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Isolation and Characterization of the Outer Membrane and Lipopolysaccharide from *Eikenella corrodens*

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The chemical composition of the outer membrane fractions (OMEs) of *Eikenella corrodens* strains 23834 and 470 as well as the strain 23834 lipopolysaccharide (LPS) was determined. The OMFs were obtained by Triton X-100 treatment of the heavier membrane fraction from sucrose density centrifugation of the total membrane fraction. The resulting OMFs of strains 23834 and 470, free of cytoplasmic membrane components, were found to contain 69.6 and 75.0% (wt/wt) protein, 4.8 and 9.2% lipid, 4.6 and 4.7% carbohydrate, and 2.0 and 4.6% muramic acid, respectively. By sodium dodecyl sulfate-polyacrylamide gel electrophoresis both OMFs contained one major peptide determined to be 33,500 daltons for the strain 23834 OMF, and 37,500 daltons for the strain 470 OMF. Analysis of the OMF fatty acids revealed hexadecanoic, hexadecenoic, octadecenoic, and lesser amounts of octadecanoic acids. Transmission electron microscopic examination of the OMFs revealed typical large sheets of membrane. Structures (10 nm in diameter) resembling pores were also evident. The *E. corrodens* LPS was found to be composed of 34.5% (wt/wt) carbohydrate and 25.0% lipid A. Only minute amounts of 2-keto-3-deoxyoctonate and heptose could be detected. Fatty acid analysis revealed primarily octadecanoic and hexadecanoic acids, with lesser amounts of octadecenoic acid. No hydroxy fatty acids were detected. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed the *E. corrodens* LPS to resemble other smooth-type LPSs. Transmission electron microscopic examination revealed a vesicle-like morphology. The *E. corrodens* LPS appears not to be a "classical," i.e., enteric, type of LPS.

Eikenella corrodens is a gram-negative, microaerophilic rod that colonizes both the upper respiratory tract and oral cavity of humans (4, 18, 34). Although it is cultured from various abscesses, most often of the head and neck (12, 13, 22, 24, 51), and is often the primary pathogen in such infections (13, 22, 24), it is also considered to be an important contributor to tissue destruction in periodontal disease (40, 50, 55).

Gram-negative bacteria are characterized by a complex cell wall consisting of a cytoplasmic membrane, a peptidoglycan layer, and an outer membrane. The outer membrane serves numerous functions for the cell and may play a primary role in pathogenesis. For example, many biological properties can be attributed to the lipopolysaccharide (LPS), the endotoxic molecule of the outer membrane. This molecule not only may be directly toxic to the host and cause pyrexia, diarrhea, prostration, and even death (49), but may also provide a means of defense for the bacterium by facilitating resistance to phagocytosis (21).

During the past decade various procedures for isolating and purifying bacterial outer membranes have been reported (32). Miura and Mizushima (36) initially used a procedure involving isopycnic sucrose density gradient centrifugation to separate the cytoplasmic membrane from the outer membrane of *Escherichia coli*. Osborn and co-workers (42) employed a similar method for the isolation of the *Salmonella typhimurium* membranes, whereas Schnaitman (48) was able to isolate the *E. coli* outer membrane and its associated proteins by treatment of the membrane fraction with Triton X-100. Using these basic techniques, a number of investigators have studied the outer membranes of a variety of gram-negative bacteria (16, 17, 30, 35, 41). Consequently, much information exists regarding the structure and macromolecular function of both the cytoplasmic and outer membranes of gram-negative procaryotes; however, less is known about their importance or role in pathogenesis. Such is the case with *E. corrodens*, with only one short report being published relevant to the LPS and its endotoxicity (2). To this end the *E. corrodens* outer membrane fraction (OMF) and LPS were isolated, and their biochemistry was studied before determining the contributions, if any, these components may make to the host's tissue destruction in peridontal disease.

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MATERIALS AND METHODS

Cultures and growth conditions. *E. corrodens* type strain ATCC 23834 (19) and strain 470 were used in this study. Strain 470 is a periodontal isolate obtained from S. S. Socransky, Forsyth Dental Clinic, Boston, Mass.

For cultivation of cells, several tryptose blood agar-cell blocks of 2- to 4-day growth were inoculated into BY broth (pH 7.2 to 7.4), which contains the following, (in grams per liter): K_2HPO_4 (1.75), KH_2PO_4 (1.36), NH_4Cl (1.00), sodium citrate · $2H_2O$ (0.50), $FeSO_4 \cdot 7H_2O$ (0.01), $CaCl_2$ (0.01), $MgSO_4$ anhydrous (0.10), KNO_3 (2.0), yeast extract (15.0), and hemin (1.5 mg per liter) (modified from D. Dusek and S. J. Badger, J. Dent. Res. **60**:332, 1981). Successive transfers of 15 to 20% inocula were made until final volumes of 6 to 12 liters were reached. All incubations were for 18 to 24 h without shaking at 37°C, at which time the cells were in the early stationary phase of growth. When labeling of the cell membranes was required, 100 μCi of [^{14}C]acetate (50 μM) was added per liter.

Isolation of OMFs. Cells were harvested by centrifugation,

washed twice with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.4), and suspended in 2 volumes of 10 mM HEPES buffer with 1 mM EDTA and 1 mg each of DNase and RNase added per g of cells. The cells were broken by three successive passages through a cold French pressure cell at 15,000 lb/in^2, $MgCl_2$ was added to a concentration of 1 mM, and whole cells and large debris were removed by low-speed centrifugation. A 5-ml sample of the low-speed supernatant (LSS) was layered onto the top of an 80 to 25% sucrose discontinuous gradient in 10 mM HEPES buffer (pH 7.4). The gradients were centrifuged at 65,000 × *g* for either 2 h (strain 23834) or overnight (14 h, strain 470). The centrifuged gradients were fractionated by conventional procedures, and the isolated membranes were washed twice in 10 mM HEPES buffer (pH 7.4). For cytoplasmic membrane isolation, the lighter (*p*, 1.13 g/ml) membrane band was removed and similarly washed. Outer membrane pellets were suspended to 2 to 6 mg of protein per ml in 2% Triton X-100 in 10 mM HEPES (pH 7.4), sonicated for 15 s, and incubated at 37°C for 1 h, after which time the membranes were cooled on ice, and the Triton X-100-insoluble fraction was recovered by centrifugation at 65,000 × *g* for 1 h. The Triton X-100-insoluble pellets were then suspended in 10 mM HEPES (pH 7.4), and cytochromes and cytochrome oxidase activities were assayed as described below. If significant activity was detected, a second treatment with Triton X-100 was performed. Any residual Triton X-100 in the membrane fraction was removed by incubating the preparation at 4°C with stirring for 12 h with 3 g of thrice-washed Amberlite XAD-2 beads per g of Triton X-100 used. After removal of the beads, a sample of the purified OMF was removed and stored at 0°C, and the remaining fraction was dialyzed against distilled water and lyophilized.

Buoyant density. The buoyant densities of membrane fractions from both linear and discontinuous gradients were calculated from the refractive index (ISCO tables; ISCO, Lincoln, Neb.) of each fraction as measured by refractometry.

Tetramethyl phenylenediamine oxidase assay. Cytochrome oxidase activity was measured by the method of Devoe and Gilchrist (8). The reaction mixture contained 0.1 each of 1.5 M NaCl and 10 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, 0.7 ml of 0.1 M Tris-hydrochloride (pH 7.5), and 0.1 ml of membrane sample. Enzyme activity was read at 520 nm in a Gilford recording spectrophotometer. All activities were adjusted for tetramethyl phenylenediamine autooxidation as measured simultaneously in the absence of sample.

Cytochromes. Cytochromes were extracted from membrane preparations by method similar to that of Osborn and co-workers (41). Briefly, membranes were suspended in 10 mM HEPES buffer (pH 7.8) to a concentration of 2 to 6 mg of protein per ml, 20 mg of sodium deoxycholate was added per ml, and the membranes were incubated for 15 min at room temperature. After centrifugation at 10,000 × *g* for 10 min, dithionite (sodium hydrosulfite)-reduced minus oxidized spectra between 390 and 600 nm were determined in a Cary model 14 recording spectrophotometer equipped with a sensitive slide wire set for 0.1 optical density units at full deflection.

Measurement of ^{14}C. The amount of ^{14}C in membrane fractions was determined after incubation of actively growing cultures in the presence of [^{14}C]acetate. The ^{14}C-incubated cultures were processed, and the membranes were separated as described above. The amount of radioactivity in samples (10 to 30 μl) of each sucrose gradient fraction or purified membrane fraction was determined with a Packard Tri-Carb liquid scintillation spectrometer with Aquasol II as the counting solution.

LPS isolation. Twice-washed, lyophilized cells of strain 23834 (14 g) were suspended to 50 mg/ml in distilled water and treated by the hot phenol-water procedure of Westphal et al. (58). The resulting crude LPS was precipitated with 2 volumes of 95% ethanol containing 20 mM sodium acetate, washed three times with 70% ethanol, and suspended in 100 ml of 50 mM Tris–1 mM $MgCl_2$ buffer (pH 4.6). DNase (100 μg) was added, and the suspension was sonicated for 30 s and incubated for 1 h at 37°C. The pH was then adjusted to 7.6, 10 mM EDTA and 100 μg of RNase were added, and the suspension was incubated for 30 min at 37°C. After 24 h of dialysis against distilled water, the LPS was treated with 2 mg of protease type II for 1 h at room temperature and overnight at 4°C, again dialyzed extensively, and finally lyophilized. The DNase, RNase, and protease treatments were repeated twice more with 50 μg each of DNase, RNase, and pronase added per ml of LPS suspension.

Lipid A hydrolysis. Cleavage of LPS and isolation and quantitation of the lipid A were as described by Kiley and Holt (27).

Phospholipid extraction and fatty acid analysis. Membrane and lipid A samples were extracted with chloroform-methanol-water by the method of Bligh and Dyer (3), and the amount of extractable material was determined gravimetrically in tared dishes. The extracted fatty acids were methylated, taken up in 100 μl of chloroform, and analyzed on Varian 3700 and 4600 gas-liquid chromatographs. Fatty acids were positively identified by cochromatography with known standards (Supelco Inc., Bellefonte, Pa.).

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) procedures were similar to those described by Laemmli (28), with either 15% or 5 to 15% gradient gels. All samples were boiled in SDS for 20 min, except LPS samples, which were boiled for 5 min. Polypeptides were stained with Coomassie blue G, whereas LPS bands were detected by a modified silver staining procedure (56). Periodic acid-Schiff staining was by the method of Fairbanks et al. (11).

Densitometric scanning of Coomassie blue G-stained gels was accomplished with a Gelman ACD-18 automatic computing densitometer. Molecular weights were determined by comparison with known molecular weight standards.

Analytical assays. Protein was determined by the Bradford–Bio-Rad procedure (1), carbohydrate was determined by the phenol-sulfuric acid procedure (10), muramic acid was determined by the acetaldehyde-*p*-hydroxy-diphenyl method of Hadzija (14), 2-keto-3-deoxyoctulosonic acid (KDO) was determined by the thiobarbituric acid method (25), and heptose was determined by the cysteine-sulfuric acid method of Wright and Rebers (58) with sedoheptulose as the standard. *E. coli* O111 LPS (Sigma Chemical Co., St. Louis, Mo.) was employed as reference LPS in the KDO and heptose assays.

Electron microscopy. Glutaraldehyde-osmium fixation, negative staining, and transmission electron microscopic (TEM) observation of membrane and LPS preparations were as described previously (44).

RESULTS

Isolation and analysis of membrane fractions. Isopycnic density gradient centrifugation of the LSS of *E. corrodens* strains 23834 and 470 resulted in the separation of two major membrane fractions (Fig. 1). The lighter-density fraction was

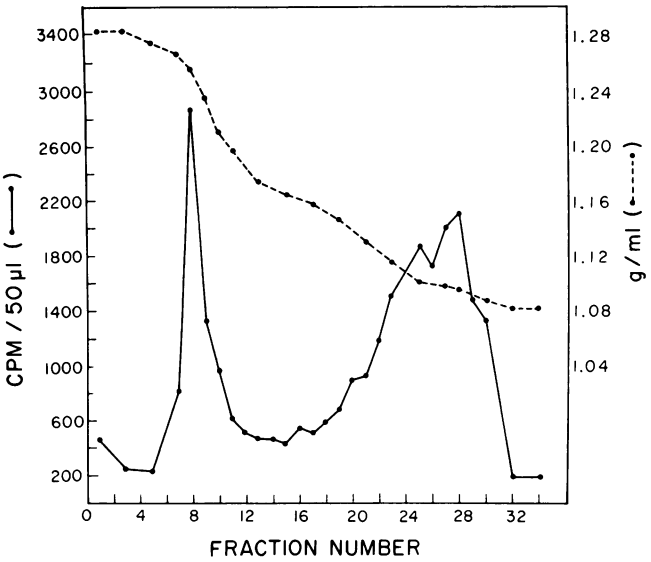

FIG. 1. Profile of discontinuous sucrose density gradient centrifugation of the LSS of *E. corrodens* 23834 grown in the presence of [^{14}C]acetate. Two membrane-enriched bands are evident. Fractions 7 through 10 and 23 through 29 were pooled and washed for analysis as outer and inner membrane preparations, respectively.

slightly yellow in appearance and more diffuse than the heavier, whitish-gray band. TEM of thin sections of these fractions are seen in Fig. 2. The lighter-density fraction of strain 23834 consisted of small, double-tracked membrane fragments and vesicles ranging in diameter from 40 to 80 nm (Fig. 2a), whereas the heavier fraction was composed of larger unit membrane vesicles ranging in diameter from 80 to 320 nm and what appears to be other nonvesicularized pieces of membrane (Fig. 2b). The distribution of membrane-associated cytochrome oxidase activity revealed the majority of the activity (82%) to be associated with the lighter-density fraction, with the remaining 18% associated with the heavier fraction. Cytochromes with absorbance maxima at 428, 552, and 563 nm were also present in both fractions. Triton X-100 solubilization removed all of the cytochromes and their associated cytochrome oxidase activity.

The purification of the OMF was also followed by negative staining of selected steps of the isolation procedure (Fig. 3, 4, and 5). The LSS (Fig. 3) consisted of a heterogeneous mixture of membrane fragments dispersed in other particulate matter and debris. After sucrose density centrifugation (Fig. 4), the heavier fraction or OMF was devoid of the particulate material; instead, the band was composed of both small and large vesicles and membrane sheets. Structures resembling membrane pores (Fig. 4) are evident in the larger membrane pieces. After Triton X-100 treatment of the OMF (Fig. 5), there was almost the complete removal of the small vesicles with the enrichment of the larger membrane sheets. Some of the large membrane sheets appeared to have rolled up onto themselves, in a "jelly roll" fashion (Fig. 5). Especially evident on several membrane sheets were the membrane pores (Fig. 5). At high magnification (Fig. 5, inset) the pores were structured, having a diameter of approximately 10 nm, and are apparently symmetrical. The pores did not appear to be arranged in an ordered fashion or array, but seemed to be distributed unevenly on the membrane surface. *E. corrodens* 470 (Fig. 6) OMF consisted of smaller membrane vesicles and fewer sheets of membrane than did that of strain 23834. In addition, there were fewer pores (Fig. 6) in strain 470 OMF. The strain 470 OMF was also devoid of cytochromes and cytochrome oxidase activity, although both were present in the strain 470 LSS and Triton X-100 supernatant.

Buoyant density. The buoyant density of the strain 23834 OMF was 1.26 to 1.27 g/ml; that of strain 470 OMF was slightly denser, banding between 1.27 and 1.28 g/ml.

Composition of the *E. corrodens* OMFs. Chemical analysis (Table 1) of the OMFs of both *E. corrodens* strains revealed them to consist of almost 75% protein, with the remainder equally distributed between lipid and carbohydrate. Muramic acid, a chemical component of peptidoglycan, varied between 2 and 5%. Although the qualitative distribution of the fatty acids of both OMFs was similar (Table 2), differences were apparent. Both OMFs contained large amounts of octadecenoic ($C_{18:1}$), hexadecanoic ($C_{16:0}$), and hexadecenoic acids, whereas strain 470 OMF also contained octadecanoic acid ($C_{18:0}$), although in a lesser amount. Thus, strain 23834 OMF contains 71.4% unsaturated fatty acids, and strain 470 OMF contains 58.8% unsaturated fatty acids. We have not been able to detect any hydroxy or branched-chain fatty acids in either whole cells or membrane fractions of the two *E. corrodens* strains by either gas-liquid chromatography or mass spectrometry. The absence of hydroxy fatty acids is especially significant since the LPS and outer membrane were both extremely active in biological assays as measured by mitogenic, bone resorption and *Limulus* lysate assays (45).

SDS-PAGE. Polypeptide analyses of the two purified membrane fractions by SDS-PAGE are shown in Fig. 7 and 9. The LSS of strain 23834 (Fig. 7, lane 2) contained at least 25 polypeptides, with a peptide of 33,500 daltons (dal) being the most abundant. Before Triton X-100 treatment (Fig. 7, lane 3), there was the loss of several of the peptides visible in the LSS and an increase in the relative amount of the 33,500-dal peptide. After Triton X-100 treatment (Fig. 7, lane 4), many of the slower-migrating polypeptides (>33,500 dal) were absent; however the faster migrating polypeptides (<33,500 dal) were present in greater amounts. Thus, purification of the OMF results in the removal of most of the higher-molecular-weight peptides and a concomitant enrichment of the lower-molecular-weight polypeptides, including the 33,500-dal polypeptide. A densitometric scan of a Coomassie blue-stained gel of the OMF of strain 23834 (Fig. 8) shows that this major 33,500-dal polypeptide accounts for about 55% of the total protein in the gel. Heating of the OMF in SDS at 70°C instead of 100°C (Fig. 7, lane 5) resulted in the polypeptides being incompletely dissociated, as evidenced by reduced amounts of each polypeptide and a densely staining smear of approximately 80,000 dal to greater than 100,000 dal. However, the 26,000- and 28,000-dal polypeptides present in the 100°C-treated OMF (Fig. 7) appeared to be missing from the 70°C-treated OMF and thus may represent heat-modifiable proteins.

An analogous situation occurred with the purification of the strain 470 OMF (Fig. 9, lane 3), except that the major outer membrane polypeptide was 38,500 dal (Fig. 9, lane 3). However, the minor peptides were nearly identical in distribution and size (Fig. 9, compare lanes 2 and 3) to that of the strain 23834 OMF. The most apparent difference between the two OMFs was that the 28,000- and 29,000-dal polypeptides of strain 23834 OMF were absent in the strain 470 OMF (Fig. 9).

Carbohydrate staining of the two outer membranes preparations by the periodic acid-Schiff technique revealed a low-

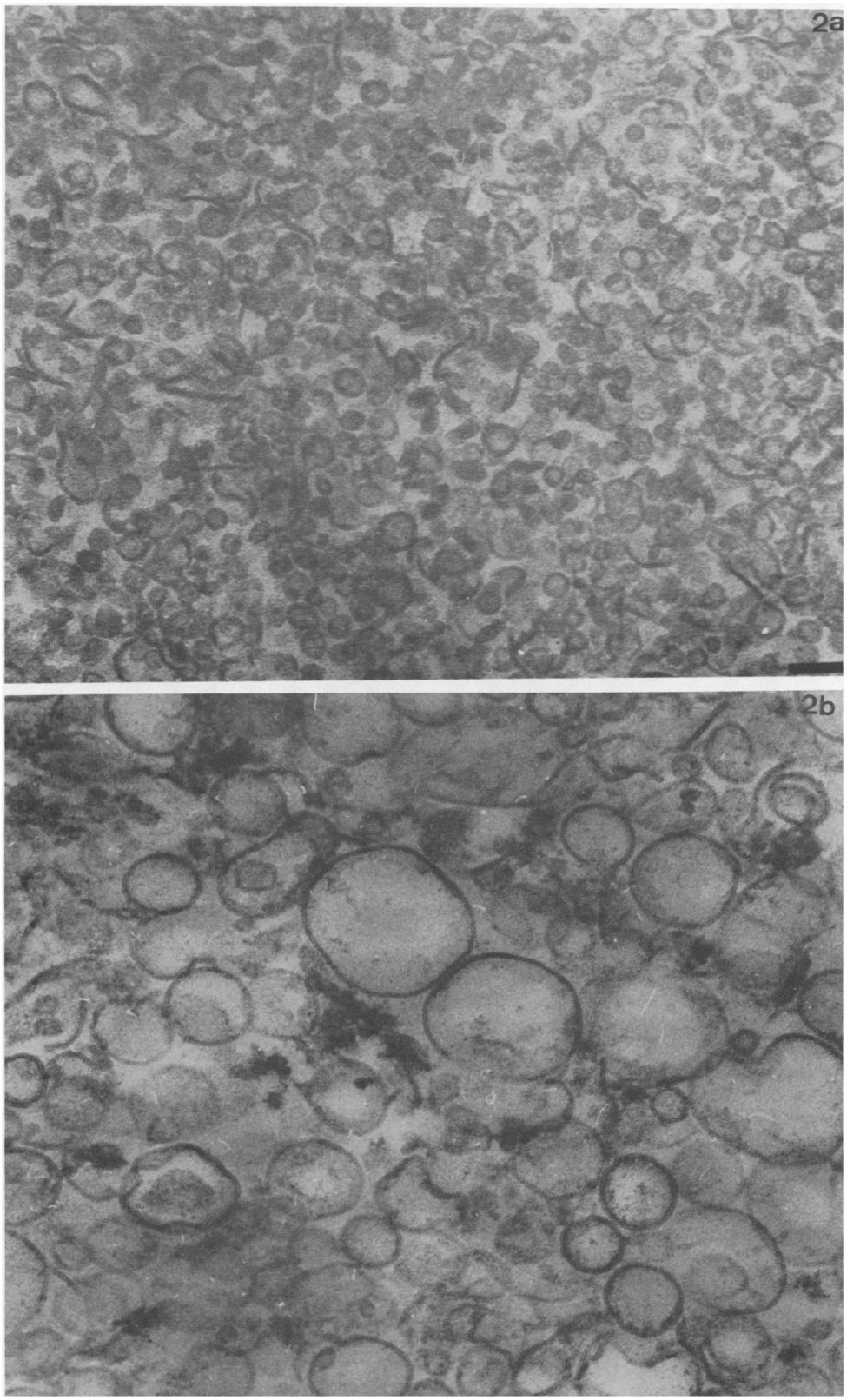

FIG. 2. TEM of thin sections of the lighter-density (a) and heavier-density (b) membrane bands resulting from discontinuous sucrose density gradient centrifugation of the LSS of French pressure cell lysate of *E. corrodens* 23834. In (a) small, 40- to 80-nm-diameter double-tracked membrane fragments and vesicles are evident, whereas in (b) the heavier membrane fraction (enriched for outer membrane constituents) consists of larger membrane vesicles and cell fragments ranging from 80 to 320 nm in diamter. Bar, 100 nm.

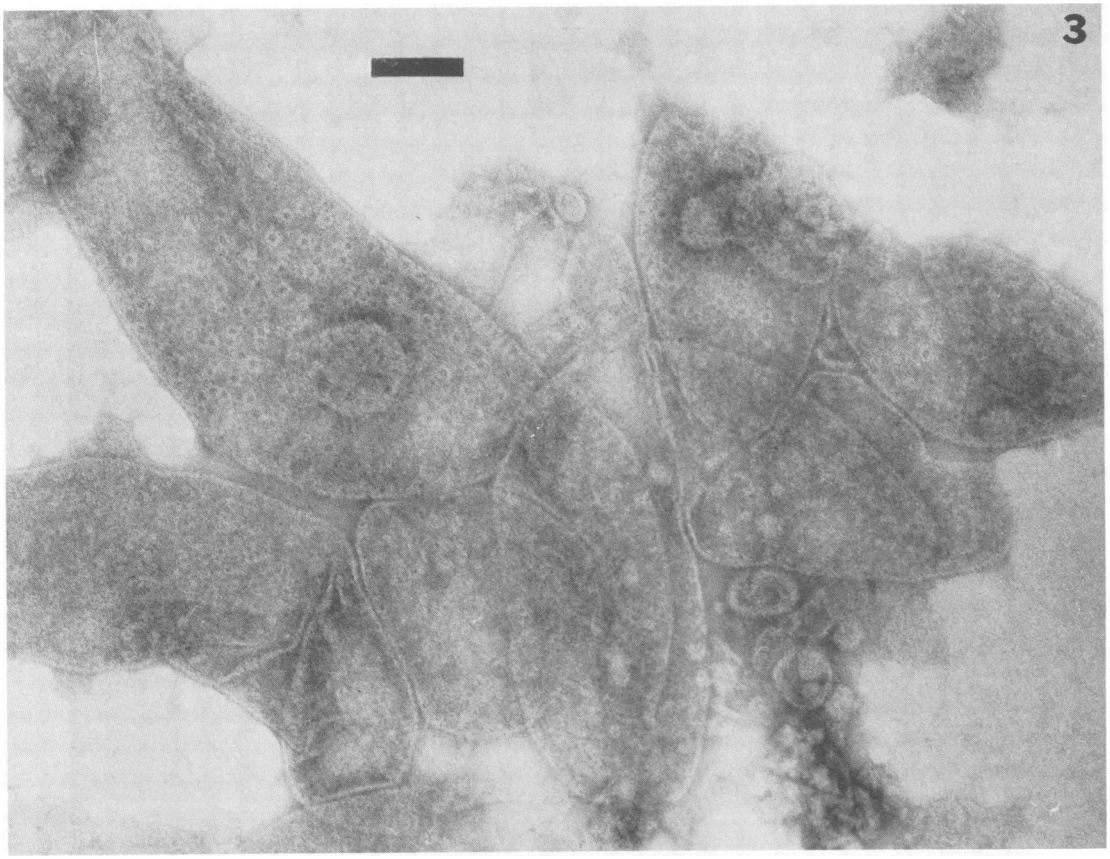

FIG. 3. TEM of negatively stained (3% phosphotungstic acid, pH 7.2), LSS from French pressure cell lysate of *E. corrodens* 23834. Large pieces of membrane, small membrane vesicles, and other nonmembranous debris are evident. Bar, 100 nm.

molecular-weight, periodic acid-Schiff–positive area at and below the skewed band (Fig. 9) of approximately 21,000 dal in the strain 23834 OMF and of 18,000 dal in the strain 470 OMF. This area also stained with the silver stain (see Fig. 11) for LPS.

Chemical composition of LPS. *E. corrodens* strain 23834 LPS contains carbohydrate, lipid, and a small amount of contaminating protein (Table 2). However, SDS-PAGE analysis of the purified LPS revealed no protein bands. The *E. corrodens* LPS consists predominantly of carbohydrate (34.5%), consistent with the LPS of other gram-negative procaryotes; however, unusually low amounts of KDO (0.4%), and heptose (0.2%) occur in the *E. corrodens* LPS. Mild acid hydrolysis of the LPS and purification of the chloroform-methanol soluble fraction released the yellow oily lipid A, which accounted for 25.0% of the total dry weight of the LPS. Gas-liquid chromatographic analysis of the fatty acids present in the lipid A (Table 3) revealed only fatty acids with an even carbon number, which included octadecanoic acid (42.0%) in the highest concentration. However, significant amounts of hexadecanoic (32.8%) and octadecenoic (22.7%) acids were also found. Only trace amounts of tetradecanoic and hexadecanoic acids were detected; as already mentioned, β-hydroxy fatty acids were not detected, and all chromatographic peaks were identified. Thus, the fatty acid content of the *E. corrodens* LPS is similar to the *E. corrodens* OMF (Table 3), but the distribution is quite different, with the LPS containing greatly reduced amounts of hexadecenoic and octadecenoic acids, but significantly increased amounts of octadecanoic acid. The LPS lipid A consequently contains considerably less unsaturated fatty acids (22.7%) than does the OMF (71.4%).

TEM of LPS. TEM of negatively stained *E. corrodens* LPS (Fig. 10) revealed small, relatively uniformly shaped vesicle-like structures that varied in size from 7 to 60 nm in diameter. The vesicles appeared to lack any detectable structure or texture to their surface, with several appearing to have collapsed inward (Fig. 10). Under no condition that we tested were the classical ribbon-and-strand configurations evident, even though *Actinobacillus actinomycetemcomitans* strain Y4 LPS (27) treated in exactly the same way stained with ribbon-and-strand configurations (micrograph not shown).

SDS-PAGE of LPS. Silver-staining of carbohyrates in SDS-polyacrylamide gels (Fig. 11) revealed that the *E. corrodens* 23834 LPS (Fig. 11, lane 5) possesses a staining pattern nearly identical to that of *E. coli* serotype O127:B8 LPS (Fig. 11, lane 4). However, the molecular weights of the two fastest-migrating doublet bands of the *E. corrodens* LPS are slightly greater than those of the *E. coli* LPS. The higher-molecular-weight bands of the *E. corrodens* LPS (Fig. 11) appear to correspond to the higher-molecular-weight bands of the *E. coli* LPS, indicating that *E. corrodens* LPS is likely a smooth LPS. Contrastingly, *Capnocytophaga gingivalis* 30N51 LPS (Fig. 11, lane 6), another periodontal isolate, appears to be a rough or semirough LPS, since all polysaccharide bands are clustered in the faster-migrating region of the gel, with none being visible in the slower-migrating region, despite the heavy concentration of LPS that was applied to the gel.

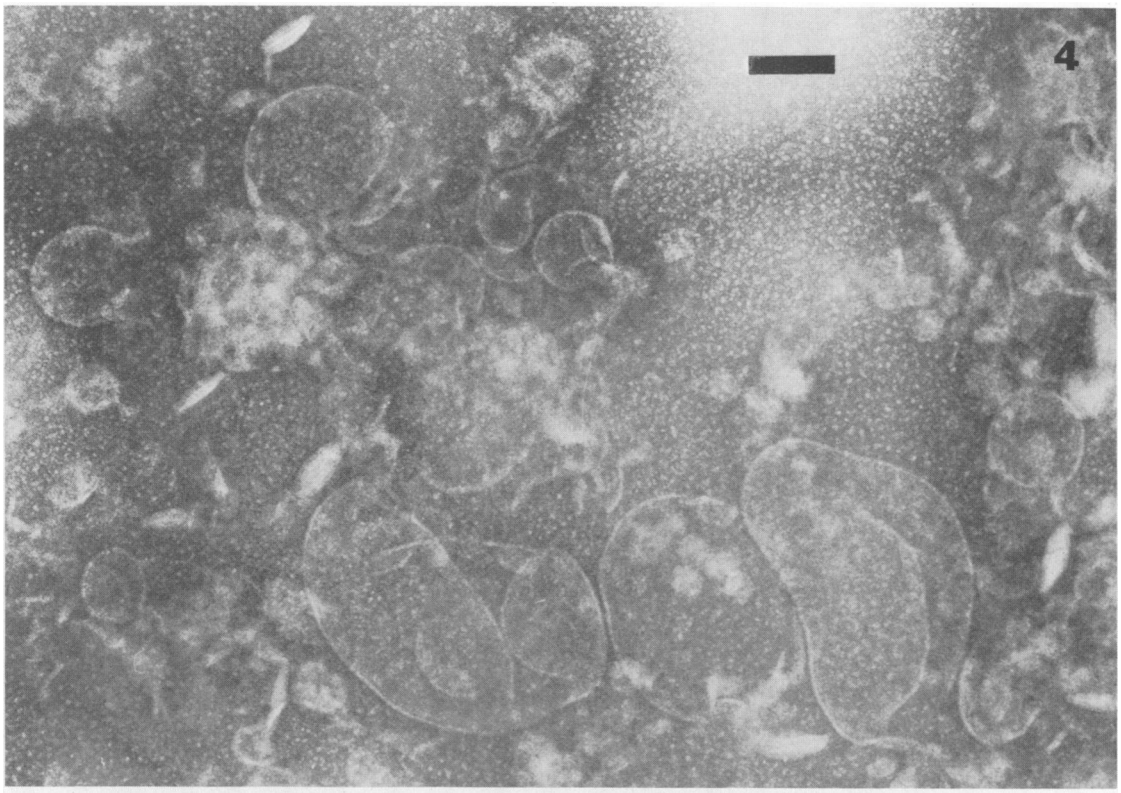

FIG. 4. Discontinuous sucrose gradient centrifugation of the LSS of *E. corrodens* 23834. The outer membrane-enriched band (the heavier of the two resulting bands), consists primarily of large sheets of membrane and several smaller membrane pieces. Note the structures that resemble pores on the large sheets of membrane. Bar, 100 nm. Negatively stained with 3% phosphotungstic acid (pH 7.2).

DISCUSSION

Our results were similar to those of several previously described membrane fractionation studies (35, 36, 42, 46, 47) of gram-negative bacteria. The outer membrane of *E. corrodens* was of a greater density than the cytoplasmic membrane and was probably similarly affected by Triton X-100. Triton X-100 has been demonstrated to not only solubilize cytoplasmic membrane, but also solubilize approximately two-thirds of the phospholipids and one-half of the LPS from the outer membrane of *E. coli* (48). However, for the biological studies for which these membrane fractions were isolated, purity of the membrane preparation was of the utmost importance, and Triton X-100 was used. The high protein content and low lipid content of the purified outer membrane may therefore be a consequence of treatment with Triton X-100. It was not possible to quantitate the amount of LPS in the OMFs due to the lack of a suitable marker for LPS, since the *E. corrodens* 23834 LPS does not contain hydroxy fatty acids and contains a minimal amount of KDO and heptose, amounts that would be undetectable in milligram quantities of OMFs.

The identification of the OMF as having been derived from the *E. corrodens* outer membrane is consistent with the enzymatic, morphological, and biochemical data. The absence of the oxidative enzyme cytochrome oxidase as well as cytochrome, which are both cytoplasmic membrane components in other procaryotic cells and separate predominantly with the LSS, indicates the absence of cytoplasmic membrane contamination. Morphologically, the membrane fractions are similar to those described by Schnaitman (48) for *E. coli* and by Osborn and co-workers (42) for *S. typhimurium*. The inner membrane forms mostly closed vesicles, generally smaller in diameter than those of the outer membrane, which are characteristically larger (some as large as 500 nm) and are often open fragments of membrane.

The protein patterns by SDS-PAGE are also consistent with those described previously for outer membranes of other gram-negative bacteria (17, 23, 46), as outer membranes have a relatively simple SDS-PAGE pattern consisting of few polypeptides in major amounts. For example, Lugtenberg and co-workers (31) have been able to resolve two to four major outer membrane proteins of 30,000 to 42,000 dal in members of the *Enterobacteriaceae*. Mizuno and Kageyama (37) have reported six major bands (50,000 dal and below) by SDS-PAGE of *Pseudomonas aeruginosa* outer membranes, and Kamio and Takahashi (23) have identified two major outer membrane proteins (40,000 and 42,000 dal) of *Selenomonas ruminantium*. These data are not absolute, since conditions of growth (6, 23), procedures of solubilization, and percentage of polyacrylamide in the gels (15) all influence the electrophoretic profiles of outer membrane proteins. Consequently, the gel pattern reported here for *E. corrodens* may be different with different polyacrylamide concentrations, protein solubilization conditions, or culture conditions. However, since the two *E. corrodens* strains in this study were treated identically, their electrophoretic patterns can be compared. The significance of the difference in the molecular migration of the major outer membrane protein of the two strains is unknown, but each

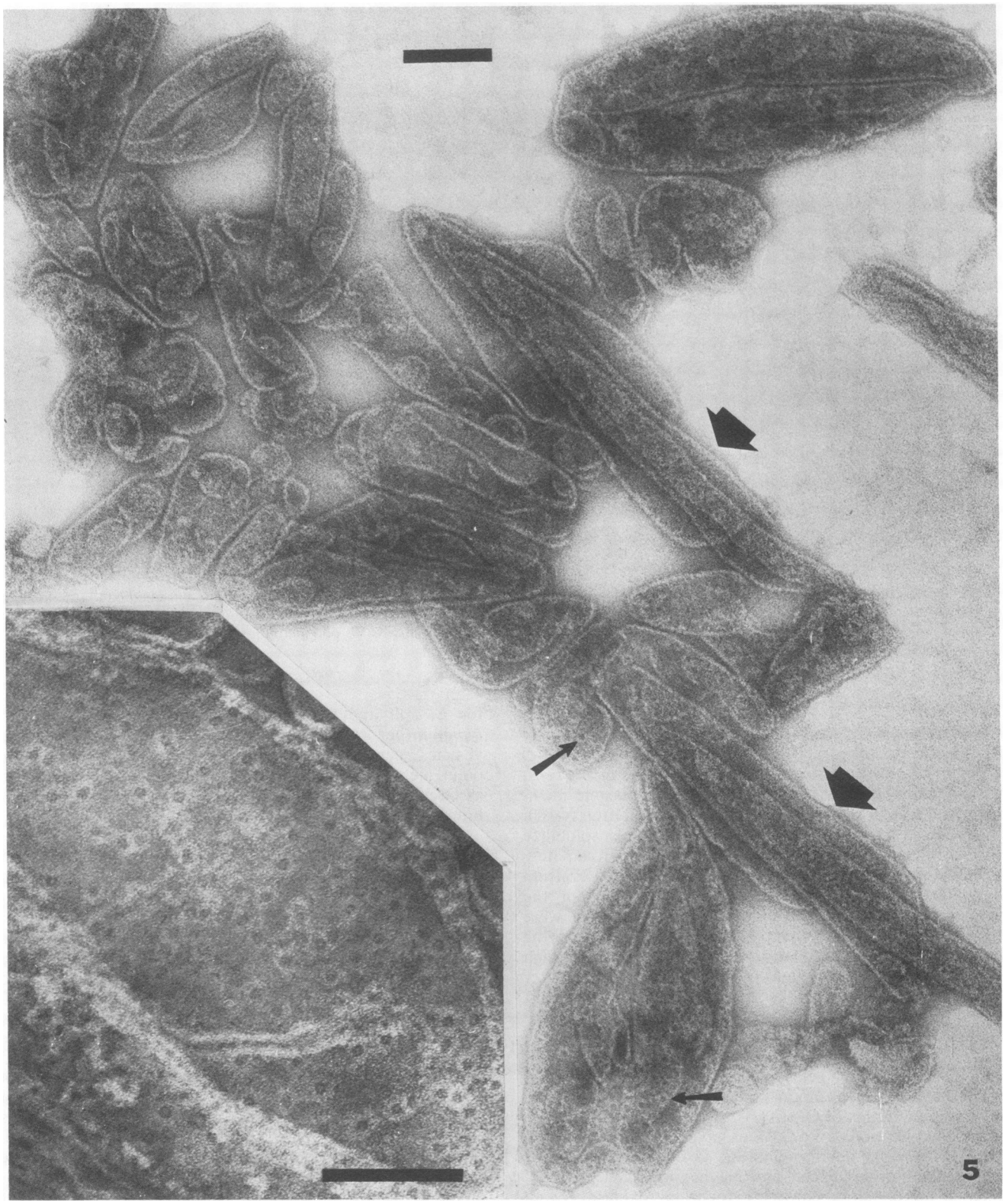

FIG. 5. Negatively stained outer membrane-enriched band of *E. corrodens* 23834 after treatment with Triton X-100. The OMF consists of large sheets of membrane, several which appear to have rolled up onto themselves (large arrows). Membrane pores are also evident (small arrows), and at a higher magnification (insert) are randomly distributed 10-nm-diameter pores. Stained with 3% phosphotungstic acid (pH 7.2). Bars, 100 nm.

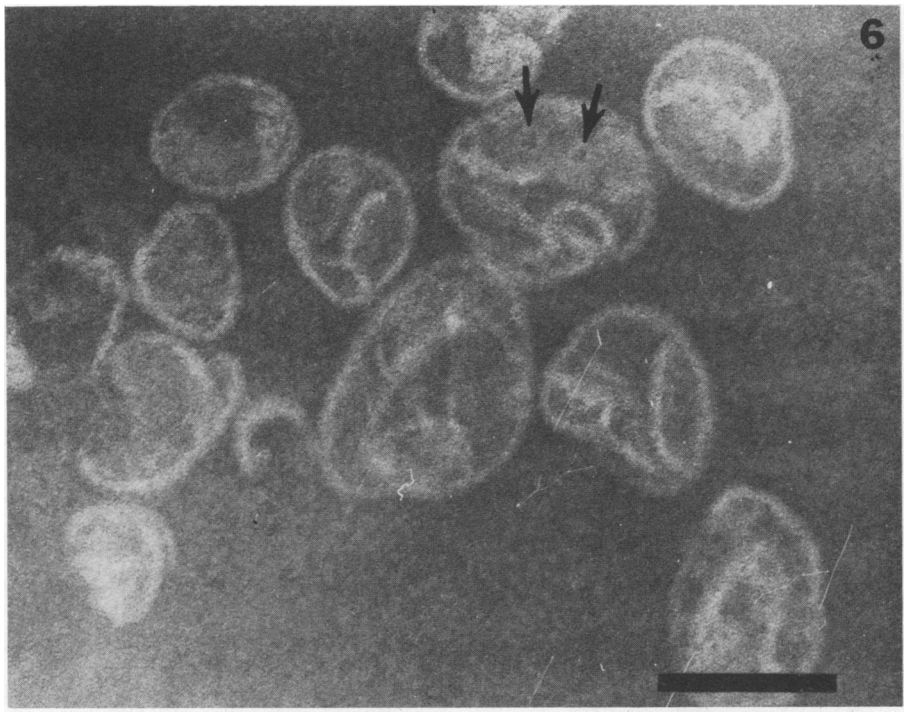

FIG. 6. TEM of negatively stained (3% phosphotungstic acid, pH 7.2) OMF of *E. corrodens* 470. Small vesicles 50 to 100 nm in diameter are apparent. Pores (arrows) occur, but are fewer in number than in the *E. corrodens* 23834 OMF (Fig. 5).

may represent a subunit of an outer membrane porin (see below).

Although the identify of the pores observed in the negative-stained electron photomicrographs of outer membrane preparations (untreated and Triton X-100 treated) is unknown, they may represent permeability channels formed by porin proteins, similar to that reported for *E. coli* and *S. typhimurium* (39). Such channels are formed through the outer membrane by proteins composed of at least three polypeptide subunits (38) in the molecular migration range of 32,500 to 38,000 dal and provide a means for free diffusion of hydrophilic molecules smaller than 650 dal through the outer membrane (7). The predominance of the 33,500- and 37,500 dal bands in the SDS-PAGE gels of *E. corrodens* 23834 and 470 OMFs, respectively, make this explanation an appealing one. The "pores" of the *E. corrodens* OMF closely resemble structurally those of the peptidoglycan matrix protein (porin) complex and purified matrix protein (36,500-dal subunits) of *E. coli* (52), except that the *E. corrodens* pores are not arranged in arrays, as are the *E. coli* porin proteins. The lack of array configurations, possibly the result of chemical charge dislocations or physical alterations during processing of the membranes, precluded any computer image analysis of the *E. corrodens* membrane preparations. However, as reported above, by direct measurement of the pores, the pore size (diameter) is estimated at 10 nm. In comparison, using computer image enhancement and optical diffraction, Steven and co-workers (52) found that the lattice constant of the porin arrays of *E. coli* was 7.7 nm.

The fatty acid profiles of the *E. corrodens* OMFs are similar to those reported by Prefontaine and Jackson (43) for cellular extracts of several other strains of *E. corrodens*. Prefontaine and Jackson (43) were able to separate strains of *E. corrodens* from other corroding bacilli (*Bacteroides ureolyticus*) and strains of *Bacteroides*, *Pasteurella*, and *Hemophilus* on the basis of their fatty acid profiles, as the *E. corrodens* strains contained a predominance of two fatty acids, hexadecanoic and octadecenoic, which accounted for approximately 70% of the total fatty acids. The six *E. corrodens* strains studied by these investigators contained 52 to 64% unsaturated fatty acids, as compared with the values reported here of 71.4% for the strain 23834 OMF and 58.8%

TABLE 1. Chemical composition of *E. corrodens* 470 and 23834 OMP and LPS

Component	% (dry wt) OMP		% LPS in strain 23834
	Strain 470	Strain 23834	
Protein	75.0	69.6	2.8
Lipid	4.8	9.2	
Carbohydrate	4.6	4.7	34.5
KDO			0.4
Heptose			0.2
Lipid A			25.0
Muramic acid	2.0	4.6	

TABLE 2. Fatty acid composition of *E. corrodens* 470 and 23834 OMP and LPS

Fatty acid	Fatty acid composition[a]		
	OMP		LPS of strain 23834
	Strain 470	Strain 23834	
$C_{12:0}$	TR	TR	ND
$C_{14:0}$	ND	ND	D
$C_{16:0}$	26.4%	28.6%	32.8%
$C_{16:1}$	15.1%	24.6%	D
$C_{18:0}$	13.8%	TR	42.0%
$C_{18:1}$	43.7%	46.8%	22.7%

[a] TR, <2%, measured as percent total fatty acids present; D, detectable (<5%); ND, not detectable.

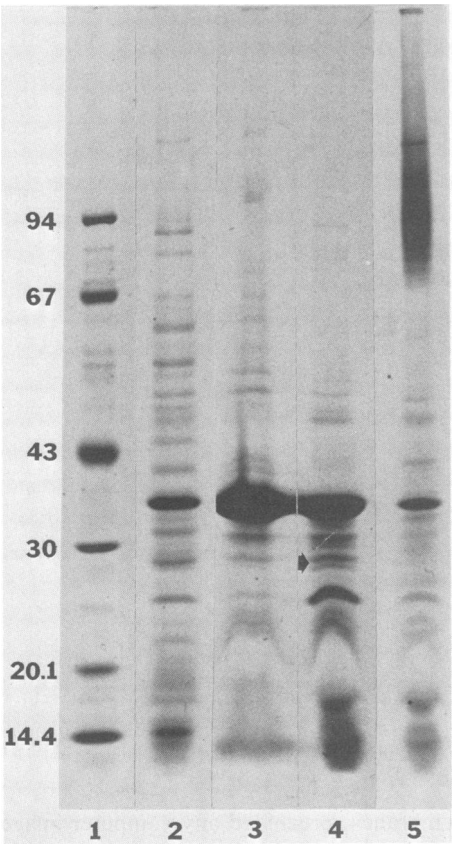

FIG. 7. SDS-PAGE (5 to 15% gradient gel) of representative membrane fractions of *E. corrodens* 23834. Lanes: 1, molecular weight standards; 2, French pressure cell lysate; 3, outer membrane before Triton X-100 solubilization; 4, outer membrane after Triton X-100 treatment (OMF); 5, OMF heated in SDS, 70°C. Lanes 2 through 5 contain 20 μg of protein per lane. Note the 26,000- and 28,000-dal bands present in the 100°C-treated OMF (arrow), but absent in the OMF heated in SDS at 70°C (lane 5).

for the strain 470 OMF. Thus, the only major difference between the results of Prefontaine and Jackson (43) and those reported here is that the *E. corrodens* 470 OMF contains a significant amount of octadecanoic acid, i.e., 13.8% of the total fatty acids present, whereas the strain 23834 OMF and all strains studied by Prefontaine and Jackson (43) contain only trace amounts of octadecanoic acid.

LPS is a unique glycolipid that is a major component of, and exists exclusively in the outer membrane of, gram-negative bacteria. The LPSs of the enteric genera, *E. coli* and *S. typhimurium*, are the best studied of all LPS molecules and have structures considered to typify classical or normal LPS, i.e., three covalently linked regions: the lipid A, the core polysaccharide, and the O-antigen. The lipid A consists of a disaccharide backbone of glucosamine substituted with aminoarabinose, ethanolamine, phosphate, and O-acylated fatty acids that include β-hydroxy myristate (57). Also linked to the glucosamine is a KDO disaccharide unit with attached heptose residues. Repeating tetrasaccharide units, which vary from serotype to serotype, make up the O-antigen, which is attached to the core polysaccharide. The hydroxy fatty acid, KDO, and heptose components are all characteristic of LPS and are used as LPS markers. The *E. corrodens* 23834 LPS is an unusual, i.e., nonclassical, LPS in that it has very low levels of (essentially no) KDO and heptose and no detectable β-hydroxy fatty acids. It is not unique however, since the LPSs of some nonenteric genera also lack one or more of these residues (26, 29, 33). For example, oral strains of *Bacteroides*, *Fusobacterium* (53), and *Capnocytophaga* (52) species have LPSs with low levels of KDO. *Neisseria meningitidis* group B LPS contains hydroxy fatty acids and KDO, but no heptose (29). Interest-

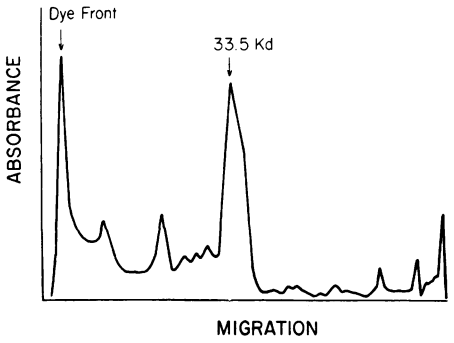

FIG. 8. Densitometric scan of Coomassie blue-stained SDS-PAGE of *E. corrodens* 23834 OMF. The major band at 33,500 daltons (33.5 kd) represent ca. 55% of the total protein.

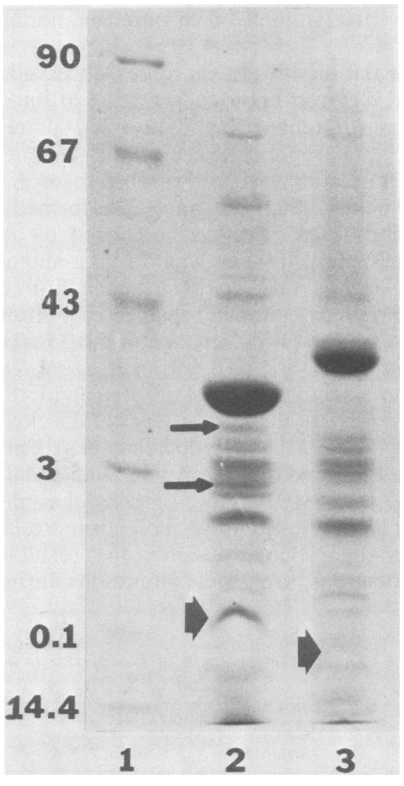

FIG. 9. SDS-PAGE (5 to 15% polyacrylamide gel) of *E. corrodens* strain 23834 OMF (lane 2) and strain 470 OMF (lane 3). A 10-mg sample of protein was applied to each lane. Two polypeptide bands visible in the strain 23834 OMF (lane 2, arrows) are absent from the strain 470 OMF (lane 3). The periodic acid-Schiff–positive band (large arrow, lane 2) appears as a lower-molecular-weight band (large arrow, lane 3).

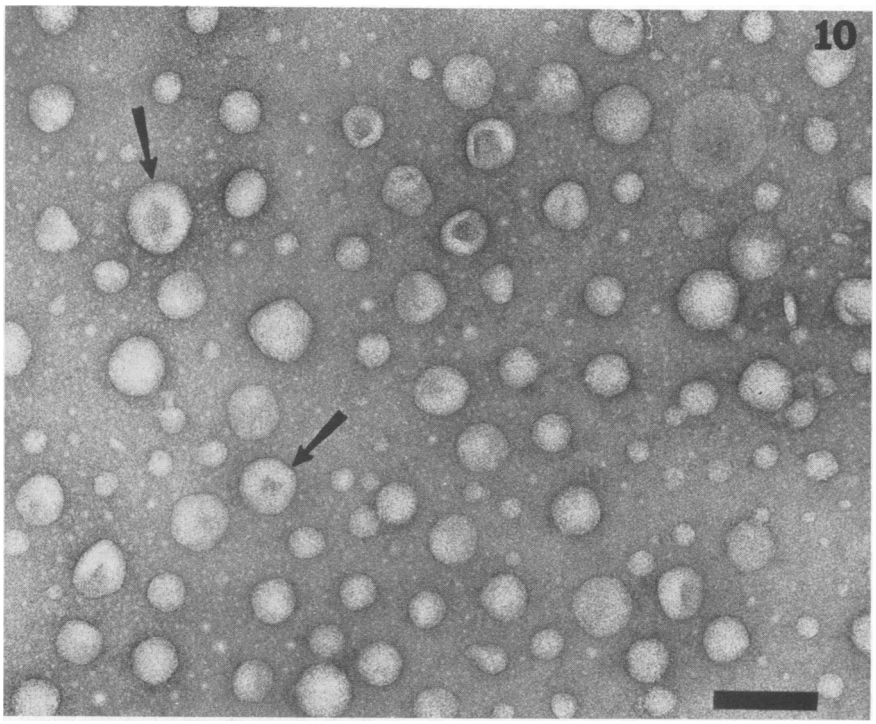

FIG. 10. TEM of negatively stained (2% phosphotungstic acid, pH 6.8) *E. corrodens* 23834 LPS. Small, 7- to 60-nm-diameter vesicle-like structures occur. Note that several appear to have collapsed toward their centers, resulting in an erythrocyte-like morphology (arrows). Bar, 100 nm.

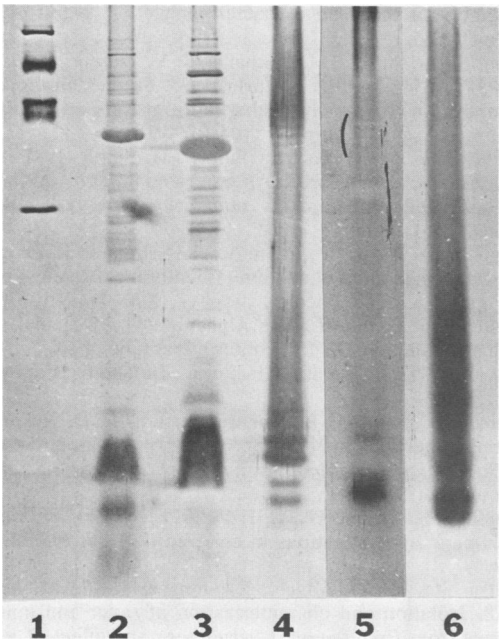

FIG. 11. Silver stain of SDS-PAGE (15% polyarylamide gel) OMFs and LPSs. Lanes: 1, polypeptide molecular weight standards; 2, 30 μg of *E. corrodens* 470 OMF; 3, 30 μg of *E. corrodens* 23834 OMF; 4, 10 μg of *E. coli* O127:B8 LPS; 5, 10 μg of *E. corrodens* strain 23834 LPS; 6, 20 μg of *Capnocytophaga gingivalis* 30N51 LPS. The *E. corrodens* LPS (lane 5) contains low-molecular-weight doublet bands and high-molecular-weight bands, similar to those of the smooth *E. coli* LPS (lane 4). Despite overloading the gel, the *C. gingivalis* LPS (lane 6) contains no visible high-molecular-weight bands. No LPS bands are apparent in the *E. corrodens* OMFs (lanes 2 and 3).

ingly, *B. fragilis* (26) and *B. melaninogenicus* (20) reportedly make LPSs that are devoid of both KDO and heptose. Although data are lacking on the distribution of hydroxy fatty acids for many of these LPS species, no hydroxy fatty acids can be detected in *Capnocytophaga* LPS (T. Poirier, personal communication). Most closely resembling the *E. corrodens* LPS, *Bacteroides gingivalis* (*B. asaccharolyticus*) LPS contains no detectable heptose, KDO, or β-hydroxy fatty acids (33). Mansheim and co-workers (33) postulate that the low endotoxic activity of this *Bacteroides* spp. LPS may be due to the absence of these components.

Perhaps the unusual morphology of negatively stained *E. corrodens* LPS, i.e., small vesicles rather than ribbons and strands, is the result of the unusual biochemistry of the molecule. Unfortunately, information regarding the morphology of the other nonclassical LPSs mentioned above is limited. However, according to Sveen (53), the KDO-limited or -deficient LPSs of *Fusobacterium* sp. and *B. melaninogenicus* stain as trilaminar disks and doughnut-shaped particles, unlike the classical LPS morphology. A comparison of the staining morphologies of the *B. gingivalis* and *E. corrodens* LPSs would be interesting, considering the similarities in their biochemistry.

Not only is the polysaccharide composition of the LPS O-antigen variable, but so also the length of the is O-antigen variable. LPSs with many repeating subunits of saccharides are known as smooth LPS, and those in which little or no O-antigen is present are termed rough LPS. A procedure for detecting bacterial LPSs in polyacrylamide gels by specifically staining the polysaccharide moiety was reported recently by Tsai and Frasch (56) and can differentiate smooth and rough LPSs by their staining patterns. Employing this staining procedure, it appears that the *E. corrodens* strain 23834 LPS is of the smooth type in that slower-migrating

polysaccharide bands (indicating long-chain polysaccharides) are visible and are comparable to those of the smooth *E. coli* LPS and similar to those reported by Tsai and Frasch (56) for smooth strains of *S. typhimurium* and *E. coli* LPSs. Interestingly, no high-molecular-weight bands are visible with *Capnocytophaga* sp. LPS, similar to the results of Tsai and Frasch (56) for a semirough LPS of *S. typhimurium*.

Since it appears that the *E. corrodens* LPS is a smooth, but atypical, LPS lacking three of the classical components of LPS, it would be interesting to determine whether the *E. corrodens* LPS possesses sufficient classical endotoxic activity, properties which would make it a potential factor in periodontal tissue necrosis.

The isolation of two highly purified preparations of *E. corrodens* surface structures, i.e., OMF and LPS, provides important cellular fractions for the assay of the biological activities of each fraction (45) and may provide information on the etiology of periodontal disease as well as other abscesses containing *E. corrodens*.

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