The isolation and characterisation of a major outer-membrane protein from *Bacteroides distasonis*

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Summary. An outer-membrane protein (OMP) was isolated from a clinical strain of *Bacteroides distasonis*. Changes in growth media did not appreciably affect the appearance of this protein in crude outer-membrane preparations examined by SDS-PAGE. However, the proportion of the protein relative to other OMPs was greater in 24-h cultures than in 48-h cultures. The protein could not be readily solubilised by various conventional detergent extraction techniques but treatment of the insoluble material at 100°C with SDS released the protein, as did overnight extraction at 37°C with SDS. This OMP was heat-modifiable, and thus was similar to the OmpA protein of *Escherichia coli*, with a faster mobility on SDS-PAGE when solubilised at 25°C than at 100°C. The critical temperature for conversion was between 80°C and 90°C. Because of the characteristic heat-modifiability, the protein was called *B. distasonis* HMP-1 (heat modifiable protein-1). Overnight exposure to EDTA or NaCl at 37°C favoured conversion of the 25°C form to the 100°C form. In intact cells, the protein was labelled by a cell-surface radio-iodination procedure, and thus is at least partially exposed at the cell surface. No reactions between the *B. distasonis* HMP-1 and antibodies to either *E. coli* OmpA or *E. coli* porin were found by Western blot analysis. A *B. distasonis* OM preparation containing predominantly HMP-1 had pore-forming ability in a liposome assay. This study is the first report of the isolation and characterisation of a heat-modifiable OMP in *Bacteroides*, and it is the first description of pore-forming activity in a *Bacteroides* OM fraction.

Introduction

The structure and function of outer-membrane proteins (OMPs) in aerobic gram-negative bacteria has been studied extensively, and our understanding of their role in dynamic cell processes has increased.^{1–3} The pore-forming molecules of *Escherichia coli* and some other aerobic bacteria have been characterised,^{3–8} and their contribution to antimicrobial resistance assessed.^{8–16} Similar research in gram-negative anaerobes has been hampered by lack of knowledge about the structure-function characteristics of their OMPs.

Studies on the development of resistance to β -lactam antibiotics in anaerobic bacteria have focused on the production of β -lactamase enzymes.^{17–20} The *Bacteroides fragilis* group of organisms (*Bacteroides sensu stricto*) are amongst the anaerobes most frequently isolated from clinical infections, and are some of the most resistant to antimicrobial agents.²¹ The OMP profiles of members of the *B. fragilis* group are much more complex than those of *E. coli*,^{22, 23} and specific permeability functions have not been assigned to any

molecular species in *Bacteroides*. Recently, a few studies^{18, 24} described permeability of the bacterial cells to β -lactams as a factor in the activity of these agents against the *B. fragilis* group, especially for *B. distasonis*,²⁴ which although one of the most resistant of the group, has one of the lowest proportions of β -lactamase-positive strains.

The purpose of this study was to investigate OMPs in *B. distasonis* and to describe the structure-function characteristics of the major OMPs. Additionally, we hoped to identify pore-forming proteins in this organism as a first step in assessing their role in resistance to β -lactam antibiotics.

Materials and methods

Preparation of crude outer membrane

B. distasonis Wadsworth Anaerobe Laboratory (WAL) strain 6779 was identified by standard methods^{25, 26} and grown for 24 h in tryptone-yeast-glucose (TYG) broth²⁷ in an anaerobic chamber (Anaerobe Systems, Santa Clara, CA, USA). The cells were harvested by centrifugation at 12 000 g for 20 min

in a Sorvall RC-5B centrifuge, and washed once in 10 mM Tris-HCl containing 10 mM MgSO₄, pH 7.4. The harvested cells were then broken by four passages through a French Pressure Cell (SLM Instruments, Urbana, IL, USA) at 12000 lb/in². The suspension was centrifuged at 2000 *g* for 5 min to remove whole cells and cell debris. The supernate contained the cell envelopes. In general, 4 L of overnight cell culture were processed and eventually resuspended to yield 108 ml of cell-envelope preparation.

Inner membrane was solubilised by adding to the cell envelope preparation, Triton X-100 2% v/v, 10 mM MgCl₂, and 10 mM HEPES (final concentrations). The mixture was incubated for 30 min at room temperature, and then centrifuged at 45000 *g* for 1 h in a Sorvall RC-5B centrifuge. The resulting pellet containing the crude outer membrane (OM) was washed once with 10 mM Tris-HCl, 10 mM MgSO₄, pH 7.4, and then frozen or lyophilised. This preparation is referred to as *B. distasonis* crude OMP.

Isolation of *B. distasonis* HMP-1

The crude OMP (64 mg) was resuspended in 50 ml of 20 mM Tris-HCl, pH 7.4, containing sodium dodecyl sulphate (SDS) 0.7% w/v, incubated for 15–30 min at 30°C, and then centrifuged at 100000 *g* (Beckman L5-65 Ultracentrifuge, Beckman Instruments, Fullerton, CA, USA) for 2 h. The pellet, containing the partially purified protein, was washed once with 20 mM Tris-HCl, pH 7.4, and recentrifuged. The resulting pellet (SDS pellet) was extracted with 4 ml of octyl-glucoside (OG; Pierce Chemical Co., Rockford, IL, USA) 3.5% w/v in 20 mM Tris-HCl, pH 7.4, overnight at 30°C, and for an additional 1–2 h at 37°C. The mixture was centrifuged at 100000 *g* and the supernate was dialysed in 20 mM Tris-HCl, pH 7.4, for 24–48 h at 4°C with frequent changes of buffer. The protein migrating at 45 kDa after solubilisation at 100°C was designated as *B. distasonis* HMP-1 because of its unusual heat-modifiable migration patterns on SDS-PAGE (see below). The dialysed preparation was concentrated *c.* four-fold with a Centricon-10 microconcentrator (Amicon, Danvers, MA, USA) and frozen at –70°C. This preparation was used for the liposome assay below.

Protein assays

The SDS-pellet and octyl-glucoside supernate preparations were first assayed with the BioRad protein assay kit (BioRad Laboratories, Richmond, CA, USA) and the Sigma protein assay kit (Sigma Diagnostics, St Louis, MO, USA). However, resulting concentrations seemed much lower than those estimated by comparing band densities on SDS-PAGE gels to band densities of bovine serum albumin standards. The *B. distasonis* HMP-1 protein was finally assayed by amino-acid analyses and by the BCA protein assay kit (Pierce Chemical Co.). With the amino-acid analysis as the standard, a “conversion

factor” was established that was subsequently used to convert the Pierce assay results to a more precise protein measurement.

SDS-PAGE

Proteins were subjected to SDS-PAGE in a modified Laemmli gel²⁸ with an acrylamide:bis acrylamide ratio of 30:0.8 and an acrylamide concentration of 10% w/v for the running gel, and an acrylamide:bis acrylamide ratio of 30:1.0 and an acrylamide concentration of 4% w/v for the stacking gel. Mol. wts were determined from standard curves constructed with mol.-wt standards (BioRad) by linear regression analysis with Symphony 2.0 statistical applications (Lotus Development Corporation, Cambridge, MA, USA).

Electro-elution of the HMP-1 molecule

SDS pellets were run on SDS-PAGE gels as indicated above but not stained. Two end lanes were cut out from the gel and bands were visualised after incubation in ice-cold 0.25 M KCl for 10 min to precipitate the proteins.²⁹ The remainder of the gel was untreated. The two end lanes served as reference lanes for cutting the appropriate bands for electro-elution, which was accomplished with a Model 422 Electro-Eluter (BioRad) following the manufacturer's directions.

Electroblotting of the HMP-1 molecule

SDS-PAGE gel solutions were filtered through a Nalgene 45-μm filter (Nalge Company, Rochester, NY, USA) immediately before pouring. Gels were stored for 24 h at room temperature and run as indicated above. Electroblotting was accomplished with a Trans-blot cell (BioRad) on to PVDF Protein Sequencing Membrane (BioRad) and stained according to the manufacturer's directions.

Amino-acid analysis of the HMP-1 molecule

Amino-acid analysis was performed by Dr Audrey Fowler at the University of California Protein Micro-sequencing Facility, Los Angeles, CA, by the Pico-Tag system (Walters/Millipore Corp., Bedford, MA, USA) after hydrolysis in 6 N HCl of electro-eluted preparations of the HMP-1 molecule transferred to BioRad PVDF protein sequencing membrane. The ratio of polar to apolar amino acids was calculated according to the method of Hatch,³⁰ and the polarity index according to the method of Capaldi and Vanderkooi.³¹

Western blot analysis of the *B. distasonis* outer membrane

Anti-*E. coli* OmpA antiserum was obtained from Dr

P. J. Bassford (University of North Carolina, Chapel Hill, NC, USA). This antiserum had been prepared in rabbits against purified OmpA. The anti-*E. coli* porin was obtained from Dr S. C. Rittenberg (University of California, Los Angeles, CA, USA) and had been prepared in the laboratory of Dr H. Nikaido (University of California, Berkeley, CA, USA) in rabbits against purified porin. Western blot analysis was performed as described previously,³² with dilutions of 1 in 250 of the respective antibody preparations.

Radio-iodination

Bacteria from 12-h cultures were washed twice with Tris buffer (10 mM Tris-HCl, pH 7.4, containing 10 mM MgSO₄) and resuspended to 0.2 g/ml (wet weight). Iodo-beads (Pierce Chemical Co.) were washed and dried according to the manufacturer's instructions. Tris buffer 100 μ l, carrier-free Na¹²⁵I (Amersham, Arlington Heights, IL, USA 500 μ Ci (5 μ l) and five Iodo-beads were added to a 20 mm $\times$ 125 mm glass tube and held for 5 min at room temperature; 1 ml of the cell suspension was added to the glass tube and the radio-iodination reaction was allowed to proceed for 15 min at room temperature with occasional gentle swirling. Radio-iodination was terminated by removing the Iodo-beads from the reaction mixture. The ¹²⁵I-labelled cells were washed three times with 20 ml of Tris buffer to remove unreacted ¹²⁵I, as well as any extraneous proteins that may have been released from lysed cells, and processed as described above to obtain OM. Samples of crude cell lysate, crude OM, and SDS pellet were removed for autoradiography.

Autoradiography

SDS-PAGE gels were stained with Coomassie Blue R250, dried on filter paper, and exposed to Kodak X-Omat XAR-5 film at room temperature for 12 h.

Reconstitution of pore-forming activity in liposomes and liposome swelling assay

Phosphatidyl choline was purified⁷ from crude egg yolk phosphatidyl choline (Sigma) and stored at -70°C. Liposomes were prepared by the procedure of Nikaido and Rosenberg.^{7,33} Briefly, 2.4 μ mol of L- α -phosphatidyl choline and 0.1 μ mol of dicetyl phosphate were added to a round-bottom glass tube and dried with a gentle stream of N₂. The dried lipids were washed with 100 μ l of benzene, dried, and then washed with 100 μ l of ethyl ether and dried again. The tubes were then stored overnight under vacuum in a desiccator jar in the dark. The desired amount of protein (dissolved in water) was added to the tubes, and the tubes were sonicated in an ultrasonic water bath (L and R Manufacturing Co., Kearny, NJ, USA) until the solution became translucent. The preparations were dried under vacuum and kept in an evacuated jar in the dark for 45 min. A solution of Dextran T-40

(Pharmacia, Piscataway, NJ, USA) 15% w/v in 5.0 mM Tris-HCl, pH 7.5, was added, and the lipid layer was wetted evenly. After incubation for 45 min at room temperature, the tubes were shaken briskly by hand to resuspend the liposomes. Liposomes with no protein added or with heat-treated protein or *E. coli* K12 crude OMP were used as controls. The liposome swelling assays were done as described by Nikaido and Rosenberg⁷ with a Beckman Model 35 Spectrophotometer. Iso-osmolar sugar solutions (c. 0.1 M) were prepared with an osmometer (Advanced Digmatic Osmometer Model 30-II, Advanced Instruments, Needham Heights, MA, USA). Rates of permeability were measured by d(1/OD)/dt, according to the method of Nikaido.³⁵

Results

Isolation of the *B. distasonis* HMP-1 protein

Initial attempts to purify the *B. distasonis* HMP-1 molecule indicated that the proportion of this protein relative to the other OMPs of similar mol. wt was not consistent when crude OM from different batches was compared in SDS-PAGE. To try to standardise the proportions of the various components formed, the OMP profiles of *B. distasonis* grown in different media, including Brucella broth (Difco), Anaerobe broth (Difco) and the TYG described above, were studied. No major differences were seen in the proportions of HMP-1. However, the proportion of HMP-1 relative to the other components in the extraction mixture was greater in a 24-h culture than in a 48-h culture. These conditions (i.e. incubation for 24 h in TYG broth) for growing the cells were then followed for the remainder of the study.

When harvested cells were broken in a French Pressure Cell and treated with Triton X-100 to remove the inner membrane,^{35,36} the HMP-1 component remained in the Triton pellet. Because of earlier reports that porin protein from other bacterial species was SDS-insoluble,³⁷ this pellet was treated with various concentrations of SDS (0.5–2.5% w/v) at 30°C to remove some of the contaminating proteins. Selective extraction of the crude OMP (Triton pellet) for either the HMP-1 protein or other contaminating proteins was also attempted with other detergents including sodium deoxycholate, Zwittergent, Nonidet, and Sarkosyl. SDS 0.7% w/v was the most efficient at selectively removing the contaminating proteins from the Triton pellet. Low concentrations extracted small amounts of the contaminating proteins, whereas high concentrations extracted many other proteins along with the HMP-1.

Solubilisation of the HMP-1 protein from the SDS pellet was first attempted with high salt (0.4 M NaCl), according to the procedure of Mitsuyama *et al.*,³⁷ but without EDTA and β -mercaptoethanol, because adding both of those components changed the distinctive heat-modifiable electrophoretic behaviour of

the *B. distasonis* HMP-1. Solubilisation with high salt was not successful. The procedure described by Hancock *et al.*³⁸ to extract the porin of *Pseudomonas aeruginosa* with a Triton X-100, EDTA solution was also attempted; no proteins were extracted from our preparation by this method. Two detergents, Chapso (Sigma) and OG, were then examined for their ability to extract the HMP-1 component. OG was the best detergent for extracting the HMP-1 from the SDS pellet, and it had the added advantage of being a small molecule (289 Da) which was easily removed by dialysis. A concentration of 3.5% w/v resulted in the best yield, although some HMP-1 remained in the insoluble pellet. Further overnight extractions at 30°C of the OG-insoluble pellet with either OG 5% w/v or SDS 2% w/v were also attempted, but did not yield significant additional amounts of HMP-1 protein. SDS-PAGE patterns of samples of various stages of the purification are shown in fig. 1. All samples run on SDS-PAGE throughout this study were solubilised at both 25°C and 100°C to detect the distinctively migrating HMP-1 band. The protein was fully released from the SDS-insoluble material by boiling the sample or by overnight extraction at 37°C in 0.1 M Tris-HCl, pH 7.4, with SDS 2% w/v (see below).

Heat modifiability of the HMP-1 protein

The HMP-1 protein had a distinctive heat-modifiable electrophoretic mobility similar to that of the OmpA protein of *E. coli* which migrates more slowly when solubilised at 100°C than when solubilised at 25°C.³⁹ The *B. distasonis* HMP-1 band at 38 kDa from preparations solubilised at 25°C was electro-eluted from an SDS-PAGE gel and then incubated at both 25°C and 100°C and re-run; the 25°C and 100°C preparations again migrated, respectively, to 38 kDa and 45 kDa (fig. 2). Electro-elution resulted in conversion, to some degree, of the 38-kDa to the 45-kDa form. However, maintaining all buffers and samples at 4°C minimised the extent of this conversion.

Fig. 3 shows an SDS-PAGE gel of the SDS pellet solubilised at various temperatures. The apparent mol. wt of the *B. distasonis* HMP-1 was 38 kDa when

solubilised at 25°C, and 45 kDa when solubilised at 100°C. HMP-1 solubilised at 55°C showed the same pattern as that extracted at 25°C, whereas the material solubilised at 80°C showed migration bands of both species, with the band at 38 kDa predominating. The material solubilised at 90°C showed migration of both species, with the band at 45 kDa predominating. The material solubilised at 100°C showed primarily the band at 45 kDa. Thus, the major shift from the 38-kDa form to the 45-kDa form occurred between 80°C and 90°C.

Metal ion effects on *B. distasonis* HMP-1 migration on SDS-PAGE

The dependence on metal ions of the SDS migration form of the *B. distasonis* HMP-1 protein was ex-

Fig. 2. SDS-PAGE patterns of *B. distasonis* HMP-1 preparations (electro-eluted, 25°C): lane 1, low-mol.-wt standards; 2, SDS pellet (solubilised at 25°C); 3, SDS pellet (100°C); 4, electro-eluted 38-kDa band cut from identical gel (as indicated by arrow) and solubilised at 25°C; 5, electro-eluted 38-kDa band cut from identical gel (→) and solubilised at 100°C. Electro-eluted band was dialysed against 10 mM Tris-HCl, pH 7.4, and concentrated in a Centricon PM10 (Amicon).

Fig. 1. SDS-PAGE gel of *B. distasonis* HMP-1 preparation: lane 1, low-mol.-wt standards; 2, cell envelope (solubilised at 25°C); 3, cell envelope (100°C); 4, Triton pellet (crude OMP) (25°C); 5, Triton pellet (crude OMP) (100°C); 6, SDS pellet (25°C); 7, SDS pellet (100°C); 8, high-mol.-wt standards; 9, octyl-glucoside supernate (OG-Sup) (25°C); 10, OG-Sup (100°C).

Fig. 3. Heat modifiability of the *B. distasonis* HMP-1 protein: SDS-pellet preparations were solubilised in sample buffer at the indicated temperatures before electrophoresis: lane 1, low-mol.-wt standards; 2, 25°C; 3, 55°C; 4, 80°C; 5, 90°C; 6, 100°C; 7, high-mol.-wt standards.

aminated. In *E. coli*, the stability of the low-temperature form of OmpA appears to involve magnesium ions.⁴⁰ Metal-ion-complexing reagents such as EDTA, phosphate ions, hydroxyl ions, the competitive cations Zn^{++} or Ca^{++} , or the reducing agent β -mercaptoethanol (β -ME) seems to cause nearly complete conversion of the *E. coli* protein to the high-mol.-wt (i.e., slower migrating) form. However, the addition of NaCl or magnesium ions favours the conversion to, or preservation of, the low-mol.-wt (i.e., faster migrating) form. In *B. distasonis* HMP-1, all of the protein migrated at the slower rate after solubilisation at 100°C, whether or not the metal-ion-complexing or competing solutions (β -ME 10% v/v, 5.0 mM EDTA, or 100 mM $CaCl_2$) were added. In contrast to the results seen with *E. coli* OmpA, 300 mM NaCl or 100 mM $MgCl_2$ did not preserve or convert any of the high-mol.-wt form to the low-mol.-wt form.

The SDS pellet was incubated overnight at 37°C with: (1) Tris buffer; (2) Tris buffer with SDS 2% w/v; (3) Tris buffer with SDS 2% w/v and 0.4 M NaCl; or (4) Tris buffer with SDS 2% w/v and 10 mM EDTA. After recentrifugation, the samples incubated in Tris alone retained about half of the HMP-1 band in the pellet fraction, and the fractions solubilised at 25°C or 100°C migrated as expected. The samples incubated with Tris and SDS released nearly all of the HMP-1 into the supernate fraction. About half of this fraction solubilised at 25°C had converted to the 100°C form (fig. 4a). The samples extracted with Tris, SDS and NaCl released about half of the HMP-1 into the supernate fraction; all of the HMP-1 (solubilised at 25°C) in both the supernate and pellet fractions migrated in the 100°C form. The sample extracted with Tris, SDS and EDTA released nearly all of the HMP-1 into the supernate fraction except that nearly all of the protein solubilised at 25°C migrated as the 100°C form (fig. 4b). We concluded from these experiments

that: (1) SDS was needed to fully release the *B. distasonis* HMP-1 from the pellet; (2) neither NaCl nor EDTA was necessary for full release of the HMP-1; (3) the addition of NaCl to the Tris and SDS seemed to inhibit full release of the protein; (4) prolonged exposure to EDTA or NaCl at 37°C favoured conversion of the 25°C form to the 100°C form.

Amino-acid analysis of the HMP-1 protein

Table I lists the amino-acid composition of the HMP-1 molecule in both 25°C and 100°C forms. The analysis was performed on an electro-eluted HMP-1 38-kDa (25°C) band that was then electrophoresed separately at 25°C and 100°C and transferred to blotting paper. The analysis was done on both the 25°C and 100°C bands to determine whether there were any obvious differences in amino-acid content between the two bands. No significant differences were seen. Tables II and III list the polarity and polarity index values of the 25°C and 100°C forms. The amino-acid composition of a heat-modifiable membrane protein from a *Fusobacterium* sp.⁴¹ is included for comparison. Capaldi and Vanderkooi³² found that membrane proteins with polarities below 40% could generally be considered "intrinsic" membrane proteins and could be separated from their respective membranes only by detergents or organic solvents, whereas 85% of the soluble proteins studied had polarities of $47 \pm 6\%$. The *B. distasonis* HMP-1 had a polarity of 43%, indicating that at least portions of the molecule are likely to be intrinsic to the membrane. The relatively lengthy incubation necessary to release this protein from the SDS pellet supports this assumption. A low polarity index, according to Hatch,³¹ indicates compact molecules with a high content of helical structure which may form heterologous interactions with other apolar materials leading to in-

Fig. 4. SDS-PAGE patterns of proteins solubilised (25°C or 100°C) from SDS pellet extracted overnight at 37°C with: (A) 10 mM Tris-HCl, pH 7.4, alone (lanes 2–5) or with SDS 2% w/v (lanes 6–9)—lane 1, low-mol.-wt standards; 2, supernate (25°C); 3, supernate (100°C); 4, pellet (25°C); 5, pellet (100°C); 6, supernate (25°C); 7, supernate (100°C); 8, pellet (25°C); 9, pellet (100°C); 10, high-mol.-wt standards; (B) 10 mM Tris-HCl, pH 7.4, with SDS 2% w/v and 0.4 M NaCl (lanes 1–4) or 10 mM EDTA (lanes 5–8)—lane 1, supernate (25°C); 2, supernate (100°C); 3, pellet (25°C); 4, pellet (100°C); 5, supernate (25°C); 6, supernate (100°C); 7, pellet (25°C); 8, pellet (100°C).

corporation of these proteins in to lipid-containing membranes. The *B. distasonis* HMP-1 has a polarity index of 1.59 (1.56 for the 100°C form), which indicates a protein of intermediate polarity with a slightly less polar nature than the heat-modifiable protein from the *Fusobacterium* sp.⁴¹ We were unable to obtain an N-terminal sequence of this protein because the N-terminus was blocked.

Radio-iodination of *B. distasonis* cell-surface-exposed proteins

The procedure used labels only cell-surface-exposed proteins because the iodinating enzyme is covalently attached to a latex bead. After iodination, the cell pellet was washed several times to remove excess

unbound ¹²⁵I, as well as any extraneous proteins that may have been released from lysed cells. Whole cells of *B. distasonis* were labelled with ¹²⁵I and then processed for purification of the HMP-1 protein. In the crude cell-envelope fraction, the major protein species labelled migrated at > 200 kDa when solubilised at 25°C (fig. 5, lane 1). At 100°C, the major band observed migrated at c. 95 kDa and another strong band was observed at c. 55 kDa (lane 2). Numerous other faint bands were observed. In the Triton pellet (i.e., OM-enriched fraction) numerous bands were observed, including those mentioned above. Additionally, the HMP-1 band (i.e., 38 kDa at 25°C and 45 kDa at 100°C) was seen clearly, indicating that at least part of the protein was exposed on the outer surface (lanes 3 and 4). In the SDS pellet solubilised at 25°C, the band of > 200 kDa was the main band labelled, and the

Table I. Amino-acid composition of electro-eluted *B. distasonis* HMP-1 38 kDa (25°C) band, solubilised at 25°C or 100°C and re-run in SDS-PAGE

Amino acid	38-kDa (25°C) form		45-kDa (100°C) form	
	Number of residues	Mol %	Number of residues	Mol %
<i>Neutral aliphatic</i>				
Gly	31	13.6	54	14.0
Ala	29	12.7	39	10.1
Val	14	6.1	24	6.2
Leu	14	6.1	28	7.2
Lle	8	3.5	15	3.9
Ser	13	5.7	20	5.2
Thr	10	4.4	16	4.1
<i>Aromatic</i>				
Phe	11	4.8	20	5.2
Tyr	8	3.5	19	4.9
<i>Sulphur-containing</i>				
Cys	0	0.0	0	0.0
Met	5	2.2	2	0.5
<i>Imino-</i>				
Pro	9	3.9	17	4.4
<i>Dicarboxylic</i>				
Asp	25	11.0	45	11.6
Glu	28	12.3	46	11.9
<i>Basic</i>				
His	2	0.9	4	1.0
Arg	11	4.8	22	5.7
Lys	10	4.4	16	4.1

HMP-1 (38-kDa) band was seen clearly (lane 5). A strong band was observed also at 25 kDa (lane 5). When the sample was solubilised at 100°C, the major bands seen migrated at 95, 66, 55, 45 (HMP-1) and 35 kDa (lane 6). The SDS pellet was also separately labelled and analysed as a control, to ensure that the HMP-1 protein could be labelled by this procedure (lanes 7 and 8).

Table II. Polarity (sum mol% of polar amino acids³¹) of electro-eluted *B. distasonis* HMP-1 38-kDa (25°C) band solubilised at 25°C or 100°C

Amino acid	Mol % of polar amino acids in		
	<i>B. distasonis</i> HMP-1		<i>F. nucleatum</i> ATCC 25586 heat-modifiable protein ⁴¹
	38-kDa (25°C) form	45-kDa (100°C) form	
Asp	11	11.6	11.5
Glu	12.3	11.9	13.3
Lys	4.4	4.1	6.8
Ser	5.7	5.2	10.5
Arg	4.8	5.7	2.8
Thr	4.4	4.1	5.9
His	0.9	1.0	2.4
Polarity	43.5%	43.6%	53.2%

Cross-reactivity with membrane proteins from *E. coli*

The antisera reacted as expected with OM preparations from *E. coli* K12, but did not react with the *B. distasonis* OM preparations (unpublished data). The anti-OmpA serum reacted with the *E. coli* preparation, staining bands at 28 and 33 kDa for preparations solubilised at 25°C and 100°C, respectively; these are the expected mol. wts for *E. coli* OmpA.⁴² The anti-porin antiserum stained one band at 35 kDa with *E. coli* preparations solubilised at 100°C (the approximate expected location for *E. coli* porin⁴), but did not react with the *B. distasonis* OM preparation. The *E. coli* porin forms aggregates at 25°C and does not enter the gel under these conditions.

Pore-forming ability of the partially purified *B. distasonis* HMP-1

The dialysed and concentrated OG-supernate frac-

Table III. Polarity index (ratio of polar to apolar amino acids³⁰) of electro-eluted *B. distasonis* HMP-1 38-kDa (25°C) band solubilised at 25°C or 100°C

Amino acid	Number of amino-acid residues in		<i>F. nucleatum</i> ATTC 25586 heat-modifiable protein ⁴¹
	<i>B. distasonis</i> HMP-1		
	38-kDa (25°C) form	45-kDa (100°C) form	
<i>Polar</i>			
Asp	25	45	11.5
Glu	28	46	13.3
Lys	10	16	6.8
Ser	13	20	10.5
Arg	11	22	2.8
Thr	10	16	5.9
Total	97	165	50.8
<i>Apolar</i>			
Val	14	24	5.5
Leu	14	28	7
Ile8	8	15	4.3
Met	5	2	1.1
Pro	9	17	5
Phe	11	20	3.4
Total	61	106	26.3
<i>Polarity index</i>	1.59	1.56	1.93

Fig. 5. Autoradiograph of SDS-PAGE gel of radio-iodinated cell-surface proteins of *B. distasonis*: lane 1, cell envelope (25°C); 2, cell envelope (100°C); 3, Triton pellet (crude OMP) (25°C); 4, Triton pellet (crude OMP) (100°C); 5, SDS pellet (25°C); 6, SDS pellet (100°C); 7, radio-iodinated SDS pellet (25°C); 8, radio-iodinated SDS pellet (100°C).

Table IV. Pore-forming activity of *B. distasonis* HMP-1 fraction in liposomes

Preparation	Protein added (μg)	Sugar	ΔOD _{min}	d(1/OD)/dt*	Permeability rate† (%)
<i>B. distasonis</i> HMP-1	20	Arabinose	0.19	0.725	100
		Glucose	0.13	0.50	69
		N-Ac-Glu‡	0.10	0.332	45
		Stachyose	0.005	0.022	3.1
	190	Maltose	0.24	0.28	22§
		Sucrose	0.19	0.207	16.5§
Heat-treated HMP-1 (negative control)	80	Stachyose	0.04	0.043	3.4§
		Arabinose	0.015	0.017	8.9
<i>E. coli</i> K-12 crude OMP	40	Arabinose	0.43	0.806	100
		Glucose	0.335	0.655	81
		N-Ac-Glu	0.225	0.428	53
		Maltose	0.06	0.112	14
		Sucrose	0.03	0.04	5.0

* d(1/OD)/dt was calculated from the formula $[1/(OD)^2] \times [d(OD)/dt]$, see Results.

† Relative to arabinose.

‡ N-acetyl-D-glucosamine.

§ Normalised to arabinose in liposomes prepared with 190 μg of protein with the proportional swelling rates in arabinose for both protein concentrations as a correction factor.

|| Normalised to arabinose with 80 μg of the same preparation of HMP-1 without heat treatment.

tion (fig. 1, lane 9) containing the *B. distasonis* HMP-1 isolated to near homogeneity (but not completely pure) was incorporated into liposomes and tested for pore-forming ability. In this assay, phospholipid liposomes were prepared containing pore-forming molecules within the bilayer membrane and an impermeable solute (in this case Dextran T-40) in the intravesicular space. When these vesicles are diluted in an isotonic solution with a test solute (i.e., the various sugars), the influx of the test solute into the liposome is followed by a rapid influx of water, and the liposomes swell, reducing their turbidity. The permeability of the

channel is assumed to be proportional to the initial swelling rate, which is proportional to $d(1/OD)/dt$.⁷ The formula, $d[1/OD]dt = [1/(OD)^2] \times [d(OD)/dt]$ was used to calculate the initial swelling rate³⁴ (where OD was the initial turbidity of the suspension, and $d(OD)/dt$ was the initial slope of the OD versus time tracing). The swelling rates of the sugars were calculated as relative permeability rates compared to the swelling rate of arabinose (100%).

The results of the liposome swelling assays are listed in table IV. Because the rate was too low to be measured for sucrose and maltose when 20 μg of *B.*

distasonis protein was used, the assays were repeated with 190 µg of protein, and the values were normalised by applying the proportional swelling rates in arabinose for both protein concentrations as a correction factor.⁷ In most assays, 20 µg of OG-supernate fractions (i.e., partially purified HMP-1) was used. The permeability coefficients for 2 µg and 10 µg of OG supernate were 0.01 and 0.065, respectively. Assays were performed with *E. coli* protein for comparison, and to serve as positive controls of the liposome preparation. For the *E. coli* sample, 40 µg of crude OMP (Triton pellet) was used. The rates of change of OD₄₀₀ were slower for the liposomes with *B. distasonis* protein than for those with *E. coli* K12 OMP. As expected, the higher mol. wt disaccharides (maltose and sucrose) had much lower permeability rates than the smaller monosaccharides. Stachyose (mol. wt 666.6) should not penetrate the pores, and was used to determine the iso-osmolar concentration for the assay. OG-supernate fractions incubated at 100°C for 10 min and then incorporated into liposomes served as negative controls, and the permeability rate of arabinose for boiled preparations was < 9% of the rate for the unboiled sample, confirming the specific protein nature of the pore-forming molecules. The pore radius was not calculated in this study because the preparations used were not completely pure, and it was possible that one or more of the contaminating proteins could have contributed to the pore formation. Electro-eluted preparations of the HMP-1 25°C band were treated as follows to remove the SDS: (1) passed through an Extracti-gel column (Pierce Chemical Co.); (2) treated by acetone precipitation alone; or (3) treated by acetone precipitation and renaturation by dilution in guanidine-HCl as described by Hager and Burgess.²⁹ None of these preparations was active in the liposome assay.

Discussion

In *E. coli*, the mobility of the major heat-modifiable OMP, OmpA, is less when solubilised at 100°C than at 25°C.³⁹ Antiserum to the *E. coli* K12 OmpA cross-reacted with the heat-modifiable proteins from all strains of Enterobacteriaceae tested in one study.⁴³ Because these heat-modifiable proteins were nearly identical in primary structure, the authors suggest that the structure, and perhaps the function, of the heat-modifiable protein is strongly conserved in Enterobacteriaceae. Proteins with similar heat-modifiable behaviour have been described in other Gram-negative bacteria.⁴³⁻⁴⁵

The *E. coli* OmpA protein is not thought to produce transmembrane channels⁴⁶ and is not thought to be important in antibiotic resistance, although recent reports have described pore-forming proteins that exhibit this particular heat-modifiable behaviour.^{38, 47} The role of porins in the permeability of gram-negative aerobic bacteria and in antimicrobial resistance has

been extensively studied^{11, 37, 48-51} and recently reviewed.^{9, 16} Diminished uptake of, and increased resistance to, particular antibiotics was seen in several species of bacteria with altered or missing porin molecules.^{11, 50-52}

In anaerobic bacteria, the role of permeability barriers in antibiotic resistance has been approached only indirectly^{53, 54} and no porin molecules have been identified in the *B. fragilis* group. Indeed, little is known about the structure and function of OMPs in anaerobes. Ionic charge, hydrophobicity, and mol. wt may influence β -lactam uptake across the *B. fragilis* OM.¹⁸ Both β -lactamase and a permeability barrier have been shown to affect the activity of cephalosporins against members of the *B. fragilis* group, except for *B. distasonis*, which showed only a permeability barrier to all antibiotics tested.²⁴

F. nucleatum is a gram-negative anaerobic bacterium commonly isolated from oral infections. Recently, Takada *et al.*⁵⁵ reported the isolation from *F. nucleatum* of a heat-modifiable protein with pore-forming activity. The protein was collected from a Sephacryl S200 column, dialysed and repeatedly acetone-precipitated to remove remaining detergent and then used in a liposome assay. In our experience, acetone precipitation destroyed the functional activity of our preparation, even when done in a procedure designed to restore some functional activity.²⁹ Electroelution may also have destroyed functional activity of our protein. The mol. wt of the porin fraction of Takada *et al.*⁵⁵ was 37 kDa when solubilised without boiling, and 41 kDa when boiled before electrophoresis. The fraction contained lipopolysaccharide and showed strong immunobiological activity.

In other studies, heat-modifiable proteins of 38-50 kDa were isolated from the OM of *F. nucleatum*^{35, 41, 56} that seem to be remarkably similar to our HMP-1 protein. Bakken *et al.*⁴¹ were unsuccessful in purifying the individual protein species from SDS solutions by gel chromatography unless proteins were first maleylated. This fusobacterium protein has been termed HM-1 (for heat-modifiable protein 1).^{35, 41, 56} HM-1 is strongly bound to the matrix, although whether it is complexed with lipopolysaccharide⁵⁵ or peptidoglycan⁵⁶ is not clear. A heat-modifiable protein from *F. nucleatum* has also been implicated as a co-aggregation receptor with *Streptococcus sanguis*,⁵⁷ although the amino-acid analysis suggests that it may be a different protein from that described by Bakken *et al.*⁴¹

We investigated the OM components of *B. distasonis* because, amongst the *B. fragilis* group, it is one of the most resistant to β -lactam antibiotics, yet it has one of the highest proportions of β -lactamase-negative strains. Thus, there must be some other resistance mechanism, such as altered targets, altered OM permeability characteristics, or low (undetectable) levels of β -lactamase combined with altered permeability. The strain used in this study was previously used to study changes in penicillin-binding proteins (PBP) of a

laboratory-induced cefoxitin-resistant mutant.⁵⁸ The strain was chosen because it did not produce β -lactamase, which made it a good candidate for producing drug-resistant mutants in a study of non-enzyme-mediated mechanisms of resistance. We found changes in the pattern of the PBP-1 protein that correlated with cefoxitin resistance but were not able to identify any OM changes.⁵⁸ Piddock and Wise⁵⁹ found two cefoxitin-resistant clinical isolates that showed OMP changes—an apparent lack of the 49–50-kDa protein and reduced affinity of PBPs for cefoxitin. In another study, no correlation between OM permeation and MICs of the cephalosporins was found in sensitive *B. fragilis* parental strains and mutants resistant to ceftazidime, cefazolin and cephalothin.⁶⁰

This study is the first report of a heat-modifiable OMP in *Bacteroides* spp., and the first report of porin activity in a bacteroides OM fraction. The *B. distasonis* HMP-1 protein exhibits a heat-modifiable behaviour in SDS gels similar to that of the *E. coli* OmpA protein, and is exposed on the outer surface of the cell. Like the pore-forming fractions in *F. nucleatum*⁵⁵ and the two *Campylobacter* species,⁴⁷ a pore-forming fraction isolated from the *B. distasonis* OM is primarily composed of a protein species with the heat-modifiable character of the *E. coli* OmpA molecule. Although the OmpA molecule is thought to be highly conserved, and cross-reactivity between OmpAs of various gram-negative aerobic enteric bacteria has been demonstrated,⁴³ the HMP-1 of *B. distasonis* did not cross-react with antiserum prepared against an *E. coli* OmpA, nor with

an antiserum to *E. coli* porin. This does not preclude the possibility that some antigenic similarity might be demonstrated with other antisera. OM preparations were made from the nine ATCC type strains of the *B. fragilis* group, and all had proteins of approximately the same size with heat modifiable behaviour similar to that of the *B. distasonis* HMP-1 (unpublished data).

Little is known about the function of the OMPs in anaerobic bacteria, or their importance in antimicrobial resistance. The expanding use of β -lactamase-stable antibiotics will probably result in the selection of anaerobic bacteria of the *B. fragilis* group able to survive the antibiotic challenge due to changes in PBPs and OM permeability characteristics. There is increasing recognition that interaction of a cell wall barrier and β -lactamase activity may result in high-level resistance to β -lactam antibiotics.^{9,61} Understanding the role of permeability barriers in antimicrobial resistance and in cell metabolism in general will require isolation and characterisation of OMPs, including pore-forming molecules, and elucidation of their structure and function.

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