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Functional Expression in *Escherichia coli* and Membrane Topology of Porin HopE, a Member of a Large Family of Conserved Proteins in *Helicobacter pylori*

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HopE is one of the smallest members of a family of 31 outer membrane proteins in *Helicobacter pylori* and has been shown to function as a porin. In this study it was cloned into *Escherichia coli* where it was expressed in the outer membrane, as confirmed by indirect immunofluorescence using HopE-specific antibodies. HopE purified from *E. coli* reconstituted channels in planar bilayer membranes that were the same size as those formed by HopE purified from *H. pylori*. A model of the membrane topology of HopE was constructed and indicated that this protein formed a β-barrel with 16 transmembrane amphipathic β-strands. The accuracy of this model was tested by linker insertion mutagenesis, assuming that, like other porins, amino acid insertions were not tolerated in the transmembrane β-strands but were tolerated in the adjoining loop regions. Generally, the results obtained with a series of 12 insertions of the sequence RSKDV and two substitutions were consistent with the topological model. The preponderance of amino acids that were conserved in the extended family of HopE paralogs were predicted to be within the membrane and comprised 45% of all residues in the membrane.

Helicobacter pylori is a curved gram-negative bacterium that has been implicated as a major cause of chronic gastritis, peptic and duodenal ulcers, and gastric carcinoma. Its genome has been sequenced from two separate isolates revealing substantative conservation of gene sequence (2, 21). One of the most striking features of these genomes is a large (32-member) family of sequence-related outer membrane proteins. This family of proteins was discovered in the pregenomic era as a series of five outer membrane proteins named HopA to HopE, which had similar N-terminal sequences, and all reconstituted channels in planar bilayer membranes (8, 9). HopE (8) was the smallest of these proteins (31,000 Da) but formed the largest channels with a single channel conductance of 1.5 nS in 1 M KCl. For this reason it was proposed to be the major nonspecific porin of the *H. pylori* outer membrane, although it had a considerably lower abundance in the outer membrane than, for example, the major porin of *Escherichia coli* (OmpF).

HopE is also one of the smallest members of the conserved family of outer membrane proteins (2, 21; R. A. Alm, J. Bina, B. M. Andrews, P. Doig, R. E. W. Hancock, and T. J. Trust, submitted for publication). Although HopA to HopE were identified on the basis of their similar N-terminal sequences (8, 9), they have extensive blocks of C-terminal sequence conservation and in fact only 21 members of the family have the N-terminal Hop motif, and 3 of these are probably not expressed due to slipped strand regulation caused by multiple CT repeats. Even these 21 proteins can be somewhat subdivided, with 11 including HopA and HopD (10; Alm et al., submitted) and the two adhesins BabA and BabB (16) being very highly conserved and having a C terminus comprising FAY (one-letter amino acid code), whereas the remaining 9, including HopB, -C, and -E, have an F residue at the C terminus, like most other β-barrel porin proteins. The 11 remaining members of the large outer membrane family do not have the typical N-terminal motif but do contain the C-terminal conserved motifs (ending in F) and have been called the Hor (Hop-related) family (Alm et al., submitted).

Overall, these 32 Hop and Hor family proteins vary substantially in size from 165 to 1,217 residues, but all contain ca. 135 to 150 conserved residues. Thus, it is of some interest to determine why these conserved residues exist. One possibility would be that the conserved sequences are required to promote homologous recombination as a mechanism for creating genomic rearrangements (21). We have previously argued against this possibility (10). Another possibility is that these sequences represent a conserved structural motif. Thus, we tested this second hypothesis here by mapping the membrane topology of HopE.

HopE, like other porins, is predicted to be a β-barrel structure like, e.g., the *E. coli* OmpF porin. The crystal structure of OmpF (and other bacterial porins) has been determined, and it was observed that it contains 16 β-strands with the general motif of alternating hydrophobic and hydrophilic residues. Interestingly, Tomb et al. (21) observed that the entire Hop and Hor families contained such alternating residues in their conserved sequences. Despite these conserved motifs, there is in fact little sequence conservation between bacterial species. Although within a species, greater conservation exists (e.g., OmpF, OmpC, and PhoE in *E. coli* are 80% identical), such minifamilies tend to be very similar in size (cf. the Hop and Hor family proteins). Also, the least-conserved regions within a species tend to be the surface loop regions (possibly due to antigenic selection) that interconnect each pair of β-strands. This latter property has been exploited to map the membrane topology of porins (1, 3), since the surface loops can tolerate the insertion or deletion of additional amino acid residues, whereas insertions into the β-strands prevent correct synthesis and secretion to the outer membrane of the mutant porin. The validity of this method has been proven by comparison to the crystal structure of *E. coli* porin PhoE (1, 4). Therefore, we

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chose this method for examining the membrane topology of HopE.

MATERIALS AND METHODS

Bacterial strains and plasmids. *E. coli* JM105 [F′ *traD36 lacIQ* Δ(*lacZ*)M15 *proA*$^+$*B*$^+$] *thi rpsL endA sbcB15 hsdR-4*(r_K^- m_K^+) Δ(*lac-proAB*) was obtained from New England Biolabs, Inc. (Mississauga, Ontario, Canada) and used as the host for transformations and also for the background strain for the expression of plasmids encoding the modified HopE proteins unless otherwise stated. The plasmid pBluescript II KS(+) was obtained from PDI Bioscience (Aurora, Ontario, Canada). *H. pylori* 22695 (21) was obtained from the TIGR Institute of Research (Rockville, Md.).

Development of plasmid pJ1. The HopE gene was amplified from *H. pylori* 22695 by using *Taq* DNA polymerase. The upstream primer 5′-AAG GAT CCG ATA GGA ATG TAA AGG AAT GG-3′ containing a *Bam*HI site and the downstream primer 5′-CCG AAT TCT AAA GGC ATG AAC GCT TGC A-3′ containing a *Eco*RI site were constructed by using a Perkin-Elmer Applied Biosystems, Inc. (ABI; Mississauga, Ontario, Canada) DNA synthesizer model 332. The resulting PCR fragment was blunt-end cloned into the *Eco*RV site in pBluescript II KS(+) in the same orientation as the *lac* promoter to give plasmid pJ1. All enzymes were purchased from Life Technologies-Gibco BRL. A MJ Research Minicycler (Boston, Mass.) was used for all PCR reactions. Plasmid pJ2 was derived in a similar fashion, assuming that the second Met codon at position +10 in the coding sequence was the start of the HopE gene.

Linker insertion mutagenesis. PCR primers were designed to insert the in-frame codons for five amino acids (RSKDV) and two unique restriction enzyme sites into the *hopE* gene, using pJ1 as the template DNA. The PCR amplification was performed with *Taq* DNA polymerase using a touchdown amplification procedure as follows. The PCR thermocycler was programmed for an initial denaturation step of 96°C for 4 min, followed by 18 cycles at an initial annealing temperature of 65°C (for 90 s), which was decreased by 0.5°C for each successive cycle, an extension step at 72°C for 6 min, and denaturation at 96°C for 1 min. Subsequent to completion of the first 18 cycles, an additional 14 amplification cycles were performed by using 72°C extension and 96°C denaturation steps with a constant 55°C annealing temperature. The resulting amplicon was extracted with phenol and chloroform, precipitated with ethanol, and made blunt by digestion with the Klenow fragment of DNA polymerase. The PCR products were digested with *Dpn*I restriction enzyme to remove the template DNA, religated, and transformed into *E. coli* JM105. Recombinant clones were identified by using oligonucleotide primer 5′-AGA TCT AAG GAC GTC-3′ plus the reverse sequencing primer in PCR amplification reactions. Identified clones were sequenced to verify that the inserted amino acids were in frame and that no errors had been introduced into the *hopE* gene.

Isolation of outer membrane proteins. *H. pylori* was grown at 37°C in an atmosphere of 10% CO_2 on chocolate agar plates (Prepared Media Laboratories, Richmond, British Columbia, Canada) overlaid with brain heart infusion broth (Accumedia, Baltimore, Md.). After an incubation period of 4 days cells were harvested from 20 plates and resuspended in 20% sucrose with 50 mg of DNase I (Boehringer Mannheim) in 10 mM Tris-HCl (pH 8.0; ICN, Aurora, Ohio). The cells were disrupted with a French pressure cell at 15,000 lb/in^2. Broken cells were overlaid on a sucrose step gradient of 1 ml of 70% and 6 ml of 70% sucrose (Fisher Scientific, Fair Lawn, N.J.) in 10 mM Tris-HCl (pH 8.0). The outer membrane fraction was collected and pelleted at 150,000 × *g*, and the pellet was resuspended in 100 μl of distilled water. *E. coli* JM105 transformants harboring the specified plasmids were selected on Luria-Bertani (Difco Laboratories, Detroit, Mich.) solid medium containing 0.4% glucose (wt/vol) and 100 μg of ampicillin (Sigma, St. Louis, Mo.) per ml. Twofold-concentrated YT medium (20) (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.) was used for liquid cultures. Ampicillin was used at a concentration of 100 μg/μl for *E. coli*. After the cells were grown to the logarithmic phase, IPTG (isopropyl-β-D-thiogalactopyranoside; Chemica Alta, Ltd., Edmonton, Alberta, Canada) was added at a final concentration of 0.1 mM, and the cell cultures were allowed to grow another 4 h before they were harvested and resuspended in 20% sucrose with 50 mg of DNase in 10 mM Tris-HCl (pH 8.0). The outer membrane fraction was isolated as described above and pelleted at 150,000 × *g*, and the pellet was resuspended in 50 μl of distilled water. The protein concentration was determined using the BCA Protein Assay (Pierce, Rockford, Ill.).

HopE purification. Outer membranes from 500 ml of log-phase culture were solubilized in 10 mM Tris-HCl (pH 8.0; Fisher Scientific)–3% *n*-octyl-polyoxyethylene (Bachem) incubated at 23°C for 1 h and centrifuged for 30 min at 173,000 × *g*. The pellet was resuspended in 10 mM Tris-HCl–3% *n*-octyl-polyoxyethylene–5 mM EDTA (pH 8.0) (Fisher Scientific), incubated at 23°C for 1 h, and centrifuged for 30 min at 173,000 × *g*, and the supernatant was collected. A Western immunoblot indicated the presence of HopE in the supernatant of the second solubilization step. The supernatant containing HopE was mixed with an equal volume of 0.125 M Tris-HCl (pH 6.8), 4% (wt/vol) sodium dodecyl sulfate (SDS), and 20% (vol/vol) glycerol (Fisher Scientific) and subjected to SDS–12% polyacrylamide gel electrophoresis (PAGE). The HopE band was excised from an unstained portion of the gel and eluted overnight at 4°C into 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), and 100 mM NaCl. The elution supernatant was run on an SDS-PAGE gel to check for purity, and a Western immunoblot was done to ensure that it was indeed HopE.

Planar lipid bilayer experiments. The basic methods used here have been reported previously (9). Membranes were made from 1.5% oxidized cholesterol in *n*-decane. Bilayers were painted across a 2-mm^2 hole in a Teflon divider separating two compartments containing 5 to 6 ml each of a bathing solution of 1 M KCl. Voltages were applied across this membrane through Calomel electrodes connected by a salt bridge, and the resultant current was boosted 10^9- to 10^{10}-fold by a current amplifier, monitored on a Tektronix model 7633 oscilloscope, and recorded on a Rikadenki R-01 strip chart recorder.

SDS-PAGE and Western blotting. Isolated outer membranes were loaded at a concentration of 15 μg/lane. Electrophoresis was carried out by SDS-PAGE on a discontinuous 12% polyacrylamide gel (11). Proteins were stained with Coomassie brilliant blue. For Western immunoblotting, unstained gels were electroblotted onto Immobilon-P membranes (Millipore, Bedford, Mass.). After blocking for 2 h at 23°C with 3% bovine serum albumin (BSA; Boehringer Mannheim)–0.1% Tween 20 (Sigma) in phosphate-buffered saline (PBS), the membranes were incubated with a 1/10,000 dilution of anti-HopE rabbit antiserum (a gift from Peter Doig, Astra Zenecca, Boston, Mass.; the antiserum was raised against denatured HopE) in 1% BSA–0.05% Tween 20 in PBS for 1 h at 37°C. The membranes were then washed with PBS and incubated with a 1/5,000 dilution of an alkaline phosphatase-conjugated secondary antibody (Bio-Rad, Richmond, Calif.) for 1 h at 37°C. The bound antibodies were detected with 5-bromo-4-chloro-3-indolylphosphate (Calbiochem, La Jolla, Calif.) and nitroblue tetrazolium (Sigma).

Indirect immunofluorescence. Detection of surface-exposed proteins and epitopes was accomplished by the method of Hofstra et al. (12). For *E. coli*, aliquots of cells (100 μl) after 4 h of IPTG induction were pelleted, washed with PBS, and incubated with a 1/100 dilution of the primary antibody in 1% BSA in PBS for 1 h at 23°C. For *H. pylori*, three to four colonies were taken directly from a solid agar plate and resuspended in PBS, the cells were then pelleted, washed with PBS, and incubated with a 1/100 dilution of the primary antibody in 1% BSA in PBS for 1 h at 23°C. Cells were then washed with PBS and incubated with a 1/2,000 dilution of a fluorescein isothiocyanate-conjugated secondary antibody (Boehringer Mannheim) for 1 h at 23°C. Cells were then washed with PBS, resuspended in 1% BSA-PBS, and dried on poly-L-lysine-coated slides purchased from Sigma. Fluorescence was monitored with a Zeiss microscope fitted with a halogen lamp, and filters were set for emission at 525 nm. All images were captured using the ELIPSE software (Carl Zeiss Canada, Don Mills, Ontario, Canada).

DNA sequencing. Plasmid DNA was sequenced with the ABI automated fluorescent sequencing system model 373. Sequencing reactions were performed using the ABI sequencing kit. PCR protocols provided by ABI were done on the MJ Research Minicycler (Boston, Mass.). Template DNA was prepared with QIAwell 8 Plasmid Kits (Qiagen, Mississauga, Ontario, Canada). Primers were synthesized on the ABI DNA/RNA Model 392 synthesizer.

RESULTS AND DISCUSSION

Cloning of HopE. There are two possible translational start sites for HopE. These comprise methionine codons three residues apart in the sequence MEFMKKF. It was felt that the second methionine was the most likely start site since the inclusion of a basic residue (E [glutamate]) anywhere in the signal sequence of any secreted protein is rare, although not unprecedented. Therefore, we cloned HopE, by PCR amplification from *H. pylori* genomic DNA, into the plasmid pT7-7, assuming that either the first (in plasmid pJ1) or second (in plasmid pJ2) methionine was the start codon when an exogenous Shine-Dalgarno sequence was supplied. Contrary to our expectations, only amplification from the first methionine led to the production of a heat-modifiable outer membrane protein that cross-reacted with antibody raised against denatured HopE protein (Fig. 1A, cf. lanes 3 and 4 and lanes 5 and 6). Plasmid pJ1 encoded this version of HopE (with its endogenous Shine-Dalgarno sequence) cloned into pBluescript II KS(+). This construct was transformed into *E. coli* JM105 and induced with 0.1 mM IPTG for 4 h. Outer membranes were isolated by sucrose density gradient centrifugation and shown to contain an outer membrane protein with an apparent molecular weight of 31,000 that comigrated with authentic HopE on SDS-PAGE and reacted with antibody to HopE (Fig. 1).

In *H. pylori* outer membranes, HopE is heat modifiable (Fig. 1A, lanes 7 and 8), a result that indicates a potential SDS-stable β-barrel structure like other porins (4, 5, 17, 18). In *E.*

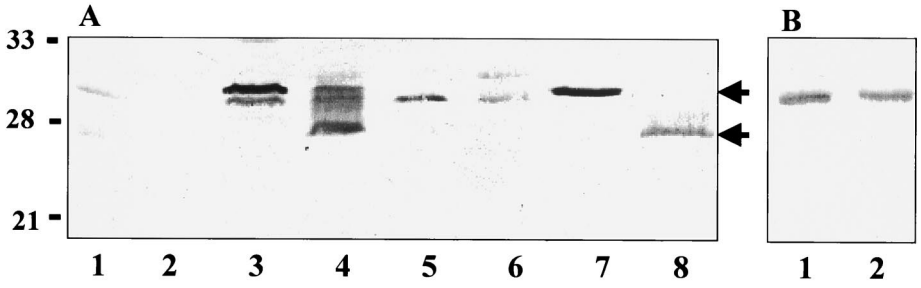

FIG. 1. (A) Western immunoblot probed with anti-HopE antibodies of outer membranes of *E. coli* JM105 clones solubilized at 100°C (heated) or 23°C (unheated). Lanes 1 and 2, JM105/pBluescript heated and unheated; lanes 3 and 4, JM105/pJ1 heated and unheated; lanes 5 and 6, JM105/pJ2 heated and unheated; lanes 7 and 8, *H. pylori* heated and unheated. Approximately 20 μg of total protein per lane was loaded. The anti-HopE antibodies were raised against denatured HopE and thus reacted more strongly to heated (denatured) HopE rather than unheated HopE in which some linear epitopes were presumably buried. (B) SDS-PAGE demonstrating the purity of HopE isolated from *E. coli* JM105/pJ1. Lane 1, solubilized at 100°C (heated); lane 2, solubilized at 23°C (unheated).

coli, however, HopE was only partially heat modifiable, with some of the protein being partly denatured at low temperature in SDS (Fig. 1A, lane 4). This could be due to the different outer membrane environment in *E. coli* (13, 14). Consistent with this observation, as HopE was purified free of LPS, it ceased to be heat modifiable (Fig. 1B). In addition, we observed a minor band of lower mobility after heating in SDS (Fig. 1A, lane 3). This band was not apparently heat modifiable (Fig. 1A, lane 4) and comigrated with the minor product observed when HopE without the first three amino acids of the signal sequence was cloned (Fig. 1A, lanes 5 and 6). We are uncertain as to what this minor band is, since although it did react with HopE antibody (and was not present in *E. coli* containing just the vector plasmid), it did not comigrate with authentic HopE (Fig. 1A, lanes 7 and 8). This could therefore represent a different processing product of HopE.

HopE was purified to apparent homogeneity (Fig. 1B, lanes 1 and 2), but it was not apparently heat modifiable. However, this does not mean that it failed to form a β-barrel structure but possibly that this β-barrel was more susceptible to SDS denaturation. Consistent with this idea, some insertion and deletion mutants of OprF (19) and OprD (13) lose their heat modification on SDS-PAGE but still form folded structures, as indicated by conformation-specific monoclonal antibodies and their ability to form channels in planar bilayers, respectively. Similarly, purified HopE was able to form channels in planar bilayer, with an average single channel conductance of 1.5 nS (Fig. 2) identical to the value obtained with HopE purified from *H. pylori* (8).

To confirm that HopE was expressed at the cell surface, we employed indirect immunofluorescence techniques to demonstrate that intact *E. coli* cells containing the plasmid pJ1 expressed HopE on their surface. The immunostaining of cells was somewhat patchy, implying that HopE might not be distributed in a random fashion in the outer membrane. However, there was no apparent concentration of HopE at the poles or septa of the cells observed.

Construction of a membrane topology model. The signal sequence cleavage site for HopE is known due to its known N-terminal sequence EGDGVYIGTNY (8). The mature HopE amino acid sequence in both sequenced genomes, strain J99 (2) and 26695 (21), was very similar, with only 6 amino acids of 250 being different. We utilized the method of Jeanteur and Pattus (14, 17) to predict the transmembrane β-strands of mature HopE (Fig. 3). This method utilizes a window of five amino acids to predict amphipathic regions of the protein comprising alternating hydrophobic and hydrophilic amino acids. In the crystallized porins, the alternation of hydrophobic and hydrophilic residues is incomplete (i.e., sometimes a polar residue appears in place of a hydrophobic residues and vice versa), in part because porins often form trimeric structures. Thus, the surfaces of the monomers that contact each other do not necessarily need to be hydrophobic like the surfaces that contact the membrane interior. Also, at least one of the surface loop regions interconnecting transmembrane β-strands (usually loop 3) inserts into the center of the barrel, and hydrophobic residues pointing into the aqueous center of the β-barrel pore can interact with these residues to form hydrophobic contacts. Thus, the averaging over a window of five residues accommodates such inconsistencies.

The model predicted by this method after refinement by experimental studies (see below) is shown in Fig. 3. It indicates a β-barrel of 16 strands with very short periplasmic turns and some long surface loop regions. This is conceptually similar to the structure of the four crystallized 16-stranded β-barrel porins, including *E. coli* OmpF (4). However, there is no sequence identity between HopE and OmpF. The HopE model places fewer amino acids at the surface, as anticipated given that it has only 250 amino acids versus 340 amino acids for OmpF. This smaller size of HopE may also explain why there are

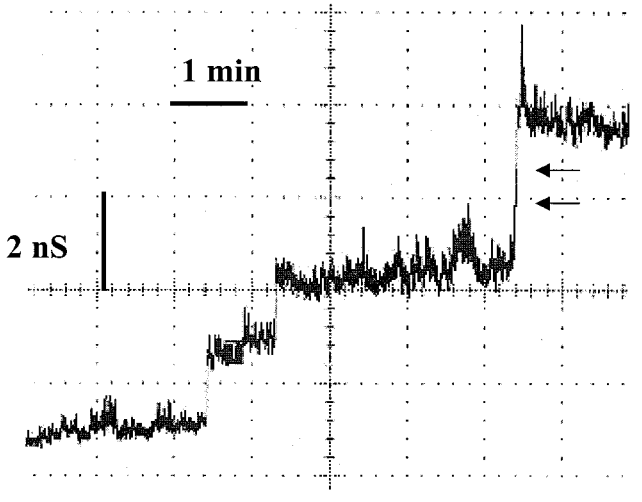

FIG. 2. Conductance trace observed after the addition of 3 ng of native HopE per ml to the aqueous phase (1 M KCl) bathing a planar lipid bilayer constituted from 1.5% oxidized cholesterol in *n*-decane. The applied voltage was 50 mV. The arrows indicate the breakpoints for three channels that entered the membrane rapidly.

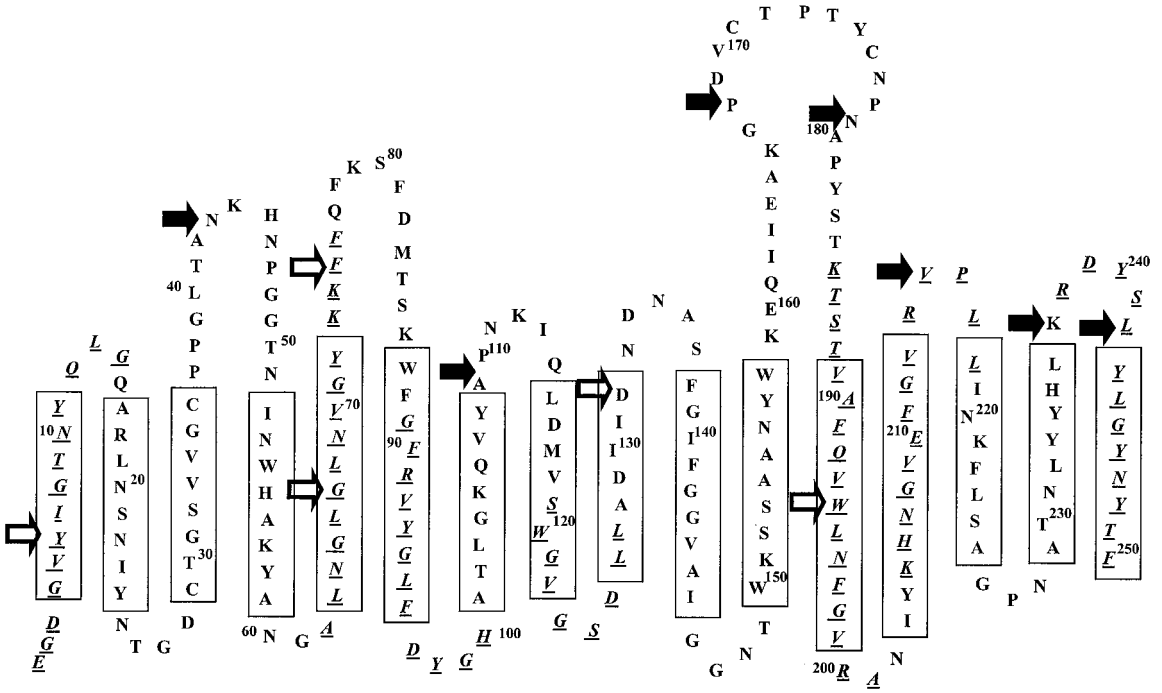

FIG. 3. Membrane topology model of HopE. Amino acids that are highly conserved throughout the Hop and Hor families of outer membrane proteins are shown in italics and underlined. Tolerated insertions are indicated by the filled arrows, and nontolerated insertions are indicated by the open arrows.

predicted to be only three surface loops longer than 6 amino acids versus seven in OmpF. One of these predicted loops, loop 6, is noteworthy since it is proposed to contain 30 amino acids including, in its middle, two cysteines bounding four amino acids. Loop 6 contains 4 of the 10 proline residues of HopE, with all of the remaining proline residues being found in loop or turn regions, as expected given that proline residues tend to distort β-strand structures.

The closest relative of HopE is a protein named HorB (Alm et al., submitted), which lacks the conserved N-terminal sequence observed for the Hop family proteins. Overall, HorB has only 29% identical amino acids. Nevertheless, it was comforting that virtually all of the stretches of misalignment (i.e., deletions and/or insertions and regions of lower similarity) could be assigned to the extramembranous regions as was also observed for the OmpF family of porins by Jeanteur et al. (17). Interestingly, the predicted HorB, which is even smaller than HopE, is maximally different from HopE in the loop 6 region, lacking all of the prolines and the cysteines of HopE and missing 17 of the 31 residues between W_{150} and Y_{182}.

Testing of the membrane topology model. Alignment of the sequenced porins has demonstrated that the β-strand regions and turns tend to be quite conserved in length, whereas the surface loops are quite variable in length (17). Consistent with this idea, insertions and deletions into the surface loop regions of individual porins are usually permissible (1, 3, 17), with the exception that some insertions into loop 3 (which folds into the interior of the β-barrel in the crystallized porins) disrupt the secretion and/or stability of the protein (1, 14). On the other hand, insertions or deletions in the β-strands and/or turn regions of porins always perturb the secretion and/or stability of the porin (1, 3, 14). Therefore, we set about testing the membrane topology model by inserting, using PCR methodology, the in-frame sequence RSKDV. The inclusion of four consecutive polar residues, three of which are charged, into HopE β-strands would be expected to disrupt this protein. A total of 12 insertions were made, with a further 2 insertions being made in which an accompanying deletion was constructed. The position of these insertions in the mature HopE sequence and their predicted locations are indicated in Table 1 and Fig. 3. The expression of HopE and its modification by heat was observed on Western immunoblots of whole-cell and outer membrane preparations (Fig. 4), and the surface expression was determined by indirect immunofluorescence with HopE-specific antibody. We considered that indirect immunofluorescence with intact cells was the best indicator of the correct localization of the HopE mutants in the *E. coli* outer membrane. Heat modification on SDS-PAGE is a signature property of many porins, but the loop 5 deletion mutant of porin OprD, for example, is more susceptible to denaturation in SDS (and thus does not demonstrate heat modification) (13) despite clear evidence that this mutant forms a native β-barrel structure (15). Consistent with this, mutant pJ18 in predicted loop 2 and mutant pJ20 in predicted loop 8 demonstrated an apparent surface location (Table 1) but were not apparently heat modifiable (Fig. 4).

Some of the mutants that failed to demonstrate surface expression, or demonstrated very weak surface expression (e.g., pJ18, pJ11, and pJ23), demonstrated very good expression on Western blots of whole-cell protein preparations. We assume that these mutants influenced secretion to some extent, and thus the corresponding mutant proteins were trapped in inclusion bodies. It was not, however, the general localization of the insertion in these proteins at the N terminus that prevented expression, since pJ30 encoding a HopE mutant with an insertion at amino acid 42 was well expressed on the surface of *E. coli*.

Overall, these data fit very well with the membrane topology model shown in Fig. 3. Insertions in predicted loop 2 (mutant pJ30), loop 4 (pJ31), loop 6 (pJ21 and pJ32), loop 7 (pJ34), and

TABLE 1. Characterization of insertional mutants in HopE

Mutant[a]	Insertional site (mature sequence)	HopE expression on Western immunoblots[b]	HopE surface expression[c]	Heat modifiability[d]	Predicted location[e]
PJ1	None	+++	+++	++	
PJ18	6	++	−	−	TM1
PJ30	42	+++	+++	−	L2
PJ11	66	+++	+	−	TM5
PJ23	74	+++	−	−	L3
PJ31	109	+++	+++	++	L4
PJ5	132	+	+	−	TM9
PJ32	168	+++	+++	++	L6
PJ21	179	+++	+++	++	L6
PJ29	Δ168–179	+++	++	++	L6
PJ17	194	+	−	−	TM12
PJ34	215	+++	+++	++	L7
PJ6	237	++	+++	++	L8
PJ20	242	+++	+++	−	L8
PJ14	Δ237–242	++	+	−	L8

[a] The following additional changes were observed from whole-gene sequencing: in PJ5 at amino acid 33, a GTA→GTG exchange leading to a neutral substitution (V33V), in PJ29 an S79T substitution, and in PJ30 an L244S substitution. The first of these was a neutral substitution, and the latter two obviously did not affect expression.
[b] HopE expression on Western immunoblots utilized whole-cell proteins.
[c] HopE surface expression was determined by indirect immunofluorescence of intact cells with HopE-specific antibodies.
[d] Heat modifiability was examined by SDS-PAGE as demonstrated in Fig. 1A.
[e] L, loop; TM, transmembrane β-strand.

loop 8 (pJ6 and pJ20) were surface expressed and thus permissive, as was the deletion of 12 amino acids from loop 6 and the insertion of 5 amino acids. In contrast, insertions in predicted transmembrane domains 1, 5, 9, and 12 were either not expressed or were very poorly surface expressed. Two results that needed clarification were those for mutants pJ23 and pJ14. The former, pJ23, was in predicted loop 3 and was neither heat modifiable nor surface expressed. A similar result was observed by us previously for deletions in the predicted loop 3 of *Pseudomonas aeruginosa* porin OprD (14) and by others for selected insertions in loop 3 (1), and we presume that the folding of loop 3 into the center of the porin β-barrel is important in the biogenesis of porins. The second construction, pJ14, involved a deletion that spanned two permissive insertion sites at amino acids 237 and 242, replacing these six residues with the inserted amino acids RSKDV. Presumably, the removal of this entire loop 8 perturbed the biogenesis of HopE, and the inserted amino acids did not repair the defect.

The loop 2 insertion mutant pJ30 was examined in more detail to confirm that it still formed a native β-barrel structure despite its inability to be modified by heat. Therefore, this mutant HopE was purified and examined for its ability to form channels in planar lipid bilayers. As seen in Fig. 5, mutant pJ30 was clearly able to form channels, but the single channel conductance in 1 M KCl was reduced to 0.63 nS compared to 1.5 nS for native HopE. Possibly, loop 2 in HopE can influence the channel properties of this porin, as previously shown for loop 2 of the *P. aeruginosa* imipenem-specific porin OprD (16). Conversely, since loop 2 in the crystallized porins reaches across to adjacent monomers and presumably stabilizes the trimer structure found in most porins, it is possible that we were analyzing the monomer for mutant pJ30 and the trimer for native HopE, which would make these proteins rather similar in trimer single-channel conductance.

As described above, HopE is a member of a large family of proteins with more than 100 conserved amino acid positions. Examining the model (Fig. 3) in more detail, it is evident that a preponderance of these conserved amino acids are predicted to be within the membrane (45% of all residues in the membrane) as opposed to outside the membrane (27% of residues). Possibly, the 30 residues not assigned to β-strands included other elements important to the construction of a β-barrel,

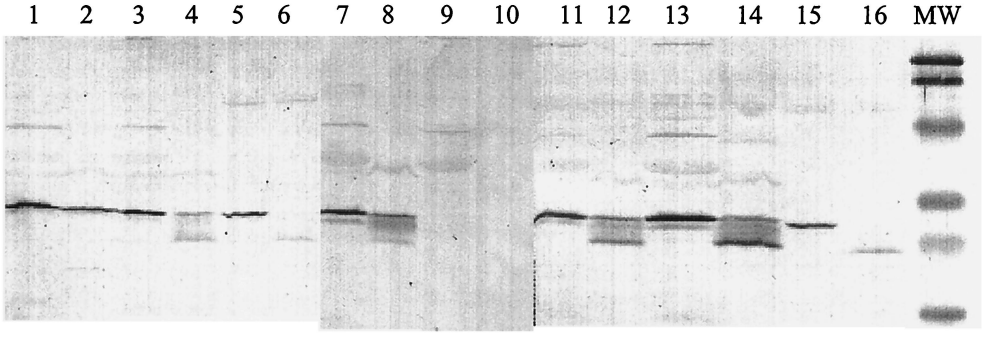

FIG. 4. Western immunoblot probed with anti-HopE antibodies of sucrose gradients of whole-cell proteins of *E. coli* JM105 clones or *H. pylori* solubilized at 100°C (odd-numbered lanes) or 23°C (even-numbered lanes). Lanes 1 and 2, JM105/pJ20; lanes 3 and 4, JM105/pJ21; lanes 5 and 6, *H. pylori* OM; lanes 7 and 8, JM105/pJ34; lanes 9 and 10, JM105/pBluescript; lanes 11 and 12, JM105/pJ31; lanes 13 and 14, JM105/pJ32; lanes 15 and 16, are *H. pylori*. Approximately 15 μg of total protein was loaded per lane.

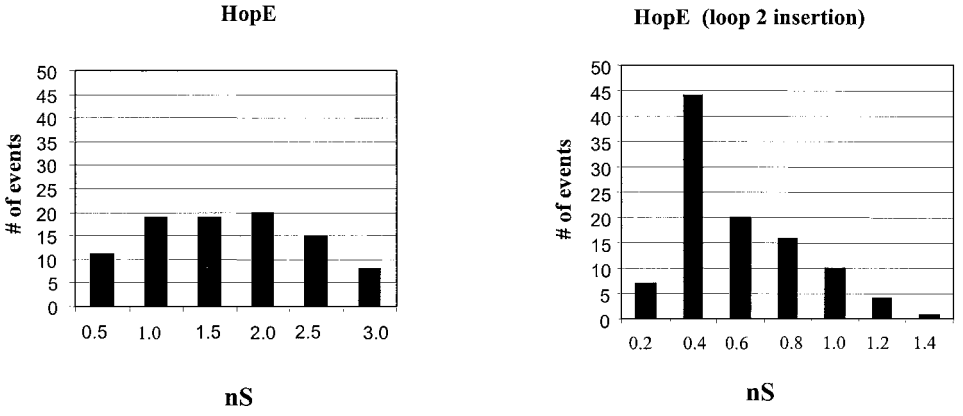

FIG. 5. Histogram showing single-channel conductance measurements in 1 M KCl under the conditions described in Fig. 3 for native HopE and HopE with the loop 2 insertion.

including β-turns at the periplasmic side (a predicted 10 residues) and parts of loop 3 (another 4 residues). Thus, the model shown in Fig. 3 is consistent with the hypothesis that the highly conserved residues of large Hop-Hor family of porins are part of a conserved scaffold for a β-barrel. We have done amphipathicity profiles on all 32 proteins related to HopE (data not shown). These proteins share 40 to 60% identity in the conserved regions of the proteins. Precedent would suggest that highly conserved regions of proteins have similar structures and/or functions, and our amphipathicity profiles are consistent with this idea. Presumably, the additional sequences (100 to 1,000 amino acids) provide the unique functions for this family of porins, such as the ability to act as adhesins (6, 7, 16).

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