Lipoteichoic Acid Is a Major Component of the *Bacillus subtilis* Periplasm

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Cryo-electron microscopy (cryo-EM) of frozen-hydrated specimens allows high-resolution observation of structures in optimally preserved samples. In gram-positive bacteria, this method reveals the presence of a periplasmic space between the plasma membrane and an often differentiated cell wall matrix. Since virtually nothing is known about the composition of its constituent matter (i.e., the periplasm), it is still unclear what structures (or mechanism) sustain a gram-positive periplasmic space. Here we have used cryo-EM of frozen-hydrated sections in combination with various labels to probe the model gram-positive organism *Bacillus subtilis* for major periplasmic components. Incubation of cells with positively charged gold nanoparticles showed almost similar levels of gold binding to the periplasm and the cell wall. On cells whose cell walls were enzymatically hydrolyzed (i.e., on protoplasts), a surface diffuse layer extending ~30 nm from the membrane was revealed. The thickness and density of this layer were not significantly altered after treatment with a nonspecific protease, whereas it was labeled with anti-lipoteichoic acid (LTA) antibodies conjugated to nanogold. Further, the LTA layer spans most of the thickness of the periplasmic space, which strongly suggests that LTA is a major component of the *B. subtilis* periplasm.

**MATERIALS AND METHODS**

**General methods.** *B. subtilis* 168 was grown in tryptic soy broth at 37°C up to mid-exponential phase (optical density at 600 nm, 0.5 to 0.8). Cell wall fragments were isolated from cells broken by a French press, essentially by boiling cell wall fragments in 4% sodium dodecyl sulfate (SDS) for 2 h to remove cytoplasmic and membrane contaminants and then washing in deionized water to remove SDS (18). Protoplasts were generated by first washing cells three times in a phosphate-buffered saline (PBS) buffer (20 mM phosphate, 50 mM NaCl, 10 mM MgCl₂ [pH 7.4]) supplemented with 10% (wt/vol) sucrose and then incubating cells in the PBS-sucrose buffer with 1 mg/ml of lysozyme at 37°C until cells rounded up (usually for 45 to 60 min; the process was monitored with a light phase-contrast microscope).

**Labeling experiments.** For labeling with positively charged undecagold (PCU; Nanoprobe), prewashed pellets of cells (75 μl), cell wall fragments (70 μl), and protoplasts (80 μl) were incubated with 150, 135, and 160 nM PCU, respectively. Cells were incubated for 5 min and then were cryoprotected and immediately frozen, whereas cell wall fragments were further washed three times in buffer before being cryoprotected and frozen (details on cryoprotection and freezing are given below). For labeling of lipoteichoic acid (LTA) on the surfaces of protoplasts, mouse monoclonal anti-LTA immunoglobulin G (IgG) (Abcam) was conjugated to mono-sulfosuccinimidyl-nanogold (MSNHS-nanogold) as recommended by the manufacturer (Nanoprobe), and anti-LTA-conjugated nanogold was separated from unbound nanogold in a microcentrifuge with a cutoff of 50 kDa (Millipore). Enough MSNHS-nanogold was added to 1.25 mg of anti-LTA IgG to give a ratio of 3.5 nanogold particles bound to each IgG molecule. To label LTA on protoplast surfaces, protoplasts were washed twice in the sucrose-PBS buffer (to remove hydrolyzed wall components) and once in a blocking buffer (sucrose-PBS buffer with 0.4% skim milk), incubated with the anti-LTA-nanogold complexes for 40 min in the blocking buffer, and further washed four times in the blocking buffer (to remove unbound IgG-nanogold complexes). As a labeling control, mouse serum IgG (Sigma) was similarly conjugated to nanogold and incubated with protoplasts.

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**Proteolytic digestion of the surface proteins of protoplasts.** Exclusively for this experiment, protoplasts were generated and further treated in buffers containing 3 mM sodium azide. Azide was added to inhibit the translocator motor SecA, thereby minimizing the translocation of proteins, especially of lipoproteins, through the major (Sec) secretion pathway of *B. subtilis* (17, 24). Protoplasts were incubated with 0.5 mg/ml of proteinase K (Sigma) (in the PBS-sucrose buffer) at 37°C for 1 h to proteolytically digest the extracytoplasmic region of membrane-anchored proteins (7, 15) and were further washed three times in the PBS-sucrose buffer to remove the protease.

**Freezing, sectioning, cryo-EM, and image processing.** Cells, cell walls, and protoplasts were cryoprotected in 17% (wt/vol) dextran, 20% dextran, and 10% dextran plus 10% sucrose, respectively (note that sucrose had already been used to generate protoplasts). Dextran was generally used for cryoprotection to ensure that frozen-hydrated sections had no to few crevasses (20, 25). In all cases, the amount of cryoprotectant added ensured good freezing (vitrification) of samples. Specimens were frozen at a high pressure and cryosectioned as described previously (20). Cryo-EM was performed under relatively low electron dose conditions (estimated electron dose, 1,000 to 2,500 e⁻ nm⁻²) in either a Leo 912AB cryo-transmission electron microscope operating at 120 kV and equipped with an in-column energy filter and a Proscan 2,048- by 2,048-pixel slow-scan charge-coupled device camera or a FEI G2 F20 cryo-transmission electron microscope operating at 200 kV and equipped with a Gatan 4,096- by 4,096-pixel slow-scan charge-coupled device camera (18, 19, 20). Images were analyzed using ImageJ software (NIH). Length measurements were corrected for compression as described previously (20).

**RESULTS AND DISCUSSION**

**Negative-charge distribution in the cell envelope.** The cell envelope of *B. subtilis* was initially probed for the distribution of negatively charged sites, a characteristic feature of its wall polymers, i.e., wall teichoic acids and peptidoglycan (which in *B. subtilis* account for almost all of the wall mass) (2, 5, 21). For that purpose, cells were incubated with PCU (total diameter of ~2.5 nm, with a gold core of 0.8 nm) (3). PCU particles were small enough to diffuse through the cell wall network and labeled negatively charged surface components from the outer face of the membrane through the periplasmic space to the outer surface of the wall (Fig. 1A and B). Note that PCU particles are too small to be seen individually, but they resulted in improved contrast of the structures to which they bound to by increasing their intrinsic densities and because of the presence of heavy atoms of gold with better electron-scattering power (compared to the low-atomic-number elements typically found in biological samples). Interestingly, these particles accumulated almost uniformly across the envelope, so that it was difficult to distinguish the interface between the periplasmic space and the cell wall, whereas they did not significantly alter the envelope thickness from that of unlabeled cells (Fig. 1A; appears ellipsoid rather than circular because of compression in the cutting direction. The long arrow indicates the cutting direction, and arrowheads point to crevasses. Contrast in frozen-hydrated sections is produced by density differences in the sample (for details about cutting artifacts and the generation of contrast, see references 1 and 10). (B) The envelope of unlabeled cells is typically seen (in frozen-hydrated sections) with the plasma membrane (PM) surrounded by a low-density periplasmic space (PS), followed by a high-density cell wall (CW). (C) An isolated cell wall fragment labeled with PCU shows increased binding of undecagold to its inner and outer surfaces in regions of good cross section (rectangle). (Inset) Density tracing taken along the marked rectangle in the image, pointing out a higher accumulation of PCU at the wall’s inner and outer surfaces (arrowheads). Bars, 200 nm (A) and 150 nm (B and C).

![FIG. 1. Negative-charge distribution in the cell envelope and cell wall of frozen-hydrated *B. subtilis*.](http://jb.asm.org/)

(A) Frozen-hydrated sections of the cell envelope labeled with PCU show an almost even accumulation of gold nanoparticles across both the periplasmic space and the cell wall, making it difficult to distinguish the interface between them. At regions of good cross section, though, it appears that slightly more PCU accumulated next to the membrane (short arrows) than in the region closer to the outer face of the envelope. This cross section of the cell wall of *B. subtilis* appears ellipsoid rather than circular because of compression in the cutting direction. The long arrow indicates the cutting direction, and arrowheads point to crevasses. Contrast in frozen-hydrated sections is produced by density differences in the sample (for details about cutting artifacts and the generation of contrast, see references 1 and 10). (B) The envelope of unlabeled cells is typically seen (in frozen-hydrated sections) with the plasma membrane (PM) surrounded by a low-density periplasmic space (PS), followed by a high-density cell wall (CW). (C) An isolated cell wall fragment labeled with PCU shows increased binding of undecagold to its inner and outer surfaces in regions of good cross section (rectangle). (Inset) Density tracing taken along the marked rectangle in the image, pointing out a higher accumulation of PCU at the wall’s inner and outer surfaces (arrowheads). Bars, 200 nm (A) and 150 nm (B and C).
TABLE 1. Thickness of cell envelope structures and compartments of B. subtilis

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Thickness (nm) of:</th>
<th>Periplasmic space/surface diffuse layer</th>
<th>Cell wall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells</td>
<td>21.1 ± 2.1</td>
<td>36.4 ± 4.7</td>
<td></td>
</tr>
<tr>
<td>Cells + PCU</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>Cell wall fragments</td>
<td>NA</td>
<td>44.0 ± 7.1</td>
<td></td>
</tr>
<tr>
<td>Cell wall fragments + PCU</td>
<td>NA</td>
<td>41.6 ± 5.8</td>
<td></td>
</tr>
<tr>
<td>Protoplasts</td>
<td>31.8 ± 2.9</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Protoplasts + anti-LTA–nanogold</td>
<td>16.4 ± 2.2</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Protoplasts + proteinase K</td>
<td>26.9 ± 3.4</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

* Each value is the average of 12 measurements ± the standard deviation, unless stated otherwise; measurements were corrected for compression as described in reference 20. ND, not determined. NA, not applicable.

** Uniform labeling of the envelope by PCU made it impossible to distinguish the interface between the periplasmic space and the cell wall (the thickness of the periplasmic space plus the cell wall was 51.7 ± 3.4 nm).

A Average of eight measurements ± standard deviation.

Table 1). Strikingly, PCU labeled the periplasm almost as intensely as it did the cell wall (Fig. 1A and B). In fact, the labeling of periplasmic components showed a slightly higher accumulation of gold nanoparticles than that observed in the central region of the wall (Fig. 1A), indicating that the periplasmic space is filled with negatively charged components readily accessible to binding by PCU.

**Negative-charge distribution in isolated cell walls.** In order to ensure that the accumulation of gold nanoparticles in the periplasmic space was in fact due to the labeling of periplasmic structures and not to the labeling of wall material (such as nascent and non-cross-linked wall material extending toward the periplasmic space), PCU was also incubated with isolated cell wall fragments (from which the membrane and the cytoplasm were removed after mechanical breakage of cells and boiling in SDS [see Materials and Methods]). Isolated cell walls showed a labeling pattern clearer than that observed in the walls of intact cells, in that undecagold accumulated to a greater extent on the inner and outer surfaces of the wall than in its middle region (Fig. 1C). This labeling pattern reflects how the cell wall grows in B. subtilis, with new wall material being added at its inner surface and the old wall being shed by autolytic enzymes at its outer surface (2, 12, 18). In these regions, the wall is less cross-linked and is thus more accessible to labeling by PCU, as observed here. The wall appeared thinner in cells than in cell wall fragments (Table 1), which is consistent with the stretching of wall fibers in the direction perpendicular to the wall surface because of high turgor pressures in cells (wall fragments are not under turgor). Importantly, labeled and unlabeled isolated cell walls had virtually the same thickness (Table 1), indicating that the periplasm, including the negatively charged periplasmic structures (shown in cells by PCU labeling), did not remain associated with the cell wall after SDS treatment.

**Fine details on the membrane surfaces of protoplasts.** Although cryo-EM has not revealed structural features in the periplasmic space of cells that could be associated with the negatively charged periplasmic components observed here (Fig. 1A and B) (18), we attempted to obtain finer details on the membrane surface by hydrolyzing the B. subtilis cell wall under osmotically stable conditions. Protoplasts were generated after hydrolysis of the wall by a lysozyme in an isosmotic solution (Materials and Methods) and, despite not seeing any visible details at a medium magnification, revealed a diffuse layer extending 32 nm from the membrane at a high magnification (Fig. 2A and B; Table 1). This membrane-associated diffuse layer could be detected only in very flat (crevasse-free) regions of frozen-hydrated sections, where cutting artifacts did not obscure fine details, and mostly in regions of maximum compression of the protoplast surface. As shown by density tracings across the envelope of protoplasts (Fig. 2B), this zone is only slightly denser than the background and must thus be very hydrated. Further, compression likely improved its detection by increasing its local density: even though both the surface layer and the background have their respective densities increased by the same factor where compression is maximal, their density differential increases slightly. Protoplasts were also incubated with PCU in an attempt to probe the charge of the diffuse surface layer, but the lack of the cell wall (and likely a strong interaction between PCU and negatively charged surface components) caused a massive rearrangement of the protoplast membranes (data not shown). Yet the presence of a diffuse zone on the surfaces of protoplasts and the intense PCU labeling of the periplasm in cells suggested that LTA developed these features. LTA is a characteristic envelope component of a large number of gram-positive bacteria with an essential role in the physiology of these organisms (11, 13, 16). LTAs are macroamphiphiles made up of long, negatively charged hydrophilic chains [poly(glycerophosphate) in B. subtilis] inserted into the outer leaflet of the membrane through a glycolipid anchor (11). Despite the fact that they occur in much smaller amounts in cells than wall teichoic acids and peptidoglycan (2, 21), their relatively high abundance in the periplasmic space could have resulted in similar levels of PCU accumulation in the periplasmic space and the entangled meshwork (of cross-linked peptidoglycan fibers with wall teichoic acids) of the cell wall.

**Labeling of LTA on the membrane surfaces of protoplasts.** To specifically label LTA at a high resolution on the surfaces of protoplasts, anti-LTA IgG molecules were conjugated to nanogold particles (nanogold has a gold core with a diameter of 1.4 nm and an organic shell that gives it a total diameter of 2.7 nm, while the IgG-nanogold complex has an overall dimension of 15 nm) (14). Immunolabeling of protoplasts with anti-LTA–nanogold caused clumping of protoplasts, which was expected because of the surface localization of LTA and the bivalent nature of IgG binding to epitopes (Fig. 2C). At a medium level of magnification, it was already possible to observe some accumulation of the nanogold complexes along the membrane surface (Fig. 2C). At a high magnification, the accumulation of nanogold complexes either between two adjacent protoplast membranes or on the membrane surface of a noncontacting protoplast clearly marked the presence of LTA in the surface diffuse zone of protoplasts and suggested a high abundance of LTA in this surface zone (Fig. 2D and E). In closely associated protoplasts, a higher-density band apparently composed of small dark dots (representing the anti-LTA–nanogold complexes) was observed between the adjacent membranes, indicating that the binding of LTA’s epitopes took place on
both membranes (Fig. 2D). This likely resulted in a pulling effect on adjacent membranes and in the aggregation of protoplasts seen at a lower magnification (Fig. 2C). Density tracings on the surfaces of noncontacting protoplasts showed a 16.4-nm-wide band, a size comparable to that of the IgG-nanogold complex (Fig. 2E; Table 1). It is possible that this thinner band (compared to the diffuse layer of unlabeled protoplasts) resulted from some limited condensation of the flexible LTA hydrophilic chains upon binding by the bivalent antibody molecules. The grayish band of anti-LTA–nanogold particles appeared slightly apart from the membrane (Fig. 2D and E). This implies either that a higher concentration of epitopes is present near the tip of LTA’s hydrophilic chains or that bulky (15-nm) bivalent IgG-nanogold complexes cross-linked adjacent LTA chains at the surface of the diffuse zone, preventing further labeling of LTA toward the membrane, or both.

**Proteolytic shaving of membrane-associated proteins on the surfaces of protoplasts.** Protoplasts were also treated with a nonspecific protease (proteinase K) in order to estimate the impact of “shaving” of membrane-associated proteins (such as lipoproteins) on the visualization of the diffuse surface layer. Lipoproteins and membrane-anchored proteins very likely represent the only other characteristic components of the *B. subtilis* envelope that could have contributed to the mass of the surface diffuse layer observed here by cryo-EM. Indeed, an increasing number of lipoproteins have been identified on the *B. subtilis* membrane, and like LTA, they also insert their proximal end into the outer leaflet of the membrane and extend their distal end toward the cell wall (23, 24). Proteinase K-treated protoplasts showed a diffuse layer only slightly thinner than that of untreated protoplasts and with a similar level of contrast (Fig. 2, compare panels B and F; Table 1), implying
that (lipo)proteins, while clearly present, are not a major component of the surface diffuse zone.

Our study shows for the first time that *B. subtilis* protoplasts possess a very hydrated, 30-nm-thick surface layer with a high LTA content. This strongly indicates that LTA is a main component of the *B. subtilis* periplasm, since the LTA surface layer is thicker than the periplasmic space (Table 1). Although specific labeling of LTA within the periplasmic space is not possible because of the large size of IgG molecules (~15 nm), another indication of the localization of LTA within this subcellular compartment was given by the intense PUC labeling of the periplasm. LTA is a characteristic envelope component of a large number of gram-positive bacteria. While many functions have been suggested for LTA, such as binding to ligands, metal cation homeostasis, and regulation of autolysis activity (2, 11, 13, 21), it is still unclear why LTA is indispensable for the viability of gram-positive bacteria that express this surface component. The presence of a continuous LTA layer on the membrane, shown here by cryo-EM, suggests a major role for LTA in the overall structural organization of the *B. subtilis* cell envelope. It is possible that interaction between adjacent LTA hydrophilic chains is important for the stability of the membrane. In contrast to the crowded and intertwined cell wall network, a very hydrated periplasm enriched in LTA could provide a medium allowing “easy” diffusion of proteins (including enzymes involved in cell wall synthesis) across and along this subcellular compartment. It is also tempting to speculate that the membrane is kept apart from the cell wall because of charge-charge repulsion between the polyanionic hydrophilic chains of LTA and the highly negatively charged wall polymers. This mechanism would be consistent with the low density observed in the periplasmic space by cryo-EM and the collapse of this compartment seen in resin-embedded sections by conventional EM. (The tight apposition between the membrane and the cell wall observed by the latter method could result from condensation of the hydrophilic chains of LTA by the organic solvents used for specimen preparation.)

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