Outer-Membrane Pore-Forming Proteins in Gram-Negative Anaerobic Bacteria

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The outer-membrane proteins (OMPs) of bacteria function as the dynamic interface between the bacterium and its surroundings and are involved in maintenance of cell structure, binding a variety of substances, adhesion to other cells, and regulation of transport of both nutrients and bactericidal agents. There is a vast amount of information about aerobic OMPs and their roles in immunogenicity, virulence, and antimicrobial resistance. Knowledge about OMPs in anaerobic bacteria is much sparser. Genetic data present in data banks regarding aerobic porins are not readily helpful in identifying or analyzing anaerobic porins because of the large phylogenetic distance between the aerobic and anaerobic organisms. We recently identified and sequenced the genes for both a porin protein complex and an OmpA protein in *Bacteroides fragilis*, and the data are summarized here. Also, recent information is presented about similar OMPs found in other gram-negative anaerobic bacteria, including *Bacteroides thetaiotaomicron*, *Bacteroides distasonis*, *Porphyromonas*, and *Fusobacterium*.

Outer-membrane proteins (OMPs) of microorganisms function as a dynamic interface between the cell and its surroundings. Some of the functions of these proteins include maintenance of cell structure, passive and active transport, adhesion to other cells, and binding a variety of substances. OMPs are also important elements of antimicrobial resistance in microorganisms. The cell membrane is the first barrier encountered by the antimicrobial agent, and the ability to penetrate the membrane is a condition, *ab initio*, of the antibiotic’s capacity to inhibit or kill the bacteria. Once it has penetrated the membrane, the antimicrobial must be able to maintain a sufficiently high intracellular concentration in order to act. Proteins that actively remove the antimicrobial from the cell (e.g., inactivating enzymes and efflux proteins) are also important factors in the organism’s development of resistance. The relative contributions of permeability changes, production of inactivating enzymes, and active efflux to antimicrobial resistance have been described extensively in aerobic organisms [1].

In anaerobes, there is little information about pore-forming OMPs and virtually none about the importance of these proteins in antimicrobial resistance. Little information can be drawn from porin proteins already characterized (e.g., from *Pseudomonas*, *Salmonella*, or *Escherichia coli*) because of the phylogenetic distance of gram-negative anaerobic rods from the *Enterobacteriaceae* [2]. Nevertheless, there has been some research on OMPs of some gram-negative anaerobes. Pore-forming OMPs described in the *Bacteroides fragilis* group organisms, *Porphyromonas*, and *Fusobacterium* will be reviewed in this report.

**B. FRAGILIS GROUP**

*B. fragilis*–group organisms are the anaerobes most frequently isolated from clinical infections [3]. They are...
one of the most antimicrobial-resistant groups of anaerobes; resistance to virtually all classes of antimicrobials has been reported [4]. Although significant resistance is due to the production of transferable β-lactamase genes [5] and to extended-action β-lactamas [4], there is evidence that other factors contribute to resistance. For example, Bacteroides distasonis is one of the most resistant species of the B. fragilis group, yet it has one of the lowest proportions of β-lactamase–positive strains. Also, changes in both penicillin-binding proteins (PPBs) and OMPs were correlated with cefoxitin resistance in B. fragilis [6]. Specifically, changes in permeability seem to contribute to β-lactam resistance [7]. Resistance due to changes in specific porin molecules have not yet been described, because these proteins are just now being identified.

In aerobes, OMPs mediate bacterial resistance not only by restricting permeability into the cell but also actively pumping the antimicrobial out of the cell. In Bacteroides, resistance due to efflux mechanisms has been described for tetracycline [8]. Miyamae et al. [9] found active efflux of norfloxacin by B. fragilis and suggested that the efflux is catalyzed by a multidrug pump similar to that of NorA/Bmr. There is no evidence, currently, of efflux proteins mediating resistance to β-lactam antibiotics. The metabolic efflux inhibitor, carbonyl cyanide m-chlorophenylhydrazone, had no effect on the MICs of several antibiotics, including cefoxitin and imipenem, in selected strains of B. fragilis (L. Piddock, personal communication), which indicates that active efflux was not the major mechanism of resistance to β-lactams in the strains tested. Of course, only a few strains were tested in those studies; there may be efflux mechanisms for β-lactams in addition to that shown for norfloxacin.

**OMPs in B. fragilis.** The isolation of pore-forming proteins in B. fragilis has been studied by 3 groups. In our laboratory, we described a 200-kDa porin protein complex (Omp200) of B. fragilis that formed pores both in liposome and black lipid bilayer studies [10]. In France, Odou et al. [11, 12] reported a 45-kDa porin-like protein from B. fragilis [11] that was more fully characterized elsewhere. The 45-kDa protein was purified by electrophoresis after SDS-PAGE and analyzed in the liposome swelling assay. Both monosaccharides and disaccharides (sucrose) were able to cause swelling of the proteoliposomes. Kanazawa et al. [13] reported 3 porin proteins (51, 92, and 125 kDa) that were active in liposome assays. The proteins were purified to apparent homogeneity and formed pores that allowed the diffusion of saccharides of M<sub>r</sub> < 340–400. A similar result was seen for the purified outer membrane of B. fragilis.

**Omp200.** Analysis of the Omp200 described in our laboratory revealed a single band (>200 kDa molecular weight [MW]) on SDS-PAGE analysis after incubation at 25°C and 2 bands (≈94 and ∼62 kDa) after boiling [10]. The larger band migrated at ∼120 or ∼94 kDa depending on electrophoresis conditions. Both incubation with copper at room temperature (during a staining procedure or before PAGE analysis) and electrophoresis (at 4°C) caused dissociation of Omp200 into 2 components, Omp121 and Omp71.

**Omp121.** Recently, we identified and sequenced the gene(s) coding for the proteins in Omp200, omp121 and omp71 (the altered nomenclature reflects the calculated MW, which is based on the deduced amino acid sequence) [14]. The C terminus of Omp121 exhibited sequence similarity with many OMPs from Enterobacteriaceae (including TonB-dependent receptors) and fulfilled the “requirements” for specific amino acids at the C terminal. We found that Omp121 was homologous to Porphyromonas gingivalis RagA (a 112-kDa protein homolog of the family of TonB-linked outer membrane receptors, described in detail below). Homology at the N terminal (in the region considered important for N terminal membrane stabilization) is also seen with the SusC protein of Bacteroides thetaiotaomicron [15] described below. Although the amino acid homology with porins from aerobic bacteria was quite low, a fold-recognition analysis program (3D-PSSM) revealed a very significant structural homology with a wide variety of porins from aerobic bacteria (including the 16-stranded Klebsiella pneumoniae OsmA, E. coli proteins OmpF and OmpC, and the 22–24 stranded ligand-gated E. coli FepA porin).

**omp71.** BLAST searching of the GenBank database did not reveal any homologs for Omp71. At this point, the function of Omp71 is unclear. We found that omp71 is located directly downstream of omp121 and that the 2 sequences are separated by at most 36 bp. These proteins are purified as a complex in the outer membrane, but we cannot rule out the possibility that that was an artifact of the purification procedure. Finding that the genes are adjacent suggests strongly that the translated proteins are indeed part of a membrane complex.

The porins found by the 2 other research groups have not been sequenced, so we cannot determine whether they are related to the porins we are studying. Although the other 2 groups used the same strain of B. fragilis used in our studies (B. fragilis ATCC 25285), the growth and purification conditions were different, and at this time it is impossible to tell whether the 125-kDa protein described is the same as the Omp121 we have described.

**omp117.** We identified the gene for an OMP of MW 117. The deduced amino acid sequence was analyzed by fold-recognition analysis, and we found the corresponding model to be the 22-stranded FepA ligand-gated porin of E. coli (E. K. Read, H. M. Wexler, and T. Tomzynski, unpublished data). Both the RagA and SusC sequences were also submitted for analysis, and the same model was predicted. The hydrophathy profiles of Omp117 and FepA are similar (data not shown). We are currently determining the function of OMP117 by analyzing disruption mutants.

**OmpA.** The OmpA protein, which is found in many spe-
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Figure 1. A 3-dimensional model of Bacteroides fragilis OmpA102-269 constructed by threading the sequence on a crystal structure of Escherichia coli OmpA21–191 (side and top views). Top view (left), side view (center), and side view with aromatic amino acids displayed as spheres (right). There are rings of aromatic amino acids at the proposed lipid/water interfaces.

cies, provides structural stability to the cell. The OmpA protein is composed of 2 domains: an N-terminal domain that forms an 8-stranded β-barrel and a C terminus that is linked to the peptidoglycan. OmpA mediates bacterial conjugation and functions as a receptor for various bacteriophages [16]. It is also required for the action of colicins K and L and stabilizes mating aggregates in conjugation. OmpA proteins (E. coli OmpA [17] and Pseudomonas aeruginosa protein F) were not originally thought to be porins [18–20]. However, Nikaido et al. [21] and Sugawara and Nikaido [22] showed that both proteins were, in fact, porins. Furthermore, E. coli OmpA was present in both closed and open forms; when liposomes with the closed and open forms of OmpA were separated, open-form liposomes had activity similar to classical E. coli porins. Current opinion is that the majority of the OmpA population is folded into an 8-stranded β-barrel that is essentially closed and that ~2% actually folds differently into a larger, many-stranded barrel that allows the permeation of organic molecules [1].

We identified and sequenced the gene for the OmpA protein of B. fragilis and analyzed the deduced protein sequence (H. M. Wexler, E. K. Read, and T. J. Tomzynski, unpublished data). Analysis of the B. fragilis ompA deduced amino acid sequence revealed striking homology at the C terminal with the OmpA signature domain of E. coli OmpA and Pseudomonas OprF; BLAST analysis of the conserved domain database, using reverse position-specific BLAST, was conducted [23] and placed the B. fragilis OmpA in the OmpA family. In addition, homology across the entire open-reading frame was seen with the 42- and 43-kDa antigens of P. gingivalis; little information is available on these proteins, aside from their nucleotide and amino acid sequences. In E. coli OmpA (GenBank accession number PO2934), the N-terminal transmembrane domain and the carboxy terminal periplasmic region are connected by a hinge region composed of an alanine-proline repeat preceded by a phenylalanine a few residues earlier. Pseudomonas OprF also has an alanine-proline repeat in approximately the same region, also preceded by a phenylalanine a few residues earlier. Although C terminals of OmpAs do not conform to the typical C-terminal patterns of many OMPs, the last membrane-spanning segment of OmpA [24] (just before the hinge region) does in fact conform to the typical C-terminal motif. Just before a potential hinge segment of B. fragilis OmpA, there is striking homology with the corresponding region (i.e., just before the hinge) in E. coli and Shigella OmpAs.

Hydropathicity profiles for B. fragilis Omp A were comparable to those for E. coli OmpA. Analysis of the amino acid sequence for the presence of β-strands was kindly done by Dr. T. Schirmer (University of Basel) [25]. This structural alignment indicates a strongly conserved structure of the N-terminal β-barrel region between B. fragilis OmpA and E. coli OmpA and Pseudomonas OprF. The structural homology of the β-barrel prediction to OmpA, coupled with the high degree of homology of the C-terminal OmpA domain signature region indicated above, provides compelling evidence that this protein is a homolog of the OmpA-like proteins in Enterobacteriaceae. A tentative model of B. fragilis OmpA is seen in figure 1. The proposed model still needs to be confirmed with experimental evidence as well as structural evidence by crystallization.

Most recently, using genomic analysis, we identified 3 more ompA genes in B. fragilis (E. K. Read, H. M. Wexler, and T. Tomzynski, unpublished data). We do not know whether or when these genes are expressed; however, the fact that the organism
has 4 gene homologs coding for very similar but not identical proteins would indicate that the OmpA protein(s) serve a very important function in the cell. The use of the multiple alignment of these sequences, rather than a single sequence, proved to be very helpful in predicting a secondary structure for these proteins mentioned above. The predicted strands are consistent with areas of conserved amino acid sequence, whereas the predicted loops have a less conserved sequence.

**Heme binding protein.** An iron-repressible 44-kDa OMP was isolated and the gene sequence determined. Analysis of the deduced amino acid sequence revealed signature heme-binding consensus motifs. The homologous protein in *E. coli* was found in the cytoplasmic but not outer membrane, whereas this protein was found in the outer membrane of *B. fragilis* [26].

**OMPs in B. thetaiotaomicron.** Salyers’s group identified a cluster of 8 starch-utilization (sus) genes in *B. thetaiotaomicron* (a member of the *B. fragilis* group). Three genes in the cluster (susA, susB, and susG) encode starch-degrading enzymes. SusR appears to be a regulatory protein that activates the promoters of the susA and the susB-G operon. SusG and SusA cleave starch into mono- and disaccharides, and SusB acts on the products of SusA and SusG. SusC, SusD, SusE, and SusF appear to be involved in binding starch to the cell surface (although the role of SusF is not yet clear) [27]. SusC appears to be a porin that allows uptake of maltodextrins, and its sequence conformed to the expected features in the C terminus of many OMPs, including a terminal phenylalanine residue and hydrophobic residues at positions −3, −5, −7, and −9 from the C terminus. Also, SusC had strong homology with other OMPs in the N-terminal region that are thought to be involved in stabilizing the protein in the outer membrane. While analyzing the *B. fragilis* Omp121 protein described earlier, we analyzed SusC by the same fold-recognition technique and found that the predicted model was the 22-stranded ligand-gated FepA porin of *E. coli*.

**OMPA-LIKE PROTEIN IN B. DISTASONIS**

In an earlier study, we also found that a nearly pure preparation of an OmpA-like protein in *B. distasonis* was also active in the liposome study [28]. The protein was heat-modifiable and tightly bound to the peptidoglycan layer. Radioiodination of surface molecules confirmed the external exposure of the protein. This protein did not react with anti-*E. coli* OmpA antisera. Outer-membrane preparations of the ATCC type strains of the *B. fragilis* group indicated that all had proteins of approximately the same size, with the heat-modifiable electrophoretic migration pattern characteristic of OmpA. These outer-membrane preparations were tested with antisera directed against *B. distasonis* OmpA; heat-modifiable proteins from *B. fragilis* and *Bacteroides uniformis* reacted specifically with this antiserum [29]. We have identified the *ompA* gene in *B. distasonis* and are currently sequencing the gene. Recent work in our group has indicated the presence of the *ompA* gene in *Bacteroides mardae* as well (unpublished data); *B. mardae* is closely related to *B. distasonis*.

**OMPS IN PORPHYROMONAS**

*P. gingivalis* is considered to be a major pathogen associated with advanced periodontal disease. *P. gingivalis* is frequently isolated in high numbers from highly inflamed subgingival lesions and is also isolated from healthy mouth tissue but in much lower numbers. Studies that have analyzed the IgG responses of patients with periodontal disease and matched healthy controls to outer-membrane preparations of *P. gingivalis* resulted in the identification of 3 immunodominant surface antigens (115, 55, and 47 kDa). Subsequently, analysis of RNA isolated from cells at 37°C, 39°C, and 41°C yielded several PCR fragments that appeared to represent temperature-regulated genes. The nucleotide sequence of one of these fragments was identified as part of the ragAB locus, which codes for both the 115- and 55-kDa immunodominant antigens (ragA and ragB, respectively). RagA is a 115-kDa OMP, homologous to the family of TonB-linked outer-membrane receptors [30]. RagB is a 55-kDa immunodominant antigen that is present in deep periodontal pockets and has been implicated in the destructive disease process of pathogenic strains of *P. gingivalis* [31]. RagA and RagB may act in concert at the surface of the bacterium to facilitate active transport, mediated through the periplasmic spanning protein, TonB, or form part of a signal transduction system. The ragAB operon has been shown to be temperature regulated, and the authors postulated that this operon, under temperature regulation, may influence the virulence potential of *P. gingivalis* strains that harbor this locus and may thus be considered a novel pathogenicity island [30]. The authors added that the temperature regulation may suggest a mechanism of evading the host response in the inflamed periodontal pocket [32].

Subsequent analysis of the 47-kDa antigen demonstrated that it is a hemagglutinin/adhesin component of a major arginine-specific protease, RgpA. Members of this family of gene products all have roles in proteolytic, adherence, or transport processes and are considered to be virulence determinants [31].

**OMPA-LIKE PROTEINS IN PORPHYROMONAS**

Recently, we found a pore-forming protein (MW 37 kDa) with the same heat-modifiable migration behavior as OmpA in *Porphyromonas asaccharolytica*, a gram-negative anaerobe [33]. Positively charged substances such as D-glucosamine diffused through this porin significantly faster than homologous un-
charged compounds; negatively charged D-glucuronic acid diffused significantly more slowly than glucose. Analysis of the B. fragilis OmpA molecule indicated strong homology with the 42- and 43-kDa antigens of P. gingivalis; little information is available on these proteins, aside from their nucleotide and amino acid sequences. We analyzed the amino acid sequences of these proteins by 3D-PSSM and found that they too have a predicted 8-stranded β-barrel structure. Very recently, a heterodimeric OMP (composed of 40- and 41-kDa proteins) was described in P. gingivalis [34] that had several similarities to OmpA-like proteins.

OMPI and OMPII protein fractions were isolated from the outer membranes of Porphyromonas endodontalis, which has been frequently isolated from peripapical abscesses and chronic root canal inflammations [35]. The OMPI fraction (mainly a 31-kDa protein with lesser amount of a heat-modifiable 40.3-kDa protein and other minor proteins) was shown to have pore-forming activity in a liposome assay. The apparent MW of the 40.3-kDa protein increased on incubation at 100°C.

**PORE-FORMING OMPS IN FUSOBACTERIUM**

A major heat-modifiable, pore-forming protein (FomA) was described in Fusobacterium nucleatum, a gram-negative anaerobic bacterium commonly isolated from oral and other infections [36, 37]. The deduced topology of FomA from the fomA gene sequence was very similar to those of other porins [38, 39]. The fomA gene was cloned and expressed in E. coli [40]. FomA was originally thought to share some sequence similarity with aerobic OmpA proteins [41]; the sequence similarity was later disproved. The deduced topology of FomA was very similar to those of other “classic” porins [38, 39]. Homology was seen on Southern blots with a probe for fomA and B. fragilis genomic DNA [42].

**ANALYSIS OF CLINICAL ISOLATES OF B. FRAGILIS FOR CHANGES IN OMPS**

Susceptibility patterns and β-lactamase activity for 3000 B. fragilis group isolates were analyzed from archival data. Strains that had been tested and found to have no discernible β-lactamase activity were analyzed further to note their patterns of cross-resistance. Strains were divided into 2 groups on the basis of susceptibility or resistance to ampicillin/sulbactam (a β-lactam–β-lactamase inhibitor combination that would not be sensitive to most β-lactamases), to further rule out any contribution of β-lactamase toward resistance. Ampicillin/sulbactam-resistant (or even moderately resistant [MIC, 8 μg/mL]) isolates were uniformly more resistant to other agents, including clindamycin (not a β-lactam agent) (data not shown). This resistance is not likely to be due to changes in PBPs, because that would not affect resistance to non–β-lactam agents. The presence of the clindamycin resistance gene in these strains is unlikely, because B. fragilis isolates with this gene have much higher MICs than those seen in these strains. In these strains, the cross-resistance appears to be due to a change in the organisms that affects multiple antibiotic classes. Certainly, it is also possible that more than one mechanism is responsible.

Strains identified in the present study as possible cross-resistant strains were studied further for changes in OMP profile.

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Figure 2. SDS-PAGE analysis of outer-membrane proteins (OMPs) of clinical strains of Bacteroides fragilis. A, Missing OmpA in strain 7503. B, Missing OMP200 bands in strain 8928. MICs of strains to ampicillin/sulbactam (2:1) are indicated. A, Lane 1, high-molecular-weight (HMW) standards; lanes 2 and 3, strain 7392 (A/S 2 μg/mL) at 25°C and 100°C; lanes 4 and 5, strain 7017 (A/S MIC 8 μg/mL) at 25°C and 100°C; lanes 6 and 7, strain 7503 (A/S MIC 8 μg/mL) at 25°C and 100°C; lanes 8 and 9, strain 7854 (A/S MIC 0.5 μg/mL) at 25°C and 100°C; and lane 10, low-molecular-weight (LMW) standards. Note the absence of OmpA bands in strain 7503. A/S, ampicillin/sulbactam. B, Lane 1, high-MW standards; lanes 2 and 3, strain 8541 (A/S MIC 0.5 μg/mL) at 100°C and 25°C; lanes 4 and 5, strain 8928 (A/S MIC 8 μg/mL) at 100°C and 25°C; lanes 6 and 7, strain 7503 (A/S MIC 1 μg/mL) at 100°C and 25°C; and lane 8, low-MW standards. Note the absence of Omp200 in strain 8928.
SDS-PAGE analysis of OMP profiles of several strains were analyzed as a first step in correlating outer membrane changes with resistance (figure 2). OmpA was missing from 1 resistant strain (7503, lanes 6 and 7) (ampicillin/sulbactam MIC, 8 μg/mL) (figure 2A). (Note: the band at the same approximate MW as OmpA [lane 7] does not have the typical migration pattern of OmpA—i.e., a lower apparent MW when solubilized at 25°C [no corresponding band in lane 6].) OmpAs were also identified by Western blot (data not shown). The MICs of ampicillin/sulbactam for strains 8541 (lanes 2 and 3) and 7854, 0.5 μg/mL. Another OMP analysis revealed the absence of Omp200 (indicated by the arrowhead) in a resistant strain (8928, lanes 4 and 5, ampicillin/sulbactam MIC, 8 μg/mL) (figure 2B). MICS of ampicillin/sulbactam for strains 8541 (lanes 2 and 3) and 7523 (lanes 6 and 7) were 0.5 and 1 μg/mL, respectively. Although analysis of the OMP profiles does reveal differences in strains of varying antimicrobial susceptibility, it is obvious from examining the complex gel patterns that it is impossible, at this point, to specifically correlate the presence or absence of a particular band with changes in susceptibility.

There is increasing evidence not only that OMPs are important in transport (including their involvement in antimicrobial resistance) but that they are critical components of the virulence mechanisms of the organism [43]. The profusion and location of cell surface porins lend them multiple functions. They may act as targets for adhesion and/or binding to cells or bactericidal compounds. The loop structure (which is the most variable) may be altered to avoid immune pressure. Antimicrobials may also regulate porin expression. Increased awareness of the multiplicity of the functions of the OMPs, coupled with our very limited knowledge of OMPs in anaerobic bacteria, underscores the need for studies to elucidate their structure and function.

References


