Cell Envelope of an Iron-Oxidizing Bacterium: Studies of Lipopolysaccharide and Peptidoglycan

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Further structural detail is presented of the cell envelope of the chemolithotroph Ferrobacillus ferrooxidans (Thiobacillus ferrooxidans). Thin sections of purified lipopolysaccharide (LPS) and peptidoglycan show structures comparable to those seen in the envelope of intact cells, whereas negative stains of LPS appear as sheets, or ribbons, or both. The sugars common to LPS, namely, heptose, glucose, galactose, mannose, and 2-keto-3-deoxyoctulosonate, were identified. The cations, iron, calcium, and magnesium, were associated with LPS. The purified LPS had a density of 1.28 and an uncorrected sedimentation coefficient of 99.9S.

Chemolithotrophic bacteria are gram-negative microorganisms possessing a multilayered cell envelope. However, little is known about the structural and chemical details of these layers, because, in chemolithotrophic bacteria, small cell yields have precluded extensive investigations.

Our laboratory has been investigating certain structure-function relationships of iron-oxidizing bacteria and has reported the general anatomy of iron-grown cells (21; Korczynski et al., 1967, Bacteriol. Proc., p. 26), glucose-grown cells (32), the peptidoglycan layer of iron-grown bacteria (31), and a possible function of the cell envelope in iron oxidation (8). The present report describes physical, chemical, and structural characteristics of lipopolysaccharide (LPS) isolated from Ferrobacillus ferrooxidans and an electron micrograph of a thin section of purified peptidoglycan (PG); thin sections were not included in an earlier publication describing the cell envelope (21) and the chemical nature of the peptidoglycan of F. ferrooxidans (31).

MATERIALS AND METHODS

Cell culture. Ferrobacillus ferrooxidans was propagated in 9K medium in 5-gal carboys under forced aeration at 28°C for 54 hr and harvested as described by Silverman and Lundgren (27). Cells at this time are in the late log phase of growth; approximately 6 g (wet weight) of cells is obtained from 96 liters of medium. Cell numbers are about 2 × 108 cells/ml and have a generation time of about 8 hr.

Electron microscopy. A previous report has given methods for chloroform-methanol extraction of cells and the isolation of peptidoglycan (31). Cells (as suspensions), PG, and LPS to be examined as thin sections were fixed with 1.5% glutaraldehyde in S-collidine-hydrochloride buffer (0.05 M, pH 7.6) for 10 min at room temperature, washed once (for 10 min) with the same buffer, and then fixed overnight at room temperature in osmium tetroxide (1.0%) in distilled water (pH 6.2). The aforementioned treatments were selected based upon results of differential staining. These appeared to give the best results. The osmolarity of the fixative was 180 milliosmol, as calculated by using the method of Maser, Powell, and Philpott (14). The fixed cells were embedded in 2.0% agar prepared with distilled water, and the agar was cut into 1-mm cubes. The embedded cells were dehydrated with ethyl alcohol (5 to 100%) and embedded in Epon 812 by the method of Luft (12). Sections were cut on an ultramicrotome with glass knives and then stained with 1.0% uranyl acetate (pH 4.5) for 30 to 60 min at 60°C, followed by lead citrate (22) for 5 min at room temperature. Purified LPS suspended in distilled water was also examined by negative staining by using methods previously described (31). These sections and negative stain preparations were examined in either an RCA EMU-2D or in a JEM-7 electron microscope.

Extraction and purification of lipopolysaccharide. LPS was extracted and purified by the classical method of Westphal (33, 34).
After centrifugation for 30 min at 3,000 X g, the aqueous upper phase was withdrawn, an equal volume of distilled water (preheated to 75 °C) was added to the remaining phases, and the extraction was repeated. The aqueous phase was again withdrawn, pooled with the initially extracted layer and dialyzed against running tap water for 6 hr and against three changes of distilled water for 20 hr. The dialyzed aqueous phase LPS was then lyophilized and weighed.

Lyophilized LPS (50 mg) was dissolved in 7.5 ml of distilled water containing 0.75 ml of 2% hexadecyltrimethylammonium bromide (Cetavlon, Eastman Organic Chemicals, Distillation Products Industries, Rochester, N.Y.); the solution was stirred at room temperature for 15 min before centrifuging at 3,000 X g for 30 min to partially remove ribonucleic acid (RNA; 3).

RNA associated with LPS was estimated to be 3% by assuming that absorbancy at 260 nm was due to nucleic acid and that 50 μg of RNA has an optical density (OD) of 1.0. The supernatant extract was collected, lyophilized, and dissolved in 3.0 ml of 0.5 M NaCl. The LPS solution was then added to 10 volumes of absolute ethanol and held at 0°C for 1 hr until a flocculent precipitate formed. The ethanol mixture was centrifuged, and the pellet was collected and suspended in 4 ml of distilled water. The solution was dialyzed against 20 ml of distilled water for 48 hr against several changes of distilled water in the cold. The dialyzed preparation was lyophilized and stored in a desiccator until used. Different batches of LPS extracted from F. feroxosidans as described above were pooled and used for the subsequent experiments.

Relative density and sedimentation velocity of LPS. The relative density of LPS was determined by moving zone centrifugation by using sucrose. Centrifuge tubes containing 50.9 to 68.0% sucrose were prepared in a total volume of 3.6 ml and allowed to equilibrate overnight. LPS (1.0 mg/1.2 ml of water) was placed on top of the gradient and centrifuged at 60,000 X g for 90 min at 20°C. After centrifugation, LPS was visible as a slight band in the gradient.

The sedimentation velocity pattern of LPS was obtained by dissolving 4 mg of LPS in 1.0 ml of 0.066 M phosphate buffer (pH 7.1). The solution was placed in a double sector cell and centrifuged at 20,410 rev/min for 33 min in a Spinco model E analytical ultracentrifuge. Photographs of schlieren optic patterns were taken on metallographic plates (2 X 10 inch plates, high green contrast, Eastman Kodak Co., Rochester, N.Y.) at various time intervals. All photographic measurements were made on a Nikon enlarger (Nippon, Kogaku, K. K., Japan) with a calibrated stage micrometer.

Chemical analyses of LPS. The phosphorus content of LPS was determined by the method of Chen, Toribara, and Warner (4), and Taussky and Shorr (29). Total nitrogen was determined by conversion of the organic nitrogen in 1 mg of lyophilized LPS to NH₃ by acid digestion and determining NH₃ with Nessler's reagent (30). Iron was determined by the method as described by Suzuki and Silver (28). Magnesium and calcium were estimated by atomic absorption spectrophotometry (Analytical Methods for Absorption Spectrophotometry, Perkin-Elmer, Norwalk, Conn.).

Heptose was determined by the procedure of Dische (7) as modified by Osborn (18). Hexose was determined by a modified anthrone method by using a 2% solution of anthrone in ethyl acetate (9). The method of Aminoff, Morgan, and Watkins (1) was used to determine 2-keto-3-deoxyoctulosonic acid. Hexosamine was determined by the method of Rondle and Morgan (23). Glucose and galactose were determined by using glucostat and galactostat kits, respectively (Worthington Biochemical Corp., Freehold, N.J.).

Gas-liquid chromatography. An F & M model 500 gas chromatograph was used equipped with a hydrogen flame ionization detector and 6-ft, U-shaped, 3% SE-30 column (60 to 90 mesh). The separation was done at 180°C. Sugars were first converted to methyl esters without directly determining yield; 2 mg of LPS was suspended in 20 ml of dry methanolic 0.5 N HCl and methanolysis was done at 80°C for 48 hr in a screw-cap test tube provided with a Teflon liner. Lipid released during methanolysis was removed by extraction with an equal volume of hexane. The extraction was repeated three times. HCl was removed by repeated evaporation of the sample to dryness under reduced pressure. The dried sample was taken up in 2 ml of methanol and passed over a column of Amberlite IR-120 resin in the H form which was pre-washed with methanol. Pooled washings were evaported to dryness under reduced pressure and redissolved in 1 ml of methanol. The final solution was dried under a gentle stream of air. Sugar-O-trimethylsilyl (TMS) derivatives were prepared by the addition of 1 ml of dry pyridine, 0.1 ml of trimethyl chlorosilane, and 0.1 ml of hexamethyldisilazane. The mixture was shaken vigorously for 5 min; 1 to 3 μl of the solution was used for gas chromatographic analysis (5).

RESULTS

Electron microscopy of the cell envelope. Figure 1 shows a thin-section profile of normal iron-grown cells possessing a multilayered cell envelope typical of gram-negative bacteria and in particular, similar to the thiobacilli (13, 26). A detailed description of the organization of the layers in the envelope of the iron-oxidizing chemioautotroph has been given (21). Chloroform-methanol extracted cells show an almost complete removal of the outer layers of the envelope (Fig. 2) except where two cells are firmly attached and apparently protected from the solvent (Fig. 3). The chloroform-methanol extraction removes LPS as determined by chemical analysis. The densely stained PG layer of the cell is still intact after chemical extraction and is identified in these micrographs based upon comparison to normal cells and on previous results of chemical extraction studies (31). Also noted in Fig. 2 and 3 are translucent areas surrounding the polyhedral bodies seen in untreated cells.
FIG. 1. Thin section of an untreated cell of F. ferroxidans showing a multilayer cell envelope. The labels are outer layer (OL) or lipopolysaccharide (LPS); middle layer (ML) or peptidoglycan (PG); polyhedral inclusion body (PB); dense body (DB). The marker bar represents 100 nm.
Fig. 2. Thin section of *F. ferrooxidans* after chloroform-methanol extraction. Nucleus (N), ribosomes (R), peptidoglycan (PG). The marker bar indicates 200 nm. All other bars in the following micrographs are the same.

(Fig. 1). The translucency is believed due to materials lost by solvent extraction. The solvent extraction seems to have damaged the cytoplasmic membrane but this is difficult to prove for even untreated cells are difficult to fix properly. Proper fixation of thiobacilli is a general problem which is still not solved (13, 26).

When phenol-extracted LPS was embedded, sectioned, and examined in the electron microscope (Fig. 4), typical unit membrane structures were seen with an approximate width corresponding to that observed in thin sections of intact cells. The triple-layered structures are generally arranged in elongated and coiled formations of various sizes. The clearly resolved areas of the section of LPS have a measured thickness of about 8 nm. A negatively stained preparation of purified LPS is shown in Fig. 5. The large perfo-
rated sheetlike structures and ribbon networks are typical of phenol-extracted LPS from *Salmonella typhimurium* (24, 25). No explanation is available for the holes in the sheets but this appearance was consistent for many preparations.

It has been reported previously that the firmly bound globular protein layer associated with the underneath peptidoglycan layer can be removed by successive treatments consisting of enzymes, formic acid extraction, phenol extraction, and a final wash with copper ethylenediamine. The insoluble material recovered is a fairly pure peptidoglycan (31). A thin section of the isolated peptidoglycan is shown in Fig. 6. This rigid layer still retains its rod shape and measures about 4.5 nm in width; this width closely agrees with the dimension of the peptidoglycan layer seen in thin sections of untreated cells (Fig. 1). The thin-section profile appearance of the rigid layer resembles that from boiled *Escherichia coli* cells, as reported by DePetris (6), and from *E. coli* B cells, as reported by Frank and Dekegel (11).

**Physical and chemical properties of LPS.** LPS extracted by phenol-water accounted for approximately 4 to 6% of the dry weight of the cells. The amount of contaminating nucleic acid initially present was estimated to be 3%; this was reduced to 1.6% after Cetavlon precipitation. This level of contaminating RNA agrees with the level of nucleic acid in purified LPS described by Burton and Carter (2).

The LPS has a relative density of 1.28 based on results of sucrose gradient centrifugation and was visible as a single band in the 59.4% sucrose fraction. The LPS had a sedimentation coefficient of 99.9S (uncorrected) and an $S_{20, w}$ of 105 when corrections were made for the viscosity and density of the buffer (Fig. 7). The latter value was based on a calculated partial specific volume (V) of 0.79 cm$^3$/g for the LPS (derived from its relative density of 1.28). The concentration dependence of the sedimentation coefficient was not checked.

Results of chemical analyses of LPS for sugars are shown in Table 1. Fig. 8 shows a profile of a gas-liquid chromatogram of the sugar components. The assignment of each sugar to a particular peak is based on the retention time of an $\alpha$-methyl-D-glucoside standard (Pfanstiehl Chemical Co., Waukegan, Ill.) given an arbitrary value of 1.0. The brackets depict the $\alpha$ and $\beta$ isomers of the sugars. The sugar components identified in
the LPS are similar to those found in the LPS isolated from gram-negative heterotrophs (19). Nitrogen and phosphorus were present in LPS as was iron which was mostly in the ferric form. Other cations present were calcium and magnesium (Table 2).

**DISCUSSION**

An understanding of structure-function relationships in the cell envelope and how they might pertain to chemolithotrophy is an overall aim of our research. Progress has been made towards an understanding of how the cell envelope is constructed (21, 31), but understanding its function requires considerably more research.

Two of the major constituents of the envelope (LPS and PG) appear to be structurally similar to those of gram-negative heterotrophs. Sections of isolated PG and LPS are consistent with the "murein sacculus" and LPS isolated by Frank and Dekegel (10, 11) and the peptidoglycan layer of *E. coli* reported by DePetris (6). The present results confirm the stainability of the PG layer in intact cell envelopes as suggested by
Negatively stained purified LPS from *F. ferrooxidans* showing the ribbons and sheets of LPS.

Murray, Steed, and Elson (16). Sections of purified LPS showed structures of various sizes often seen in trilamellar arrangements comparable to those seen in intact cells. Negatively stained preparations showed ribbon-like structures which assume various conformations depending upon the physical environment. The long ribbon-like structures are similar to LPS structures reported by Shands, Graham, and Nath (25) except for the trilamellar structures which were absent in negative stains.

Preliminary results of chemical assays of sugars, detected by both gas chromatography and chemical analysis of LPS, indicate a similar composition...
of the basic sugars to LPS from *E. coli* and various *Salmonella* (19). However, at this time we know relatively little else about the sugar components and how they are organized in LPS.

Physically, the LPS macromolecule, as isolated by phenol-water extraction, is a large molecule of a molecular weight in excess of a million. This value, along with its density of 1.28, agrees with that of purified LPS isolated from a heterotrophic bacterium (17). Iron, mostly in the ferric form, was associated with the LPS, suggesting that LPS might serve as the initial binding site for the energy substrate (Fe$^{3+}$) of the organisms. This idea is currently under investigation. Other cations as magnesium and calcium were also associated with LPS but their function is unknown.

**ACKNOWLEDGMENTS**

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FIG. 7. Sedimentation velocity pattern of phenol extracted LPS from F. ferrooxidans. The sample was centrifuged at 20,410 rev/min at 20 C. Approximately 4 mg of LPS was dissolved in 0.066 M phosphate buffer (pH 7.1). Time (min) after attaining speed: (A) 16, (B) 20, (C) 24, (D) 28. The bar angle was 65 C. The sedimentation coefficient of the LPS was corrected for the viscosity and density of the buffer. The partial specific volume ($\bar{V}$) of the LPS was calculated from the relative density of the macromolecule (1.28), where $1/1.28 = \bar{V}$.

TABLE 1. Sugar composition of lipopolysaccharide (LPS) from Ferrobacillus ferrooxidans

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>LPSa (%)</th>
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</thead>
<tbody>
<tr>
<td>Hexose (total)</td>
<td>39.2</td>
</tr>
<tr>
<td>Heptose</td>
<td>12.7</td>
</tr>
<tr>
<td>Hexosamine</td>
<td>4.5</td>
</tr>
<tr>
<td>2-Keto-3-deoxyoctulosonate (KDO)</td>
<td>8.7</td>
</tr>
<tr>
<td>Glucose</td>
<td>3.0</td>
</tr>
<tr>
<td>Galactose</td>
<td>6.5</td>
</tr>
</tbody>
</table>

* Values of analyses here and in Table 2 represent averages determined from at least three separate determinations which were done on samples of LPS extracted from different batches of cells.

+ Assumed that when OD$_{445}$ minus OD$_{425}$ = 1.07, then l-glycero-d-mannoheptose = 1.0 mole.
+ Assumed KDO had a molar extinction coefficient of $72 \times 10^{-4}$ and a molecular weight of 236.

LITERATURE CITED


Fig. 8. Sugar components of LPS from F. ferroxidans. Sugars were determined by gas chromatography in an instrument (model 500) from the F & M Scientific Corp., Avondale, Pa., by using a 6-ft, U-shape, 3% SE-30 column, at 180 C. One microliter of sample was injected, the sensitivity range was 10, and the attenuation control was set at 32. No KDO was detected by this method, for the sugar is destroyed during methanolysis.

**Table 2. Elemental analysis of lipopolysaccharide (LPS) from Ferrobacillus ferroxidans**

<table>
<thead>
<tr>
<th>Element</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrogen</td>
<td>9.25</td>
</tr>
<tr>
<td>Phosphorus</td>
<td>3.89</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.13</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1.31</td>
</tr>
<tr>
<td>Fe⁺⁺ and Fe⁺⁵⁺</td>
<td>83.00</td>
</tr>
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</table>