \) in the \textit{B. subtilis} CW affects the folding and stability of the secreted proteins (Hyyryläinen \textit{et al.}, 2000) and increases susceptibility to antibiotics that target the CW (May \textit{et al.}, 2005). Therefore, we examined whether \( \text{d}-\text{alanylation} \) affected the expression or localization of \textit{LytF}. The results indicated that the \textit{dltA} mutation did not affect either \textit{LytF} expression (Fig. 1b, 3g) or septum localization of \textit{LytF} during vegetative growth (Fig. 3e). In addition, no significant differences in expression or localization of \textit{LytF} were observed in the \textit{tagE} null mutant strain (Fig. 1b, 3f–g), which has a defect in glycosylation of the major WTA (Allison \textit{et al.}, 2011; Lazarevic \textit{et al.}, 2002). A previous report has demonstrated that \textit{TagE} is only able to use a poly(GroP) polymer as a substrate (Allison \textit{et al.}, 2011). Therefore, it is thought that WTA polymer synthesis is initiated by \textit{TagF} prior to glycosylation. Based on this finding, defects in WTA glycosylation might not affect expression and localization of \textit{LytF}.

These observations suggest that \( \sigma^D \)-dependent \textit{lytF} transcription is considerably repressed in the \textit{ltaS} mutant, which affects the main chain biosynthesis of LTA during exponential growth, or is slightly delayed in mutants involved in the glycolipid anchor synthetic pathways. By contrast, the expression and localization of \textit{LytF} was not altered in mutants involved in the modification, \( \text{d}-\text{alanylation} \) or glycosylation, pathway of teichoic acids. Taken together, it appears that accumulation of irregular LTA, both in the main chain and glycolipid anchor, on
the cell surface affects the temporal activation of lytF transcription.

**σ^D-dependent lytF transcription is significantly repressed in multiple mutants of ItaS and its homologues**

Next, we examined how mutations in other ItaS homologues, in the ItaS mutant background, affected lytF transcription. A previous report demonstrated that LtaS, YfnI, and YggS are LTA synthases that function in the main chain polymerization, while YvgJ is an LTA primase that attaches the initial GroP subunit to the glycolipid anchor (Wörmann et al., 2011). As indicated in Fig. 1(a), we found that lytF transcription was not altered in the triple mutant (yfnI yggS yvgJ). In addition, LytF localization in the triple mutant was similar to that in the WT strain (Fig. 2f). By contrast, the P_{lytF}-lacZ activities in the double mutants (ltaS yggS or ltaS yvgJ) were very similar to that in the ltaS single mutant (Fig. S1b). Moreover, we found that lytF transcription was almost abolished in only the ltaS yfnI mutant among double mutants involving ltaS (Fig. 1a, S1b). Likewise, LytF-6×FLAG in the CW fraction was significantly decreased and the foci were not observed

![Image](http://mic.sgmjournals.org)
**Subcellular localization of LytF-6×FLAG in mutants of glycolipid anchor synthesis, d-alanylation, and glycosylation.**

(a–f) DIC and IFM analyses of LytF-6×FLAG were performed with strains carrying a P\text{ure}-lytF-6×flag fusion gene at the lytF locus. Cultures were inoculated at a starting OD\textsubscript{600} of 0.001. After incubation for 3.5 h (OD\textsubscript{600}, 0.5–0.7), cells were harvested and fixed. The exposure times were 0.04 s for the DIC images and 0.8 s (gain 2) for the Cy3 images. IFM images were superimposed on the DIC images. Scale bars, 10 μm. Maximum mean value of fluorescence intensity together with calculated SD and the frequency of the LytF-6×FLAG foci at septum and poles (in percentage and raw values) are indicated. (a) MH1022 (WT), (b) HY1305 (ugtP), (c) YK2175 (gtaB), (d) YK2174 (pgcA), (e) YK2199 (ditA), and (f) YK2176 (tagE). (g) Western blot analysis of LytF-6×FLAG in mutants of glycolipid anchor synthesis, d-alanylation, and glycosylation. Culture conditions and sampling point of Western blot analysis were similar to those of the IFM images (a–f). Lane 1: MH1022 (WT), lane 2: HY1305 (ugtP), lane 3: YK2175 (gtaB), lane 4: YK2174 (pgcA), lane 5: YK2199 (ditA), and lane 6: YK2176 (tagE). Proteins from the cell surface were equivalent to 0.05 OD\textsubscript{600} cells in each lane. The molecular masses of the protein standards (Bio-Rad) are indicated on the left.

(Fig. 2g, j). Furthermore, LytF expression during the exponential growth phase was almost abolished in the triple (l\text{ta}S y\text{fn}l y\text{g}g\text{S}) and quadruple mutants (Fig. 1a, 2j). Taken together, these results suggest that YqgS and YvgJ may not complement the roles of LtaS and Yfnl during vegetative growth. Supporting these observations, LytF septal localization in chained and bent cells was also absent in the triple and quadruple mutants (Fig. 2h, i).

**LTA prevents LytF sidewall localization**

We previously reported that WTA prevents LytF localization in the cylindrical part of the cell (Yamamoto \textit{et al.}, 2008b). We examined here whether LTA also hindered sidewall localization of LytF. As described in the previous section, we demonstrated that lytF expression was significantly repressed in the l\text{ta}S mutant or absent in the multiple LTA synthase mutants (Fig. 1a). Therefore, we placed a lytF-6×flag fusion gene downstream of an IPTG-inducible P\text{spac} promoter at the amyE locus. As shown in Fig. 4(a, c), the fluorescent intensity of LytF-6×FLAG at the septa and poles in the l\text{ta}S mutant were slightly enhanced compared with those in the WT cell. In addition to the septa and poles, LytF was weakly localized to the sidewall (Fig. 4c–ii, g). Supporting these results, the amount of LytF bound to the cell surface was clearly increased in the l\text{ta}S mutant compared with the WT strain (Fig. 4b). Moreover, the mean chain length (17.6 μm ± 8.5) of LytF-expressing cells in the l\text{ta}S mutant background was shorter than that (33.2 μm ± 15.4) of non-expressing ones, suggesting that cell separation was partially restored by an artificial induction of LytF.

Next, we examined how multiple mutations in the l\text{ta}S homologues affected LytF localization. Interestingly, LytF localized on the cylindrical part, with patterns of distributed dots and tilted bands across the long axis of the cell in the double (l\text{ta}S y\text{fn}l) mutant (Fig. 4d, S2d iv–vi). Moreover, the signal intensity of the LytF foci along the sidewall (Fig. 4g), and those at septum and poles, were increased as compared to those in the l\text{ta}S mutant (Fig. 4c, d), whereas those in the triple (l\text{ta}S y\text{fn}l y\text{g}g\text{S}) or quadruple mutants were almost similar to the double mutant (Fig. 4d–g). These observations were consistent with the results from the Western blot analysis (Fig. 4h). These results suggest that LTA controls LytF localization not only at the septum, but also at the cylindrical part of the cell together with WTA. In addition, LytF induction in cells lacking LTA partially restored cell separation but not the bent cell shape (Fig. 4d–f).

**YqgS alone complements the physiological roles of LtaS**

It is thought that LtaS plays a principal role in LTA synthesis during vegetative growth because only an l\text{ta}S mutation affected both cell division and morphology (Wörmann \textit{et al.}, 2011). The other three homologues, Yfnl, YqgS, and YvgJ, appear to be required for adaptation to environmental stress or developmental conditions (Hashimoto \textit{et al.}, 2013; Schirner \textit{et al.}, 2009). However, it is unclear whether these homologues are able to complement these LtaS functions during vegetative growth.
LTA regulates expression and localization of LytF

Fig. 4. Localization patterns of induced LytF-6×FLAG in LTA mutants. Localization patterns of induced LytF-6×FLAG in (a) YK2118 (WT), (b) YK2259 (sigD), (c) YK2154 (itaS), (d) YK2181 (itaS yfnI), (e) YK2182 (itaS yfnI yqgS) and (f) YK2179 (itaS yfnI yqgS yvgJ). DIC and IFM analyses of LytF-6×FLAG were performed with strains carrying a Pspac-ltyF fusion at the amyE locus. Cultures were inoculated at starting OD600 of 0.001 (YK2118, YK2259 and YK2154), 0.002 (YK2181 and YK2182), or 0.003 (YK2179) in LB medium with the addition of 0.2 mM IPTG. After incubation for 3.5 h (OD600, 0.5–0.6), cells were harvested and fixed. The exposure times were 0.04 s for the DIC images (i) and 0.35 s (gain 2) for the Cy3 images (ii). Maximum mean value of fluorescence intensity together with calculated SD of the LytF-6×FLAG foci at septum and poles is indicated below the IFM images (a–ii–f–ii). Scale bars, 10 μm. (g) Fluorescence intensity of LytF-6×FLAG on the sidewall in the itaS homologue mutants. Averaged fluorescence intensities (relative units, RU) of the LytF foci along the sidewall (a–ii–f–ii) were quantified. Error bars represent SD of fluorescent intensity measurements. (h) Western blot analysis of LytF-6×FLAG in the itaS homologue mutants. Western blot analysis of LytF-6×FLAG bound to the CW. Culture conditions and sampling point were similar to those of IFM (a–f).

Lane 1: YK2118 (WT), lane 2: YK2154 (itaS), lane 3: YK2181 (itaS yfnI), lane 4: YK2182 (itaS yfnI yqgS), lane 5: YK2179 (itaS yfnI yqgS yvgI), lane 6: YK2259 (sigD). Proteins from the cell surface were equivalent to 0.05 OD600 cells in each lane. The molecular masses of the protein standards (Bio-Rad) are indicated on the left.

DISCUSSION

We found that ltyF transcription was significantly decreased in single and multiple mutants of itaS, which encodes a principal LTA synthase, and slightly reduced in mutants of ugtP, gtaB, and pgcA, which encode enzymes involved in the glycolipid anchor biosynthesis (Fig. 1). Previous reports revealed that SigD regulon expression was decreased in the ugtP mutant and mutants with altered membrane composition (Salzberg & Helmann, 2008), and that lipid composition was considerably changed in the itaS mutant (Hashimoto et al., 2013). In addition, in the S. aureus ugtP mutant, the lipid modification by LTA was reduced and directly attached to diacylglycerol without its typical glycolipid anchor (Fedtke et al., 2007; Kiriukhin et al., 2001). Moreover, since it has been reported that the septum localization of GFP-UgtP was abolished in B. subtilis pgaA or gtaB mutants (Weart et al., 2007), the function of UgtP as the metabolic sensor seems to be impaired in mutants involved in the glycolipid anchor synthesis. Therefore, reduction in ltyF transcription in the itaS, ugtP, gtaB, and pgcA mutants might result from an alteration in the lipid modification in the cytoplasmic membrane. Furthermore, ltyF transcription was almost abolished in the double (itaS yfnI), triple (itaS yfnI yqgS), and quadruple mutants (Fig. 1a). Interestingly, we also found that σD-dependent transcription of ltyF was
significantly repressed in tagO null mutant cells, which lack WTA (Fig. S1d). Similar reduction was observed in the lytD gene (Fig. S1c, d), which is one of the SigD regulon and encodes N-acetylglucosaminidase (Margot et al., 1994; Rashid et al., 1995). These results suggest that LTA and WTA, both of which are similar anionic polymers in the

**Fig. 5.** Complementation test with the ltaS homologues. Cultures were inoculated at a starting OD_{600} of 0.001 into LB medium without (a, b) or with 0.5 mM IPTG (c–f). After incubation for 3.5 h (OD_{600} 0.5–0.7), cells were harvested and fixed. Cell morphology and LytF localization were examined in strains carrying IPTG-inducible ltaS homologue genes, (a) MH1022 (WT), (b) KY1201 (P_{spac}-ltaS), (c) RY1649 (ltaS P_{spac}-yqgS), (d) RY1648 (ltaS P_{spac}-yfnl), (e) RY1650 (ltaS P_{spac}-yvgJ), and (f) KY1222 (ltaS). The exposure times were 0.04 s for the DIC images and 0.8 s (gain 2) for the Cy3 images. IFM images were superimposed on the DIC images. Scale bars, 10 μm. Maximum mean value of fluorescence intensity together with calculated SD and the frequency of the LytF-6×FLAG foci (in percentage and raw values) are indicated below of the superimposed images (a–f). (g) β-galactosidase activity of P_{lytF-lacZ} in the complementation experiment. Cultures were inoculated at a starting OD_{600} of 0.001 into LB medium with 0.5 mM IPTG. YK2093 (○: WT without IPTG), YK2105 (■: ltaS without IPTG), YK2268 (□: P_{spac}-ltaS), YK2274 (△: ltaS P_{spac}-yfnl), YK2275 (◆: ltaS P_{spac}-yqgS), and YK2276 (×: ltaS P_{spac}-yvgJ). Growth (OD_{600}) and β-galactosidase activity (units (mg protein)^{-1}) are shown by dotted and solid lines, respectively. The experiments were performed three times with similar results. (h) Western blot analysis of LytF-6×FLAG in the complementation experiment. Culture conditions and sampling point were similar to those of IFM (a–f). Proteins from the cell surface were equivalent to 0.05 OD_{600} Cells in each lane. The molecular masses of the protein standards (Bio-Rad) are indicated on the left. Lane 1: MH1023 (WT), lane 2: KY1222 (ltaS), lane 3: KY1201 (P_{spac}-ltaS), lane 4: RY1649 (ltaS P_{spac}-yqgS), lane 5: RY1648 (ltaS P_{spac}-yfnl), lane 6: RY1650 (ltaS P_{spac}-yvgJ).
poly glycerol-phosphate backbone of the \textit{B. subtilis} CW, are also required for temporal expression of the SigD regulon. By contrast, it has been reported that \textit{sigM} was activated in the \textit{ltaS} mutant (Hashimoto \textit{et al.}, 2013) and that mRNA levels of the four extracytoplasmic function (ECF) sigma factors (\textit{sigM}, \textit{sigX}, \textit{sigW}, and \textit{ytaC}) were increased in the double (\textit{ltaS yfnI}) and triple (\textit{ltaS yfnI yqgS}) mutants. Finally, six ECF sigma factors, except for \textit{sigZ}, are activated in the quadruple mutant (Hashimoto \textit{et al.}, 2013). Further experiments are needed to explain the opposing regulatory aspects between the SigD regulon and the ECF sigma factor regulons in the LTA-defective strains.

We also found that the $\sigma^{III}$ repression, abnormal cell division, and chained cell morphology observed in the \textit{ltaS} mutant cells were almost completely suppressed by induction of \textit{yqgS} alone, among the three \textit{ltaS} homologues (Fig. 5). This is supported by previous findings where YqgS expressed from a multicopy plasmid complemented a \textit{S. aureus ltaS}-depletion strain (Wöremann \textit{et al.}, 2011), suggesting that induction of \textit{YqgS} during vegetative growth might synthesize the normal LTA levels that are normally produced by LtaS. By contrast, only cell separation was partially restored by induction of LytF, whereas irregular cell division and the bent cell morphology were not (Fig. 4). These results suggest that the LTA polymers synthesized by LtaS or YqgS are required for proper septum placement and the normal rod-shaped morphology.

A previous report indicated that \textit{yfnI}, which is involved in the SigM regulon (Eiamphungporn \& Helmann, 2008; Jervis \textit{et al.}, 2007), is induced by $\sigma^{III}$ in the \textit{ltaS} mutant, and that YfnI synthesizes a longer LTA polymer than that produced by LtaS (Wöremann \textit{et al.}, 2011). When \textit{lytF} transcription was induced in the \textit{ltaS} mutant, LytF was localized to a sidewall, in addition to the septum and poles (Fig. 4c). This suggests that the longer LTA polymer synthesized by YfnI in the \textit{ltaS} mutant may not sufficiently complement the function of the normal LTA polymer produced by LtaS, especially with regard to the LytF-controlled localization. Therefore, the LytF-attachable region, which predominantly appears to be the naked PG (Yamamoto \textit{et al.}, 2008b), may remain at the cylindrical part of the cell. By contrast, YqgS is able to synthesize the normal length LTA polymer, similar to that produced by LtaS. It has been reported that YqgS retains LTA primase and polymerase activities and synthesizes a similar LTA in length as produced by LtaS in the stationary phase (Wöremann \textit{et al.}, 2011). Therefore, when \textit{yqgS} was induced during the vegetative growth phase, its product could sufficiently complement LtaS function in cell morphology, cell division, and proper expression and localization of LytF (Fig. 5c, g–h). In \textit{S. aureus}, it has been demonstrated that LtaSSA$^{III}$-depletion, which causes growth arrest and aberrant cell morphology, is functionally complemented by expression of a single copy of \textit{ltaS} or a multicopy of \textit{yqgS} (Gründling & Schneewind, 2007a; Wöremann \textit{et al.}, 2011).

The amount of LytF that attached to the CW gradually increased when \textit{lytF} was induced in the single (\textit{ltaS}) and double (\textit{ltaS yfnI}) mutants (Fig. 4h). However, no significant difference in the LytF amounts was observed among the double (\textit{ltaS yfnI}), triple (\textit{ltaS yfnI yqgS}), and quadruple mutants (Fig. 4h). Interestingly, LytF localization along the sidewall in the multiple mutants appeared to be a helix-like pattern with distributed dots (Fig. S2d–f; iv) and tilted bands (Fig. S2d–f; iv, vi). By contrast, when LytF was artificially expressed in a sigD mutant, the amount and localization of LytF on the cell surface was very similar to those of the WT strain (Fig. 4b, g–h). These results suggest that LytF sidewall localization mainly results from reduction or defect of LTA, but not loss of SigD-dependent transcription. It is still unclear why LytF is localized in the helix-like pattern along the sidewall on the cell surface lacking LTA. In a previous report, we observed a similar helix-like pattern on the WTA-reduced cell surface, but LytF was evenly distributed on the WTA-depleted cell surface (Yamamoto \textit{et al.}, 2008b). This difference in the control of localization by LTA and WTA may result from the location of these polymers on the CW layer; i.e. LTA is tethered to the cytoplasmic membrane and is mainly located in the periplasmic space (Matias & Beveridge, 2008), while WTA is attached to the PG and is distributed over the CW layer. Further work is necessary to determine the roles of these anionic polymers in the various CW functions.

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