Pore-Forming Ability of Major Outer Membrane Proteins from *Wolinella recta* ATCC 33238

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Three major outer membrane proteins with apparent molecular masses of 43, 45, and 51 kDa were purified from *Wolinella recta* ATCC 33238, and their pore-forming abilities were determined by the black lipid bilayer method. The non-heat-modifiable 45-kDa protein (Omp 45) showed no pore-forming activity even at high KCl concentrations. The single-channel conductances in 1 M KCl of the heat-modifiable proteins with apparent molecular masses of 43 kDa (Omp 43) and 51 kDa (Omp 51) were 0.49 and 0.60 nS, respectively. The proteins formed nonselective channels and, as determined by experiments of ion selectivity and zero-current potential, were weakly anion selective.

The surface of gram-negative prokaryotes, commonly referred to as the cell envelope, consists of two membranes, an outer membrane and an underlying inner or cytoplasmic membrane. The outer leaflet of the outer membrane is constructed almost exclusively of the amphiphilic lipopolysaccharide with associated proteins, while the inner leaflet contains phospholipids in addition to protein. The nature of this chemical distribution results in the formation of an asymmetric outer membrane. The inner leaflet of the outer membrane and cytoplasmic membrane encloses the periplasmic space which contains the peptidoglycan and a multitude of hydrolytic and proteolytic enzymes that function to hydrolyze both extracellular and intracellular macromolecules for use in cellular metabolism (26). The peptidoglycan may be covalently attached to the outer membrane through a lipoprotein (22).

The outer membranes of gram-negative bacteria play important roles in determining the molecules that are taken up into the cell and those that are excreted into the environment (14). In addition, since in many bacteria the outer membrane is the outermost layer, it can play a role in the interaction with antibodies and other proteins as well as in the interaction with other cells and surfaces in the vicinity of the bacterium. One of the major species of proteins in the outer membrane comprises the porins (24). Porins form water-filled channels traversing the outer membrane. Since these channels have distinctive diameters, they give to the outer membrane a molecular sieving function whereby the rate of trans-outer-membrane movement of small hydrophilic molecules is determined in part by the size of the molecule relative to the size of the channel. Consequently, porins are the main uptake route for, but considerably impede the uptake of, many commonly used antibiotics (24). Also, they influence nutrient uptake and exclude large molecules from the environment, including hydrolytic enzymes and toxins. In addition to their role in permeability, specific porins have been reported to have several other important properties in pathogenesis, including mitogenicity for B cells (27); a direct influence on phagocytic function (8), including toxicity for murine macrophages and inflammatory activity (29); a function in the adherence promoting uptake

of intracellular organisms (10); prominent immunogenicity in certain infections (8, 12, 29, 30); and vaccine potential (21).

Many porins have been described to date and their general features are quite well conserved (15), but there is as yet no absolute set of rules to permit identification of porins without direct isolation and characterization by model membrane methods. For example, several well-known porins are sodium dodecyl sulfate (SDS) stable and are purified as functional trimeric units; however, monomers derived from these are nonfunctional. There are, however, a small number of porins that are clearly functional as monomers (33, 35).

Although porins from several enteric organisms and aerobic or facultative anaerobic pathogens have been described, very few have been described for anaerobes and/or oral pathogens (2, 27, 31). *Wolinella recta* is a significant member of the periodontopathic microbiota of several of the periodontal diseases, including refractory disease, and those diseases associated with immunocompromised individuals (9, 23, 28, 32). In a previous report, we described (19) the purification and biochemical characterization of three major outer membrane proteins (Omps) from *W. recta* ATCC 33238, with apparent molecular masses of 43, 45, and 51 kDa (Omp 43, Omp 45, and Omp 51), which are also present in fresh clinical isolates (see reference 5 cited in Kennell and Holt [19] and Kennell et al. [20]). In the study presented here, we report that two of these, Omp 43 and Omp 51, can function as porins.

MATERIALS AND METHODS

Bacterial strain and growth. *W. recta* ATCC 33238 was grown in mycoplasma-formate-fumarate broth under anaerobic conditions as described previously (13).

Isolation of outer membranes and porin proteins. The Omps of *W. recta* ATCC 33238 were prepared as described previously (19). Briefly, whole cells were harvested from the stationary growth phase by ultrafiltration through a 0.45-μm-pore-size filter cassette (Millipore Corp., Bedford, Mass.). After cell disruption in a cold French pressure cell, the membranes were isolated by ultracentrifugation. Omps were solubilized with 5% (wt/vol) Zwittergent 3-14 (Calbiochem Corp., San Diego, Calif.) in 20 mM Tris-HCl (pH 8.0) plus 0.5 M $CaCl_2$ at 37°C for 3 h. The solubilized proteins were precipitated with 4 volumes of ice-cold ethanol and resolu-

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bilized in 0.5% (wt/vol) Zwittergent 3-14–50 mM Tris-HCl (pH 8.0)–10 mM EDTA–0.02% (wt/vol) NaN$_3$ at 37°C for 16 h. The proteins of interest (Omp 43, Omp 45, and Omp 51) were separated by DEAE-Sephacel (Pharmacia, Inc., Piscataway, N.J.) ion-exchange chromatography. The final protein purification was performed by H$_2$O extraction of single bands from SDS-polyacrylamide gels of unheated fractions. The individual proteins were determined to be pure by analysis of SDS-polyacrylamide gels loaded with at least 15 µg of protein per lane and staining with Coomassie brilliant blue R-250 and silver nitrate (17).

Black lipid bilayer experiments. Determination of the porin activity of the three purified *W. recta* Omps (Omp 43, Omp 45, and Omp 51) was carried out by using the black lipid bilayer procedure of Benz et al. (3). Briefly, the black lipid membranes were formed in Teflon chambers from 1.5% (wt/vol) diphytanoyl phosphatidylcholine (oxidized cholesterol) in *n*-decane. A salt bridge was established for the measurement of electrical conductance by immersing Calomel electrodes on each side of the Teflon chamber which was separated by a perforated divider with a 2-mm^2 hole over which the lipid membrane was painted. For conductivity measurements, the unheated Omps were added to one chamber at different concentrations. The resultant current changes were boosted 10^9-fold by a Keithley 427 current amplifier (Cleveland, Ohio), and the output was monitored on a Tektronix 5111A storage oscilloscope (Beaverton, Oreg.) and recorded on a strip chart recorder. The average single-channel conductance in each salt solution was calculated by counting 100 or more single events.

The measurements of zero-current potential of the *W. recta* Omps were made by forming an equilibrium membrane containing 100 or more porin channels in the presence of 0.1 M KCl as described by Benz et al. (4). The zero-current potential was then measured by reducing the applied voltage to zero and subsequently increasing the KCl concentration in one chamber in a stepwise fashion. The voltage increase due to the balancing of the concentration gradient by the electrochemical potential created by preferential movement of one of the two ions, K$^+$ or Cl$^-$, was recorded and used for determination of selectivity by applying the Goldman-Hodgkin-Katz equation for the calculation of the zero-current potential (2).

RESULTS

As described in an earlier report (19), the three Omps of *W. recta* ATCC 33238 were purified to greater than 95% homogeneity by using selective solubilization by the zwitterionic detergent Zwittergent 3-14 and then by using gel elution (Fig. 1). No contaminating lipopolysaccharide was revealed by loading at least 15 µg of the purified protein preparations onto each lane after the preparations were heated at 75°C for 5 min and subsequently staining the gel with silver nitrate. In the SDS-polyacrylamide gel stained with Coomassie brilliant blue R-250, the 45-kDa protein did not demonstrate heat-modifiable behavior at any temperature between 25 and 100°C, whereas the 43-kDa polypeptide (Omp 43) was the result of heat modification of a 34-kDa polypeptide and the 51-kDa polypeptide (Omp 51) was the result of heat modification partly of a 38-kDa polypeptide and partly of aggregates of an apparent size of >150 kDa at 70°C or higher (data not shown).

The addition of small amounts of Omp 43 and Omp 51 to the aqueous-phase bathing planar phospholipid bilayer membranes resulted in increases in the conductance of these
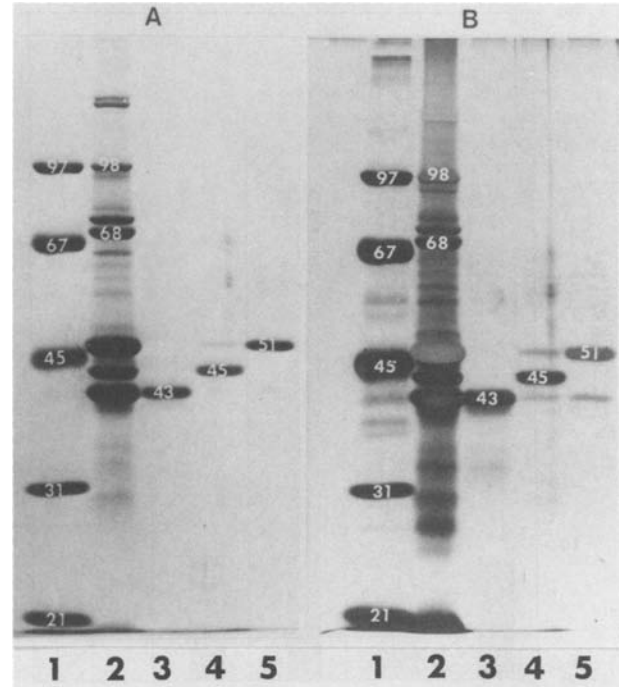

FIG. 1. SDS-PAGE of *W. recta* ATCC 33238 Omp. Lanes: 1, low-molecular-weight standards (Bio-Rad) used to calculate molecular masses by linear regression; 2, 0.5% Zwittergent 3-14-soluble proteins; 3, Omp 43; 4, Omp 45; 5, Omp 51. Each lane of a 4% (wt/vol) acrylamide stacking gel and a 10% (wt/vol) acrylamide resolving gel was loaded with 15 µg of protein after heating at 75°C for 10 min. Gel A was stained with Coomassie brilliant blue R-250; gel B is gel A after additional staining with silver nitrate by the method of Hitchcock and Brown (17). The contaminating proteins visible in gel B, lanes 4 and 5, did not interfere with the interpretation of the results as stated in the text.

bilayers upon imposition of a voltage (Fig. 2). The magnitude of increase was dependent upon the concentration of protein added. In general, it required 1,000-fold more Omp 43 than Omp 51 to result in the same levels of increase in membrane conductance. When protein concentrations in the aqueous phase were reduced to very low levels (25 ng/ml for Omp 43 and 30 pg/ml for Omp 51), the conductance increased in a steplike manner (Fig. 2), representing the progressive incorporation of single pore-forming units into the membrane. These increases were not uniform in size but clustered around a mean (Fig. 3). Thus, the average single-channel conductances in 1 M KCl were 0.60 ± 0.19 nS for Omp 51 and 0.49 ± 0.16 nS for Omp 43. In contrast, Omp 45 exhibited only a few inconsistent conductance steps, even
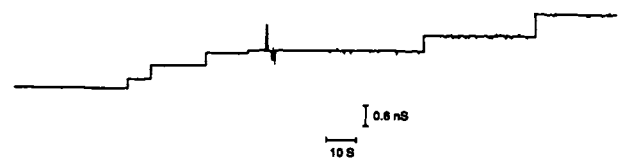

FIG. 2. Membrane conductance increase after the addition of 30 pg of Omp 51 from *W. recta* ATCC 33238 per ml of aqueous 1 M KCl bathing a lipid membrane formed from 1.5% oxidized cholesterol in *n*-decane. The applied voltage was 10 mV.
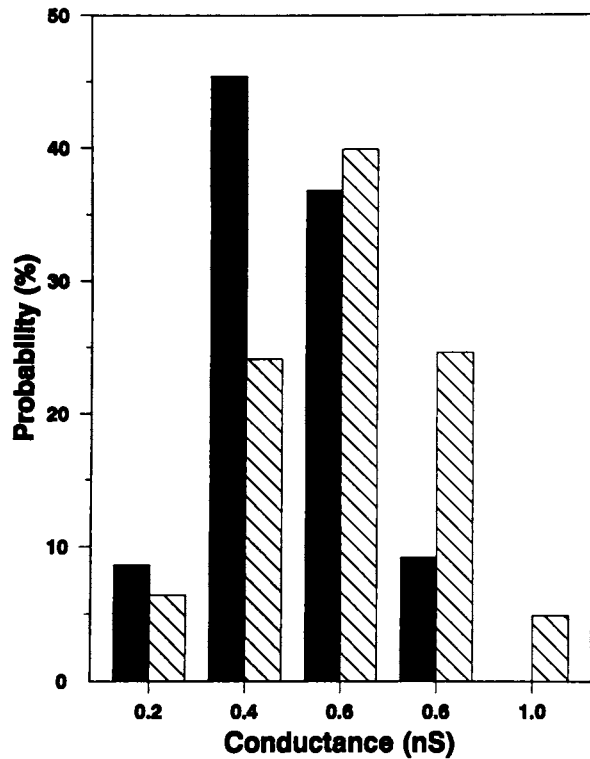
FIG. 3. Histogram of single-channel conductance steps (in nanosiemens) formed by *W. recta* ATCC 33238 Omp 43 (■) and Omp 51 (▨) in 1 M KCl across a lipid membrane. The voltage applied was 10 mV. The mean conductance of 174 events of Omp 43 is 0.49 nS (±0.16), and the mean conductance of 203 events of Omp 51 is 0.60 nS (±0.19).

when as much as 64 ng of protein per ml was present in the solutions bathing the lipid bilayer membrane.

The single-channel conductance of Omp 51 was a linear function (correlation coefficient, 0.997) of the KCl concentration bathing the lipid bilayer (Table 1). This suggested that the channel was water filled and only weakly ion selective. Similar experiments could not be performed for Omp 43 because of the lack of channels reconstituted at lower salt concentrations. (Since incorporation of porins into the lipid bilayer is dependent on hydrophobic interactions, it is favored at high salt concentrations and inhibited at low salt concentrations.)

To test the hypothesis that the Omp 51 was weakly ion

TABLE 1. Average single-channel conductances (G) of *W. recta* ATCC 33238 porins Omp 51 and Omp 43

Omp	Salt (concn [M])	G (nS) (avg ± SD)	Monomer pore diam (nm)	n^a
Omp 51	KCl (1.0)	0.60 ± 0.19	0.65	203
	KCl (0.7)	0.41 ± 0.14	0.63	106
	KCl (0.4)	0.27 ± 0.14	0.68	155
	KCl (0.1)	0.08 ± 0.03	0.69	149
	LiCl (1.0)	0.43 ± 0.17	0.68	220
	KCH₃COO (1.0)	0.29 ± 0.13	0.56	136
Omp 43	KCL (1.0)	0.49 ± 0.16	0.58	174
	LiCl (1.0)	0.31 ± 0.13	0.58	172

a Number of events.

selective, we examined the conductance in salts for which one of the ions, K^+ or Cl^-, was substituted by a more bulky ion, either Li^+ (because of its large hydration shell) or acetate. Omp 51 single-channel conductance was somewhat more affected by changing the anion size (for KCH_3COO) than by changing the cation size (LiCl). This suggested that the channel was weakly anion selective. Similarly, Omp 43 conductance was not greatly affected by changing the cation size, indicating that it was also a weakly selective channel. To specifically examine this, experiments of zero-current membrane potential were performed for both porins, and the permeability ratio of Cl^- to K^+ (P_{Cl^-}/P_{K^+}) was calculated by the Goldman-Hodgkin-Katz equation (2). This indicated that P_{Cl^-}/P_{K^+} values were 1.87 for Omp 43 and 1.46 for Omp 51, demonstrating that both channels were weakly anion selective.

DISCUSSION

The results presented here indicate that *W. recta* contains at least two porin proteins, Omp 43 and Omp 51. There are several possible explanations for the differences in protein concentrations required to increase the level of membrane conductance. (i) Omp 43 may have an intrinsically lower affinity for lipid bilayers, resulting in low incorporation rates. (ii) The purification methodology may have led to partial destruction of the pore-forming ability of Omp 43. (iii) The pore-forming activity of the Omp 43 preparation may have been due to contamination by small amounts of Omp 51 (not visible on Fig. 1). We do not favor the last explanation since the mean single-channel conductances measured for Omp 43 were significantly different ($P < 0.05$ by Fisher's exact test) from those observed for Omp 51 in both 1 M KCl and 1 M LiCl. It is also possible that the contaminating proteins detected essentially only in the silver-stained gel were compromising the pore-forming activity analyses with respect to pore-forming activity of the 41-, 45-, and 51-kDa proteins. However, the 43-kDa contaminant in the 51-kDa protein preparation (Fig. 1B, lane 5) would have had no interfering effect since 1,000-fold the amount of the 43-kDa protein was necessary to detect its pore-forming activity. If these minute protein contaminants were affecting the 45-kDa preparation (Fig. 1B, lane 4), also a major Omp in the porin region, it should have presented pore-forming activity but it did not (i.e., the contaminants were either too small or at too low a concentration). Although one must be cautious about making conclusions about a lack of activity for given proteins (because of the requirements for detergents in the purification protocols and the resultant potential for denaturation of these proteins), it is noteworthy that porin activity correlated with heat-modifiable behavior on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). All known porins demonstrate heat modifiability because they contain a substantial β-sheet structure which traverses the membrane and represents the lining of the porin channels (14, 18, 24). This orderly array of β-sheets is resistant to SDS attack at low temperatures but not at high temperatures, thus giving porins their different mobilities (because of their different configurations) when solubilized at low or high temperatures.

Interestingly, most porins when solubilized at a low temperature in SDS migrate on SDS-PAGE as trimers (18, 24). Denaturation of the trimers to the monomeric form by a variety of methods results in loss of porin activity (2). In contrast, the *W. recta* Omp 43 and Omp 51 appeared to migrate as folded monomers with apparent lower molecular

masses (i.e., higher mobility) on SDS-PAGE when solubilized at low temperatures (19). Since the final step in purification of these proteins was extraction from SDS-polyacrylamide gels, we can be fairly sure that these porins are in fact functional as monomers. There is only one other precedent for a porin that definitely functions as a monomer, OprF of *Pseudomonas aeruginosa* (16, 34), although other porins with similar heat-modifiable behavior have been observed (27). Thus, Omp 43 and Omp 51 fit into an unusual subclass of porins.

Both Omp 43 and Omp 51 formed weakly anion-selective porins in lipid bilayer membranes. Their channel sizes can be estimated by analogy with the well-characterized porins of *Escherichia coli* (5). Monomeric single-channel conductances of 0.49 and 0.60 nS for Omp 43 and Omp 51, respectively, correspond to trimeric conductances of 1.47 and 1.80 nS. These are the approximate conductances of the OmpC and OmpF trimers from *E. coli*. Thus, Omp 43 would be expected to have a channel diameter similar in size to that of OmpC (i.e., 1.08 nm in diameter), whereas Omp 51 would have a size close to that of OmpF (i.e., 1.16 nm in diameter). However, unlike these *E. coli* porins (5), Omp 43 and Omp 51 exhibit selectivity for anions rather than cations.

Although porins have been found in all gram-negative bacteria in which they have been sought, there is still no absolute formula for predicting which proteins in outer membranes are porins. For example, Omp 43 and Omp 51 lack one of the more common identifying characteristics of porins, i.e., a stable trimeric structure when solubilized in SDS at a low temperature (2, 24). In addition, Omp 45 falls within the usual size range of porins but is evidently not a porin. Thus, given the importance of porins in pathogenesis (see above), we feel that it is essential to identify and characterize these Omps, especially as they might relate to pathogenesis (see below). In particular, little is known about the porins of either oral pathogens or anaerobes (27, 31). Only two porins have been examined from these categories. Takada et al. (27) isolated a 41-kDa Omp from *Fusobacterium nucleatum*. The porin was heat modifiable from a size of 37 to 41 kDa and demonstrated porin activity in liposome swelling experiments. However, no detailed analysis of this channel was performed.

The amino-terminal amino acid sequence of Omp 43 has a 33% sequence homology to Omp P.69 from *Bordetella pertussis* (7, 19). This is of some interest since P.69 is a prominent component of the new-generation acellular pertussis vaccines (6, 7, 25). It will therefore be interesting to see if P.69 forms a porin, especially since the only porin characterized to date from *B. pertussis*, the 40-kDa porin, is anion specific and thus does not have the channel properties that one might predict for a general porin like OmpF, OmpC (1), Omp 43, and Omp 51.

There are an increasing number of reports regarding the immunobiological activity of Omps, porins, lipoproteins, and peptidoglycans on immunologically competent cells in in vitro assays (see Galdiero et al. [11] and Takada et al. [27] for appropriate references). Porins, in addition to being able to kill murine macrophages, are also able to interfere with phagocytic activity and intracellular killing by leukocytes (29), produce a dose-dependent inflammatory response in rat footpads, which is inhibitable by the nonsteroidal and steroidal anti-inflammatory agents indomethacin and dexamethasone, and give a dose-dependent release of histamine from macrophages (11). Porin proteins cause the inhibition of phagocytosis through activation of adenylate cyclase (8) and also are able to activate the complement system.

Whether the porins from *W. recta* described in this study possess similar activities and whether they are capable of functioning as virulence factors in vivo remain to be determined.

The goal of this study was to characterize the three Omps of *W. recta* ATCC 33238 as to their pore-forming abilities. The heat-modifiable Omp 43 and Omp 51 displayed pore-forming ability, whereas the non-heat-modifiable 45-kDa protein did not. The function of Omp 45 in the outer membrane remains to be determined. Functional studies of the Omp 43 and Omp 51 porins such as permeability and cross-linking experiments, the activity in animal models of inflammation, and their immunobiological response would provide information on the structure and size of the pores and their potential biological activity in mammalian hosts, ultimately indicating their possible role in inflammatory diseases such as periodontal disease.

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