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Biochemical Properties of the Outer Membrane of *Treponema denticola*

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The outer membranes (OMs) from serovars a, b, and c of *Treponema denticola*, originally isolated from periodontal patients, were prepared. Dialysis of the OMs against 20 mM $MgCl_2$ yielded the aggregable (A) and the nonaggregable (NA) moieties of the OMs. The absence of muramic acid, adenosine triphosphatase, hexokinase, and nucleic acid as well as electron microscopy indicated that the OM preparations were homogeneous. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the A and NA moieties of the OMs showed approximately 25 Coomassie brilliant blue R-250 stain-positive bands or 47 silver-stained polypeptides. The relative molecular masses ranged between 14 and 97 kDa. The electrophoretic polypeptide profiles of the A and NA moieties shared many similarities among serovars a, b, and c. However, they exhibited variation in the overall pattern, intensity, or location of the polypeptide stained zones. This was especially true for serovar b. Two-dimensional electrophoretic studies showed an excess of 100 silver-stained spots with isoelectric points of 4.6 to 7.0 and relative molecular masses in the 14- to 97-kDa range. The OMs contained simple proteins, glycoproteins, and lipoproteins. The NA moieties of the OMs contained 4 to 6, 10 to 12, and 4 to 6 glycopeptides as well as two, seven, and two lipoprotein bands for serovars a, b, and c, respectively. The A moieties of the OMs showed 7 to 9, 11 to 13 and 5 to 6 glycopeptides as well as four, five, and three lipoprotein bands for serovars a, b, and c, respectively. Lipopolysaccharide was detected in the OMs of the three serovars following removal of proteins with proteinase K, pronase and silver staining of sodium dodecyl sulfate-polyacrylamide gels, or removal of lipopolysaccharide from the OMs by hot phenol extraction. The 66- and 53-kDa bands were present in serovars b and c, while a band with a relative molecular mass of 45 kDa was present only in serovar c. Endotoxin-like activity was also shown in the OMs of the three serovars by the *Limulus* amebocyte clotting assay and the chick embryo lethality test. This is the first report on selected biochemical properties of the OM macromolecules of three known serovars of *T. denticola*.

An acknowledged cause of periodontal disease is the bacteria found in the dental plaque (26). Intermediate-size oral spirochetes, including *Treponema denticola*, have been associated with acute necrotizing ulcerative gingivitis (27, 31). The three serovars a, b, and c of *T. denticola* have been described previously (7, 34). Using monoclonal antibodies developed against serovar "b," Simonson et al. (33) provided evidence of a positive relationship between *T. denticola* serovar "b" and severe periodontitis. This spirochete was present at a significantly elevated level in plaque samples collected from deep subgingival pocket sites of patients with severe periodontitis (33). *T. denticola* produces immunosuppressive substances (3), tissue-damaging enzymes (30), and metabolic products and antigens which are potentially harmful (27).

The structure of *T. denticola* is, in general, similar (5) to that of the other spirochetes and consists of a typical cytoplasmic membrane composed of phospholipids and proteins or enzymes such as adenosine triphosphatase, periplasmic flagella, a cell wall containing muramic acid, and an outer membrane (OM), or outer sheath, which can be removed by treatment with 1.4 mM sodium dodecyl sulfate (20). Limited information is available on the macromolecular composition and function of the OM of oral spirochetes. For example, by using intact cells, cell lysates, and immunoelectron microscopy, a 53-kDa antigen has been found in the outer membrane of *T. denticola* serovar c (34, 40). In the present investigation, the macromolecular profiles and some biological properties of clean OM preparations of *T. denticola* serovars a, b, and c are presented.

MATERIALS AND METHODS

Isolation of the OM. *T. denticola* ATCC 35405 (serovar a), ATCC 33521 (serovar b), and ATCC 35404 (serovar c) were used in this study. These strains were originally isolated from human periodontal pockets. The spirochetes were cultured anaerobically at 35°C for 5 to 8 days in the prereduced medium of Blakemore and Canale-Parola (2) which was supplemented with 5% (vol/vol) heat-inactivated rabbit serum. Cells from 15 liters of cultures of the assay treponemes in the late logarithmic phase of growth were removed by centrifugation at 12,000 × *g* for 30 min, washed two times with deionized doubly distilled water, and lyophilized. The OMs were removed from the spirochetes by treatment for 15 min with 1.4 mM sodium dodecyl sulfate (SDS) and subsequently isolated as described by Johnson et al. (20). The adopted protocol involved the following modifications. Phenylmethylsulfonyl fluoride (1 ml of a 100 mM solution) was added to the OM isolation mixture to prevent protein degradation by serine proteases. Furthermore, fol-

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lowing the 15-min exposure of the spirochetes to 1.4 mM SDS and the removal of cells by centrifugation, the supernatant was passed through a 0.22-μm-pore-size filter instead of a 0.45-μm-pore-size Millipore filter. This was done to avoid contamination of OMs by intact spirochetes, which can pass through 0.45-μm-pore-size filters. Dialysis of the outer membranes against 20 mM $MgCl_2$ yielded the aggregable (A) and the nonaggregable (NA) moieties of the OM. $MgCl_2$ treatment was designed for the enrichment of the OM macromolecules. In contrast to the previous isolations of spirochetal OMs (20), in this study the OMs were assayed for possible contamination by using DNA, RNA, and hexokinase as markers of cytoplasmic constituents; ATPase as a cytoplasmic membrane marker; and muramic acid as a peptidoglycan marker (4, 6, 11, 12, 21, 23). Furthermore, the morphology of the OMs and the spirochetes from which the OMs were removed was determined by electron microscopy (20). Briefly, preparations containing 100 to 300 μg of the A and NA moieties of the OMs were used for biochemical assays. DNA was determined by the diphenylamine method, using calf thymus DNA as the standard (4). RNA was determined by the method of Griswold et al. (11), using yeast RNA (type III; Sigma Chemical Co., St. Louis, Mo.) as a standard. Agarose gel electrophoresis was also used to assay for DNA (19). Hexokinase was measured by an NADP-linked assay (21), which measured the increase in NADPH A_{340}. The assay mixture contained 0.1 ml of phosphate buffer (0.1 M, pH 7.5), 0.2 ml of 0.15 M glucose, 40 μl of 1 M $MgCl_2$, 100 μl of 13 mM NADP, 2 U of glucose 6-phosphate dehydrogenase, 100 to 300 μg of the NA or A moiety of the OM, and 81 mM ATP; and the volume was brought to 2 ml with deionized distilled water. Baker's yeast hexokinase (sulfate-free; EC 2.7.1.1; type F 300; Sigma) was used as a standard. Controls consisted of reaction mixtures in which glucose 6-phosphate dehydrogenase, the source of hexokinase, or glucose was omitted. ATPase was measured by the liberation of P_i (6, 23) in 1 ml of reaction mixture containing 2.5 mM ATP (vanadate-free), 100 mM KCl, 4 mM $MgCl_2$, and 50 mM Tris-acetate buffer (pH 6.5). The reaction was started by the addition of the NA or A moiety of the OM containing 100 to 300 μg of protein, and it was terminated by the addition of 0.5 ml of 1.5 M perchloric acid. The controls included the reaction mixture to which the sample was added after perchloric acid treatment. Muramic acid was determined by the colorimetric *p*-hydroxybiphenyl method of Hadzija (12). Positive controls included mixtures to which known quantities of muramic acid were added. Negative controls included mixtures without the recommended reagents. The muramic acid used as a standard was treated identically as the NA and A moieties of the OMs were.

Protein estimations of samples containing mercaptoethanol were performed by the protocol of Hill and Straka (13), which uses complex formation of bicinchoninic acid with copper and iodoacetamide binding of free mercaptoethanol. The A moieties were solubilized with EDTA at a final concentration of 5 to 8 mM. The OM moieties that did not contain mercaptoethanol were processed directly with bicinchoninic acid reagent (35).

Electron microscopy. Spirochetal preparations were fixed with 1% OsO_4, dehydrated, and embedded in Epon. Sections were cut on an LKB III ultratome and collected on Parlodion-covered copper grids. The sections were stained with 2% uranyl acetate and examined with an Hitachi HV-IIB electron microscope operated at 75 kV (28).

Electrophoretic studies. SDS-polyacrylamide gel electrophoresis (PAGE) was done on 12.5% (wt/vol) polyacrylamide gels essentially by the method of Laemmli (24). However, 0.8% piperazine diacrylamide (Bio-Rad, Richmond, Calif.) replaced bisacrylamide to reduce background staining, increase physical gel strength, and reduce gel swelling (15, 16). Charcoal treatment of acrylamide and piperazine diacrylamide was also used to remove some impurities in the gels. Finally, SDS was omitted from the separating gel, but the concentration of SDS was increased to 0.2% in the running gel buffer to reduce interference by SDS during treatment with the silver staining reagents (15, 16, 29, 37). SDS-PAGE was performed at 4 to 6°C in a Hoeffer SE 600 gel chamber with a current of 15 mA per gel until the tracking dye reached the separating gel, and then a constant current of 30 mA per gel was used for approximately 4 h. Generally, the following proteins (Bio-Rad and Sigma) were subjected to electrophoresis as described above for SDS-PAGE and served as relative molecular weight standards: β-galactosidase, 116,250; phosphorylase *b*, 97,400; bovine serum albumin, 66,000; ovalbumin, 45,000; glyceraldehyde-3-phosphate dehydrogenase, 36,000; carbonic anhydrase, 29,000; trypsinogen, 24,000; trypsin soybean inhibitor, 20,100; and lactalbumin, 14,200. Gels were stained with 0.2% (wt/vol) Coomassie brilliant blue R-250 in 50% ethanol–5% acetic acid or with an ammoniacal silver nitrate solution (1, 16, 17).

Periodic acid-Schiff staining in SDS-polyacrylamide gels. Following electrophoresis, the gels were processed as described by Fairbanks et al. (8) for glycoprotein detection. Samples to be compared for glycoprotein staining were electrophoresed in the same slab so that a side-by-side comparison could be made of the protein components. A known glycoprotein control was electrophoresed and processed simultaneously. The periodate step was omitted as an additional control. The Schiff reagent was freshly prepared.

Apolipoprotein staining. Detection of apolipoprotein was made by comparison of the lipase-treated and untreated samples following SDS-PAGE. To samples containing 150 μg of protein, 8.5 U of yeast lipase type VII (EC 3.1.1.3; Sigma) was added to a reaction mixture consisting of the sample, lipase, 50 mM Tris hydrochloride (pH 7.4), and 10 mM $MgCl_2$. The reaction mixture was incubated at 37°C for 2 h. Then, usually 30 ml of 5× Laemmli sample loading gel buffer was added, and the samples were processed for SDS-PAGE (24). Beta-serum lipoprotein fraction III (National Biochemical Products, Cleveland, Ohio) was used as an apolipoprotein standard. Following SDS-PAGE, the gels were placed for 24 h in a solution composed of 0.4% Sudan black dissolved in ethylene glycol–15% acetone–12% glacial acetic acid. The gels were destained with the solution described above, but without the Sudan black dye (10).

Detection of LPS. Two approaches were used to detect lipopolysaccharide (LPS) in the A and NA moieties of the OM. The first method consisted of pronase or proteinase K digestion of the OM moieties prior to SDS-PAGE, periodate oxidation of LPS, and silver staining of the gels (30). Briefly, samples containing 100 μg of protein were placed in the sample lysing buffer and prepared for SDS-PAGE (24). For protein digestion, 0.25 μg of proteinase K per μg of the sample dissolved in the lysing buffer was added and incubated at 60°C (14). Following SDS-PAGE, the gels were fixed in a solution containing 40% ethanol and 5% acetic acid. LPS oxidation was then attempted for 10 min with 0.7% periodic acid dissolved in a mixture composed of 40% ethanol–5% acetic acid (39). The gels were washed three times with deionized water for 20 min each time and stained with silver nitrate. The second method for LPS detection

was a slight modification of the procedure described by Kido et al. (22), in that a hot phenol extraction (65°C for 10 min) instead of the cold phenol treatment was used to extract the LPS from the OMs. *Escherichia coli* endotoxin O55:B5 (Sigma), untreated or treated, was used as a standard. Two-dimensional PAGE was performed as described previously (16, 30).

***Limulus* test.** Experiments designed to detect LPS in the A and NA moieties of the OMs were conducted by the method of Levin and Bang (25). New disposable plasticware found to be free of endotoxin was usually used. Sterile and pyrogen-free glassware, when needed, was prepared by autoclaving it for 45 min, followed by heating it for 3 h at 175°C in a dry oven. All tests were conducted in new disposable polystyrene culture tubes (10 by 75 mm), which were immediately capped after the reaction mixtures were added. All endotoxin-free reagents required for the performance of the *Limulus* test, which was supplied in a kit with assay instructions known as E-Toxate (*Limulus* amebocyte lysate), were purchased from Sigma. Optional endotoxin-free reagents, such as 0.1 N HCl and 0.1 N NaOH for pH adjustment of the samples to be tested at the optimum range of pH 6.8 to 7.5, were also purchased from Sigma. E-Toxate had a sensitivity of 0.025 to 0.1 endotoxin unit per ml toward the standard *E. coli* O55:B5 LPS. Dilutions of test and control samples (*E. coli* LPS in pyrogen-free water) were incubated in tubes with amebocyte lysate at 37°C for 1 h, and then the tubes were inverted. Any tube which did not contain a hard gel that did not adhere to the bottom of the tube was scored as negative.

Chick embryo lethality. Large white eggs obtained from a single flock of Illinois hens were used in all experiments. The eggs were incubated at 37.0 ± 0.5°C and rotated daily. On day 11 of incubation, the eggs were candled, the chorioallantoic membrane was dropped, and the substance to be tested was inoculated onto the choriallantoic membrane (36). The eggs were broken 18 to 24 h later and examined for dead embryos. As was the case with the *Limulus* assays, only sterile pyrogen-free plasticware and glassware were used during the chick embryo assay. Dilutions of test and control samples were made in pyrogen-free water (Sigma). Bacterial contamination of the eggs was rare, and experiments in which it occurred were discarded. The experiments described here were repeated three to seven times and were reproducible, and the typical data depicted in Fig. 1 to 7 or Tables 1 to 3 were obtained.

RESULTS

Release of the OM. Electron microscopy of serovars a, b, or c indicated that exposure of *T. denticola* serovar a, b, or c to 1.4 mM SDS for 15 min removed the outer membranes of the assay spirochetes without disturbing the typical protoplasmic cylinder. The electron micrographs indicate that the periplasmic flagella remained attached to protoplasmic cylinders. Others may have been removed with the protoplasmic cylinders during the separation of the OMs from the spirochetes. Also, electron microscopy of the A and NA moieties of the OM preparations did not show any periplasmic flagella (Fig. 1). Similarly, biochemical analysis for DNA, RNA hexokinase, ATPase, and muramic acid indicated that neither the A nor the NA moiety of the OM contained any detectable cytoplasmic contents, cell membrane, or peptidoglycan markers (Table 1). The yield of the OMs ranged between 4 and 7% of the cell dry weight and was approximately equally divided between the A and NA moieties.

OM polypeptide characterization by SDS-PAGE. The NA moieties of the OMs of *T. denticola* serovars a, b, and c showed 10, 8, and 12 Coomassie brilliant blue R-250-stained polypeptides, respectively. Upon SDS-PAGE, the A moieties revealed 15 to 22 Coomassie brilliant blue R-250-stained polypeptides for serovars a, b, and c. The majority of the polypeptides had a relative molecular mass that ranged between 14 and 97 kDa. Some faint bands had a relative molecular mass of 10 kDa. The electrophoretic polypeptide profiles of both moieties of the three serovars showed many similarities. A band with a relative molecular mass of approximately 50 kDa was found in both the A and NA moieties of the three serovars. However, upon close examination, they revealed slight variations. For example, the A moiety of serovar b had more bands near the 35- to 66-kDa range than serovars a or c did, and it also formed a band at the 20-kDa-molecular-mass scale (Fig. 2).

Detection of the OM polypeptides was greatly improved by silver staining of the SDS-polyacrylamide gels. A protein load of 15 μg resolved approximately 47 silver-stained polypeptides for both the A and NA moieties of the OMs of serovars a, b, and c. The relative molecular masses ranged between 14 and 97 kDa. The electrophoretic polypeptide profiles of the A and NA moieties remained constant, and they shared many similarities, forming common bands at the 50- to 53-kDa-molecular-mass range. However, they exhibited variation in the overall pattern, intensity, or location of the stained zones of polypeptides. For example, the A moiety of serovar a showed a well-spaced ladder-like electrophoretic profile and contained fewer bands than the A moieties of serovars b or c did. Furthermore, a 17-kDa band present in the NA moieties of the three serovars was absent in the A moieties of serovars a, b, and c (Fig. 3).

Glycoprotein staining. An effort was made to assess the OMs for the presence of glycoproteins. Therefore, following SDS-PAGE of the protein samples of the OM proteins, the gels were processed for periodic acid-Schiff staining of glycopolypeptides (8). The experimental protocol revealed 7 to 9, 11 to 13, and 5 to 6 stained bands of glycopolypeptides for the A moiety of the serovars a, b, and c, respectively. The NA moieties of the OMs of serovars a, b, and c contained 4 to 6, 10 to 12, and 4 to 6 faintly stained Schiff-positive bands, respectively. The glycopolypeptides of the OMs of the three serovars were found to have relative molecular masses of 15 to 66 kDa (Fig. 4). It is recognized that in the SDS-PAGE system, glycoprotein electrophoretic mobilities may not reflect reliable molecular masses (41). A known glycoprotein, horseradish peroxidase, was used as a positive control for the periodic acid Schiff reagent reaction. Omission of either the periodic acid treatment or Schiff reagent yielded negative results. The A moieties of the OMs of the three serovars showed common bands at approximately the 31-, 38-, and 55-kDa-molecular-mass scale. The NA moieties of the OMs of serovars a, b, and c had common bands at the 43- and 70-kDa scale.

These experiments also indicated that there is variation in terms of number, location, or concentration of glycopolypeptides in the A and NA moieties of each serovar as well as between the three serovars of *T. denticola*. For example, there appears to be a higher level of a 31-kDa glycopolypeptide in the A moiety of serovar b than in the A moieties of serovars a and c. Similarly, the 26-, 34-, 48-, and 60-kDa glycopolypeptides were found only in the A moiety of serovar b. The A moiety of serovar c also lacked the 66-kDa glycopeptide.

Apolipopolypeptide staining. To determine the presence of

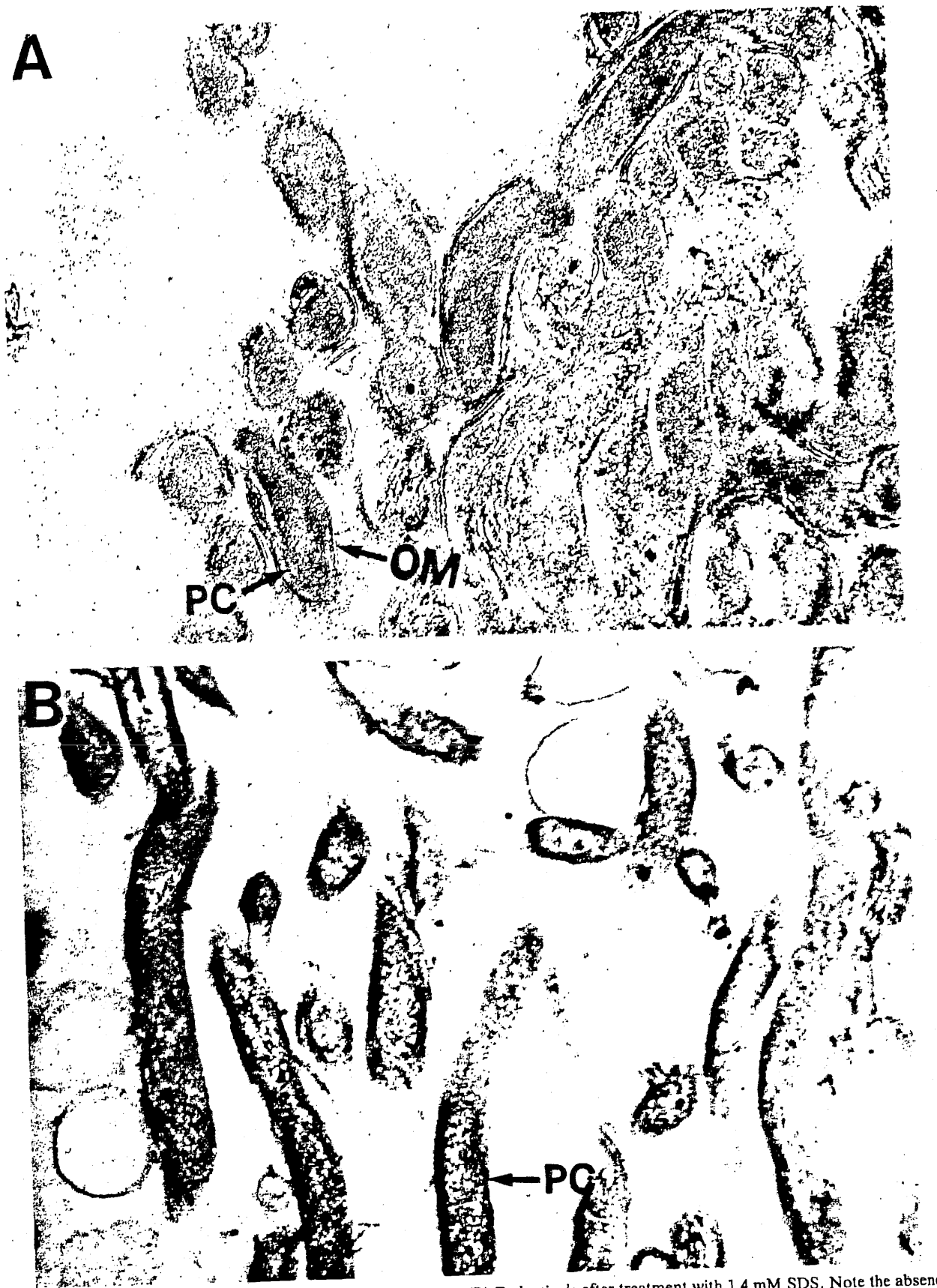

FIG. 1. (A) Normal *T. denticola* cells a, b, or c (section) serovar. (B) *T. denticola* after treatment with 1.4 mM SDS. Note the absence of the OM on the protoplasmic cylinder. (C) The A moiety of the OM obtained by dialysis against 20 mM $MgCl_2$. (D) The NA moiety of the OM of *T. denticola*. OM, outer membrane; PC, protoplasmic cylinder. Magnifications, ×67,200.

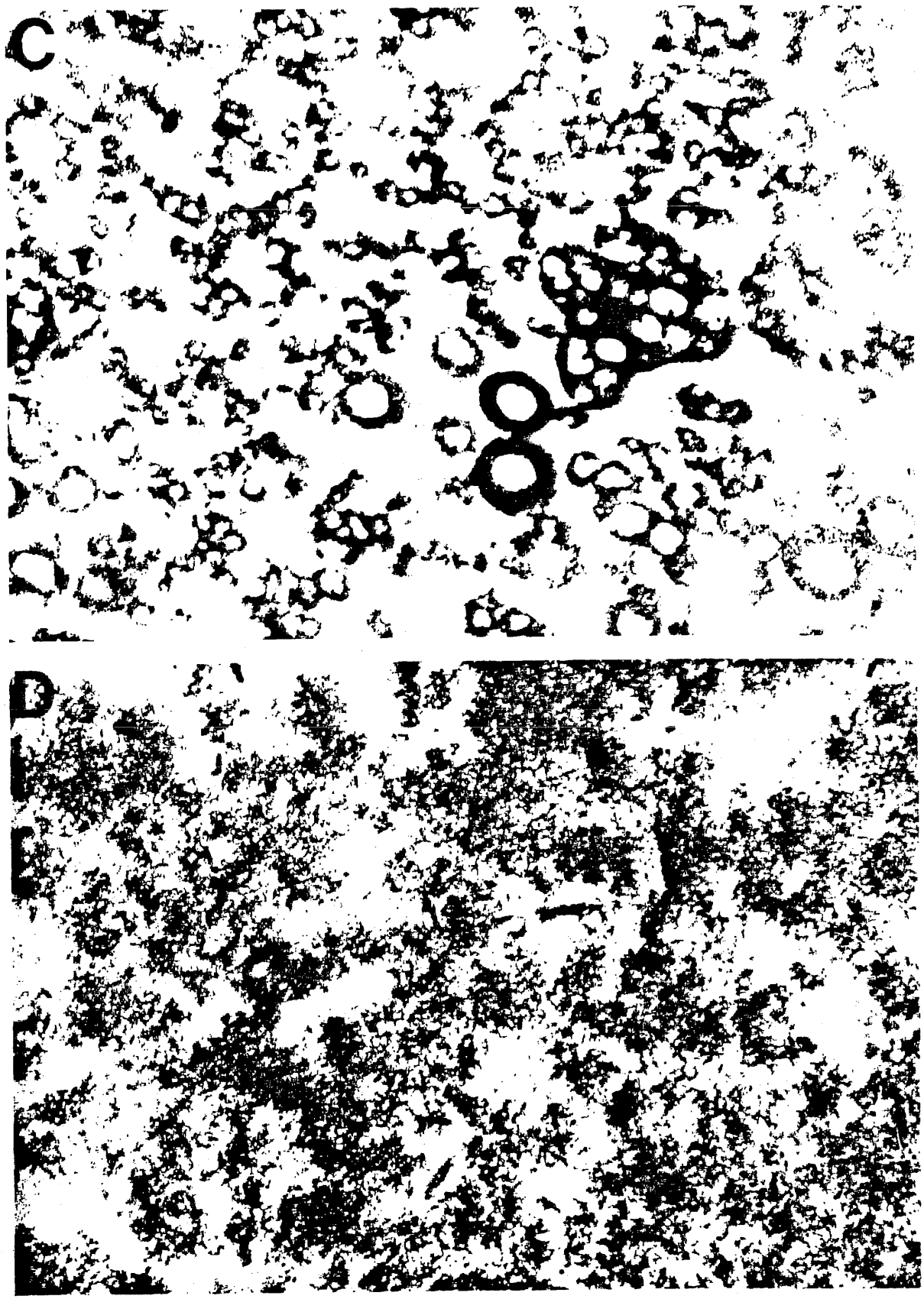

FIG. 1—*Continued.*

TABLE 1. Homogeneity of the OMs of *T. denticola* serovars a, b, and c

Possible source of contamination	Marker[a]	Sample size[b] (μg of protein)	Sensitivity of assay
Cell wall	Muramic acid	300	5 μg
Cytoplasmic membrane	ATPase	300	0.15 μg of released P
Cytoplasmic contents	DNA	200	2.5 μg (diphenyl reaction)
	DNA	100	10 ng (electrophoresis)
	RNA	300	10 μg (orcinol reaction)
	Hexokinase	300	1 μM

[a] None of the markers was detected.
[b] Equal quantities of the A and NA moieties of the OMs were tested in five consecutive OM preparations.

apolipopolypeptides in the OM of *T. denticola*, the resolved polypeptide components were stained with Sudan black following SDS-PAGE. Other investigators (10), using Sudan black for the identification of serum lipoproteins, have demonstrated the usefulness of this stain.

The NA moieties of the OMs of serovars *a*, *b* and *c*, upon SDS-PAGE and staining of the gels, yielded two, seven, and two Sudan black-positive bands, respectively. The bands appeared faint. The A moieties of the OMs of *T. denticola* contained four, five, and three Sudan black-positive bands for serovars a, b, and c, respectively. Lipase treatment of the OM moieties prior to SDS-PAGE reduced greatly or eliminated the Sudan black-positive bands. With both the A and NA moieties of the OMs of the three serovars, the Sudan black-positive bands appeared within the 15- to 66-kDa relative-molecular-mass range. The NA moiety of serovar b formed faint Sudan black-positive bands at the 16-, 29-, 36-, 42-, and 48-kDa relative-molecular-mass scale, while the same moiety of serovar a formed only faint Sudan-positive bands at the 36- and 42-kDa area. The A moieties of the OMs of the three serovars had common Sudan black-stained bands at the 29-, 36-, and 48-kDa scale. Serovars a and b shared a 66-kDa band. However, serovar b had an additional 42-kDa band.

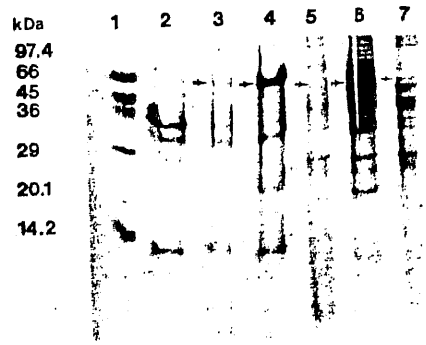

FIG. 2. SDS-PAGE analysis of the OM polypeptides of *T. denticola* serovars a, b, and c stained with Coomassie blue. Lane 1, molecular mass markers; lanes 2 through 4, the NA moieties of the OMs of serovars a, b, and c, respectively; lanes 5 through 7, the A moieties of the OMs of serovars a, b, and c, respectively. Arrows indicate the polypeptides described in the text.

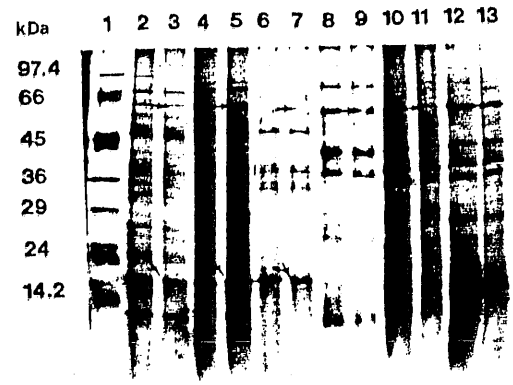

FIG. 3. SDS-PAGE analysis of the outer membrane of *T. denticola* serovars a, b, and c stained with silver nitrate. Lane 1, molecular mass markers; lanes 2 and 3, 4 and 5, and 6 and 7, charged with samples of 15 or 7.5 μg of protein of the NA moieties of the OMs of serovars a, b, and c, respectively; lanes 8 and 9, 10 and 11, and 12 and 13, charged with samples of 15 or 7.5 μg of protein of the A moieties of the OMs of serovars a, b, and c, respectively. Arrows indicate the polypeptides described in the text.

LPS staining. Pronase or proteinase K digestion of the NA moieties of serovars a, b, and c yielded about two, six, and five LPS bands, respectively, with relative molecular masses of 15 to 66 kDa. The 66- and 53-kDa bands were present in serovars b and c but were absent in serovar a, while a band with an approximate relative molecular mass of 45 kDa was present in serovar c but not in serovars a or b. The 66- and 53-kDa bands of serovar b were more prominent than those for serovar c were. The 66- and 53-kDa LPS bands were found in the three serovars of the A moieties, but were so

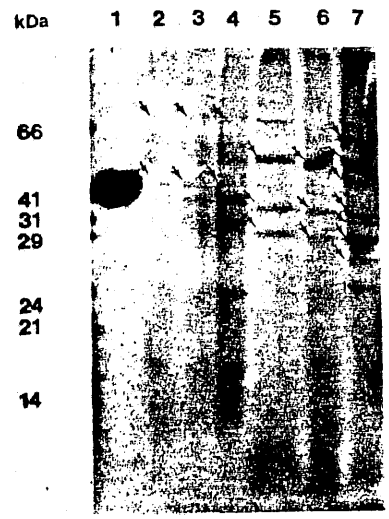

FIG. 4. Periodic acid-Schiff staining of the OM polypeptides of *T. denticola* serovars a, b, and c following SDS-PAGE. Each lane was charged with a 150-μg protein sample. Lane 1, a known glycoprotein control, peroxidase; lanes 2 through 4, charged with the NA moieties of the OMs of serovars a, c, and b, respectively; lanes 5 through 7, charged with the A moieties of the OMs of serovars a, c, and b, respectively. Arrows indicate the polypeptides described in the text.

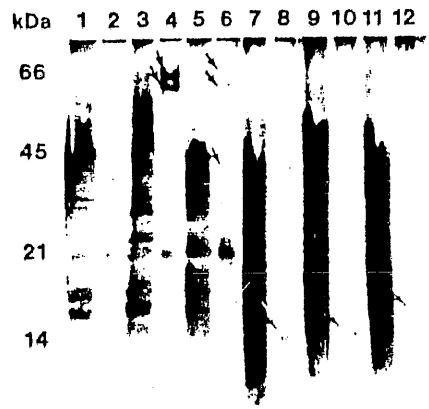

FIG. 5. LPS silver staining after pronase digestion of the outer sheath of *T. denticola* serovars a, b, and c. Lanes 1 and 2, 3 and 4, and 5 and 6, charged with samples containing 15 μg of protein of the pronase-untreated and -treated NA moieties of the OMs of serovars a, b, and c, respectively; lanes 7 and 8, 9 and 10, and 11 and 12, charged with samples containing 15 μg of protein of the pronase-untreated and -treated A moieties of serovars a, b, and c, respectively. Arrows indicate the polypeptides described in the text.

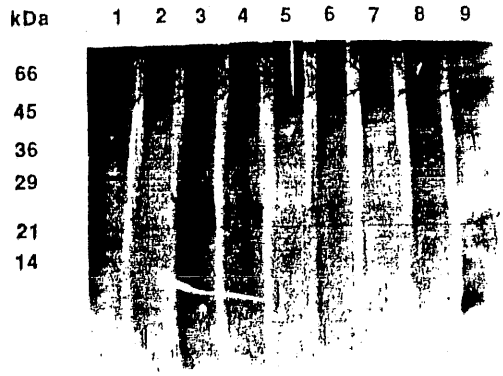

FIG. 6. LPS silver staining after hot phenol extraction of the OMs of *T. denticola* serovars a, b, and c. Lane 1, molecular mass standards; lanes 2 through 4, LPS extracted from samples containing 200 μg of protein of the NA moieties of the OMs of serovars a, b, and c, respectively; lanes 5 through 7, LPS extracted from samples containing 200 μg of protein of the A moieties of the OMs of serovars a, b, and c, respectively; lane 8, charged with 0.4 μg of *E. coli* O55:B5 LPS that had been extracted with hot phenol; lane 9, charged with 0.4 μg of *E. coli* O55:B5 LPS. Arrows indicate the polypeptides described in the text.

faint that they could not be photographed. Several bands with relative molecular masses of 10 to 15 kDa were very prominent in the A moieties of the three serovars, and they appeared very faint or absent in the NA portion of the outer membranes of serovars a, b, and c (Fig. 5).

To improve the resolution of the LPS bands described above, hot phenol extraction of the LPS and SDS-PAGE experiments were conducted. The NA moiety of serovar b had a distinct band with a relative molecular mass of 24 kDa that was absent from serovars a and c. The 66- and 53-kDa bands were found in both the NA and A moieties of serovars a, b, and c as well as the *E. coli* LPS, which was used as a control. It should be pointed out that the hot phenol LPS extraction procedure eliminated the 10-kDa band found in LPS preparations involving the use of proteolytic enzymes (Fig. 6).

Two-dimensional protein electrophoretic studies. To obtain a more precise account of the number, the isoelectric points, and the relative molecular masses of the OM polypeptides, two-dimensional polypeptide analysis experiments were conducted. These experiments showed an excess of 100 silver-stained polypeptides with isoelectric points of 4.2 to 6.9 and relative molecular masses of 14 to 97.4 kDa (Fig. 7). Similar relative molecular masses and isoelectric point profiles of the OM polypeptides were observed for serovars a and c.

***Limulus* assays.** Gelation of the *Limulus* amebocyte lysate has been used as a sensitive means for the detection of endotoxin (25). Since the electrophoretic studies indicated that LPS was present in the A and NA moieties of the OMs of *T. denticola*, *Limulus* tests were conducted to obtain additional evidence for the presence of endotoxin in the OM of *T. denticola*. Table 2 summarizes the results of these experiments. It is apparent that both the A and NA moieties of the OMs of serovars a, b, and c showed *Limulus* amebocyte lysate clotting activity. The A moieties had gelation endpoints in the 5-ng range, while the NA moieties, with the exception of serovar b, had gelation endpoints of 5 to 15 ng. The *E. coli* endotoxin control had a gelation endpoint of 0.5 to 1 ng.

Chick embryo lethality assays. Chick embryos have been used to assay the biological properties of endotoxins (36). We conducted chick embryo assays to extend our work on the LPS found in the OMs of *T. denticola*. The data obtained from these assays are given in Table 3. With the exception of serovar c, doses of 48 to 80 μg of the NA moieties and 200 to over 400 μg of the A moieties of the OMs of *T. denticola* were required to kill the 11-day-old chick embryos. *E. coli* LPS had a lethal dose close to that found for the A moieties of serovars a and b.

DISCUSSION

The macromolecules of the spirochetal cell surface may be used for the identification of the spirochetes. Furthermore, they may be toxic to gingival cells, or they may be involved in the attachment of spirochetes to the oral tissue. The biochemical properties of the OMs of *T. denticola* serovars a, b, and c and their moieties remain unknown. Mild treatment of *T. denticola* with 1.4 mM SDS produced OMs with undetectable contamination from the cytoplasm, cell membrane, cell wall, or other recognizable spirochetal components, as judged by chemical, enzymatic, and electron microscopic examinations (Table 1, Fig. 1).

Exposure of oral spirochetes to 1.4 mM SDS solubilized the OM. However, aggregation of the OM by 20 mM $MgCl_2$ depends upon the microorganism under study (20). Thus, to avoid errors in the polypeptide profile of the OM, electrophoretic experiments with both the A and the NA moieties of the OM were conducted.

The data presented in this report indicate that the OM of the assay oral spirochetes contain simple proteins as well as glycoproteins and lipoproteins. Although the OM polypeptide profiles of the three serovars of *T. denticola* were similar in many respects, there appeared to be a certain diversity with regard to the concentration, number, or location of the various polypeptides within the electropherograms. Isolation of these unique polypeptides may provide us with some macromolecules that could be used in studies concerned

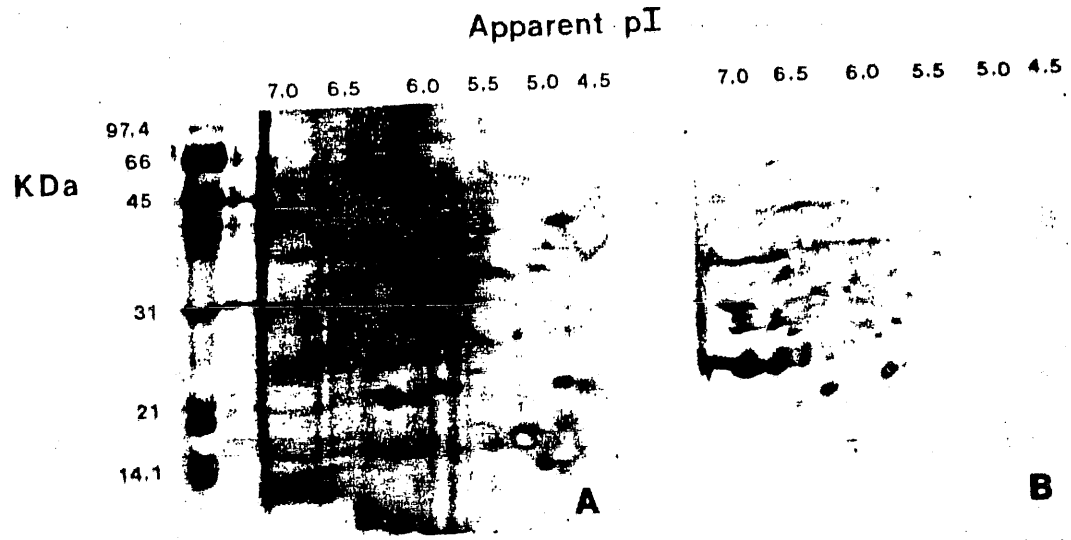

FIG. 7. Silver-stained two dimensional polypeptide profile of the A (A) and the NA (B) moieties of the OM of *T. denticola* serovar b. Sample concentration, 250 μg of protein.

with the biological or diagnostic functions of the OM polypeptides.

SDS-PAGE followed by Coomassie brilliant blue R-250 staining of whole-cell lysates of *T. denticola* serovar c by Umemoto et al. (40) revealed a 53-kDa antigen that was absent in whole-cell lysates of *T. denticola* serovars a and c. The polypeptide bands of the whole-cell lysates ranged from approximately 15 to 140 kDa. *T. denticola* serovars a and c had similar Coomassie brilliant blue R-250-stained SDS-PAGE profiles, with the main bands being approximately 50 kDa. Immunoelectron microscopic studies suggested that the 53-kDa antigen of *T. denticola* serovar c may originate from the OM. The use of monoclonal antibodies and immunoelectron microscopy has also resulted in the demonstration of a surface-specific antigen for *T. denticola* serovar "b" by Simonson et al. (34). This antigen appeared to be specific for *T. denticola* serovar b because the monoclonal antibody raised by Simonson et al. (34) did not cross-react with *T. denticola* serovar c by enzyme-linked immunosorbent assay (40).

A common observation that was made during the electrophoretic studies of the OM of *T. denticola* was the tendency of *T. denticola* serovar b to have more polypeptides (Fig. 1 to 6) or electrophoretic polypeptide profiles slightly different from those of *T. denticola* serovars a or c.

The finding that the OM of *T. denticola* serovars a, b, and c contained periodic acid Schiff-positive reagent bands (Fig. 4) suggests that glycopeptides may be present in the OMs of *T. denticola* serovars a, b, and c and that they may be involved in the attachment of the assay spirochetes to human gingival cells. *T. denticola* has been shown to adhere to various human cells (32, 42). Weinberg and Holt (42) have shown that *T. denticola* GM-1 and MS25 were more adherent to human gingival fibroblasts than *T. denticola* TD-4 (serovar c) was. Polyclonal antibodies to strain GM-1 inhibited GM-1 adherence by 70%, while strains MS25 and TD-4 showed different degrees of cross-reactive inhibition, indicating common but not identical epitopes on the surfaces of the three *T. denticola* strains. Pretreatment of the three strains with trypsin did not inhibit adherence; however, exposure to sugars and lectin pretreatment of the human

TABLE 2. *Limulus* amebocyte lysate clotting activity of the outer membrane of *T. denticola*[a]

Sample concn (ng)	*Limulus* lysate clotting activity[b]					
	Serovar a		Serovar b		Serovar c	
	A moiety	NA moiety	A moiety	NA moiety	A moiety	NA moiety
225	+	+	+	+	+	+
113	+	+	+	+	+	+
52	+	+	+	+	+	+
26	+	+	+	+	+	−
11	+	−	+	+	+	−
5.5	+	−	+	+	−	−
2.8	−	−	−	+	−	−
1.4	−	−	−	−	−	−
0.7	−	−	−	−	−	−
0	−	−	−	−	−	−

[a] Data were obtained from seven determinations.
[b] +, solid clot in the bottom of the tube; −, no clot.

TABLE 3. Chick embryo lethality of the outer membrane of *T. denticola*

Material inoculated[a]	No. inoculated	Concn (μg) yielding 100% mortality
A moiety, serovar a	27	210–315
NA moiety, serovar a	22	68–80
A moiety, serovar b	28	316–407
NA moiety, serovar b	25	48–60
A moiety, serovar c	23	>400
NA moiety, serovar c	21	300–409
E. coli LPS	19	50–100
Control (no OM)	17	No deaths

[a] The indicated material was diluted in pyrogen-free water and was inoculated on the chorioallantoic membrane of 11-day-old chick embryos.

gingival cells inhibited adherence. These results implicate a lectin-like adhesion on *T. denticola* surface with activity for sugars on the human gingival fibroblasts (42).

Both the A and NA moieties of the OM of *T. denticola* serovar b contained more apolipopeptides than *T. denticola* serovar a or c did, as judged by Sudan black staining. The differences in the number of apolipopeptides observed could not be explained on the basis of differences in the staining properties of the apolipopeptides of the various serovars, because apolipoprotein staining with Sudan black appeared to be generally modest. Therefore, some of the diversity in the apolipopeptide profiles observed is probably serovar specific.

The presence and role of LPS in spirochetes remains conjectural (9). There is evidence for the presence of LPS in the *Treponema refringens* Nichols strain (18). The presence, location, or function of LPS in *T. denticola* is not known.

The detection of LPS-like components (Fig. 5 and 6, Tables 2 and 3) in the OM of *T. denticola* is another interesting finding brought out by this study. An LPS that is also known as endotoxin has been used for diagnostic purposes, and it may elicit a wide variety of pathophysiological effects during an infection (38). However, further investigations will be required to define the precise role of the LPS-like component of the OM of *T. denticola*.

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