Cell Wall and Lipid Composition of Isosphaera pallida, a Budding Eubacterium from Hot Springs

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Isosphaera pallida is an unusual gliding, budding eubacterium recently isolated from North American hot springs. Electron micrographs of ultrathin sections revealed a cell wall atypical of eubacteria: two electron-dense layers separated by an electron-transparent layer, with no evident peptidoglycan layer. Growth was not inhibited by penicillin. Cell walls were isolated from sheared cells by velocity sedimentation. The rigid-layer fraction, prepared from cell walls by treatment with boiling 10% sodium dodecyl sulfate, was hydrolyzed and chemically analyzed for muramic acid. This essential component of peptidoglycan was absent. Amino acid analysis demonstrated a proteinaceous wall structure. Pitlike surface structures seen in negatively stained cells and thin sections were correlated with periodically spaced perforations of the rigid sacculus. An analysis of the lipid composition of I. pallida revealed typical ester-linked lipids with unbranched fatty acids, in contrast to the isoprenyl ether-linked lipids of archaea, which also have proteinaceous cell walls. Capnoids, unusual sulfonolipids which are present in gliding bacteria of the Cytophaga-Flexibacter group, were absent.

Isosphaera pallida is a recently described aerobic, heterotrophic bacterium found in hot springs (S. J. Giovannoni, E. Schabtach, and R. W. Castenholz, Arch. Microbiol., in press). Before it could be cultured, this organism was known as a cyanobacterium (Isocyistis pallida Woronichin [22]) and later as a yeast (7, 8), on the basis of morphology and habitat.

In numerous respects I. pallida is phenotypically unusual. It is the only budding bacterium known to glide and the only heterotrophic bacterium known to be phototactic. Under some conditions cells form motile, macroscopic aggregates, or “comets” (4). I. pallida also has an unusual cell wall ultrastructure and is resistant to β-lactam antibiotics, suggesting that it does not have a peptidoglycan cell wall.

On the basis of traditional phenetic taxonomies, it has been difficult to infer an appropriate taxonomic position for I. pallida; no described group of budding bacteria shares the unusual constellation of traits and morphology found in this organism. However, the Planctomyces group of budding bacteria is known to have protein cell walls from which the macromolecular heteropolymer peptidoglycan is absent.

Among procaryotes, nonpeptidoglycan walls have, until recently, been regarded as a feature of the archaeabacterial kingdom (16). It is now known that members of the genus Chlamydia as well as of the Planctomyces group (15) lack muramic acid, a key structural component of peptidoglycan. 16S rRNA oligonucleotide catalogs from members of the Planctomyces group show low homologies to catalogs of other eubacterial groups, leading to speculation that these organisms might have diverged from the eubacterial line of descent before the evolutionary appearance of peptidoglycan (19).

Thus far, precise relationships among the major eubacterial groups (i.e., branching orders) have not been resolved by comparative sequence analyses, although such analyses are likely to be forthcoming. Additional biochemical information, as well as comparative sequence analyses, will be needed before the identification of ancestral eubacterial groups can be achieved.

With these problems in mind, we have examined the structure and composition of the I. pallida cell wall in detail. We have also investigated the lipids of this organism to determine if phytyl glycerol ether lipids, similar to those of archaea, or capnoids, lipids implicated in the mechanism of gliding motility in the Cytophaga-Flexibacter group of bacteria (1), are present.

MATERIALS AND METHODS

Culture conditions. I. pallida 1SI1, isolated from Kah-neetah Hot Springs in Oregon, was used in all experiments, unless otherwise indicated. Cultures were grown as described elsewhere (Giovannoni et al., in press) and harvested in the exponential growth phase. Lyophilized cells of Methanobacterium thermoautotrophicum were kindly provided by Ralph Wolfe.

For sulfonolipid determination, cells were uniformly labeled with Na235SO4 in a defined medium containing glucose (medium IMG with carrier sulfate omitted [Giovannoni et al., in press]). Each 250-ml culture contained 0.5 mM of Na235SO4 at a specific activity of 0.5 mCi/mmol.

Isolation of whole-cell-wall and rigid-layer fractions. Cell pellets were suspended in 2 volumes of 50 mM sodium phosphate buffer (pH 7.5) with 1 mM MgCl2 and 5 μg of DNase I (Sigma Chemical Co.) per ml. Cells were broken by a single passage through a French pressure cell at 21,000 lb/in². The whole cell wall and rigid-layer fractions were prepared as described by Schrader et al. (18), except that the pronase treatment was omitted.

Chemical analysis of the cell wall. Samples of the cell wall rigid layer, with and without added muramic acid, were hydrolyzed in 6 N HCl in vacuo at 108°C for 20 h. Amino acid analysis was performed on a Dionex single-column amino acid analyzer equipped with a computing integrator. Muramic acid was also assayed colorimetrically after degra-
ation to lactic acid by alkaline hydrolysis as described by Hadzija (13).

Electron microscopy. Cell wall samples and whole cells were negatively stained with 2% uranyl acetate. Specimens were examined and photographed with a Philips 300 transmission electron microscope. Thin sections were prepared as described elsewhere (Giovannoni et al., in press).

Extraction and purification of lipids. The extraction and analysis of sulfolipids were carried out as described by Godchaux and Leadbetter (10, 11).

The method of Bligh and Dyer (3) was used for the extraction of other lipids. Lyophilized cells were suspended in chloroform-methanol-water (1:2:0.8 [vol/vol]; 114 mg/l of cells [dry weight]) and stirred at room temperature for 3 h. Cell residue was collected by filtration through Whatman no. 1 filter paper and reextracted with a second portion of the solvent. Chloroform (44 ml), water (22 ml), and saturated NaCl solution (5.5 ml) were added to the combined filtrates, which were then centrifuged to separate the phases. The chloroform phase was dried under a stream of N2, and the residue was taken up in 15 ml of chloroform-methanol-water (60:30:4.5 [vol/vol]). Nonlipid contaminants were removed by passage through a Sephadex G-25 column (21).

Total lipids were separated into polar lipids, neutral lipids, and glycolipids by chromatography on a silicic acid column (Sigma SIL-LC silicic acid, 325 mesh, prewashed with petroleum ether). Neutral lipids were eluted with chloroform, glycolipids were eluted with acetone, and polar lipids were eluted with methanol. Following elution, lipid fractions were dried under a stream of N2 in tared tubes and exposed to a high vacuum until their weight was constant.

Acid methanolysis of lipids. Polar lipid fractions from M. thermoautotrophicum and I. pallida were heated overnight at 90°C in a solution of 2.4 M HCl in methanol. The solution was then extracted twice with equal volumes of hexane. The hexane was removed under a stream of N2.

Thin-layer chromatography. Silica Gel G layers, 250 μm thick and developed with chloroform-methanol-water (130:50:8), were used for the identification of lipids other than capnoids. Plates were activated by being heated at 100°C for 1 h prior to use. Chromatographic methods for the detection of sulfolipids were as described by Godchaux and Leadbetter (9, 10). Rhodamine 6G was used as a spray reagent (14).

Whole-cell fatty acids. Fatty acid methyl esters were analyzed by gas chromatography and mass spectrometry as previously described (10).

Infrared spectroscopy. A Nicolet Fourier transform infrared spectrometer was used to examine polar lipid fractions from M. thermoautotrophicum and I. pallida. Dried samples were taken up in a small amount of chloroform and applied to NaCl plates. The solvent dried before scanning was done.

RESULTS

Cell wall. The cell wall of I. pallida, as observed in ultrathin sections by transmission electron microscopy, was similar in appearance to a unit membrane; two electron-dense layers were separated by an electron-transparent layer (Giovannoni et al., in press). The thickness of the entire trilaminar structure was about 11 nm, whereas the width of a unit membrane is typically 7 to 8 nm. Whole cell walls isolated by velocity sedimentation retained their shape and were readily distinguishable from membrane vesicles, which sometimes were observed adhering to cell wall fragments. I. pallida did not appear to have a lipopolysaccharide layer, although a 40-nm-thick surface layer resembling the glycolyces of gram-negative bacteria was evident.

Transmission electron microscopy of the sodium dodecyl sulfate-extracted rigid-layer fraction revealed intact sacculi, suggesting that these are indeed covalently bound, rigid macromolecules (Fig. 1). Regularly spaced perforations in the rigid layer were observed. These conformed in size and spacing to “crateriform” structures, surface ultrastructural features observed in negatively stained cells (Giovannoni et al., in press).

From a 2.1-g (wet weight) cell pellet, 5.6 mg (dry weight) of rigid layer was isolated, corresponding to about 1.4% of the dry weight of the cells. Two samples of the rigid-layer fraction, one a control containing added muramic acid, were separately hydrolyzed.

No diaminopimelic acid or muramic acid peaks were observed in the hydrolyzed rigid-layer fraction, although muramic acid was quantitatively recovered from the control sample. No muramic acid was detected in the rigid-layer fraction by a chemical assay. A chemical assay of the control sample indicated that degradation of muramic acid did not occur during hydrolysis.

The amino acid composition of the rigid layer is shown in Table 1 and Fig. 2. Recovered amino acids accounted for 99% of the weight of the rigid layer. Cysteine, methionine, proline, and tryptophan were not determined.

Lipid composition. M. thermoautotrophicum, an archaeabacterium of known lipid composition (20), was coanalyzed with I. pallida to provide a source of phytanyl glycerol ether lipids. A total of 11.3% of the dry weight of I. pallida was recovered in the lipid fraction, of which 8% was neutral lipid, 33% was glycolipid, and 59% was polar lipid. Total lipids extracted from M. thermoautotrophicum accounted for 4% of its dry weight; 50% of the total lipid was phosholipid.

The polar lipid fraction from I. pallida contained two major species with Rf values of 0.41 and 0.22 and a minor species with an Rf value of 0.12 (Fig. 3). The polar lipid fraction from M. thermoautotrophicum contained three major species with Rf values of 0.44, 0.29, and 0.1.

A thin-layer chromatogram of the hexane-soluble components released by acid methanolysis of the polar lipid fractions is shown in Fig. 3. Acid methanolysis hydrolyzes ester but not ether linkages. A single major species (Rf 0.5) which cochromatographed with C14- and C18-saturated fatty acid methyl ester standards appeared in the I. pallida sample following acid methanolysis. The mobility of a major species present in the M. thermoautotrophicum sample was changed by acid methanolysis (Fig. 3, lane b); however, the products did not cochromatograph with the species present in the material from I. pallida (Fig. 3, lane d). The major hexane-soluble acid methanolysis products in M. thermoautotrophicum are diphytanyl glycerol diether and diphytanyl glycerol monoether (26). It is clear that these are absent or nearly absent from I. pallida.

Nicolet Fourier transform infrared spectroscopy was used to further investigate the hexane-soluble acid methanolysis products from I. pallida and M. thermoautotrophicum (Fig. 4). A major peak at 1,740 cm⁻¹ (the carbon-oxygen stretching of esters) was observed in the I. pallida sample. This peak was barely detectable in the M. thermoautotrophicum sample. A major absorption band was present at 1,700 cm⁻¹ in the M. thermoautotrophicum sample, indicating the presence of ethers; the I. pallida sample did not have a major band at this position. Minor peaks were detected at 1,110 and 1,170 cm⁻¹ in the I. pallida sample. These peak heights
were difficult to ascertain because of the presence of multiple absorption peaks. Hence, the major polar lipids of *I. pallida* are ester linked and do not resemble the predominantly ether-linked lipids of archaebacteria.

The whole-cell fatty acid composition of *I. pallida* was determined by subjecting cells to acid methanolysis and analyzing the hexane-soluble extract by gas chromatography and mass spectrometry. Three major and two minor peaks were detected by gas chromatography. A minor peak at a retention time of 18.1 min was identified as phthalate from its mass spectrum (data not shown); it was probably a contaminant.

A peak at a retention time of 14.2 min which contained 2.1% of the total lipid yielded a mass spectrum with a major peak at *m/z* = 103. This fragment was characteristic of 3-hydroxy fatty acid methyl esters and resulted from a break between C-3 and C-4 (5). The chain length was 18, as deduced from a comparison of the retention times with those of standards.

A peak present at a retention time of 12.6 min which contained 21.7% of the total lipid yielded a mass spectrum with a major peak at *M* = 298, indicating a C16 fatty acid methyl ester. Major fragments characteristic of hydroxy fatty acids were not observed. A comparison of the retention times with those of standards indicated that this was a normal fatty acid.

A peak at a retention time of 12.4 min which contained 47.5% of the total lipid yielded a mass spectrum typical of unsaturated fatty acid methyl esters. *M*+ was at *m/z* = 296, indicating a C18:1 compound. The position of the carbon-carbon double bond was deduced from the presence of a peak at *M* − 74. A comparison of the retention times with those of standards indicated a nonbranched-chain structure.

When *I. pallida* was grown in a minimum medium containing Na235SO4, 7.9% of the incorporated 35S was found in the lipid fraction. The whole lipid fraction was chromatographed, and labeled components were detected autoradiographically. Nonlabeled lipids in the sample as well as standards (capnine and N-acylcapnine) were detected by rhodamine staining. A single labeled lipid (*Rf*, 0.48) was found (data not shown). This compound did not correspond to either N-acylcapnine (*Rf*, 0.6 to 0.7) or capnine (*Rf*, 0.33). The labeled zone was found to contain 70% of the radioactivity from the crude lipid fraction. It is likely that the remaining radioisotopically labeled material, which did not move from the origin, was not lipid.

Whole lipid extract from *I. pallida* was decylated and chromatographed against capnine and N-acylcapnine. Again, the label did not migrate with the capnine standard, indicating that capnine is not among the deacylation products (as would be the case with capnoids). It can be concluded that the labeled lipid present in *I. pallida* is not a capnoid. Indeed, most of the label originally found in the lipid zone at an *Rf* of 0.48 remained at the origin as a consequence of methanolysis. This behavior is characteristic of sulfate esters (as opposed to sulfonates such as capnoids). This sulfur-containing lipid, present in the cells at about 2 to 3 μmol/g of cells (wet weight; estimated as previously described [10]), represented a substantial fraction of the total lipid found in *I. pallida* (e.g., for a lipid with a molecular weight of 333, about 0.5% of the cell dry weight or about 5% of the total lipid). It is probable that the compound is a sulfated ester.

**DISCUSSION**

The rigid-layer wall cell of *I. pallida* appears to be composed entirely of amino acids and does not contain muramic acid, a key structural component of peptidoglycan. Diaminopimelic acid, which is found in most eubacterial peptidoglycans, is also absent. Some other eubacteria also have nonpeptidoglycan cell walls, as do archaebacteria. Members of the genus *Chlamydia* (2, 6) and of the *Planctomyces* group (15) have been shown to lack peptidoglycan. The molar ratios of amino acids reported for the cell wall of *Planctomyces* strain IFAM 1313 differ significantly

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**TABLE 1. Amino acid and amino sugar contents of the hydrolysate of the rigid-layer fraction from *I. pallida***

<table>
<thead>
<tr>
<th>Amino acid or amino sugar</th>
<th>Content (μmol/mg of cell wall)</th>
<th>Molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>0.62</td>
<td>2.6</td>
</tr>
<tr>
<td>Thr</td>
<td>0.82</td>
<td>3.4</td>
</tr>
<tr>
<td>Ser</td>
<td>0.66</td>
<td>2.8</td>
</tr>
<tr>
<td>Glu</td>
<td>0.94</td>
<td>3.9</td>
</tr>
<tr>
<td>Gly</td>
<td>1.15</td>
<td>4.8</td>
</tr>
<tr>
<td>Ala</td>
<td>0.94</td>
<td>3.9</td>
</tr>
<tr>
<td>Val</td>
<td>0.78</td>
<td>3.2</td>
</tr>
<tr>
<td>Ile</td>
<td>0.10</td>
<td>4.2</td>
</tr>
<tr>
<td>Leu</td>
<td>0.23</td>
<td>1.0</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.55</td>
<td>2.3</td>
</tr>
<tr>
<td>Phe</td>
<td>0.36</td>
<td>1.5</td>
</tr>
<tr>
<td>Lys</td>
<td>0.26</td>
<td>1.1</td>
</tr>
<tr>
<td>His</td>
<td>0.24</td>
<td>1.0</td>
</tr>
<tr>
<td>Arg</td>
<td>0.39</td>
<td>1.6</td>
</tr>
<tr>
<td>MUR</td>
<td>&gt;0.01</td>
<td></td>
</tr>
<tr>
<td>DAPA</td>
<td>&gt;0.01</td>
<td></td>
</tr>
</tbody>
</table>

*a* MUR, Muramic acid; DAPA, diaminopimelic acid. Proline, methionine, tryptophan, and cysteine were not determined.

*b* Relative to histidine.
from those that we found in \textit{I. pallida}; thus, it is unlikely that the walls of these organisms are highly similar.

We observed pitlike ultrastructural features in the cell wall of \textit{I. pallida}. Negative stains of whole cells revealed rings with an outer diameter of 27 nm evenly distributed across the cell surface at intervals of about 120 nm. The pits could be seen in thin sections of whole cells and isolated cell walls where the trilaminar wall structure periodically tapers inwards and disappears. The sodium dodecyl sulfate-insoluble rigid-layer fraction had perforations of about the same size and spacing; these are almost certainly an underlying structural aspect of the pits seen in negatively stained whole cell walls. The functional significance of these structures is unknown; however, structures of similar appearance, termed crateriform structures, have been seen in \textit{Planctomyces} and \textit{Pirella} strains (17). In \textit{Pirella} strains, the pits are distributed evenly over the cell surface, whereas in \textit{Planctomyces} strains, they are arranged at the cell poles. The phylogenetic evidence would suggest that these are homologous structures. However, perforations of the rigid layer similar to those described here have also been observed in phylogenetically unrelated species (e.g., the cyanobacterium \textit{Pseudanabaena} sp. [12]).

The polar lipids of \textit{I. pallida} contained ester-linked aliphatic side chains, and the total fatty acid composition of \textit{I. pallida} was also typical of eubacteria. Phytanyl ether side chains were not present, thus further distinguishing it from the archaeabacteria.

The mechanism(s) underlying gliding motility among prokaryotes is poorly understood. It seems probable that there is more than one mechanism of gliding motility among phylogenetically diverse organisms (4, 10). Capnoids (2-amino-3-hydroxy-15-methylnonadecane-1-sulfonic acid and its \textit{N}-acylated derivatives), which are distinctive lipids found in the \textit{Cytophaga-Flexibacter} group of gliding bacteria, have been implicated in the mechanism of gliding motility within this group (1). \textit{I. pallida}, the only budding bacterium known to glide, lacks capnoids.

Budding bacteria with proteinaceous cell walls, including \textit{I. pallida}, remain an enigmatic group surrounded by fundamental question. It will be of immediate interest to determine the phylogenetic relationships of this group with greater accuracy. While cell wall architecture may turn out to be a keystone of prokaryotic evolution, it is evident that peptidoglycan content alone cannot be used as a chemotaxonomic marker to distinguish archaeabacteria from eubacteria.

**ACKNOWLEDGMENTS**

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