Characteristics of \textit{Bacteroides fragilis} lacking the major outer membrane protein, OmpA

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OmpA1 is the major outer membrane protein of the Gram-negative anaerobic pathogen \textit{Bacteroides fragilis}. We identified three additional conserved \textit{ompA} homologues (\textit{ompA2–ompA4}) and three less homologous \textit{ompA}-like genes (\textit{ompA}s 5, 6 and 7) in \textit{B. fragilis}. We constructed an \textit{ompA1} disruption mutant in \textit{B. fragilis} 638R (WAL67\textit{ompA1}) using insertion-mediated mutagenesis. WAL67\textit{ompA1} formed much smaller colonies and had smaller, rounder forms on Gram stain analysis than the parental strain or other unrelated disruption mutants. SDS-PAGE and Western blot analysis (with anti-OmpA1 IgY) of the OMP patterns of WAL67\textit{ompA1} grown in both high- and low-salt media did not reveal any other OmpA proteins even under osmotic stress. An \textit{ompA1} deletant (WAL186\textit{ompA1}) was constructed using a two-step double-crossover technique, and an \textit{ompA} ‘reinsertant’, WAL360 + \textit{ompA1}, was constructed by reinserting the \textit{ompA} gene into WAL186\textit{ompA1}. WAL186\textit{ompA1} was significantly more sensitive to exposure to SDS, high salt and oxygen than the parental (WAL108) or reinsertant (WAL360 + \textit{ompA1}) strain. No significant change was seen in MICs of a variety of antimicrobials for either WAL67\textit{ompA1} or WAL186\textit{ompA1} compared to WAL108. RT-PCR revealed that all of the \textit{ompA} genes are transcribed in the parental strain and in the disruption mutant, but, as expected, \textit{ompA1} is not transcribed in WAL186\textit{ompA1}. Unexpectedly, \textit{ompA4} is also not transcribed in WAL186\textit{ompA1}. A predicted structure indicated that among the four OmpA homologues, the barrel portion is more conserved than the loops, except for specific conserved patches on loop 1 and loop 3. The presence of multiple copies of such similar genes in one organism would suggest a critical role for this protein in \textit{B. fragilis}.

INTRODUCTION

\textit{Bacteroides fragilis} is a major component of the gastrointestinal flora and the most frequent anaerobic pathogen (Finegold & Wexler, 1996). Until the publication of the genome sequence in 2004–5 (Cerden˜o-Ta´rraga \textsuperscript{et al.} (Finegold & Wexler, 1996). Until the publication of the intestinal flora and the most frequent anaerobic pathogen

Abbreviations: Ct, cycle threshold; OM, outer membrane; OMP, outer membrane protein.

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composed of eight β-sheets and a peptidoglycan-linked periplasmic domain. OmpA has been implicated in maintaining cell structure, in biofilm formation, as having adhesin, invasin and evasin properties, as being a receptor for colicins and bacteriophages (Smith et al., 2007), in macrophage activation (Souslas et al., 2000; Wang & Kim, 2002; Prasadarao et al., 1996) and in virulence (Huang et al., 2000). Although OmpA1 is the major OMP of B. fragilis, its function in this genus has not been described. The purpose of this work was to study the function of the OmpA1 protein in B. fragilis by constructing and characterizing strains with defective or absent OmpA1 and comparing them with the parental strain.

PATIENTS AND METHODS

Growth of B. fragilis. The bacterial strains, plasmids and primers used are listed in Table 1. B. fragilis 638R is a clinical isolate and is the strain generally used in research laboratories for genetic manipulations. The published B. fragilis sequence is that of B. fragilis NCTC 9343 (Cerdeno-Tarraga et al., 2005 and supplementary data therein; Kuwahara et al., 2004). Sequencing data for B. fragilis 638R were produced by the Bacteroides fragilis Sequencing Group at the Sanger Institute and can be obtained from ftp://ftp.sanger.ac.uk/pub/pathogens/bf/BF638R.xls. B. fragilis 638R and pFD516 (a gift of Dr C. J. Smith, East Carolina University, Greenville, NC) were used for construction of the disruptant WAL67ompA. B. fragilis ADB77 (a thy-deficient mutant of B. fragilis 638R and named WAL108 in our laboratory) and the suicide plasmids pYT102 and pADB242b (used for construction of the deletant and reinsertant, respectively) were a kind gift from Dr Michael Malamy, Tufts University, Boston, MA. Strains were grown as described previously (Pumbwe et al., 2006) in brain heart infusion (BHI) broth supplemented with thymine (Baughn & Malamy, 2002) or in anaerobic minimal medium with 0.5% glucose (AMMgluc) (Baughn & Malamy, 2002). Thymine (50 μg ml⁻¹) was added for growth of thy⁻ strains (i.e. WAL108 and its derivatives).

Construction of WAL186 ompA1 (ompA1 deletion mutant). An in-frame deletion of ompA1 was constructed by a two-step double-crossover technique with pYT102 (Baughn & Malamy, 2002). Briefly, 800 bp fragments of the upstream and downstream regions (including ~50–100 bp of the beginning and end of ompA1) were amplified using specific primers, to which appropriate restriction sites were added for subsequent cloning into pYT102 (Table 1). pYT102 was digested with BamHI and HindIII and gel-purified. PCR amplions were digested with BamHI/HindIII and gel-purified. PCR amplions were blunt-end ligated into pYT102 in a three-part ligation, as described by Pumbwe et al. (2006).

Chemo-tropism resistant colonies were screened to confirm that they were Tet-sensitive, and further screened by PCR with sets of both internal and junction primers to confirm that they were the desired deletion resolution products. Deleted genes were verified by DNA sequencing of the deletion junction. The ompA1 deletant was named WAL186ompA1.

Construction of WAL360+ompA1 (ompA1 reinsertant). The full-length B. fragilis ompA1 gene (including about 800 bp upstream and downstream of the gene) was cloned in the suicide vector pADB242b. The recombinant plasmid was verified by DNA sequencing. E. coli DH5α/pADB242b::ompA1updown and E. coli DH5α/pRK231 were mated with B. fragilis WAL186ompA1, as described by Baughn & Malamy (2002), and the cointegrants were selected as described above. Cointegrants were plated on minimal media with thymine and trimethoprim to select for the second recombination event. Reinsertants containing full-length ompA1 were confirmed by sequencing and the ompA ‘reinsertant’ was named WAL360+ompA1.

Cloning and expression of B. fragilis ompA1 in E. coli, purification of OmpA1 from inclusion bodies, and production of anti-OmpA1 IgY. Recombinant OmpA1 was produced as described by Wexler et al. (2002b). Briefly, B. fragilis ompA1 was cloned into pET-27b (+) (Novagen). Purified plasmid DNA was used to transform BL21 (DE3) pLysS (Novagen) according to the manufacturer’s instructions. Cells were grown and inclusion bodies prepared according to manufacturer’s directions. Gel-purified OmpA1 was submitted to Aves Lab (Tigard, OR) for production of anti-OmpA1 IgY.

Cells from a 500 ml overnight culture were harvested by centrifugation at 6000 g for 20 min in a Sorvall RC-5B centrifuge, and washed once in 10 mM Tris/HCl containing 10 mM MgSO₄. Cells were broken by four passages through a French pressure cell (SLM Instruments) at 12 000 p.s.i. (82.8 MPa). The suspension was centrifuged at 6000 g for 5 min to remove whole cells and cell debris. The supernatant contained the cell envelopes and cytoplasm. The inner membrane was solubilized by adding 2% Triton X-100 containing 10 mM MgCl₂ and 10 mM HEPES (final concentration) to the supernatant and incubating for 30 min at room temperature, and then centrifuging at 45 000 g (1 h). The resulting pellet containing the crude OM was washed once with 10 mM Tris/HCl, 10 mM MgSO₄, pH 7.4, and then frozen.
Table 1. Strains, plasmids and primers used in this study

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SDS-PAGE and Western blot analysis of \textit{B. fragilis} OM preparations. OMs were prepared as described by Weder \textit{et al.} (2002b). SDS-PAGE was performed as described by Gallagher (1987) using a modified Laemmli gel (Laemmli & Favre, 1973). Samples were incubated in SDS sample buffer at either room temperature or 100 °C for 5 min. Molecular masses were based on molecular mass standards (Sigma). The gel was stained by a rapid technique and destained extensively with several changes of destain buffer (Wong \textit{et al.}, 2000). The Western blot was performed as described by Ausubel \textit{et al.} (1987) using a 1:5000 dilution of the anti-OmpA IgY.

RNA extraction. Total cellular RNA was isolated from strains cultured in BHI broth containing 5 % thymine using the RNeasy-RNA Protect (Qiagen) method with on-column DNase treatment. Strains were incubated for 2 h under anaerobic conditions to the mid-exponential phase of growth (OD600 0.4). Aliquots (3 ml) were mixed with an equal volume of RNA-ProTect and the extraction was continued according to the manufacturer’s instructions. A standard PCR confirmed that the RNA was free of chromosomal DNA contamination. The integrity of the extracted RNA was confirmed by agarose gel electrophoresis and by spectrophotometric analysis ($A_{260}/A_{300}$). Samples were quantified by $A_{260}$ measurement and the measurement was converted to ng μl$^{-1}$.

RT-PCR. DNA-free total RNA was isolated from \textit{B. fragilis} 638R using the RNeasy Mini kit and RNase-Free DNase Set (Qiagen) according to the manufacturer’s instructions. Primers pairs specific for each of the \textit{B. fragilis} ompA gene homologues (Table 1) and total \textit{B. fragilis} RNA (10 ng per reaction) were used in the OneStep RT-PCR kit (Qiagen) to amplify specific transcripts according to the manufacturer’s instructions. Amplification products were separated on a 1.5 % (w/v) agarose-Tris/acetate/EDTA (TAE) gel containing 5 g ethidium bromide ml$^{-1}$.

Quantification of gene expression by quantitative comparative real-time RT-PCR. Briefly, two-step real-time PCR was performed with the Cepheid SmartCycler amplification and detection instrument using the Quantitect SYBR Green one-step RT-PCR kit (Qiagen). Primers were designed to amplify products of 130–170 bp in size and were added to the reactions at a final concentration of 1.0 μM each. RNA samples were added to the reactions to result in 200 ng per reaction, except for the 16S RNA samples, which were added to a final amount of 200 pg per reaction. Expression levels were measured as an amount of cDNA as extrapolated by a cycle threshold (Ct) value from the standard real-time PCR growth curve. The Ct was the cycle number at which the growth curve attained exponential growth and was thus the highest concentration of template. In order to rule out any non-specific products resulting from primer-dimers, melting-curve analysis of the amplified products was performed. RNA expression was normalized to the parental strain by using 16S RNA. Expression results were quantified by the comparative cycle threshold approximation method (Stintzi \textit{et al.}, 2003), using the assumption that the PCR growth curve efficiency for all reactions was 100 % and that the DNA concentration doubled at each cycle: 
\[
\Delta Ct \text{ (fold-change in expression)} = 2(Ct_{\text{parental}} - Ct_{16S \text{ rRNA}}) - 2(Ct_{\text{deletant}} - Ct_{16S \text{ rRNA}})
\]

Data were analysed by Student’s $t$ test, and a value of $P<0.05$ was considered significant. A greater than twofold change in expression compared to the parental strain was considered significant.

SDS, acid and high-salt sensitivity assays. Challenge with SDS, acid and high salt was performed as described for \textit{E. coli} OmpA (Wang, 2002) using media and incubation conditions appropriate for \textit{B. fragilis}. Bacteria were grown in BHIS broth to OD600 0.6, diluted in 0.154 M NaCl (equivalent to physiological saline: 0.9 % NaCl) to $10^6$ c.f.u. ml$^{-1}$, and plated on BHIS agar containing various concentrations of SDS. Plates were incubated anaerobically for 48 h at 37 °C, and c.f.u. were counted. For acid survival, the exponential-phase bacteria were diluted 30-fold in PBS. A one-tenth volume of the suspension was mixed with BHIS containing acetic acid to a final pH of 3.8, and incubated at 37 °C for 20 min. Plates were incubated anaerobically for 48 h at 37 °C, and c.f.u. were counted. For the high-osmolarity challenge, a 1:30 bacterial suspension was mixed with an equal volume of either 0.154 or 5 M NaCl and incubated at room temperature for 2 h. Plates of varying dilutions were incubated anaerobically for 48 h at 37 °C, and c.f.u. were counted.

Oxygen sensitivity assay. The oxygen sensitivity of the ompA deletant was measured in an agar tube assay (Rocha \textit{et al.}, 2007). Strains were grown in BHIS/thy anaerobically at 37 °C. One hundred microlitres of overnight (stationary phase) culture was mixed with 5 ml BHIS/thy and 0.4 % agar in a clear polystyrene tube and incubated aerobically at 37 °C for 48 h. The distance between the top of the agar and the visible growth within the agar was measured.

Genomic and proteomic analyses. \textit{B. fragilis} OmpAs were aligned with \textit{B. fragilis} OmpA1 using the \textit{BLAST} 2 program (http://blast.ncbi.nlm.nih.gov) and E values were generated by the program (E values are a measure of the probability that the alignment is due to chance; lower E values indicate a greater significance of the alignment). The signal sequence cleavage site was predicted by SignalP V2.0. SignalP comprises two signal peptide prediction methods, SignalP-NN (based on neural networks) and SignalP-HMM (based on hidden Markov models) (Nielsen \textit{et al.}, 1997). The \textit{PSORT} algorithm was used to analyse a submitted sequence for signal sequences, cleavage sites, amino acid composition and potential transmembrane regions, and then to predict the subcellular location of the protein being analysed. \textit{CLUSTALW} 1.8 (http://www.ebi.ac.uk/clustalw) (Thompson \textit{et al.}, 1994) was used to generate the alignments.

β-Sheet prediction and model prediction. The prediction of β-sheets was kindly done by Dr Tilman Schirmer (University of Basel) according to his published method (Schirmer & Cowan, 1993). The multiple alignment was done with \textit{MUSCLE} (http://www.ebi.ac.uk/Tools/muscle/index.html) with output in a \textit{CLUSTAL} format. The alignment was threaded on the \textit{E. coli} OmpA β-barrel (1bxwa) structure; regions of conservation were analysed by surface-mapping of phylogenetic information using the program \textit{CONSURY} (http://consurf.tau.ac.il/index.html) (Landau \textit{et al.}, 2005; Glaser \textit{et al.}, 2003).
and visualized with POLYVIEW-3D (http://polyview.cchmc.org/polyview3d.html). Specific orientation of the molecule was done with Jmol within the POLYVIEW-3D program.

RESULTS

Identification of \textit{ompA} homologues in \textit{B. fragilis}

In studies completed before the publication of the \textit{B. fragilis} genome sequence, we identified the major OMP gene (\textit{ompA1}) in \textit{B. fragilis} (Wexler et al., 2002b) and subsequently identified three additional \textit{ompA} genes using a TBLASTN search against genomic data from the \textit{B. fragilis} sequence data (http://www.sanger.ac.uk/Projects/B_fragilis/). The amino acid sequences for OmpA1, OmpA2 and OmpA4 are identical for \textit{B. fragilis} strains ATCC 25285 and \textit{B. fragilis} 638R. There are two amino acid differences between \textit{B. fragilis} 25285 OmpA3 and \textit{B. fragilis} 638R OmpA3 (F19\textsuperscript{A}L, K225\textsuperscript{A}R). According to our model, F19 is in the leader sequence, before the cleavage site, and K225 is in the periplasmic portion, just after the last \(\beta\)-strand; thus, the barrel portion of the four OmpA homologues is completely conserved in these two strains. Subsequent analysis of the annotated \textit{B. fragilis} sequence revealed three additional \textit{ompA} family homologues (OmpAs 5, 6 and 7) that are somewhat removed from the OmpA1–4 cluster but do contain the OmpA signature domain at the C terminal (http://expasy.org/prosite/PDOC00819). OmpA5 is approximately the same length as OmpAs 1–4 (372–399 aa), OmpA6 has 224 aa and OmpA7 has 616 aa. OmpAs 1–7 correspond to \textit{B. fragilis} NCTC9343 (ATCC 25285) genes BF 3810, 1689, 1285, 1681, 1988, 1959 and 3801, respectively.

Conservation of \textit{B. fragilis} OmpAs and homology to OmpAs from other organisms

MUSCLE (http://www.ebi.ac.uk/Tools/muscle/index.html) (multiple sequence comparison by log-expectation) was used to generate a phylogram that includes \textit{B. fragilis} OmpAs 1–7, \textit{P. gingivalis} 42 kDa antigen (OmpA-like), \textit{Pseudomonas marginalis} OprF and \textit{E. coli} OmpA (Fig. 1). \textit{B. fragilis} OmpAs 1–4 exhibited considerable homology throughout the protein sequence (E values of OmpAs 2, 3 and 4 with respect to OmpA1 are \(2e^{-48}\), \(1e^{-46}\) and \(3e^{-41}\), respectively, with homology across the entire length of the protein, and particularly in the predicted \(\beta\)-strands). OmpAs 2 and 4 were the most homologous (identities 331/373 (88%), positives 351/373). OmpAs 5 and 6 showed less homology (E values \(7e^{-10}\) and \(9e^{-10}\), respectively, compared to OmpA1) and OmpA7 shared some homology in the \(\beta\)-strand region. All seven homologues shared the characteristic C-terminal OmpA domain (data not shown).

Characterization of WAL67\textit{ompA1}

Amplification and sequencing of the recombination junction verified the disruption of the \textit{ompA1} gene in WAL67\textit{ompA1}. The absence of the OmpA1 protein in WAL67\textit{ompA1} was confirmed by SDS-PAGE analysis of the OM and by Western blot analysis conducted with anti-OmpA1 IgY antisera (data not shown). WAL67\textit{ompA1} grew much more slowly than both \textit{B. fragilis} 638R and other unrelated \textit{omp} mutants constructed with the pFD516 suicide vector at the same time. After 48 h, only pinpoint colonies were seen, compared to robust 1–2 mm colonies for the other disruptants and the wild-type strain.

\textbf{Fig. 1.} Phylogenetic comparisons of \textit{B. fragilis} OmpAs with other members of the OmpA-domain family. The phylogram was based on an alignment using MUSCLE (http://www.ebi.ac.uk/Tools/muscle/index.html) and the tree drawn with Phylodendron (http://iubio.bio.indiana.edu/treeapp/treeprint-form.html). BFOmpA5, \textit{B. fragilis} OmpA5; ShortOmpA6, 224 aa OmpA6; Pgingivalis42kD, \textit{P. gingivalis} 42 kDa antigen; BFompA3, \textit{B. fragilis} OmpA3; BFompA1, \textit{B. fragilis} OmpA1; BFompA2, \textit{B. fragilis} OmpA2; BFompA4, \textit{B. fragilis} OmpA4; LongOmpA7, 616 aa OmpA7; OmpAEcoli, \textit{E. coli} OmpA; OprFPMarginalis, \textit{Pseudomonas marginalis} OprF.
Examination of Gram stains revealed that WAL67ompA1 cells were shorter and rounder than those of the other strains. The geometric mean cell lengths for BF638R and WAL67ompA1 were: 638 parental, 1.5 μm; WAL67ompA1, 0.79 μm; strains carrying disruptions in nanH or other unrelated omp genes did not show similar changes.

OmpA homologues are not induced in WAL67ompA1 under conditions of osmotic stress

Since OmpA and OprF are important for stabilizing cells in hypo-osmolar media in E. coli and Pseudomonas aeruginosa, respectively, we grew B. fragilis 638R and WAL67ompA1 under normal and low-salt conditions to see whether one of the other ompAs was expressed in the mutant under a stress condition. Cells were grown in salt-free medium with and without added salt (200 mM NaCl), and OMs were analysed by SDS-PAGE and Western blotting with anti-OmpA IgY antisera. No OmpA-like proteins were seen in the mutant, regardless of the salt concentration in the growth medium (data not shown). It is possible that they are expressed at such a low level that we could not detect them in SDS-PAGE and/or that the organism might compensate for the loss of OmpA1 by increasing the transcription of one of the other ompAs expressed in the mutant or that the organism might compensate for the loss of OmpA1 by increasing the transcription of one of the other OmpAs expressed in the mutant, regardless of the salt concentration in the growth medium (data not shown). It is possible that they are expressed at such a low level that we could not detect them in SDS-PAGE and/or that the anti-OmpA1 IgY antisera did not recognize them, despite the considerable homology in the β-strand region among the homologues (since the antisera were prepared using gel-purified OmpA1 as antigen, antibodies to all of the regions, including β-strands, would be expected to be present in a polyclonal antiserum).

Construction and characterization of WAL186ompA1

Sequence analysis confirmed the deletion of the ompA1 gene and SDS-PAGE analysis confirmed the lack of the OmpA1 protein in WAL186ompA1 (Fig. 2). No other OmpAs were detected in WAL186 or WAL186ompA1 using silver stain analysis (data not shown). We believe that although ompAs 2, 3 and 4 are transcribed in WAL108 and WAL186ompA1, OmpAs 2, 3 and 4 are not expressed at sufficient levels to be detected in the SDS-PAGE analysis of the OM preparation. Colonies of WAL186ompA1 were much smaller than those of WAL108 (parental) or WAL360 + ompA1; these results echoed those seen with WAL67ompA1.

Resistance of WAL108 and WAL186ompA1 to osmotic stress

WAL186ompA1 was more sensitive than WAL108 to exposure to both SDS and high salt. Exposure of WAL108 to 5 M NaCl for 2 h resulted in a three-log reduction in growth (1 × 10^6 to 5 × 10^3); WAL186ompA1 did not grow at all after exposure to high salt. Similarly, growth of WAL108 on media containing 0.05–0.2 % SDS resulted in a three-log reduction in growth as compared to growth on media without SDS (1 × 10^6 to 5.3, 5 and 4.5 × 10^5 on 0.05, 0.1 and 0.2 % SDS, respectively); WAL186ompA1 did not grow at all on media containing even 0.05 % SDS. No change in growth between WAL108 and WAL186ompA1 was seen after exposure to low pH.

Transcription of ompA homologues in B. fragilis 638R, WAL108, WAL67ompA1 and WAL186ompA1

Transcription levels of the ompA homologues in the B. fragilis constructs are shown in Table 2. The major transcribed homologue was ompA1, followed by ompA3, ompA2 and ompA4. Our studies with WAL67ompA1 had already indicated that B. fragilis OmpA1 is important in maintaining cell structure; therefore, we initially assumed that the organism might compensate for the loss of ompA1 by increasing the transcription of one of the other ompA homologues. However, we found that transcription of ompA4 was significantly reduced in WAL186ompA1, suggesting the presence of a positive regulatory mechanism to control ompA4 transcription that is dependent on ompA1. Interestingly, the same effect was not seen in WAL67ompA1. We speculated that the truncated ompA1 gene or gene product in the disruption mutant could fulfill the function of the full-length product in regulating ompA4 transcription.

Response of ompA transcription levels to high salt

Exposure of WAL108 and WAL186ompA1 to 200 mM NaCl significantly reduced transcription of all four ompA
homologues in WAL108, and of ompAs 2, 3 and 4 in WAL186ΔompA1 (Table 2). Gram stain analysis indicated similar morphology in WAL108 and WAL186ΔompA1 grown on standard media (somewhat pleomorphic Gram-negative rods). Gram stain analysis of the strains grown overnight with 200 mM NaCl revealed that both WAL108 and WAL186ΔompA1 assumed very small, round forms under these conditions.

B. fragilis WAL186ΔompA1 is more sensitive to oxygen than WAL108

WAL186ΔompA1 was more sensitive to oxygen stress than either WAL108 or WAL360 + ompA1 (the ompA reinsertant), indicating that the absence of the ompA1 gene, and not some downstream effect or other random mutation, was responsible for the change in sensitivity to oxygen (Fig. 3). The average measurements between the top of the agar and the visible growth within the agar were: B. fragilis 638R, 6.8 mm; WAL108, 9.2 mm; WAL186ΔompA1, 14 mm; WAL360 + ompA1, 9.8 mm. Incubation for an additional 24 h did not affect the results.

B. fragilis OmpA1 does not appear to be important for transport of antimicrobials into the cells

Susceptibility testing was performed for a wide variety of antimicrobials, including β-lactams (ampicillin, cefoperazone, cefoxitin, cephalixin, cefotizoxime), carbapenems (doripenem, ertapenem, faropenem, imipenem, meropenem), quinolones (ciprofloxacin, gatifloxacin, norfloxacin, levofloxacin, moxifloxacin), chloramphenicol, metronidazole, clindamycin, Erm and Tet. No significant change was seen in MICs between WAL108 and WAL186ΔompA1.

Nucleotide sequences and genetic organization of B. fragilis ompAs

Potential promoters upstream of the start codon for B. fragilis ompAs were identified based on the consensus promoter sequences (Bayley et al., 2000). ompA2 and ompA4, which share the most homology of the four genes, are separated by ~1000 bp, are in inverse orientation and may be the result of a duplication event. Both genes have very conserved upstream sequences that have a low level homology (E value 1e−5) to Vibrio cholerae otnG (involved in cell wall polysaccharide biosynthesis.) Also, pairwise BLASTN analysis of the upstream sequences revealed a highly conserved 200 bp region (E value 5e−50) upstream of the otnG-like sequences.

Amino acid sequences and signal peptide sequences of B. fragilis OmpAs

The homology of OmpAs 1–4 extends throughout the entire ORF with 30 to 34% identity and 49 to 50% similarity. OmpA2 and OmpA4 are the most homologous pair (84% identity and 89% similarity). The homology is

Table 2. Quantitative RT-PCR of ompAs 1–4

<table>
<thead>
<tr>
<th>Gene</th>
<th>Expression Ct for strain:</th>
<th>108</th>
<th>WAL186ΔompA1</th>
<th>WAL67ΔompA1</th>
<th>108 + 200 mM NaCl</th>
<th>186 + 200 mM NaCl</th>
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<tr>
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<tr>
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<tr>
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<td>37.59</td>
<td>31.89</td>
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<td>0</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of oxygen stress on B. fragilis. The agar tube assay measures the ability of B. fragilis to grow when exposed to oxygen; the most anaerobic environment is at the bottom of the tube, with increasing oxygen exposure toward the surface. The distance between the top of the agar and the visible growth within the agar was measured; a smaller distance indicates a greater ability to grow with some oxygen exposure. Left to right: WAL638R; WAL108, parental; WAL186ΔompA1 (deletant); WAL360 + ompA1 (reinsertant).
more marked in the C-terminal region, and all have significant and similar homology to the conserved domain database entry for the OmpA family. In both E. coli OmpA and Pseudomonas OprF, the N-terminal transmembrane domain and the C-terminal periplasmic region are connected by a hinge region (Chen et al., 1980) composed of an alanine-proline (A-P) repeat preceded by a phenylalanine a few residues earlier (Vogel & Jahnig, 1986; Woodruff & Hancock, 1989). While no A-P repeat was seen in B. fragilis OmpA, there is an arginine-proline-methionine-proline (RPMP) segment, preceded by two phenylalanines (two and six bases earlier). This segment may serve the same function as the OmpA hinge. When we examined the sequence just before the potential RPMP hinge in B. fragilis OmpAs 1–4, we found striking similarity to the corresponding regions in E. coli and Shigella OmpAs (Fig. 4).

The B. fragilis OmpA1–4 sequences have a ‘terminal’ phenylalanine as the last amino acid of the last β-sheet of the β-barrel, which is considered essential in many OMPs (Struyve et al., 1991) and is consistent with OmpAs from other species. In addition, homologues of OmpAs 1–4 have hydrophobic amino acids at positions –3, –5, –7 and –9 relative to the terminal phenylalanine, and this is also characteristic of porin proteins. The corresponding region in Pseudomonas is different, although completely conserved in four different species. Alignment of this region of B. fragilis OmpA1–4 with the last β-sheet of E. coli OmpA was helpful in constructing the alignments used in the structural predictions for B. fragilis OmpA. The C-terminal 17–27 aa of the B. fragilis OmpA homologues do not align with the OmpA domain consensus and may reflect the phylogenetic distance of B. fragilis from the constituents that define the OmpA family.

Signal sequences and cleavage sites for each of the homologues predicted by both SignalP and PSORT were in agreement. OmpA1, OmpA2 and OmpA4 have the typical A-X-A sequence preceding the cleavage site (von Heijne, 1985). Other residues were found, often in conjunction with an alanine, as seen with OmpA3 (V-F-A). Also, the bulky aromatic residue at the X position (i.e. phenylalanine) that is often present (von Heijne, 1983) is seen in OmpA3. In addition, the PSORT algorithm predicted, with varying degrees of certainty, an OM location for each of the homologues. Interestingly, the algorithm also confirmed, at a lower level of certainty, a periplasmic location for the B. fragilis, as well as the E. coli, OmpAs, presumably a reflection of the periplasmic OmpA domain.

**Proposed secondary structure of B. fragilis OmpAs**

B. fragilis OmpAs 1–4 were submitted for 3D-PSSM fold recognition analysis [http://www.sbg.bio.ic.ac.uk/3dpssm/index2.html; a method using 1D and 3D sequence profiles coupled with secondary structure and solvation potential information (Kelley et al., 2000)]; the predicted structures of all four homologues were transmembrane β-barrels. Dr Tilman Schirmer (University of Basel) kindly analysed these proteins for the presence of β-sheets (Schirmer & Cowan, 1993). Using the multiple alignment of these sequences, rather than a single sequence, proved to be very helpful in predicting secondary structure for these proteins. In the putative β-barrel portion of the alignment, the algorithm predicted eight β-sheets in regions where amino acid conservation was the highest. The areas of greatest homology were those of the predicted eight β-sheets. A schematic diagram of B. fragilis using the amino acid sequence from B. fragilis OmpA1 is shown in Fig. 4. Interestingly, gaps in the alignment of the β-barrel domain fell within loops predicted to be exposed on the outer surface of the bacterium, possibly reflecting different biological functions or simple genetic drift.

**Fig. 4.** Schematic diagram of the proposed OmpA transmembrane fold. A 2D representation of the eight-strand β-barrel of B. fragilis OmpA1 spanning the OM based on a CLUSTALW alignment of the sequences of the four B. fragilis OmpAs, E. coli OmpA, and P. aeruginosa OprF. The left panel shows the ‘hinge region’ in the four B. fragilis OmpAs, E. coli OmpA, P. aeruginosa OprF and several other OmpA homologs. The N-terminal residue after signal cleavage is Q20 and the C-terminal residue remains K394. β-Strands are labelled and are represented by arrows with the first and last predicted amino acid indicated. Loops are indicated by arcs between adjacent β-barrels. Elongated circles represent predicted z-helices. The OmpA domain is located in the periplasm and starts at position V293.
Proposed model of *B. fragilis* OmpA

Analysis of the predicted 3D structures based on these sequences was accomplished by threading an alignment made from the four *B. fragilis* OmpA sequences, *E. coli* OmpA and *Pseudomonas* OprF onto the *E. coli* crystal structure (Fig. 5). The barrel structure was coloured according to amino acid conservation. The barrel portion was more conserved than the loops, especially in the ‘lower’ half facing the periplasm. Both loop 1 and loop 3 had conserved patches (not visible in the figure).

DISCUSSION

Publication of the *B. fragilis* genome sequence in 2004 has facilitated studies of *B. fragilis* OMPs (Cerden˜o-Ta´rraga et al., 2005; Kuwahara et al., 2004). The *B. fragilis* genome is relatively large (~5.3 × 10^6 vs 4.2 × 10^6 bp for *E. coli* K12) and there are multiple homologues of many genes, particular membrane protein genes. Significant work has been accomplished on the genes involved in capsule formation, and their regulation and importance in immune modulation and host colonization (Comstock & Kasper, 2006; Cassel et al., 2008; Coyne et al., 2008; Liu et al., 2008). Most of the phenotypic and functional description of other *B. fragilis* OMPs was completed at least a decade before the sequence became available, and it is difficult or impossible to identify the proteins described with their respective genes. Odou and colleagues described a 45 kDa porin protein in *B. fragilis* (Odou et al., 1998), and later described a complex form of this protein that migrates at ~210 kDa when electrophoresed before boiling (Odou et al., 2001). Kanazawa and colleagues isolated three proteins (51, 92 and 125 kDa) with porin activity (Kanazawa et al., 1995). Unfortunately, neither of these two groups identified the proteins by either genetic or proteomic methods.

![Fig. 5. Conservation of amino acids in the *B. fragilis* OmpA barrel. *B. fragilis* OmpA alignments are threaded onto the *E. coli* OmpA crystal structure using CONSURF and visualized with POLYVIEW-3D. Upper panel, outside surface of barrel (front and back); lower panel, slab view indicating inside surface of barrel. Conservation is colour-keyed as indicated (royal blue/turquoise = least conserved to red/maroon = most conserved).](image)
We reported the identification, gene sequence and characterization of \emph{B. fragilis} \textit{omp200}, a porin gene (Wexler \textit{et al.}, 2002a), and \emph{B. fragilis} \textit{ompA} (Wexler \textit{et al.}, 2002b) in 2002.

\section*{OmpAs in other organisms}

The role of OmpA as a porin molecule that would allow passage of nutrients and/or antibiotics into the cell has been hotly debated (Smith \textit{et al.}, 2007; Nakamura & Mizushima, 1976; Nikaido & Vaara, 1985; Gotoh \textit{et al.}, 1989; Yoshihara & Nakae, 1989). Nikaido proposed that the different channel sizes seen could be explained by different conformations assumed by both OmpA and OprF (Brinkman \textit{et al.}, 2000; Sugawara & Nikaido, 1994). Recent understanding is that both the full-length and the N-terminal domains of OmpA (and OprF) can form pores of varying sizes (Arora \textit{et al.}, 2000; Brinkman \textit{et al.}, 2000; Sugawara & Nikaido, 1994; Zakharian & Reusch, 2003, 2005) and that the larger conformation can, in fact, function as a porin (Smith \textit{et al.}, 2007; Sugawara & Nikaido, 1994; Nikaido \textit{et al.}, 1991). Thus, for \textit{Pseudomonas}, in which OprF is the major porin, the low permeability of the main conformation of OprF accounts for the low permeability of the OM, and decreased expression of OprF has been implicated in antimicrobial resistance (Pumbwe & Piddock, 2000). In \emph{E. coli}, however, there are other ‘classical’ trimeric porins, OmpF and OmpC, that allow passage of solutes and are implicated in antimicrobial resistance (Cohen \textit{et al.}, 1988). Therefore, in \emph{E. coli}, the low permeability of the majority of the OmpA molecules does not affect the permeability as significantly (Nikaido, 2001). Other examples of OmpA homologues can be found that are (Zhang \textit{et al.}, 2008) or are not (Bratu \textit{et al.}, 2008) implicated in antimicrobial resistance; whether the OmpA serves as the major porin for the cell may be a factor (McGowan, 2006).

We found that \emph{B. fragilis} constructs lacking OmpA1 were smaller in size and less resistant to both SDS and high salt than the parental strain. Similarly, \textit{P. aeruginosa} OprF is important in maintaining the structural integrity of the cell and is required for growth at low osmolarity (Woodruff & Hancock, 1989); truncation of the C-terminal domain results in altered cellular morphology (Rawling \textit{et al.}, 1998). In \emph{E. coli} also, OmpA is implicated in withstanding stresses due to SDS, an acidic environment and high osmolarity (Wang, 2002). Besides functioning in bacterial conjugation, and as a phage and colicin receptor (Morona \textit{et al.}, 1984), \emph{E. coli} OmpA is implicated in the invasion of brain microvascular endothelial cells (Huang \textit{et al.}, 2000) and in activating human macrophages (Soulas \textit{et al.}, 2000; Wang & Kim, 2002; Prasadaraar \textit{et al.}, 1996). The OmpA homologues in \emph{P. gingivalis} are being evaluated as possible prophylactic agents for \emph{P. gingivalis}-associated periodontitis (Veith \textit{et al.}, 2001). The role(s) of \emph{B. fragilis} OmpA homologues in these processes have not yet been investigated.

\section*{Multiple copies of \textit{ompA} genes in other organisms}

\emph{E. coli} OmpA and \textit{Pseudomonas} OprF are the best studied of the OmpA homologues. The \emph{E. coli} K12 MG1655 genome indicates only one full-length OmpA protein, with multiple shorter membrane proteins and lipoproteins that have the OmpA-like consensus domain, which is important in attachment to the peptidoglycan layer (Ullstrom \textit{et al.}, 1991; Rawling \textit{et al.}, 1998). A search of the \textit{Pseudomonas} PAO1 genome with the OprF sequence indicated that there was also only one full-length OprF-like protein, while there were multiple shorter proteins (150——270) that contained the OmpA-like consensus domain. In contrast, two OmpA homologues are found in \textit{Aeromonas salmonicida} and in \textit{Haemophilus ducreyi} (Klesney-Tait \textit{et al.}, 1997; Costello \textit{et al.}, 1996).

Among anaerobes, \textit{ompA} homologues have been studied in two \textit{Porphyromonas} species, \emph{P. gingivalis} (Yoshimura \textit{et al.}, 2009; Iwami \textit{et al.}, 2007; Nagano \textit{et al.}, 2005; Imai \textit{et al.}, 2005; Murakami \textit{et al.}, 2002) and \textit{Porphyromonas asaccharolytica} (Magalashvili \textit{et al.}, 2007). In \textit{P. gingivalis}, two OmpA homologues have been described. Studies in \textit{P. gingivalis} have used different strains (ATCC 33277, W83 and W50) and the OmpA homologue ‘pairs’ are variably named: Pgm6/7 (Nagano \textit{et al.}, 2005), 42 and 43 kDa immunoreactive antigens (Nelson \textit{et al.}, 2003) and Omp40/41 (Veith \textit{et al.}, 2001), respectively. The lack of uniform nomenclature somewhat confuses comparisons among them. \textit{P. gingivalis} mutants lacking the OmpA homologues Pgm6/7 have less stable membranes than wild-type cells, as evidenced by the wavy and irregular OM structures seen by transmission electron microscopy, and more vesicles are released from cells (Iwami \textit{et al.}, 2007). In \textit{P. asaccharolytica}, an OmpA homologue (Omp-PA) with pore-forming ability has been isolated from the OM. Further characterization has revealed that this porin consists of two different fractions: a heat-modifiable fraction, which in its denatured form migrates on SDS-PAGE as a protein with a molecular mass of 41 kDa, and a heat-resistant fraction, which does not change its migration on SDS-PAGE after boiling. A liposome swelling assay reveals that only the heat-resistant fraction is able to transport sugars after its incorporation into the liposomes, although it does not discriminate between differently sized sugars. The authors hypothesize that the heat-modifiable fraction corresponds to the ‘closed’ conformer of Omp-PA, whereas the heat-resistant fraction corresponds to the ‘open’ conformer of the protein (Magalashvili \textit{et al.}, 2007).

Both Pgm6 and 7 (encoded by genes PG 0695 and 0694) and Omp40/41 are contiguous genes and form single operons. In contrast to the genetic arrangements of the \textit{ompA} homologues in \textit{Porphyromonas}, the \textit{ompA} homologues in \textit{B. fragilis} are not adjacent and do not constitute a single operon. \textit{B. fragilis} OmpA2 and OmpA4, the most similar \textit{ompA} homologues, are divergently transcribed and separated by ~1000 bp. Heterodimers have been observed.
with Omp40/41 in *P. gingivalis* W50 (Veith *et al.*, 2001), and more recently, heterotrimeric with Pgm6 and Pgm7 in *P. gingivalis* ATCC 33277 (Nagano *et al.*, 2005). Our earlier analyses with purified *B. fragilis* OmpA showed that arabinose could pass through the OmpA1 pore in a liposome assay; also, black lipid bilayer experiments indicated that *B. fragilis* OmpA forms channels of multiple sizes of 0.1–0.3, 0.6 and 0.9 ns (which would be consistent with monomer, dimer and trimer forms). However, we did not find any changes in MICs to any antimicrobials tested, supporting our assertion that *B. fragilis* OmpA1 does not act as a major porin for the organism.

Further investigations of the function of OmpA1 in *B. fragilis* and of the functions of the four surface-exposed loops are under way. Microarray data (unpublished data) indicate that transcription of many *B. fragilis* OMPs is affected by the deletion of *ompA1*; thus, the assignment of a function to a single protein might prove difficult. To date, we are not aware of any other bacterium that has four conserved *ompA* homologues (and an additional *ompA* that is somewhat less conserved). Thus far, we are unable to find stress conditions that increase transcription of the other *ompA* homologues, and their function in the bacterium remains unclear.

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