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Pore-Forming Properties of the Major 53-Kilodalton Surface Antigen from the Outer Sheath of *Treponema denticola*

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A 53-kDa protein from the outer sheath of the oral spirochete *Treponema denticola* was purified to homogeneity and shown to reconstitute channels in black lipid bilayer model membranes. The channel had a single-channel conductance of 1.8 nS in 0.1 M KCl, making this the largest porin channel observed to date (estimated diameter, 3.4 nm). Electron micrographs of 53-kDa-protein-containing outer sheaths of *T. denticola* showed a regular hexagonal array of darker staining pits.

Treponema denticola is an oral spirochete that has been reported to be closely associated with periodontitis (6, 20). It possesses a wide variety of potential virulence factors, including motility due to periplasmic flagella (3), at least two proteases (5, 16), a factor that inhibits polymorphonuclear leukocyte function (3), and an ability to attach to host cells and tissues (6, 17). Like other spirochetes, *T. denticola* contains a sheathlike outer membrane. Recently, the protein content of the outer sheath was investigated (6). A single major protein with an apparent molecular weight of 53,000 was identified and demonstrated to mediate binding to fibronectin, laminin, and fibrinogen but not to gelatin or bovine serum albumin (6).

This protein was demonstrated to form sodium dodecyl sulfate (SDS)-stable oligomers, to be localized by immunoelectron microscopy on the surface of *T. denticola*, and to have an acidic pI. Since these were properties consistent with those of porins, a class of outer membrane proteins which form transmembrane channels (7), we examined the ability of the purified protein to reconstitute channels in model membranes. The data demonstrate that the 53-kDa protein is a porin with a very large channel diameter.

MATERIALS AND METHODS

Bacterial strain. *T. denticola* serovar a (ATCC 35405) and serovar c (ATCC 35404) were maintained as described before (6). The purity of the culture was checked under phase-contrast microscopy before harvest.

Detergent extraction of porin. Approximately 15 g (wet weight) per 8-liter culture of *T. denticola* ATCC 35404 cells from the late-logarithmic-phase growth was harvested by centrifugation at 5,000 × *g* for 1 h at 4°C. The cells were washed twice with phosphate-buffered saline (PBS; pH 7.2) and then once with double-distilled H_2O (10,000 × *g* for 45 min at 4°C). Washed cells were suspended to a final volume of 200 ml in double-distilled H_2O. Nonidet P-40 was added to the *T. denticola* cell suspension to a final concentration of 0.1%. The mixture was stirred gently overnight at 4°C. The undissolved material was pelleted by centrifugation at 10,000 × *g* for 45 min at 4°C. Tris-NaCl-SDS buffer was added to the supernatant to a final concentration of 50 mM Tris-HCl, 200 mM NaCl, and 0.1% (3.5 mM) SDS (pH 7.5). The mixture was incubated in a 37°C water bath for 1.5 to 2 h. (The extraction mixture contained a chymotrypsin-like protease [5] that digested away unwanted proteins in the 30- to 60-kDa regions.) After autoproteolysis, the solution (total volume, ~200 ml) was again centrifuged at 10,000 × *g* for 45 min at 4°C. The supernatant obtained was concentrated at 4°C to a volume of approximately 5 ml by Amicon ultrafiltration with an XM50 filter. The protein content of the concentrate (approximately 0.1 g) was measured by the bicinchoninic acid microassay method (11). The specimen was then dialyzed against 10 mM Tris (pH 7.5). The protein without the detergent precipitated inside the dialysis bag. The precipitate was pelleted by centrifugation at 10,000 × *g* for 45 min at 4°C, washed once with double-distilled H_2O, and then subjected to fast protein liquid chromatography (FPLC).

FPLC purification of partially purified 53-kDa porin. Before the partially purified sample was applied to the FPLC (Pharmacia, Uppsala, Sweden) Mono Q (HR 5/5) column, it was concentrated fivefold by using an Amicon microconcentrator and rediluted in column buffer (10 mM Tris-HCl [pH 8], 1% octyl polyoxyethelene; Bachem, Switzerland]. The protein was eluted from the Mono Q column by applying a gradient of 0 to 0.4 M NaCl in 10 mM Tris-HCl (pH 8.0)–1% octyl polyoxyethylene at a flow rate of 1 ml/min. Fractions (1 ml) containing the 53-kDa porin were rechromatographed under the same conditions. Peak B contained pure 53-kDa protein (Fig. 1). Analysis of all fractions was performed by SDS-polyacrylamide gel electrophoresis (PAGE) as previously described (10).

Black lipid bilayer analysis. Single-channel conductance (1), macroscopic conductance inhibition (8), and zero-current membrane potential experiments (1) were performed as described previously.

Electron microscopy. Twenty milliliters of 3-day-old cultures of *T. denticola* ATCC 35405 was harvested and washed as described above. The washed cell pellet was suspended in PBS (pH 7.2) and subjected to mild ultrasonication (1-min pulse sonication at 20% duty cycle and output of 2 with a Sonifier Cell Disrupter 350; Branson Sonic Power Co., Danbury, Conn.). After sonication, the suspension was diluted with PBS to an optical density at 660 nm of 0.2 and applied to a Parlodion film-covered, carbon-stabilized copper grid. Routine negative staining was carried out with 4% uranyl acetate (pH 4.5) (13). The location of the 53-kDa

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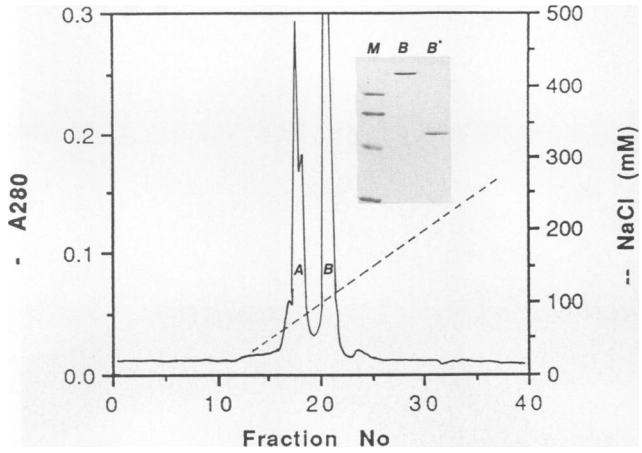

FIG. 1. FPLC purification of the 53-kDa porin, which was eluted from the column with 110 mM NaCl (peak B). The insert represents SDS-PAGE of unheated (lane B) and heated (lane B*) samples of peak B. The molecular mass markers (lane M) used were 94, 67, 43, and 30 kDa.

protein on isolated sheaths of *T. denticola* was studied by immunoelectron microscopy by using the methods described previously (6). Specimens were examined with a Phillips 300 electron microscope operating at 60 or 80 kV.

RESULTS

Purification and model membrane characterization of the 53-kDa protein. The 53-kDa protein was previously demonstrated to be localized to the outer sheath, to be the most predominant protein in *T. denticola* whole cells, and to be well solubilized in Nonidet P-40 (6). It was purified to homogeneity by using a procedure outlined previously (6), with the exception of inclusion of a detergent exchange step prior to ion-exchange FPLC (Fig. 1). The protein ran on SDS-PAGE as a single oligomer band with an apparent molecular weight of 160,000 when it was not heated prior to electrophoresis and with an apparent molecular weight of 53,000 when it was heated in SDS to 100°C for 10 min prior to electrophoresis, which confirmed earlier results (6).

Addition of the purified 53-kDa protein to the salt solution bathing a lipid bilayer membrane led to step increases in membrane conductance (Fig. 2). By analogy with other model membrane studies (1, 7), these step increases were considered single-channel events because of the progressive incorporation of pore-forming units into the planar lipid bilayer membrane separating two aqueous compartments. Consistent with this, the sizes of the step increases in, e.g., 1 M KCl showed a rather narrow range, with few steps larger than 12 nS or smaller than 10 nS (Fig. 3). Other salts demonstrated similar histograms of channel size distribution. As a control, we utilized outer membrane protein OprH of *Pseudomonas aeruginosa*. Consistent with published data (23), no channels were observed.

The 53-kDa channel was very large, as indicated by the

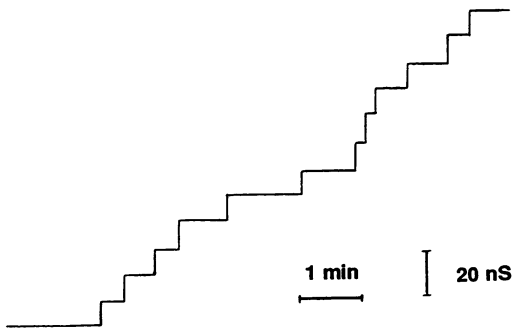

FIG. 2. Analysis of single-channel conductance events due to incorporation of the 53-kDa porin into black lipid membranes. Lipid membranes were formed from a 1% solution of diphytanoyl phosphatidylcholine in *n*-decane, and a voltage of 10 mV was applied. The average single-channel conductance was calculated to be 10.9 nS.

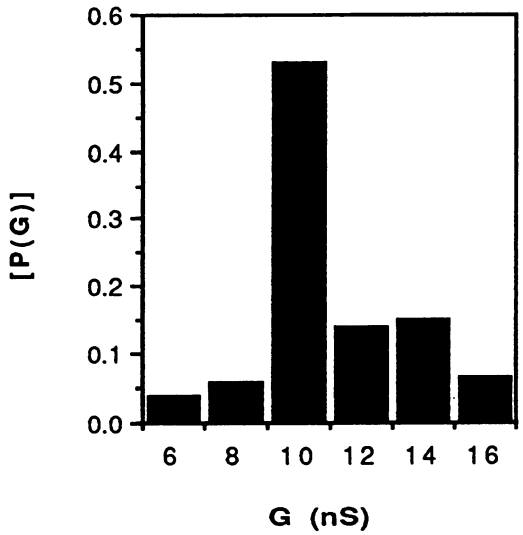

FIG. 3. Histogram of single-channel conductance increases observed for the purified 53-kDa membrane protein after it was added to the aqueous phase (1 M KCl) bathing a lipid bilayer membrane. A total of 216 events was recorded for this histogram, which shows the probability of a given single-channel conductance increment [P(G)] as a function of single-channel conductance (G). The single-channel conductance was determined to be 10.9 nS.

TABLE 1. Average single-channel conductance of *T. denticola* 53-kDa porin[a]

Salt	Concn (M)	*G* (nS)	G/σ (10^{-8} cm)	*n*
KCl	1	10.9	10.6	318
	0.1	1.8	15	145
	0.05	1.4	20	77
	0.01	0.2	14	108
LiCl	1	9.2	13	162
NaCl	1	11.8	14	129
$K^+CH_3COO^-$	1	9.8	14	165
KI	1	18.8	15	195
KBr	1	13.3	12	112
KF	1	15.0	19	149
$Tris^+Cl^-$	1	7.1	23	224
$Tris^+Hepes^-$ (pH 8)	1	0.5	7.3	200
Na^+Hepes^- (pH 9)	1	2.8	15	215

[a] The pH of the salt solutions was 7 if not otherwise indicated. A constant voltage of 10 mV was applied to the diphytanoyl phosphatidylcholine membrane. Average conductance increments (*G*) were determined by recording a large number (*n*) of conductance steps and averaging them. σ is the specific conductance of the aqueous salt solution.

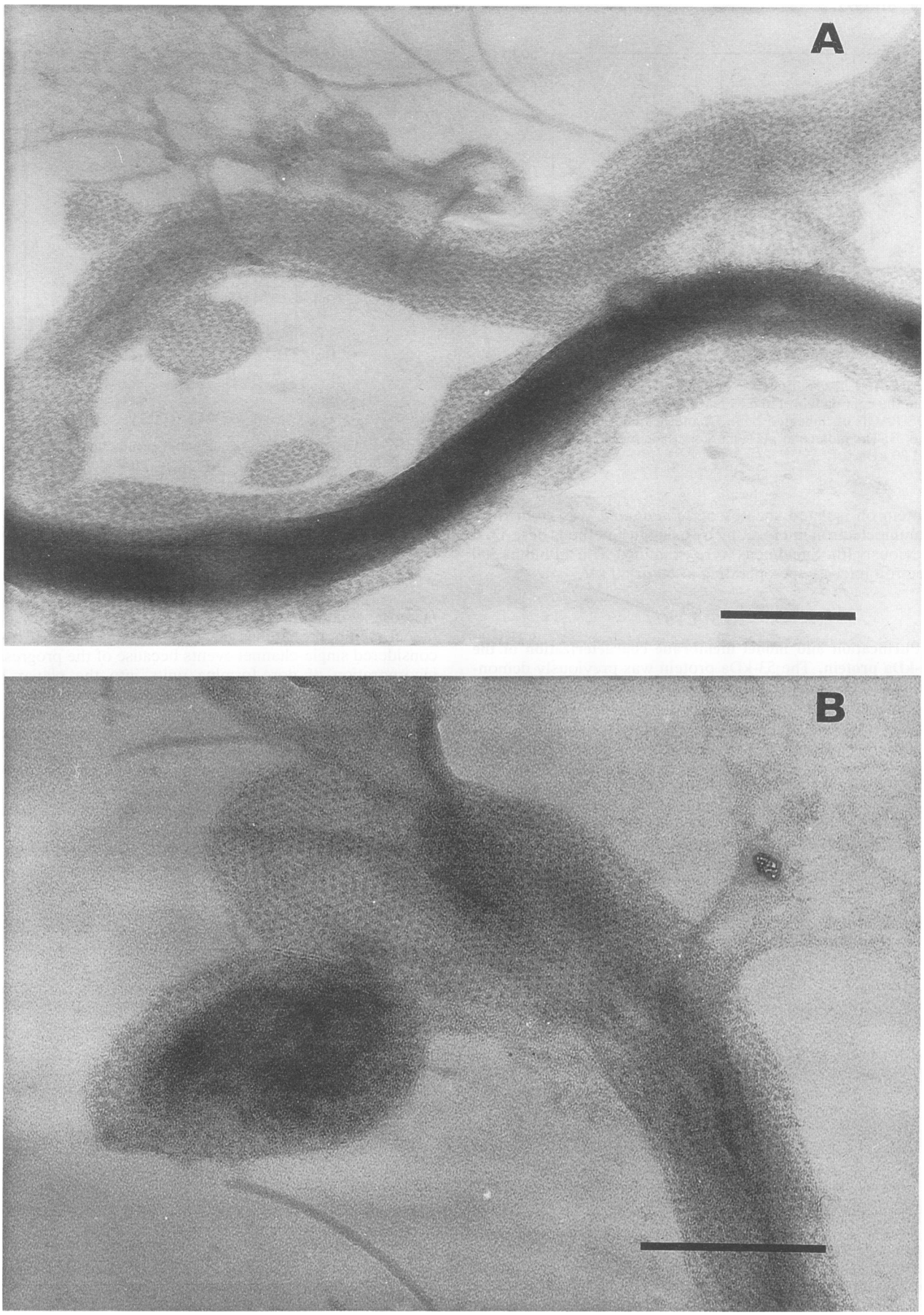
A
B

FIG. 4. Electron micrographs of sonicated 3-day-old *T. denticola* ATCC 35405 cells. (A) Darkly staining cell bodies, periplasmic flagella, and outer sheaths in the form of both vesicles and extended sheaths are evident. (B) Outer sheath of *T. denticola* ATCC 35405, showing a definite hexagonal array. Bars = 300 nm.

large single-channel conductance. Although the single-channel conductance was influenced by the specific conductance properties of the salt used, the ratio of single-channel conductance (G) to specific conductance (σ) was little affected by salt or ion composition (Table 1). This means that these ions moved through the channel in a fashion similar to their movement through a similar salt solution that separated two electrodes across which voltage had been applied. Thus, this result was consistent with the 53-kDa protein forming a large, relatively nonselective channel. When both the anion and the cation were replaced by the very large ions $Tris^+$ (0.67-nm diameter) and $Hepes^-$ (an ellipsoid molecule 1.4 by 0.6 by 0.5 nm), there was a small decrease in the G/σ ratio observed. Thus, only in this case did interaction of the interior of the channel with these large ions (which are even bulkier because of the hydration shell they carry) restrict ion movement, and even in this case, the effect was smaller than that observed for other porins. This is consistent with the 53-kDa porin forming the largest porin channel observed to date.

The single-channel conductance experiments did not indicate a strong preference for any ions. In zero-current membrane potential experiments (1), a KCl concentration gradient was imposed between the two chambers separated by a membrane containing 100 channels of the 53-kDa porin. Preferential movement of Cl^- was indicated by the formation of a negative potential which balanced the osmotic potential caused by the concentration gradient. Application of the Goldman-Hodgkin-Katz equation to these data indicated that the channel was weakly anion selective, with a ratio of permeability to Cl^- over permeability to K^+ of 1.6. Decreasing the salt concentration resulted in an approximately linear decrease in conductance (Table 1). However, the slope of the conductance-concentration curve was only 0.5. A similar result for *Escherichia coli* hemolysin (2) has been interpreted as being due to surface charges at the pore mouth which result in substantial surface potentials. In the case of the 53-kDa protein, these charges would be assumed to be due to cationic (basic) amino acids that would tend to attract anions and repel cations.

Previous studies indicated that the 53-kDa protein binds fibronectin and fibrinogen. To test whether the binding site was within the channel, macroscopic conductance inhibition experiments (8) were performed. Addition of human fibrinogen (final concentration, 0.2 mg/ml) or bovine fibronectin (final concentration, 0.1 mg/ml) to the solutions bathing a membrane loaded with approximately 500 channel-forming units of the 53-kDa protein had no effect on conductance of KCl through these channels. The inability to block KCl movement suggested either that the binding affinity of the putative binding site is low or that the binding site is so located that it does not influence the channel properties.

Morphology studies. The 53-kDa protein formed by far the most conductive porin channels observed to date. This fact led us to examine whether these channels were observable in isolated sheaths. In intact *T. denticola* cells, the cell body

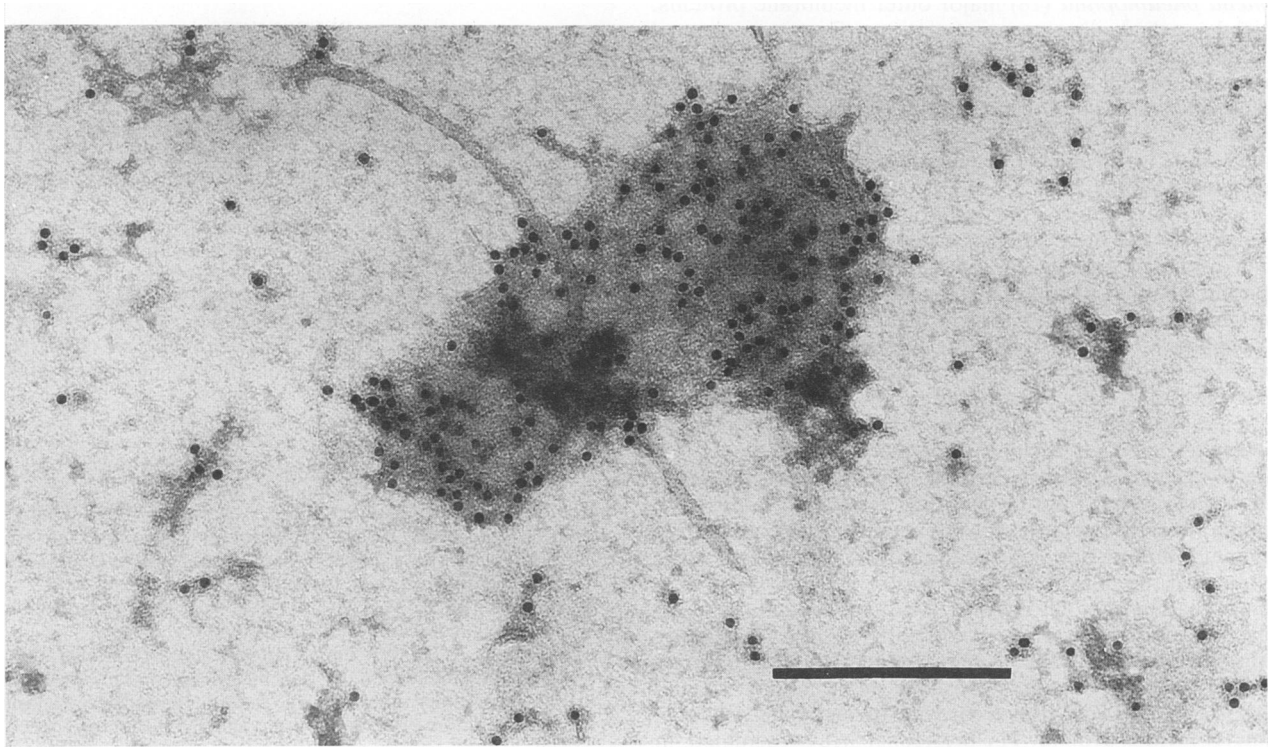

FIG. 5. Electron micrograph of isolated outer sheath from *T. denticola* ATCC 35405 decorated with gold-labeled specific antibody against the 53-kDa protein. Bar = 300 nm.

obscured observation of potential arrays. Various techniques to separate the outer sheaths from the rest of the spirochetes were attempted. These included vortexing for 5 min with 1- to 5-μm-diameter glass beads, freezing and thawing 40 times, and mild ultrasonication of cultures of various ages. Sonication of 3-day-old cultures gave by far the most adequate result. Such preparations demonstrated isolated sheaths, small vesicles that derived from these sheaths, and bacterial darkly staining bodies that were sometimes surrounded by sheath material (Fig. 4A). The sheath material demonstrated an apparent regular distribution of darker staining regions with an approximate center-to-center distance of 20 nm and darker stained areas approximately 4 to 5 nm in diameter. In some micrographs, areas with clear hexagonal arrays were observed (Fig. 4B).

The abundance of the 53-kDa protein in the *T. denticola* cell and its localization to the outer sheath (6) were consistent with this hexagonal array consisting of 53-kDa oligomers. As further evidence, the 53-kDa protein was localized to the fragments of outer sheath by immunogold electron microscopy (Fig. 5). During preparation for immunogold electron microscopy, the hexagonal pattern of staining disappeared. This could have been due to treatment with specific antibody or to the 1% bovine serum albumin used to block nonspecific binding. However, the distance between the most closely spaced gold particles (Fig. 5) was similar to the distance between the darker staining regions in Fig. 4B.

DISCUSSION

The data presented here clearly indicate that the 53-kDa protein of *T. denticola* is a porin. Taken together with its previously proposed function as an adhesin for certain matrix proteins (6), the 53-kDa protein joins a select group of porins, including the *Chlamydia trachomatis* (22) and *Legionella pneumophila* (18) major outer membrane proteins, which have dual adhesin-porin functions. The single-channel conductance of the 53-kDa protein was 1.8 nS in 0.1 M KCl. This value can be used to estimate the channel diameter by the formula $G = \sigma\Pi r^2/l$ (where G is the single-channel conductance, σ is the specific conductance, r is the channel radius, and l is the channel length, assumed to be 6 nm [10]). This gives a diameter of 3.4 nm, by far the largest observed to date. Interestingly, the previous largest channel was observed for the only other characterized spirochete porin, the 36.5-kDa protein of *Spirochaeta aurantia*, which had an apparent channel diameter of 2.3 nm (10), whereas the *E. coli* OmpF porin has an apparent diameter of 1.17 nm. This is consistent with the concepts that the spirochete is a primitive filter feeder and that the outer sheath serves as a crude filtration device permitting relatively free flow of nutrients through the periplasm during the movement of spirochetes through their environment. Interestingly, despite the rather exotic appearance of spirochetes, the properties of the 53-kDa porin, including resistance to protease degradation and detergent denaturation, formation of native oligomers, and an apparent acidic pI, match those of other members of the porin family.

Examination of isolated outer sheaths revealed a hexagonal array. Evidence was consistent with this hexagonal array being due to the 53-kDa protein. Indeed, the approximate magnitude of the regularly arrayed, more highly stained areas (4 to 5 nm) was consistent with the estimated channel diameter for this protein. The hexagonal array resembled, in appearance, an S layer. Nevertheless, available information in the literature does not suggest the presence of an extra layer outside the *T. denticola* outer sheath (4) as assessed by freeze-substitution transmission electron microscopy. Similarly, freeze-etching of an oral treponeme (E-21) which has the regular surface hexagonal array of a 62-kDa protein (13) did not reveal the presence of an S layer (15). Thus, the 53-kDa protein is probably an example of what Messner and Sleytr (14) call a cooperative assembly. Interestingly, other porins are also arranged in hexagonal arrays, e.g., OmpF from *E. coli* (21) and the major porins of *Thermatoga maritima* (19) and *Bordetella pertussis* (9), and in the last two cases, these hexagonal arrays can be visualized in the native outer membrane.

It was previously demonstrated that the 53-kDa protein bound to fibronectin, fibrinogen, and laminin. One possibility is that this binding helps the association of *T. denticola* with periodontal tissues (6, 17). However, given that *T. denticola* produces, in its outer sheath, a chymotrypsin-like protease which is capable of degrading laminin and fibronectin (5), it is possible that this binding serves to concentrate a potential food source near the porin channel to permit uptake of peptides produced by proteolytic cleavage. The large channel of the 53-kDa protein and its predicted large exclusion limit for peptides would be advantages in this regard. Such a function would be equivalent to the NosA porin of *Pseudomonas stutzeri*, which has a binding site for Cu^{2+} outside the actual ion-conducting channel (12).

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