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Isolation of the Outer Membrane and Characterization of the Major Outer Membrane Protein from *Spirochaeta aurantia*

A. M. KROPINSKI,[1] T. R. PARR, JR.,[2] B. L. ANGUS,[2] R. E. W. HANCOCK,[2] W. C. GHIORSE,[3] AND E. P. GREENBERG[3]*

Department of Microbiology and Immunology, Queen's University, Kingston, Ontario, Canada K7L 3N6[1]; Department of Microbiology, University of British Columbia, Vancouver, British Columbia, Canada V6T 1W5[2]; and Department of Microbiology, New York State College of Agriculture and Life Sciences, Cornell University, Ithaca, New York 14853[3]

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The outer membrane of *Spirochaeta aurantia* was isolated after cells were extracted with sodium lauryl sarcosinate and was subsequently purified by differential centrifugation and KBr isopycnic gradient centrifugation. The purified outer membrane was obtained in the form of carotenoid-containing vesicles. Four protein species with apparent molecular weights of 26,000 (26K), 36.5K, 41K, and 48.5K were readily observed as components of the vesicles. The 36.5K protein was the major polypeptide and constituted approximately 90% of the outer membrane protein observed on sodium dodecyl sulfate-polyacrylamide gels. Under mild denaturing conditions the 36.5K major protein exhibited an apparent molecular weight of approximately 90,000. This, together with the results of protein cross-linking studies, indicates that the 36.5K polypeptide has an oligomeric conformation in the native state. Reconstitution of solubilized *S. aurantia* outer membrane into lipid bilayer membranes revealed the presence of a porin, presumably the 36.5K protein, with an estimated channel diameter of 2.3 nm based on the measured single channel conductance of 7.7 nS in 1 M KCl.

The spirochetes are a particularly ancient group of bacteria, and they represent 1 of about 10 recognized sublines of eubacteria (20, 44). They are characterized by a distinctive morphology and unique motility (11–13, 30). Generally, cells are helical and possess a protoplasmic cylinder that comprises the nuclear and cytoplasmic regions, as well as the cytoplasmic membrane-peptidoglycan layer. Wrapped around the protoplasmic cylinder are structures called periplasmic flagella (13, 22, 30). One end of each flagellum is inserted near one pole of the protoplasmic cylinder, and the other end is not inserted. Both the periplasmic flagella and the protoplasmic cylinder are enclosed within the outer membrane (also known as the outer sheath). Thus, with the exception of the location of flagella within the periplasmic space, spirochetes possess a gram-negative cell surface. Members of this group, however, are no more closely related to other gram-negative bacteria than they are to gram-positive bacteria (44).

The gram-negative outer membrane is of considerable importance in that it constitutes the first diffusion barrier between the cell and the external environment, and it also allows the existence of an extracytoplasmic aqueous cellular compartment, the periplasmic space. While a great deal is known about the chemistry and permeability of outer membranes from bacteria such as *Escherichia coli* (38, 41) and *Pseudomonas aeruginosa* (24, 25, 55), far less is known about spirochete outer membranes. Much of the available information concerns identification of cell surface antigens of pathogenic, or at least host-associated, spirochetes (4, 17, 33, 39, 42, 49). For example, Barbour et al. (5) recently identified the major surface (outer membrane) protein antigens of the Lyme disease spirochete *Borrelia burgdorferi*. In part because it is impractical to obtain sufficient cell mass for detailed biochemical studies of the outer membrane from most spirochetes (because of low growth yields and complex nutritional requirements [13]), little is known about the permeability of spirochete outer membranes.

During ongoing studies of the free-living facultative anaerobe *Spirochaeta aurantia*, it has become apparent that information about the outer membrane is important to our general understanding of transport, motility, and chemotaxis of this organism (22). Furthermore, this spirochete is relatively easy to culture en masse because it has simple nutritional requirements and the growth yields are reasonable (9). In this report we describe a method for the isolation of outer membrane vesicles from *S. aurantia* and the characterization of a major surface protein.

MATERIALS AND METHODS

Bacterial strains and culture conditions. Two strains of *S. aurantia* were used in this study: *S. aurantia* M1 (9) and a nonflagellated mutant, strain NP-7, which was derived from strain M1 (43). Cultures were grown in 10 liters of GTY broth (23) contained in a 19-liter carboy. The incubation temperature was 30°C, and air was bubbled through the medium at a rate of 600 ml/min. The inoculum was 10% (vol/vol) of a late-logarithmic-phase culture, and after 24 h cells were harvested by centrifugation at 4°C. The cell pellets (1 to 1.4 g/liter) were stored at −80°C.

Membrane solubilization studies. *S. aurantia* cells were suspended to a final concentration of 0.1 g (wet weight) per ml in 25 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonate (HEPES buffer) (pH 7.5) containing 50 μg of DNase 1 per ml. Samples of 5 ml were incubated in a water bath at 20°C for 5 min; and then 5 ml of prewarmed deionized water, sodium lauryl sarcosinate (Sarkosyl; CIBA-GEIGY Corp., Summit, N.J.), or sodium dodecyl sulfate (SDS) was added dropwise with continuous mixing. Following 15 min of incubation, the samples were centrifuged at 200,000 × *g* for 1 h at 20°C. The pellet and supernatants were separated, and the pellets were suspended in 10 ml of HEPES buffer before further analysis.

* Corresponding author.

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Isolation of the spirochete outer membrane. *S. aurantia* NP-7 cells (13.8 g [wet weight]) were suspended to 110 ml with HEPES buffer containing 50 μg of DNase 1 per ml, and an equal volume of freshly prepared 1% (wt/vol) Sarkosyl was added slowly with stirring. After a 15-min incubation period the lysed cell suspension was centrifuged at 130,000 × *g* for 1.5 h at 4°C. The pelleted material was suspended in HEPES buffer and centrifuged at 20,000 × *g* for 15 min. The pellet from this low-speed centrifugation contained peptidoglycan and peptidoglycan-associated material. This peptidoglycan-containing fraction was washed twice with HEPES buffer, and the supernatant fluids from each wash were combined. Solid KBr was then added to the combined supernatant fluids to a final concentration of 0.3 g/ml, and the material was centrifuged at 130,000 × *g* for 12 h at 4°C. The higher density outer membrane-containing band was collected, diluted with HEPES buffer, and concentrated by centrifugation at 130,000 × *g* for 3 h.

Electron microscopy. Purified outer membranes in suspension were negatively stained by a modification of the method described by Racker et al. (45). Copper grids (300 mesh) supporting carbon-coated Formvar films were floated film-side down on a drop of 3% ammonium molybdate (pH 7) or on a drop of 4% uranyl acetate (pH 4). Excess stain was removed with filter paper, and the grids were examined with a transmission electron microscope (model 301; Phillips) operated at 80 kV. Electron photomicrographs were taken on Kodak electron image film at an instrument magnification of 20,520.

Gel electrophoresis. Except where indicated, SDS-polyacrylamide gel electrophoresis was through 1.5-mm-thick slab gels of 12.5% acrylamide overlayed with 4.5% stacking gels prepared by the procedures described by Laemmli (35). Routinely, electrophoresis was carried out at room temperature and at a constant 100 V until the tracking dye entered the resolving gel, at which time the voltage was increased to 180V. The protein molecular weight markers (Bio-Rad Laboratories, Richmond, Calif.) were lysozyme (14,400), soybean trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), bovine serum albumin (66,200) and phosphorylase *b* (92,500).

To investigate the heat modification of outer membrane proteins, samples were incubated in electrophoresis sample buffer (1% SDS, 2% 2-mercaptoethanol, and 10% glycerol in 0.0625 M Tris [pH 6.8]) at 4 to 100°C for 10 min. For two-dimensional (unheated and heated) SDS-polyacrylamide gel electrophoresis, samples of outer membrane proteins incubated in electrophoresis sample buffer at 4°C were run on SDS-polyacrylamide gels as described above, and when the dye approached the bottom of the gel, a gel strip containing the separated outer membrane protein was excised. This gel strip was placed in a tube containing sample buffer, and the tube was placed in a boiling water bath for 10 min. The excised strip was then sealed on top of a second SDS-polyacrylamide slab gel with molten 1% agarose and electrophoresed as described above. Non-heat-modifiable proteins appeared on the diagonal, and heat-modifiable proteins appeared to the left or right of the diagonal.

For intermolecular and intramolecular disulfide bridge studies, the techniques described by Allore and Barber (2) were used.

For two-dimensional (isoelectric focusing and SDS) polyacrylamide gel electrophoresis, isoelectric focusing was performed in slab gels of 5% acrylamide with an acrylamide-bisacrylamide ratio of 28:1.5. The pH gradient ranged from approximately 3.0 to 6.0, and the gel contained 2% Triton X-100. Sample protein in 50 mM Tris hydrochloride (pH 6.8) was solubilized by incubation with 1% SDS–0.2% 2-mercaptoethanol at 100°C. After solubilization and before gels were loaded, Triton X-100 (1%), urea (4 M), and ampholines were added to the samples. Electrophoresis was carried out for 24 h at 400 V to be sure that the acidic proteins focused. Gel strips were cut from the first-dimension gel and frozen at −70°C until use. Strips were sealed in place with 0.8% agarose on top of SDS-polyacrylamide gels prepared by the procedure described by Lugtenberg et al. (37), and electrophoresis in the second dimension was carried out at 150 V.

Gels were stained with either Coomassie blue or silver. Unless otherwise specified, staining was with silver. The Coomassie stain was composed of Coomassie brilliant blue R250 dye (0.5 mg/ml) in 20% methanol–7.5% acetic acid. Silver staining was accomplished by using a modification of the procedure described by Wray et al. (54). Gels were incubated overnight with shaking in 50% methanol, stained with silver (0.5% [wt/vol]) in solution with 0.75% (wt/vol) NaOH–1.4% (vol/vol) NH_4OH, rinsed extensively with distilled water, and developed in an aqueous solution of 0.0005% (wt/vol) citric acid–0.019% (vol/vol) formaldehyde.

Chemical cross-linking of outer membrane proteins. The cross-linking agent, dithiobis (succinimidyl) propionate (DSP; 7.5 μg of DSP dissolved in 1 μl of dimethyl sulfoxide) was added to 15 μl of a suspension of outer membranes (0.5 mg/ml) in 0.2 M triethanolamine buffer (pH 8.5) by the method of Reithmeier and Bragg (46). Cross-linking was allowed to proceed for 2 min and was then stopped by the addition of 5 μl of 1 M Tris hydrochloride (pH 8.5). The cross-linked preparations were diluted 1:1 in a solution of 4% (wt/vol) SDS–20% (vol/vol) glycerol–0.5 M Tris hydrochloride (pH 6.8) and analyzed by one- or two-dimensional (unreduced and reduced) SDS-polyacrylamide gel electrophoresis (3).

Chemical analysis of the outer membrane. Prior to chemical analysis the outer membrane preparation was dialyzed against several changes of deionized water at 4°C. The protein content was assayed by the Folin reaction (27) in addition to the commercial Bradford assay (Bio-Rad). Total carbohydrate was quantitated by the procedure described by Dubois et al. (16), with glucose used as the standard. Fatty acid content was estimated by gas-liquid chromatography of samples transesterified in 2 M HCl–methanol, as described previously (34), except that methyldodecanoate was used as the internal standard.

Production and assay of antisera. Mouse anti-outer membrane serum was obtained by intravenous injection of 40 μg of *S. aurantia* outer membrane into several 25-g female B6D2 mice. The mice were boosted intraperitoneally with 25 μg of outer membranes on days 4, 16, and 34. Antisera, collected 3 days after the final immunization, produced an enzyme-linked immunosorbent assay (40) titer, using either *S. aurantia* outer membranes or whole cells of *S. aurantia* M1 as antigens, of 1:3,200 to 1:6,400. Rabbit anti-periplasmic flagella serum was a gift from B. Brahamsha.

Black lipid bilayer experiments. The preparation of the protein used in this study consisted of outer membranes solubilized in 2% Triton X-100–10 mM Tris hydrochloride (pH 7.4). The 36,500-molecular-weight outer membrane protein (36.5K protein) in this preparation was judged to be 90% pure on the basis of densitometer tracings of SDS-polyacrylamide gel electrophoretograms. The protein was kept in aqueous solution at 4°C during the investigation of its pore-forming characteristics. For the calibration of our in-

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strumentation, OmpF porin of *E. coli* B, which was isolated as described previously (8), was used.

The methods used for the characterization of the pore-forming ability of the *S. aurantia* protein have been described previously in detail (7, 8). The lipid used to form the membrane in the Teflon (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) chamber was 1.5% (wt/vol) oxidized cholesterol in *n*-decane, which was a generous gift from R. Benz, Universität Konstanz, Federal Republic of Germany. The experiments were done at room temperature (24°C). Bilayer formation was recognized by the fact that the membrane appeared black when viewed by incident light. Electrical measurements were made by immersing the Ag-AgCl electrodes into the aqueous solutions on either side of the Teflon divider which was perforated with a small hole (0.1 to 2 mm^2 in diameter) over which the membrane was painted. Voltage was applied across the membrane with a voltage source (Omnical 200; W-P Instruments, Inc.). Current fluctuation experiments were monitored with a preamplifier (model 427; Keithley) connected to a storage oscilloscope (model 5115; Tektronix; plug-in amplifier model 5A22; Tektronix). Conductance data were recorded on a strip chart recorder for further analysis. Bulk conductance experiments were monitored using an electrometer (model 610C; Keithley).

RESULTS

Isolation of outer membranes from *S. aurantia*. One method for isolating outer membranes from bacteria involves the selective detergent solubilization of one or the other of the membranes (18, 31, 33, 48, 51). Sarkosyl and Triton X-100 have been employed to selectively solubilize cytoplasmic membranes from a variety of gram-negative bacteria; this process leaves behind outer membrane vesicles which can be harvested by centrifugation (18, 48). At low concentrations, SDS has been used to selectively solubilize outer membranes of spirochetes belonging to the genera *Treponema* (31, 51) and *Borrelia* (33). The solubilized spirochete outer membrane can then be reconstituted by dialysis to remove the detergent (31, 33, 51). With *S. aurantia*, SDS did not selectively solubilize either cytoplasmic or outer membranes but Sarkosyl proved effective. The addition of Sarkosyl to suspensions of *S. aurantia* resulted in a decrease in turbidity and an increase in viscosity due to DNA release (because viscosity decreased on the addition of DNase 1 to 50 μg/ml). Examination by dark-field microscopy revealed that at low Sarkosyl concentrations (0.02 to 0.08% [wt/vol]) cells are spherical. At higher Sarkosyl concentrations (0.1 to 0.6% [wt/vol]) only amorphous material was observed. The amount of protein in the supernatant fluid after high-speed centrifugation increased with increasing Sarkosyl concentration, up to about 0.3 to 0.4% (wt/vol) Sarkosyl. Above this concentration the relative amount of solubilized protein (80 to 90% of the total protein) increased only slightly. As indicated by SDS-polyacrylamide gel electrophoresis, only a limited number of polypeptides remained with the Sarkosyl-insoluble pellets from high-speed centrifugations (Fig. 1). When similar experiments were performed with the *S. aurantia* mutant strain NP-7, which does not have flagellar filaments, fewer polypeptide bands appeared in the Sarkosyl-insoluble fraction (Fig. 1). Because the bands present only in the wild type exhibit apparent molecular weights identical to those of the *S. aurantia* flagellar polypeptides (B. Brahamsha and E. P. Greenberg. Abstr. Annu. Meet. Am. Soc. Microbiol. 1986, I-120), it appears that flagella

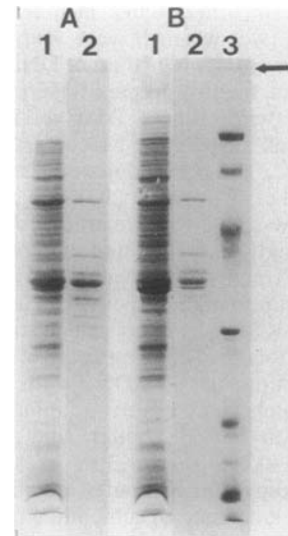

FIG. 1. SDS-polyacrylamide gel of Sarkosyl-insoluble material from *S. aurantia* M1 (A) and the nonflagellated mutant strain NP-7 (B). The arrow indicates the top of the resolving gel. Lane 1, no Sarkosyl added; lane 2, 0.6% (wt/vol) Sarkosyl. The molecular weight standards described in the text are shown in lane B3. The gel was stained with Coomassie blue.

copurified with other Sarkosyl-insoluble material. Because this could confound attempts to characterize membranous material, the nonflagellated mutant was used in all subsequent experiments. It should be noted, however, that we cannot rule out the possibility that there are other undetected differences between *S. aurantia* M1 and the mutant strain NP-7.

Sarkosyl-insoluble material was fractionated further by the procedure described above. Essentially, the bulk of the peptidoglycan was removed from suspended Sarkosyl-insoluble material by low-speed centrifugation, and the remaining supernatant fluid was subjected to KBr density gradient centrifugation. This allowed resolution of two distinct bands. There was a thin orange-red pellicle at the top of the gradient and a wide reddish orange (outer membrane) band in the lower third of the centrifuge tubes. Extraction of pigment and spectral analysis demonstrated that the color resulted from the *S. aurantia* carotenoid dihydro-hydroxytorulene (23). Carotenoids have been shown to occur in the outer membranes of other bacteria (47). Each fraction was analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2). The polypeptides in the pellicle fraction were also present in the peptidoglycan fraction that was obtained by low-speed centrifugation. The major peptidoglycan-associated polypeptide had an apparent molecular weight of 37,500. Analysis of the wide red-orange band from KBr gradients revealed one major polypeptide with an apparent molecular weight of 36,500 and several minor polypeptides.

The material from this higher density band was concentrated by centrifugation for further study. Electron microscopy revealed that it was composed of membrane vesicles of different sizes (Fig. 3). Experiments with specific antisera supported the conclusion that these vesicles represented the outer membrane. Antiserum raised against these vesicles reacted in ELISA enzyme-linked immunosorbent assays to give identical titers of 1:6,400 with both whole cells and membrane vesicles of *S. aurantia* and, furthermore, was able to agglutinate viable cells. As a control titers of anti-

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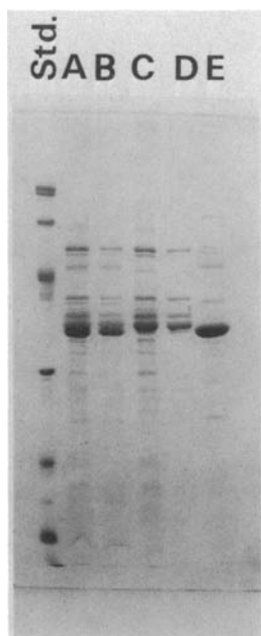

FIG. 2. SDS-polyacrylamide gel of fractions obtained during purification of outer membranes from Sarkosyl-insoluble material. Lane A, bulk insoluble material; lane B, supernatant fluid from low-speed centrifugation; lane C, peptidoglycan-associated material pelleted by low-speed centrifugation; lane D, KBr pellicle fraction; lane E, wide red-orange band obtained from KBr density gradient centrifugation. Std., molecular weight standards. The gel was stained with Coomassie blue.

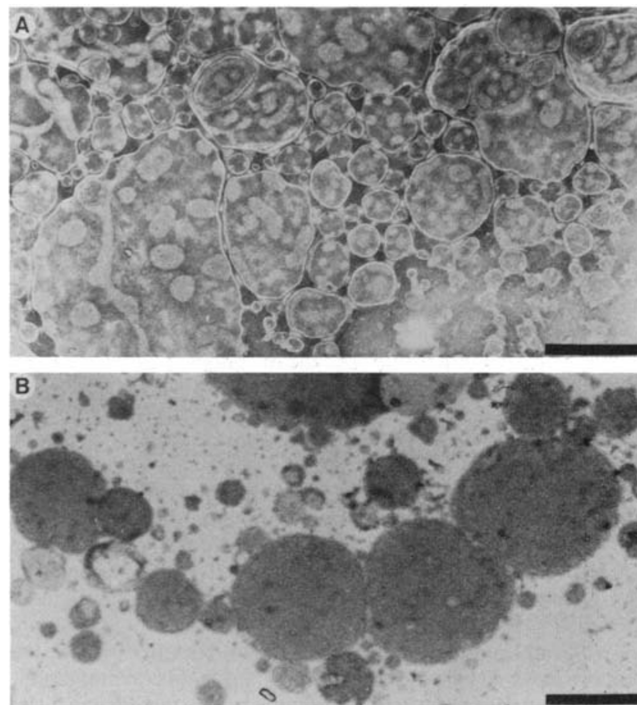

FIG. 3. Electron micrographs of the outer membrane preparation from *S. aurantia* NP-7. (A) Stained with ammonium molybdate; (B) stained with uranyl acetate. Bars, 0.5 μm.

serum raised in rabbits against the periplasmic flagella were determined against whole cells and a sonicated, broken cell suspension of *S. aurantia* M1. The titer was 1,000-fold greater when assayed with the broken cell suspension.

A chemical analysis of the outer membrane vesicles showed a composition of 13% protein, 31% carbohydrate, and 20% fatty acid. The low recovery (64%) could be due to the presence of lipid head groups or carbohydrates such as amino sugars that are refractory to phenol-H_2SO_4 analysis or to membrane proteins refractory to the protein assays that were used in this study (50).

Characterization of the outer membrane proteins from *S. aurantia*. As mentioned above, there was one major band on SDS-polyacrylamide gels of outer membranes that appeared to constitute about 90% of the total protein (Fig. 2, lane E). This 36.5K protein also appeared as a major band in electrophoretic analyses of the total cell proteins (Fig. 1). Three other prominent minor outer membrane protein bands had apparent molecular weights of 48,500, 41,000, and 26,000 (Fig. 2, lane E). Aside from the 36.5K polypeptide band these three polypeptides were the most abundant, but each represented less than 5% of the total membrane protein, as indicated by band intensity.

One characteristic of bacterial outer membrane proteins observed in many species studied is their so-called heat-modifiable character. That is, unless the solubilization is at a high enough temperature, the protein remains in an oligomeric or compact, partially folded form (19, 24, 52). The 36.5K polypeptide from the *S. aurantia* outer membrane was heat modifiable. At solubilization temperatures of up to 30°C the 36.5K polypeptide band was not observed and the major band had an apparent molecular weight of approximately 90,000 (Fig. 4), which is typical of bacterial outer membrane porin proteins. The relationship of the 90K protein band to the 36.5K protein band was confirmed by two-dimensional (unheated and heated) SDS-polyacrylamide gel electrophoresis (data not shown). To demonstrate that the 90K band represented an oligomer, rather than an aggregate, of the 36.5K protein, the proteins of the outer membrane were cross-linked with DSP, a 1.2-nm, cleavable cross-linker. Similar to other porins (3) this analysis revealed that the

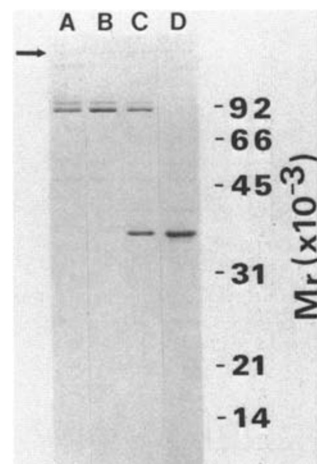

FIG. 4. Heat modification of *S. aurantia* outer membrane proteins. Vesicle preparations were incubated in the sample buffer described by Laemmli (35) for 10 min at 4°C (lane A), 20°C (lane B), 40°C (lane C), or 100°C (lane D). Samples were then electrophoresed at 4°C and subsequently stained with Coomassie blue.

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36.5K protein could be cross-linked to dimers and trimers (data not shown). Like the *E. coli* PhoE porin (3), intramolecular cross-links were also observed. A further electrophoretic analysis (2) indicated that there was no intermolecular disulfide bridging in the *S. aurantia* outer membrane proteins (data not shown), unlike in *Chlamydia* (6) and *Legionella* (10, 21) outer membrane proteins.

Two-dimensional (isoelectric focusing and SDS) polyacrylamide gel electrophoretic analyses of outer membrane proteins from a variety of gram-negative species have revealed that the majority of these proteins have acidic pIs (38). In particular, the porin proteins of *Salmonella typhimurium* and *P. aeruginosa* have pIs in the range of 4.0 to 4.8, although slight smearing of these polypeptides is often observed, making precise determination of pIs difficult (28). The major 36.5K outer membrane protein of *S. aurantia* also displayed some smearing on two-dimensional (isoelectric focusing and SDS) polyacrylamide gel electrophoresis and an acidic pI estimated to be between 3.3 and 3.5 (data not shown). Because of their considerably smaller quantities in the outer membrane, it was more difficult to analyze the 48.5K, 41K, and 26K polypeptides by two-dimensional gel electrophoresis. Nevertheless, spots with apparent molecular weights of 41,000 and 26,000 were seen at acidic pIs (3 to 4) in some analyses. The 48.5K polypeptide was never observed in these two-dimensional electrophoretic analyses.

Porin function of the *S. aurantia* outer membrane. The 36.5K major outer membrane protein apparently existed as a native oligomer (possibly a trimer) in the outer membrane. The pore-forming porin proteins of other gram-negative cells have molecular weights ranging from about 28,000 to 47,000 (41) and usually are thought to occur as trimers in the outer membrane. Therefore, we examined the pore-forming function of solubilized *S. aurantia* outer membranes.

When small quantities (final concentration, approximately 3 ng/ml) of Triton X-100-solubilized outer membrane (containing predominantly the 36.5K protein) were added to the aqueous solutions bathing a black lipid membrane, conductance increased in a stepwise fashion (Fig. 5). These discrete

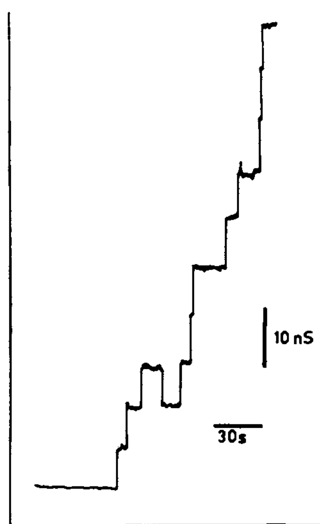

FIG. 5. Step increases in conductance after the addition of Triton-X-100-solubilized outer membranes (final concentration, 3 ng of protein per ml) to the aqueous phase (1 M KCl) bathing a lipid bilayer membrane. The recording starts on the left.

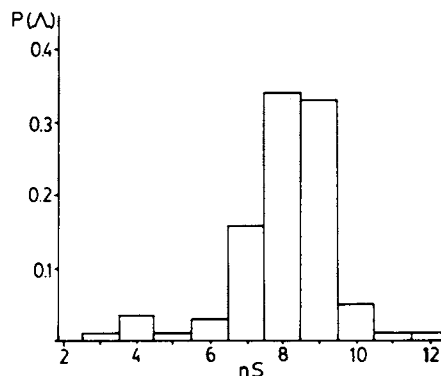

FIG. 6. Histogram of the single channel conductance increases observed for the Triton-X-100-solubilized outer membrane preparation after it was added to the aqueous phase (1 M KCl) bathing a lipid bilayer membrane. A total of 217 events were recorded for this histogram, and the probability of a given single channel conductance increment [P(Λ)] as a function of Λ is shown. The average single channel conductance, $\bar{\Lambda}$, in this experiment was determined to be 7.8 nS.

conductance increases did not occur on the addition of boiled protein or detergent alone. Nearly all conductance increases were directed upward, although some decreases in conductance of equal magnitude were noted. By analogy with other lipid bilayer experiments, each conductance increment was considered to be due to the incorporation of a single porin channel-forming unit into the membrane. These conductance increments were not all of equal size but were distributed over a range, as has been reported for other porins (7, 8). Histograms of the probability distribution of conductance increases showed a typical distribution in the size of the conductance steps and few step increases of less than 6 nS (Fig. 6). The average single channel conductance increase for the *S. aurantia* porin in 1 M KCl was 7.7 nS. After the membranes had incorporated a significant number of pore-forming units, transmembrane current was measured as a function of the applied voltage (over a range of 10 to 180 mV). The current to voltage plot passed through zero and was linear, indicating that the pores formed by the protein were not regulated, gated, or induced by voltage (7, 8).

The average conductance increment ($\bar{\Lambda}$) was also determined for a variety of salts and concentrations (Table 1). In each case, at the concentrations shown, the ratios of the single channel conductance Λ to the bulk conductance (σ) were higher than for other porins that have been characterized to date (7, 8). Furthermore, with the exception mentioned below, the ratio of $\bar{\Lambda}$ to σ was substantially affected only when the large cation Tris was used as part of the salt solution (an observation that could be explained if the channel was cation selective). Thus, these data are consistent with the proposal that this *S. aurantia* porin forms large, water-filled channels.

Other reported bacterial porins typically show a linear relationship between the salt concentration and $\bar{\Lambda}$. The *S. aurantia* porin did not. The single channel $\bar{\Lambda}$ decreased with decreasing salt concentration; however, there was significant deviation from the expected consistent ratio of $\bar{\Lambda}$ to σ, beginning at concentrations below 200 mM salt. For example, extrapolation of the data in Table 1 for KCl to 10 and 100 mM KCl would predict single channel conductances of 0.09 and 0.83 nS, respectively, instead of the values of 0.5 and 1.6 nS, respectively, that were obtained experimentally (average

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TABLE 1. Average conductance increments[a]

Salt	Concn (M)	$\bar{\Lambda}$ (nS)[b]	σ (mS/cm)	$\bar{\Lambda}/\sigma$ (10^8 cm^{-1})	*n*
KCl	1.00	7.7	110	7.0	797
KCl	0.50	3.6	56	6.4	208
KCl	0.20	1.7	22	7.8	173
LiCl	1.00	3.5	71	5.0	300
NaCl	1.00	5.3	84	6.3	159
MgCl	1.00	5.1	128	4.2	165
MgCl	0.50	3.3	64	5.1	86
Tris-HEPES	0.50	0.2	7.2	2.6	161
Tris-Cl	0.50	0.6	30	2.1	100
Sodium-HEPES	0.50	1.7	18	9.3	138

[a] Increments were measured on membranes made from 1.5% oxidized cholesterol in *n*-decane in the presence of Triton X-100-solubilized *S. aurantia* outer membrane proteins.
[b] $\bar{\Lambda}$, Average single channel conductance from the number (*n*) of measured single-channel events.
[c] σ, Bulk conductance of the given salt in the absence of a membrane.

from 451 and 559 single channel events recorded). Similar results were observed with NaCl (data not shown).

DISCUSSION

In this report we have described a method for isolating outer membrane vesicles from *S. aurantia*. The method involved treatment of cells with Sarkosyl to solubilize components other than the outer membrane and isopycnic KBr gradient centrifugation to purify the Sarkosyl-insoluble outer membrane vesicles. This procedure differs from those described previously for the isolation of outer membranes from other spirochetes in that most of these procedures involve selective solubilization of the outer membrane followed by (re)aggregation of membranous material. One exception is the procedure developed by Masuda and Kawata (39), which involved freeze-thaw fragmentation of an oral treponeme followed by differential centrifugation to obtain membrane vesicles (31, 33, 51). We estimate that the material isolated by our procedure accounted for approximately 10% of the total cell dry weight (data not shown). Similar estimates for two other spirochetes, *Leptospira pomona* (56) and *Borrelia hermsii* (33), are 4.7 and 20.9%, respectively. Our 10% estimate was consistent with the observation that the 36.5K outer membrane protein is one of the most abundant proteins in *S. aurantia* cells (Fig. 1).

The isolation of *S. aurantia* outer membrane afforded considerable technical problems. The classical technique for separating cytoplasmic membrane from outer membrane, which simply involves cell breakage followed by sucrose density gradient centrifugation (19, 26, 29, 32, 38), proved unsuccessful (data not shown), as did variations on the classical technique, although this in itself is not without precedent (1, 14, 36). Furthermore, techniques developed to selectively solubilize outer membranes from other spirochetes (31, 33, 51) proved inapplicable to *S. aurantia*. One reason for the difficulty in applying standard membrane separation techniques may involve the apparent lack of a classical lipopolysaccharide in *S. aurantia* (A. Kropinski, P. Hitchcock, and E. P. Greenberg, unpublished data). This caused an additional problem in that lipopolysaccharide is generally used as a marker in identification of outer membranes from other organisms (38, 41, 53). Because we could not use lipopolysaccharide as a marker, we obtained three other lines of evidence that favor the conclusion that the Sarkosyl-insoluble vesicles represent the outer membrane of *S. aurantia*. First, these vesicles contained as the major polypeptide the 36.5K protein which had a variety of properties similar to outer membrane porin proteins of other bacteria (see below). Second, the *S. aurantia* outer membrane contained a functional porin. Third, antiserum raised to the outer membrane vesicles agglutinated fresh *S. aurantia* cells, indicating that the serum recognized surface-exposed epitopes. Nevertheless, despite their identification as outer membrane vesicles, it should be noted that the purification procedure did involve detergent treatment, and it is possible that this resulted in extraction of some outer membrane components. Also, several polypeptides were associated with the peptidoglycan fraction. It is possible that in whole cells such peptidoglycan-associated proteins are also associated with outer membranes since outer membrane proteins from other organisms are often associated with peptidoglycan (25, 38).

The spirochetes constitute 1 of about 10 major subgroups of eubacteria, and they are very distantly related to bacteria like *E. coli* (20, 44). Nevertheless, the outer membrane of *S. aurantia* contains at least one porin protein (Fig. 5). Porins form hydrophilic channels across the outer membrane, and in all gram-negative bacteria that have been studied to date they have the following general properties: they are abundant; they have an oligomeric, often trimeric, structure; the monomer molecular weight is in the range of 27,000 to 48,000; and the pI is acidic (38, 41). The 36.5K protein in the outer membrane vesicles from *S. aurantia* had similar properties. The 36.5K protein was one of the most abundant cellular proteins (Fig. 1), its molecular weight fell within the range of those of other porins, the pI was about 3.4, and it appeared to form trimeric associations (on the basis of the minimal possible oligomeric structure that is consistent with both the cross-linking data and the apparent molecular weight of the major outer membrane protein when it is not heat modified).

The black lipid bilayer data presented in this report strongly support the notion that the *S. aurantia* 36.5K major outer membrane protein is a porin. Although the porin activity was not exhaustively purified, the Triton X-100-solubilized outer membrane preparation used in this study contained at least 90% pure 36.5K protein, which is a purity similar to that of other porins utilized in in vitro characterizations of porin function. The *S. aurantia* porin formed transmembrane channels larger than those of any other bacterial porins that have been reported (37, 41). The single channel conductance in 1 M KCl of 7.7 nS is comparable with that of *P. aeruginosa* porin protein F (5.6 nS) (7) and the *E. coli* OmpF porin (1.9 nS) (8). From the average conductance in 1 M KCl (7.7 nS), assuming that the pore is a

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cylinder with a spherical cross section and that it is filled with an aqueous solution of the same conductance as the external bulk phase, the radius of the pore can be calculated by the following equation: $\bar{\Lambda} = \sigma \Pi r^2/l$, where $\bar{\Lambda}$ is the average single channel conductance, σ is the bulk conductance of the aqueous phase, r is the radius of the pore, and l is the length of the pore, which is assumed to be 6 nm (15). By using this equation, the average pore diameter for the *S. aurantia* porin is estimated to be approximately 2.3 nm. These data support the assumption that the aqueous channel of the porin described here is considerably larger than that of other porins that have been described to date (37, 41). This large size may well be one explanation for the high susceptibility of *S. aurantia* to many antibiotics (9). By using the assumption that the size of sugars varies with the cube root of their molecular weight (55), an estimate of the exclusion limit for uncharged sugars for the *S. aurantia* porin would be greater than 4,000 daltons, compared to ~3,000 daltons for *P. aeruginosa* and 600 daltons for *E. coli*. It is important to remember that such values are comparative values rather than absolute numerical statements of pore sizes.

The data presented here are consistent with the assumption that the porin of *S. aurantia* formed large, water-filled channels. The unique characteristic of this porin preparation as compared with those of other bacterial pores studied is that its $\bar{\Lambda}$ to σ ratio increased with decreasing salt concentrations. Although a number of experiments were performed in an attempt to explain these observations, no definitive explanation arose. We consider that further purification of the *S. aurantia* porin and testing in the lipid bilayer system, vesicle swelling systems, or both would be of interest, as would a study of the outer membrane exclusion limit in whole cells of *S. aurantia* under osmotic conditions that support culture growth.

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