Teichoic Acid Polymers Affect Expression and Localization of DL-Endopeptidase LytE Required for Lateral Cell Wall Hydrolysis in Bacillus subtilis

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ABSTRACT
In Bacillus subtilis, the DL-endopeptidase LytE is responsible for lateral peptidoglycan hydrolysis during cell elongation. We found that $\sigma^D$-dependent transcription of lytE is considerably enhanced in a strain with a mutation in ltaS, which encodes a major lipoteichoic acid (LTA) synthase. Similar enhancements were observed in mutants that affect the glycolipid anchor and wall teichoic acid (WTA) synthetic pathways. Immunofluorescence microscopy revealed that the LytE foci were considerably increased in these mutants. The localization patterns of LytE on the sidewalls appeared to be helix-like in LTA-defective or WTA-reduced cells and evenly distributed on WTA-depleted or -defective cell surfaces. These results strongly suggested that LTA and WTA affect both $\sigma^D$-dependent expression and localization of LytE. Interestingly, increased LytE localization along the sidewall in the ltaS mutant largely occurred in an MreBH-independent manner. Moreover, we found that cell surface decorations with LTA and WTA are gradually reduced at increased culture temperatures and that LTA rather than WTA on the cell surface is reduced at high temperatures. In contrast, the amount of LytE on the cell surface gradually increased under heat stress conditions. Taken together, these results indicated that reductions in these anionic polymers at high temperatures might give rise to increases in SigD-dependent expression and cell surface localization of LytE at high temperatures.

IMPORTANCE
The bacterial cell wall is required for maintaining cell shape and bearing environmental stresses. The Gram-positive cell wall consists of mesh-like peptidoglycan and covalently linked wall teichoic acid and lipoteichoic acid polymers. It is important to determine if these anionic polymers are required for proliferation and environmental adaptation. Here, we demonstrated that these polymers affect the expression and localization of a peptidoglycan hydrolase LytE required for lateral cell wall elongation. Moreover, we found that cell surface decorations with teichoic acid polymers are substantially decreased at high temperatures and that the peptidoglycan hydrolase is consequently increased. These findings suggest that teichoic acid polymers control lateral peptidoglycan hydrolysis by LytE, and bacteria drastically change their cell wall content to adapt to their environment.

The cell wall (CW) of Gram-positive bacteria is responsible for maintaining cell shape and bearing environmental stress. The CW consists of mesh-like peptidoglycan (PG) and covalently linked anionic polymers such as teichoic acids (TAs) (1). PG has a basic mesh-like structure composed of long glycan strands cross-linked by peptide side chains. Anionic polymers are either covalently attached to the PG (wall teichoic acid [WTA]) or anchored to the membrane lipids (lipoteichoic acid [LTA]) (1, 2). In Bacillus subtilis 168, the WTAs are composed of major and minor forms, and both are tethered to peptidoglycan via a linkage unit (3). The main chains of the major and minor WTAs consist of glycerol phosphate (GroP) and glucosyl-N-acetylgalactosamine 1-phosphate (GlcGalNAcP) repeats, respectively.

LTA in B. subtilis consists of poly(GroP) polymers linked on the cytoplasmic membrane via a glycolipid anchor, diglycosyl-diacylglycerol (Glc$_2$-DAG) (4, 5). The anchor synthesis is catalyzed by the glycosyltransferase UgtP, and the main chain is mainly synthesized by LtaS (5). The loss of LtaS activity in B. subtilis impacts cell division, cell morphogenesis, and divalent cation homeostasis (6). In addition to these phenotypes, a quadruple ltaS homolog mutant shows a loss of LTA (7), an aberrant twisted morphology, slower growth (6), and reduced adsorption of rare earth elements (8, 9). Moreover, we reported that $\sigma^D$-dependent transcription of lytF, which encodes a DL-endopeptidase that functions in cell separation, is reduced in an ltaS mutant and is nearly absent in multiple mutants of ltaS and its homologs (10). Interestingly, we also found that lytF transcription was repressed in tagO-null mutant cells, which lack WTA. These results indicate that LTA and WTA, which are similar anionic polymers with a poly(GroP) backbone located on the B. subtilis cell surface, are required for temporal $\sigma^D$-dependent lytF expression. Moreover, both WTA and LTA hinder LytF localization in the cylindrical part of the cell.
of the cell (10, 11). In addition to septum PG digestion by LytF and CwlS, sidewall PG hydrolysis, which is required for nascent PG incorporation, is catalyzed by LytE and CwlO during vegetative growth (12, 13). Both LytE and CwlO are in the WalRK regulon (14). In addition, lytE transcription is regulated by σI, which is required for heat shock adaptation (15, 16) and is enhanced at high temperatures (17). Moreover, the β-lactamopeptidase activity of LytE is essential for survival at high temperatures (17). It is thought that LytE digests not only septum PG but also sidewall PG to allow newly synthesized PG precursors to be incorporated (12). The sidewall localization of LytE appears to be governed by an interaction between the C-terminal catalytic domain of LytE and the actin homologue MreBH (12). Furthermore, it has been reported that a colO ΔlytE double mutant strain is not viable and that cells lacking LytE and depleted for CwlO exhibit defects in lateral CW synthesis and cell elongation (14). In addition, recent reports have revealed that CwlO is also localized along the sidewall (13, 18). Interestingly, the localization and activity of CwlO are controlled by an ABC transporter-like component, FtsEX, located on the cytoplasmic membrane (18, 19).

Previous reports have shown that cells lacking LTA grow slowly and have an aberrant twisted chain morphology (6, 10). In addition, when WTA was abolished, cells grew slowly, showed a swelled morphology, and formed clumps (20). Thus, we presumed that the loss of LTA or WTA affects not only cell division and separation but also lateral CW elongation. In this study, we investigated the effects of the anionic polymers LTA and WTA on the expression and localization of the β-lactamopeptidase LytE, which is required for lateral PG hydrolysis during cell elongation. Moreover, we found that cell surface decorations with LTA and WTA are decreased at high temperatures.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table S1 in the supplemental material. For the localization analysis of a FLAG tag fusion of LytE, B. subtilis WECF6 (22), was used as the parent strain throughout this study. B. subtilis strains were grown in Luria-Bertani (LB) medium (21) at 37°C unless otherwise noted. When necessary, chloramphenicol, kanamycin, spectinomycin, tetracycline, and erythromycin were added at final concentrations of 5, 5, 100, 10, and 0.3 μg/ml, respectively. To culture the conditional mutants of lytE, mreBH, sigI, and sigH, the expression and localization of the β-lactamopeptidase LytE, which is required for lateral PG hydrolysis during cell elongation, was confirmed by PCR to obtain strain WECF6FL (wprA epr lytE-6×FLAG).

To construct the PamyE-lacZ, PamyO-lacZ, and PamyC-lacZ fusions at the amyE locus, the 5′-flanking regions of lytE, mreBH, and cwlO were amplified with two sets of primers, PhytE-EF and PIE-SmR for lytE, PmreBH-LHZ-EF and PmreBH-LHZ-SmR for mreBH, and PcwlO-Ef and PcwlO-SaSr for cwlO using B. subtilis 168 chromosomal DNA (chrDNA) as the template. After the amplified lytE, mreBH, and cwlO fragments were digested with EcoRI and Smal, they were cloned into the corresponding sites of pDHAFBLZ (24) to generate pPEIzL, pPmreBH-LHZ, and pPCOLz, respectively. The plasmids were linearized with Scal and transformed into B. subtilis to construct strains YK3001 (PamyE-lacZ), YK3003 (PamyO-lacZ), and YK3004 (PamyC-lacZ).

To construct disruption mutants in sigI and sigH, the internal regions of these genes were amplified with primers SIGId-HF and SIGId-Br for sigI and SH-HF and SH-BR for sigH using B. subtilis 168 chrDNA as the template. After digestion with HindIII and BamHI, the sigI and sigH fragments were cloned into the corresponding sites of pM4AZ (10) to generate pM4ΔZsigId and pM4ΔZsigHd, respectively. The resulting plasmids were transformed into B. subtilis to generate strains sigIdΔ (sigI:: pM4ΔZsigId) and sigHdΔ (sigH:: pM4ΔZsigHd).

To construct an mreBH mutant, a DNA fragment containing the upstream and downstream regions of mreBH was amplified with primers mreBHSalF and mreBHBSmr using pBmreBH (11) as the template. After digestion with SalI and Smal, the fragment was cloned into the corresponding sites of pMAD (25) to generate pMADmreBH. To obtain an mreBH mutant (TK2202) without any antibiotic resistance genes, we used a previously described efficient allelic replacement method (25). The mreBH locus of the clean mutant was checked by PCR and sequencing with primers mreBH-UP and mreBH-DN.

To construct a colO-null mutant, an internal region of colO was amplified by PCR using B. subtilis 168 chrDNA as the template and primers pQEcEc-Fw and KR-YVCE. The amplified fragment was digested with EcoRI and KpnI, and the digested fragment was cloned into the corresponding sites of pM4 (25) to generate pM4mreBHF. The kanamycin resistance cassette of pDG780 (digested with HindIII) was cloned into the HindIII site of pB-cwlo to obtain pB-cwlo. The resulting plasmid was linearized with BamHI and transformed into B. subtilis to obtain the OKD (colO-kan) strain.

β-Galactosidase assay. After the cells were cultured at 37°C, samples were withdrawn at various time points to assay the β-galactosidase activity. The β-galactosidase activity (expressed as units per milligram of protein) was measured and calculated as described by Shimotsu and Henner (26). One unit of β-galactosidase activity was defined as the amount of enzyme necessary to release 1 nmol of 2-nitrophenol from O-nitrophenyl-β-D-galactopyranoside (ONPG) in 1 min at 28°C. The assay was performed at least three times independently.

Immunofluorescence microscopy. The sample preparation for immunofluorescence microscopy (IFM) was described previously (10, 23) and used with minor modifications, as follows. An anti-DYKDDDDK tag monoclonal antibody (1:400; Wako Pure Chemical Industries) was used as the primary antibody, and an Alexa Fluor 555 F(ab′)2 fragment of goat anti-mouse IgG (H+L) (1:1,200; Life Technologies) was used as the secondary antibody. The lysosome treatment just before the sample was spotted onto a poly-l-lysine-coated microscope slide (23) was omitted to avoid exfoliation of the LytE-6×FLAG protein. Fluorescence microscopy was performed as described previously (10) with an Axio Imager M1 microscope, a Plan-Apochromat Fluorite differential interference objective (magnification, ×63; numerical aperture, 1.4), and standard filter sets for visualizing rhodamine (for Cy3 or Alexa Fluor 555). The exposure time was 0.04 s for differential interference contrast microscopy and 0.005 to 0.1 s (gain 2) for Alexa Fluor 555. The cells were photographed with a charge-coupled device camera (AxioCam MRm; Carl Zeiss) using AxioVision software (version 4.6; Carl Zeiss). The three-dimensional deconvolution utility of the AxioVision software was used for z-axis imaging. All
images were processed with AxioVision and Adobe Photoshop. ImageJ v.1.47 (National Institutes of Health) was used for a quantitative analysis. Fluorescence was quantified as described in a previous report (18). Fluorescent images were basically taken with the same acquisition settings, but the exposure time was appropriately varied so that the fluorescence was not saturated. The averaged value of the fluorescence intensity was calculated in segments of equal size on the lateral walls or the septa of the cells (>50 cells).

SDS-PAGE and Western blot analysis of the cell surface proteins and LTA. Cell surface proteins were prepared as described previously (27). Briefly, 200 μl of 2 × SDS-PAGE sample buffer (28) was added to the cell pellet, corresponding to 1 unit at optical density at 600 nm (OD600). The cell suspension was boiled for 10 min, and the proteins were separated by SDS-PAGE in 12% (wt/vol) polyacrylamide gels as described by Laemmli (28). After electrophoresis, Western blot analysis of the LytE-6×FLAG fusion protein was performed as described previously (23). An anti-DYKDDDDK tag monoclonal antibody (Wako Pure Chemical Industries) was used as the primary antibody, and an anti-mouse IgG, horseradish peroxidase (HRP)-linked whole antibody (GE Healthcare) was used as the secondary antibody (both at 1:10,000 for FLAG-6×FLAG). Western Lightening ECL Pro kit (PerkinElmer), according to the manufacturer’s instructions.

Preparation of CW, PG, and WTA. The cell wall (CW) and peptidoglycan (PG) of the B. subtilis strains were prepared as described previously (11, 29, 30). The amount of PG was calculated by measuring the OD600 value. One (OD600/μl) unit is equivalent to 6.45 mg/ml of PG (11). Cell wall teichoic acid (WTA) was extracted using two methods with either trichloroacetic acid (TCA) or NaOH (31). The phosphorus content was determined by methods described in previous reports (32, 33) and was converted into the amount decorating 1 mg of PG. Polyacrylamide gel electrophoresis of WTA was performed as described previously (31). After electrophoresis, WTA was visualized by the alcian blue-silver stain method (34).

RESULTS

lytE transcription is enhanced in mutants defective for LTA or WTA. As described in our previous report, σ32-dependent transcription of lytE was repressed in mutants lacking LTA or WTA (10). B. subtilis produces another Nα-endopeptidase, LytE, which is responsible for not only cell separation but also cell elongation during vegetative growth (12, 23, 35, 36). Interestingly, lytE transcription is enhanced when the ltaS gene is disrupted. The β-galactosidase activity driven by the lytE promoter was at least 2-fold higher in the ltaS mutant than in wild-type B. subtilis 168 cells (Fig. 1A). Previous reports demonstrated that the lytE gene has two promoters, PsigH and PsigL (17, 35). Here, we tried to determine which promoter is required for induction of lytE expression in ltaS-deficient cells. To address this, we introduced a sigH mutation into an ltaS-null mutant background. As a result, lytE induction was almost completely lost (see Fig. S1B in the supplemental material). In contrast, a sigH mutation in the ltaS mutant had little effect on lytE transcription during early vegetative growth. These results suggest that σ2-dependent lytE transcription is elevated in the ltaS mutation. Moreover, transcription from PsigL was slightly affected in a triple mutant strain with mutations in three ltaS homologues (yfnI, yqgS, and yvgJ) (Fig. 1A), suggesting that the LTA polymer synthesized by LtaS is a main regulator of vegetative lytE transcription. We also examined lytE expression in strains harboring mutations in pgcA, gtaB, and ugtP, which are involved in the glycolipid anchor biosynthesis pathway (4, 37–39). As shown in Fig. S1A in the supplemental material, lytE transcription was clearly increased in the pgcA, gtaB, and ugtP mutants compared with the expression in the wild-type strain.

Next, we tested whether multiple mutations in ltaS and its homologues influence lytE expression. As shown in Fig. 1A, the peak lytE expression in a double ltaS yfnI mutant was reduced and occurred earlier during vegetative growth than the expression in an ltaS single mutant. This suggests that the longer LTA polymers produced by YfnI in the ltaS mutant cells (7) also affect lytE tran-

FIG 1 Effect of mutations in genes involved in the teichoic acid biosynthetic pathway or the modification pathway on lytE transcription. Cultures were inoculated at a starting optical density at 600 nm (OD600) of 0.001, unless otherwise noted. Growth (OD600) and β-galactosidase activity are represented by dotted and solid lines, respectively. The experiments were performed three times with similar results. (A) β-Galactosidase activity of PsigL-iaaZ in the wild type and ltaS homologue mutants: YK3001 (wild type; ○), YA1715 (ltaS; □), YK2307 (ltaS yfnI; ●), YK2236 (ltaS yfnI ygS; △), YK2184 (ltaS yfnI ygS ygF; ▲), and YK2157 (yfnI ygS ygF; ■). For the multiple mutants, cultures were inoculated at OD600 of 0.002 (YA1715, YK2307, and YK2236) or 0.003 (YK2164) because they grew slowly. (B) β-Galactosidase activity of PsigH-iaaZ in the wild type and WTA biosynthesis mutants: YK3001 (wild type; ○), YK2234 (tagE; ●), YK2377 (ggaaAB; □), and YK2145 (tagO; △). For the tagO-null mutant (YK2145), the culture was inoculated at a starting OD600 of 0.005 because it grew slowly.
scription. In addition, the transcriptional activities from the P_{SIG} promoter in triple ltaS yfnI yggS and quadruple mutants were slightly enhanced compared with the expression in the ltaS yfnI double mutant (Fig. 1A). Moreover, lytE transcription in a tagO-null mutant, in which WTA is absent, is approximately 4-fold higher than that in the wild-type strain (Fig. 1B). Likewise, transcription of mreBH, which is another \( \sigma^1 \)-dependent gene (40), was enhanced in the LTA and WTA mutants (see Fig. S2A in the supplemental material). These results suggest that impairment of cell surface decoration by anionic polymers greatly influences \( \sigma^1 \)-dependent lytE transcription. In contrast, lytE transcription was barely altered in cells absent minor WTA (gguAB mutant) and glycosylation (tagE mutant) and only slightly reduced in cells missing \( \beta \)-alanation (dltA mutant) (Fig. 1B; see also Fig. S1A in the supplemental material).

Both LTA and WTA control the cell surface localization of LytE. We showed that LTA or WTA deficiency results in considerable enhancement of lytE transcription from the \( \sigma^1 \)-dependent promoter (Fig. 1; see also Fig. S1A and B in the supplemental material). Next, we examined whether these enhancements affect the cell surface localization of LytE. To test this, we analyzed the localization of 6×FLAG-tagged LytE in the cell surface fraction by Western blotting. The results clearly showed a larger amount of LytE in the cell surface fraction of the ltaS mutant than that of wild-type cells and the amounts of LytE in the double, triple, and quadruple mutants were gradually increased compared to that in the ltaS single mutant (Fig. 2A). To confirm these observations, we carried out an immunofluorescence microscopy (IFM) experiment with cells expressing the LytE-6×FLAG fusion protein. As described in previous reports (12, 13), LytE localized not only to the septum and the poles but also to the sidewall in a helix-like manner across the long axis (Fig. 2B and C). Interestingly, a considerable increase in the LytE foci was observed in the ltaS mutant (Fig. 2B), and the helix-like pattern was not altered (Fig. 2C). In addition, the signal intensities of LytE foci in the multiple gene mutants were slightly increased compared to that in the ltaS single mutant (Fig. 2B), which was consistent with the results of the Western blot analysis (Fig. 2A). However, the localization patterns in the multiple mutants have appeared not to alter compared to that in the ltaS mutant (Fig. 2C). Next, we examined LytE localization in the glycolipid anchor biosynthesis pathway mutants. In the ugpP mutant, the amount of LytE-6×FLAG on the cell surface was higher than that in the wild-type strain (Fig. 2A). Likewise, IFM showed that the LytE foci in the ugpP mutant were stronger than those in the wild-type cells (Fig. 2D). Very similar increases in the LytE amount were observed in the gtaB and pgcA mutants (Fig. 2A and D). Moreover, a large amount of LytE-6×FLAG was detected in tagO-null mutant cells lacking WTA (Fig. 3A). An IFM image of the tagO-null mutant showed that the LytE foci are fairly evenly distributed on the cell surface (Fig. 3B). However, it was difficult to clearly observe the LytE localization in the tagO-null mutant since these cells formed clumps. Therefore, we performed similar localization analyses with a tagO conditional mutant strain. The results clearly showed that the LytE levels gradually increased as TagO expression was reduced (Fig. 3A). Moreover, we found that the increased fluorescence of the LytE foci were unevenly localized on the TagO-depleted cell surfaces (0.08 and 0.04 mM IPTG) (Fig. 3C), but the foci were fairly evenly distributed on the TagO-deficient cell surfaces (0 mM IPTG) (Fig. 3C). Taking our results together, we concluded that both LTA and WTA affect the amount of LytE on the cell surface at the level of transcription and localization.

Increased LytE localization in the ltaS mutant is largely independent of MreBH and is suppressed by YqgS induction. A previous report showed that sidewall LytE localization depends on the physiological interaction between the C-terminal DLL-endopeptidase domain of LytE and the actin homologue MreBH (12). In addition, both lytE and mreBH are in the SigI regulon (17, 40). In the previous section, we showed that \( \sigma^1 \)-dependent transcription of lytE and mreBH were activated in an ltaS mutant (see Fig. S1B and S2A in the supplemental material). In contrast, an mreBH mutation did not affect lytE transcription (T. Kondo, unpublished data). Thus, we examined whether the increased LytE localization along the sidewall of the ltaS mutant is controlled in an MreBH-dependent manner. Very interestingly, the amount of LytE in the cell surface fraction of the ltaS mreBH double mutant was very similar to that of the ltaS mutant (Fig. 4A). In addition, no distinguishable difference was observed in the localization patterns along the sidewall in these mutants (Fig. 4B). Only the LytE foci along the sidewall in the ltaS mreBH double mutant were slightly reduced compared to the foci in the ltaS mutant, whereas the foci at the septum and poles were slightly increased (Fig. 4B). Taken together, these results appear to suggest that increased LytE localization in the ltaS mutant might be largely governed in an MreBH-independent manner.

Our previous report revealed that artificial expression of YqgS, which has both LTA primase and LTA synthase activities (7), in an ltaS mutant restored the normal rod-shape morphology, SigD-dependent expression, and septal localization of LytF (10). Thus, we examined whether artificial expression of YqgS also suppressed the enhanced SigI-dependent expression and lateral localization of LytE in the ltaS mutant. The results indicated that the transcription level of lytE in YqgS-induced cells in an ltaS genetic background was suppressed to that in wild-type cells but those in YfnI- and YvgJ-induced cells were not (see Fig. S2B in the supplemental material). In addition, Western blotting and IFM analyses indicated that the amount of localized LytE in the ltaS genetic background was restored only by YqgS induction (Fig. 4C and D). Taken together, these results clearly indicate that the LtaS paralog YqgS is able to complement the physiological roles of LtaS in lateral PG hydrolysis.

Cell surface decorations with LTA and WTA were decreased at high temperatures. In this report, we found that defects in LTA or WTA enhance SigI-dependent lytE transcription (Fig. 1; see also Fig. S1B in the supplemental material). A similar enhancement of lytE transcription has been reported under heat stress (17, 41). Therefore, we assessed LytE localization at high temperatures and found that the amount of LytE in the cell surface fractions gradually increased under heat stress (Fig. 5B). Moreover, IFM showed that the LytE foci on the sidewall at 52°C were obviously stronger than those at 37°C and were gradually enhanced as the culture temperature increased (Fig. 5A). The localization pattern of LytE at 52°C appeared to resemble the patterns in the ltaS mutant and the TagO-reduced cells (Fig. 2B and C and 3C). These results suggest that cell wall decorations by LTA or WTA might be reduced at high temperatures. To verify this, the amount of phosphate in the CW was measured when cells were grown at different temperatures. The results showed that the amounts of phosphate in the CW at 42°C and 47°C were lower. 
than that at 37°C and the amount at 52°C was considerably (approximately 45%) lower (Fig. 5C). To confirm these findings, we performed a native PAGE analysis of the WTA polymer. As shown in Fig. 5D, the length and amount of the WTA polymer from cells cultured under heat stress were lower than those from cells grown at 37°C. Surprisingly, in addition to the reduced WTA, Western blot analysis of LTA showed that LTA decreased as the culture temperature increased (Fig. 5E). Notably, LTA in cells cultured at 52°C was mostly lost. These results indicate that cell surface decorations with WTA and LTA are clearly lower when cells are cultured at high temperatures.
In this study, we found that lytE transcription was considerably increased in a strain containing a mutation in \( ltaS \), which encodes a principal LTA synthase, as well as in strains containing multiple mutations in \( ltaS \) and its homologs (Fig. 1A). The enhancement of lytE transcription observed in the \( ltaS \) mutant appeared to be regulated in the \( \sigma^H \)-dependent manner (see Fig. S1B in the supplemental material). A similar enhancement was observed in single mutants of \( ugpP \), \( ggaB \), \( gtaB \), and \( pgcA \), which encode the enzymes required for synthesis of the LTA glycolipid anchor (see Fig. S1A in the supplemental material), or \( tagO \), which encodes the first enzyme required for synthesis of the linkage unit of WTA (Fig. 1B). These findings suggest that defects in the poly(GroP) main chains of LTA and WTA lead to considerable activation of \( \sigma^H \)-dependent transcription. Interestingly, transcription of \( cwlO \) (YK3004), which encodes another \( \Delta^L \)-endopeptidase required for cell elongation, was little altered in the \( ltaS \) mutant (YK2237) (Y. Kiriyama, unpublished data). This divergence on transcription between lytE and cwlO in the \( ltaS \) mutant may result from a difference in \( \sigma^H \) dependence (42). In cells lacking LTA or WTA, the lateral PG hydrolytic activity of LytE might be insufficient during cell elongation. Therefore, these cells may respond by upregulating lytE transcription. A previous report revealed that the Mg\(^{2+}\) dependence of an \( mbI \) mutant is suppressed by mutations in \( ltaS \) and \( rsgI \), which encodes an anti-sigma factor for SigI (43). In this report, we found that a mutation in \( ltaS \) enhanced \( \sigma^H \)-dependent transcription of lytE and \( mreBH \) (Fig. 1A; see also Fig. S1B and S2A in the supplemental material). Since both mutations occur in an

**DISCUSSION**

In this study, we found that lytE transcription was considerably increased in a strain containing a mutation in \( ltaS \), which encodes a principal LTA synthase, as well as in strains containing multiple mutations in \( ltaS \) and its homologs (Fig. 1A). The enhancement of lytE transcription observed in the \( ltaS \) mutant appeared to be regulated in the \( \sigma^H \)-dependent manner (see Fig. S1B in the supplemental material). A similar enhancement was observed in single mutants of \( ugpP \), \( ggaB \), \( gtaB \), and \( pgcA \), which encode the enzymes required for synthesis of the LTA glycolipid anchor (see Fig. S1A in the supplemental material), or \( tagO \), which encodes the first enzyme required for synthesis of the linkage unit of WTA (Fig. 1B). These findings suggest that defects in the poly(GroP) main chains of LTA and WTA lead to considerable activation of \( \sigma^H \)-dependent transcription. Interestingly, transcription of \( cwlO \) (YK3004), which encodes another \( \Delta^L \)-endopeptidase required for cell elongation, was little altered in the \( ltaS \) mutant (YK2237) (Y. Kiriyama, unpublished data). This divergence on transcription between lytE and cwlO in the \( ltaS \) mutant may result from a difference in \( \sigma^H \) dependence (42). In cells lacking LTA or WTA, the lateral PG hydrolytic activity of LytE might be insufficient during cell elongation. Therefore, these cells may respond by upregulating lytE transcription. A previous report revealed that the Mg\(^{2+}\) dependence of an \( mbI \) mutant is suppressed by mutations in \( ltaS \) and \( rsgI \), which encodes an anti-sigma factor for SigI (43). In this report, we found that a mutation in \( ltaS \) enhanced \( \sigma^H \)-dependent transcription of lytE and \( mreBH \) (Fig. 1A; see also Fig. S1B and S2A in the supplemental material). Since both mutations occur in an
activation of the SigI regulon, the \textit{ltas} mutation appears to suppress the lethal phenotype of the \textit{mbl} mutant. In addition, enhancement of \textit{lytE} expression in the \textit{ltas} mutant was only suppressed by artificial induction of another LTA synthase, \textit{YqgS} (Fig. 4C and D; see also Fig. S2B in the supplemental material), which produces normal-length LTA polymer as \textit{Ltas} (7). In contrast, it has been reported that the LTA synthase \textit{YfnI} synthesizes the longer LTA polymer and that \textit{Ltas} primase \textit{YvgJ} transfers one glycerol phosphate subunit onto the glycolipid anchor (7). In our previous study, we showed that \textit{YqgS} induction completely restored \textit{dlt}-dependent expression and septal localization of \textit{LytF}, which functions as a principal vegetative cell separation enzyme, in an \textit{ltas} mutant (10). These results appear to suggest that induction of \textit{YqgS} in \textit{ltas} mutant cells complements not only the \textit{LytF}-induced cell separation but also the lateral PG hydrolysis catalyzed by \textit{LytE}.

Transcriptional enhancement of \textit{lytE} in \textit{LTA}- or WTA-defective mutants caused the increased amount of \textit{LytE} localized on the cell surface (Fig. 2 and 3). This elevation in \textit{LytE} may result from a synergistic effect of both transcriptional enhancement and an increased \textit{LytE}-attachable region. Interestingly, the patterns of \textit{LytE} localization were helix-like in cells lacking LTA or reduced WTA (Fig. 2C and 3C) and evenly distributed in cells absent WTA (Fig. 3B and C). These localization patterns resemble those of another DL-endopeptidase, \textit{LytF}. Our previous reports revealed that \textit{LytF} was localized to the septum and poles in the wild-type strain (23) and was also localized to the sidewalls in \textit{LTA}- or WTA-deficient mutants (10, 11). The N-terminal LysM motifs of \textit{LytE} are required for specific attachment to PG, and both \textit{LTA} and WTA control the sidewall localization of \textit{LytF} (10, 11). In addition, Hashimoto et al. (13) reported that chimeric proteins containing the N-terminal LysM domain of \textit{LytE} and the C-terminal DL-endopeptidase domains of \textit{LytF} or \textit{CwlS} complemented the important functions of \textit{LytE} in lateral PG hydrolysis. Since cell wall attachment of \textit{LytE} also appears to depend on three tandem LysM motifs in the N-terminal cell wall-binding domain, \textit{LytE} localization may be controlled by \textit{LTA} and WTA in the same manner as \textit{LytF}. This idea is supported by the finding that an \textit{mreBH} mutation barely affected \textit{LytE} localization in the \textit{ltas} mutant (Fig. 4B), suggesting that \textit{LytE} might be localized to the sidewall in a largely \textit{MreBH}-independent manner when \textit{Ltas} is absent. Actually, the \textit{LytF} foci along the sidewall were slightly reduced in the \textit{ltas mreBH} double mutant, whereas the foci at the septum and poles were slightly increased compared with these foci in the \textit{ltas} mutant (Fig. 4B). This suggests that the specific interaction between \textit{LytE} and \textit{MreBH} is dispensable for the localization and function of \textit{LytE} in the \textit{ltas} mutant cells. As support for this presumption, we found that a \textit{cwlo ltas mreBH} triple mutant (JK2553) exhibited growth similar to that of a \textit{cwlo ltas} double mutant (JK2552) (J. Kasahara, unpublished data). This result indicates that the increased \textit{LytE} observed along the sidewall in the triple mutant is fully functional for the lateral PG hydrolysis required for cell elongation in the absence of \textit{MreBH}. These observations appear to be supported by a previous report that depletion of either \textit{CwlO} or FtsEX in cells lacking \textit{MreBH} did not affect cell growth (19).

It is thought that the SigI-dependent genes are mainly involved in heat stress responses (15, 16). Notably, SigI is required for most \textit{lytE} transcription, and the DL-endopeptidase activity of \textit{LytE} is essential for survival under heat stress (17, 41). In this report, we found that loss of the poly(GroP) main chains of \textit{LTA} or WTA at 37°C affects the expression of \textit{lytE} and \textit{mreBH}, which are part of the SigI regulon (Fig. 1; see also Fig. S2A in the supplemental material). In addition, the amount of \textit{LytE} on the wild-type cell
surface gradually increased as the culture temperature increased (Fig. 5A and B). Moreover, the LytE localization pattern at high temperatures is very similar to that in LTA-deficient or WTA-reduced cells (Fig. 2C, 3C, and 5A). These observations suggest that cell surface decorations with LTA and/or WTA might be reduced at high temperatures. Actually, the amount of phosphorus in CW prepared from cells cultured at 52°C was considerably lower (approximately 55%) than that from cells cultured at 37°C (Fig. 5C). The reduced amount of phosphorus appeared to result from decreases in both the total amount and the length of WTA polymer (Fig. 5D). Moreover, LTA in wild-type cells was gradually reduced at increased temperatures and was nearly lost at 52°C (Fig. 5E). Taken together, these findings strongly suggest that transcriptional enhancement of the SigI regulon at high temperatures may result from reduced cell surface decorations with WTA and LTA. In addition, it appears that *B. subtilis* cells drastically change their cell wall content to adapt to the high temperature conditions. More detailed studies are needed to explain why the cell surface decorations with LTA and WTA affect expression of the SigI-dependent transcription and are decreased at high temperatures.
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