First publ. in: Journal of Biological Chemistry 272 (1997), 21, pp. 13614-13621

Porins of *Haemophilus influenzae* Type b Mutated in Loop 3 and in Loop 4*

(Received for publication, December 27, 1996, and in revised form, March 23, 1997)

Ramakrishnan Srikumar‡, David Dahan‡, Francis F. Arhin§, Paul Tawa, Kay Diederichs¶, and James W. Coulton‖

From the Department of Microbiology and Immunology, McGill University Montreal, Québec H3A 2B4, Canada and ¶Fakultät für Biologie, Universität Konstanz, Universitätsstrasse 10, D-78434 Konstanz, Germany

Porin (341 amino acids; mass of 37,782 Da) in the outer membrane of *Haemophilus influenzae* type b (Hib) permits diffusion into the periplasm of small solutes up to a molecular mass of 1400 Da. Molecular modeling of Hib porin identified its structural similarities to OmpF of *Escherichia coli* and disclosed for Hib porin a shorter length of loop 3 and a longer length of loop 4. By site-directed mutagenesis of the porin gene *ompP2*, mutant porins were constructed to contain 6 or 12 amino acid deletions either in loop 3 or in surface-exposed loop 4. Wild type Hib porin and mutant porins were expressed in a nontypeable *H. influenzae* strain deleted for the *ompP2* gene. The mutant porins were purified and reconstituted into planar bilayers, tested for channel formation and compared with wild type Hib porin. Mutant *Haemophilus* porin possessing a 6-amino acid deletion in loop 3 displayed a broad distribution of single channel conductance values, while deletion of 12 amino acids from the same loop destabilized the porin channel. By comparison, deletion of 6 or of 12 amino acids from loop 4 of Hib porin resulted in an increased single channel conductance (1.15 and 1.05 nanosiemens, respectively) compared with wild type Hib porin (0.85 nanosiemens). The C3 epitope of the poliovirus VP1 capsid protein was inserted either into loop 3 or into loop 4 of Hib porin. By flow cytometry, the C3 epitope was detected as surface-exposed in strains expressing C3 insertion in loop 4; in strains expressing C3 insertion in loop 3, the epitope was inaccessible. We propose that loop 4 of Hib porin, although surface-accessible, is oriented toward the central axis of the pore and that deletions in this loop increase the single channel conductance by widening the pore entrance.

The outer membrane of Gram-negative bacteria forms a selective permeability barrier to substances that are present in their environment. Solutes such as sugars, amino acids, nucleosides, and small antibiotics diffuse across the outer membrane, whereas substances such as proteins, detergents, and large antibiotics do not readily gain access to the periplasm. Porins are trimeric proteins located in the outer membrane and are largely responsible for the molecular sieve properties of this bilayer. They form water-filled channels, which allow the diffusion of hydrophilic molecules into the periplasm; large antibiotics are excluded from this compartment (1, 2). The maximum size of a solute molecule that can permeate the pores defines a value termed the molecular mass exclusion limit. Solutes lower in molecular mass than this value are considered to diffuse through porins into the periplasm; solutes whose molecular mass exceed the value of the exclusion limit are apparently impeded in their passage. The variety of porins and their exclusion limits differ from one bacterial genus to another (3).

Hib[1] is an encapsulated Gram-negative bacterium that until recently was the leading cause of meningitis in infants under 18 months. The most abundant protein in the outer membrane of Hib is porin, encoded by the *ompP2* gene. Sequencing of the *ompP2* gene (4, 5) revealed an open reading frame for a signal sequence of 20 amino acids followed by 341 amino acids of the mature protein. Whereas the outer membrane of *Escherichia coli* contains at least three proteins (OmpF, OmpC, and PhoE) that are diffusion channels (6), only one of the six major outer membrane proteins from Hib apparently has channel activity. Hib porin has a molecular mass exclusion limit of 1400 Da (7), considerably larger than the value of 600 Da (6) for the pore formed by OmpF of *E. coli*.

Significant advances in our understanding of porin functions derive from the crystal structures of *Rhodobacter capsulatus* porin (8), *Rhodopseudomonas blastica* porin (9), and *E. coli* OmpF, PhoE (10), and LamB (11) porins. From these analyses at atomic resolution, the folding pattern of bacterial porins was demonstrated to be 16 (or 18, in the case of specific porins) anti-parallel β strands that traverse the outer membrane and loops that connect the β strands on both sides of the membrane. The whole structure forms a β barrel. The connecting loops on the extracellular surface are longer than the turns on the periplasmic surface. In all five porin structures, loop 3 folds back into the channel and produces a constriction of the channel. This structural feature is considered to determine pore sizes and thus the molecular mass exclusion limits of porins.

Based on parameters of hydrophilicity and amphiphilicity, we exploited computer-assisted algorithms to generate a model for the secondary structure of Hib porin (12). Even though the amino acid sequences of porin from nontypeable *Haemophilus*

* This work was supported in part by Grant MT-6911 (to J. W. C.) from the Medical Research Council, Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Recipient of a fellowship from the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche, Québec, Canada.

§ Recipient of financial support from "For the Love of Kids Foundation," Montreal, Canada.

‖ Portions of this work were conducted during a sabbatical leave at the Institut für Biophysik, Universität Freiburg (sabbatical supported by the Bilateral Exchange Program: Natural Sciences and Engineering Research Council (Canada)-Deutsche Forschungsgemeinschaft and the Alexander von Humboldt-Stiftung, Bonn). To whom correspondence should be addressed: Dept. of Microbiology and Immunology, McGill University, 3775 University St., Montreal, Québec H3A 2B4, Canada. Tel.: 514-398-3929; Fax: 514-398-7052; e-mail: jwcoulton@microimm.mcgill.ca.

[1] The abbreviations used are: Hib, *H. influenzae* type b; mAb, monoclonal antibody; PBS, phosphate-buffered saline; Hi, *H. influenzae*; PAGE, polyacrylamide gel electrophoresis; nS, nanosiemen(s).

did not show extensive homology to any known porins (3), our model of Hib porin is in agreement with an emerging consensus for the channel-forming motif of porins. We predicted 16 antiparallel β strands that traverse the outer membrane and eight long loops that connect the β strands on one side of the membrane. Experiments that used a panel of nine mAbs against Hib porin (13, 14) supported our computer-assisted predictions of secondary structure and allowed for orientation of the Hib porin model; the eight long, connecting loops were assigned to the extracellular surface. These studies also identified two surface-exposed regions in Hib porin, amino acids 162–172 and amino acids 318–325, which were assigned to the fourth loop (loop 4) and to the eighth loop (loop 8) in our secondary structure model. Two regions between amino acids 112–126 and 148–153 were buried or inaccessible at the surface of the outer membrane. We proposed (12) that residues 112–126 contribute to the third loop (loop 3) of Hib porin and that the inaccessibility of these residues at the cell surface was due to loop 3's forming a constriction inside the pore. This paper describes experimental strategies for isolation of an *Haemophilus influenzae* strain that is deleted for the *ompP2* gene and the construction of mutant Hib porins by site-directed mutagenesis. Furthermore, we propose a homology-based model of Hib porin that provides the structural framework for our discussion of the channel-forming activity of these mutant Hib porins.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Media

The strains and plasmids used in this study are listed in Table I. Nontypeable *H. influenzae* (Hi) strain DB117 is a recombination-deficient derivative from parent Hi strain KW20. *Haemophilus* strains were grown routinely on chocolate agar plates (36 g·liter^{-1} GC base (Difco), 10 g·liter^{-1} hemoglobin, and 20 ml·liter^{-1} Vitox supplements (Oxoid)) containing 150 μg·ml^{-1} of bacitracin. Liquid cultures of *Haemophilus* strains were propagated in brain heart infusion (Oxoid) broth supplemented with hemin (10 μg·ml^{-1}) and NAD$^+$ (10 μg·ml^{-1}); this medium is designated supplemented brain heart infusion. Media for *E. coli* strains have been described (15). Antibiotic concentrations used for selection of chromosomal or plasmid markers after transformation of *Haemophilus* and *E. coli* were 20 μg·ml^{-1} of kanamycin and 10 μg·ml^{-1} of tetracycline. For selection of plasmid-encoded resistance in *E. coli*, ampicillin concentration was 100 μg·ml^{-1}.

Molecular Biological Techniques

Restriction endonuclease digestions, ligations, and DNA manipulations were performed as described by Sambrook *et al.* (15). *E. coli* strain DH5α or Hi strain DB117 were used as hosts for large scale isolation of plasmid DNA using the plasmid maxi kit from QIAGEN. DNA was extracted from agarose gels using the GeneClean kit (Bio 101). To transform *E. coli*, cells were made competent with calcium chloride (15). *Haemophilus* strains were made competent for DNA uptake using calcium chloride; alternatively, *Haemophilus* strains were induced to be naturally competent (16).

Construction of a Nontypeable Hi Strain Deleted for Its Porin Gene

The entire Hib porin gene (*ompP2*) together with upstream sequences (1.1 kb at the 5′-end of the gene) and downstream sequences (0.37 kb at the 3′-end of the gene) are contained in the shuttle vector pEJH39-1-35 as an *Eco*RI-*Pst*I fragment. This plasmid was linearized with *Pvu*II and then subjected to partial digestion with *Mlu*I. The digestion products were electrophoresed on an agarose gel; the 11.6-kb *Pvu*II-*Mlu*I DNA fragment was isolated. A double-stranded DNA adaptor with *Pvu*II and *Mlu*I ends and containing an internal *Sal*I site (underlined) was constructed by annealing two single-stranded oligonucleotides: 5′-CTG GTC GAC A-3′ and 5′-CG CGT GTC GAC CAG-3′. Ligation of the adaptor to the 11.6-kb DNA fragment therefore introduced a novel *Sal*I site. The ligation mixture was used to transform Hi strain DB117; selection was for tetracycline resistance. Plasmid DNA was isolated from transformants and was designated pRS02. Plasmid pRS02 was deleted for 98% of the coding sequences for Hib porin, and it retained sequences flanking the excised porin gene. To overcome *Sal*I site modification in Hi strain DB117, pRS02 was used to transform *E. coli* strain DH5α and plasmid DNA was re-isolated. *E. coli*-passaged pRS02 was digested with *Sal*I and ligated to a 1.1-kb *Sal*I-restricted DNA fragment containing a kanamycin resistance gene derived from transposon *Tn*903. The ligation mixture was used to transform *E. coli* strain DH5α with selection for both tetracycline and kanamycin resistances. Plasmid pRS21 harbors the kanamycin resistance cassette with its own promoter in the same orientation as the Hib *ompP2* promoter. Plasmid pRS21, digested to completion with *Bam*HI, was used to transform naturally competent Hi strain KW20; selection was for kanamycin resistance. Transformants were screened for absence of porin in outer membrane preparations. From Hi strains that were missing porin, chromosomal DNA was subjected to Southern blotting using as probes the *ompP2* gene and the kanamycin resistance gene.

Southern Hybridization

Genomic DNA was isolated from *Haemophilus* strains by a microscale procedure (16). DNA restriction fragments were separated electrophoretically in 0.7% agarose gels containing TAE (0.01 M Tris acetate plus 0.01 M EDTA) buffer and transferred to Nytran hybridization membranes (Schleicher & Schuell). Enzymes and the digoxigenin DNA labeling kit (The Genius system) for Southern hybridization were obtained from Boehringer Mannheim. Southern hybridizations were carried out with digoxigenin-labeled probes at 25 ng·ml^{-1} of hybridization solution. Hybridizations were at 68 °C and a final wash with 0.1 × SSC containing 0.1% SDS at 68 °C for 15 min was included to reduce background.

Mutagenesis of Cloned Hib Porin

Plasmid pEJH39-1-35 was digested with *Pvu*II and *Ssp*I. The 1.1-kb *Pvu*II-*Ssp*I DNA fragment was isolated; it contained only the coding sequences for the mature form of Hib porin. This DNA fragment was ligated to the 2.5-kb *Pvu*II fragment from pBluescript SK(−) and used to transform *E. coli* strain DH5α; selection was for ampicillin resistance. A recombinant plasmid in which the *Pvu*II-*Ssp*I DNA fragment from pEJH39-1-35 was cloned in the same orientation as the *lac*Z gene in pBluescript SK(−) was designated pFFA02. It was used for mutagenesis experiments.

Deletion Mutagenesis—Site-directed deletions in Hib porin were constructed with the Muta-Gene phagemid *in vitro* mutagenesis kit, version 2 (Bio-Rad), using single-stranded mutagenic oligonucleotides. Two regions in Hib porin were selected for mutagenesis (Fig. 1), and they correspond to loop 3 and to loop 4 in the proposed topological model for Hib porin (12). Plasmid pFFA02 was used to transform *E. coli* strain CJ236. After infection of a transformant with helper phage M13K07, uracil-containing single-stranded phagemid DNA was isolated and used for the mutagenesis. Two mutagenic oligonucleotides created deletions of 6 amino acids and 12 amino acids in loop 3: 5′-ACA AGT GCA GAA GAT AAA GAG CTC GAC TAT ATT CCT ACT AGT-3′ and 5′-GAT GGC ATA ACA AGT GCA GAG CTC CCT ACT AGT GGT AAT ACC-3′, respectively. Two mutagenic oligonucleotides created deletions of 6 amino acids and 12 amino acids in loop 4: 5′-AAG CGT GAG GGT GCA AAA GAG CTC AAG GCT GGT GAA GTA CGT-3′ and 5′-TTA GCA CAA AAG CGT GAG GAG CTC GAA GTA CGT ATA GGT GAA-3′, respectively. Each mutagenic oligonucleotide also incorporated a unique *Sac*I site (underlined). After *in vitro* mutagenesis, the reactions were used to transform *E. coli* strain MV1190. Plasmids were isolated from transformants and mapped using restriction enzyme digestions. All candidates for each mutation were confirmed by DNA sequencing of plasmid DNA. Plasmids (pFFA05 to pFFA08, Table I) were digested to completion with *Pvu*II and *Mlu*I, and the 1.0-kb *Pvu*II-*Mlu*I DNA fragments containing the mutations were isolated. These fragments were ligated to the 11.6-kb *Pvu*II-*Mlu*I DNA fragment from pEJH39–1-35 and used to transform competent Hi strain RSFA21, with selection for kanamycin and tetracycline resistances.

C3 Insertion Mutagenesis—Plasmid pFFA02 contains unique sites for the restriction enzymes *Spe*I and *Sna*BI within the coding sequences for the mature form of Hib porin. Restriction sites for *Spe*I and *Sna*BI are found within sequences encoding amino acids of loop 3 and loop 4 of Hib porin, respectively. These sites were used to construct in-frame insertions of the C3 epitope of the VP1 protein of poliovirus (17, 18) in Hib porin. Plasmid pFFA02, digested with either *Spe*I or *Sna*BI, was ligated to double-stranded oligonucleotides. Linker *Spe*I-C3 was constructed by annealing two single-stranded oligonucleotides: 5′-CT AGT GAT AAC CCG GCG TCG ACC ACT AAC AAG GAT AAG A-3′ and 5′-CT AGT CTT ATC CTT GTT AGT GGT CGA CGC CGG GTT ATC A-3′. Linker *Sna*BI-C3 was constructed by annealing two single-stranded oligonucleotides: 5′-GT GAT AAC CCG GCG TCG ACC ACT

TABLE I
Bacterial strains, phages, and plasmids used in this study

Strain, phage, or plasmid	Relevant characteristics	Source or reference
E. coli strains		
DH5α	*supE44*Δ*lacU169*(ϕ*80 lacZ*Δ*M15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Life Technologies, Inc.
CJ236	*dut1 ung1 thi-1 relA1*/*pCJ105*(*cam*^{r}F′)	Bio-Rad
MV1190	Δ(*lac-proAB*) *thi supE*Δ(*srl-recA*)*306*::Tn*10*(*tet*^{r}) F′[*traD36 proAB lac1*^{q} *lacZ*Δ*M15*]	Bio-Rad
Haemophilus strains		
ATCC9795	Wild type Hib subtype 1H *ompP2*$^+$	(7)
KW20	Wild type Hi Rd *ompP2*$^+$ *rec-1*$^+$	(30)
DB117	KW20 *rec-1*	(31)
DL42	Wild type Hib subtype 1H *ompP2*$^+$ *rec-1*$^+$	(4)
DL42/2F4$^-$	DL42 *ompP2*	(24)
RSFA21	KW20 Δ*ompP2 kan*^{r}	This study
RS01	RSFA21 containing pEJH39-1-35	This study
RS03 to RS08	RSFA21 containing pRS03 to pRS08	This study
Phage		
M13K07	M13 carrying a mutation in gene II	Bio-Rad
Plasmids		
pBluescript SK(−)	Phagemid *bla*$^+$	Stratagene
pUC-CI	pACYC184 Ω(*Eco*RI::*Eco*RI pUC4K 1.1-kb *kan*^{r}	(32)
pEJH39-1-35	pGB103 Ω(*Pst*I::*Eco*RI-*Pst*I DL42 2.5-kb *ompP2*$^+$)ColE1 Hi Rep	(24)
pFFA02	pBluescript SK(−) Ω(*Pvu*II::*Pvu*II-*Ssp*I pEJH39-1-35 (1-kb sequences coding for mature Hib porin)	This study
pFFA03 to pFFA08	pFFA02 carrying mutations in sequences coding for mature Hib porin	This study
pRS02	pEJH39-1-35 Ω(*Pvu*II-*Mlu*I::*Pvu*II-*Sal*I-*Mlu*I adaptor)	This study
pRS03 to pRS08	pEJH39-1-35 carrying mutations in sequences coding for mature Hib porin	This study
pRS21	pRS02 Ω(*Sal*I::*Sal*I pUC-CI 1.1-kb *kan*^{r})	This study

AAC AAG GAT AAG C-3′ and 5′-G CTT ATC CTT GTT AGT GGT CGA CGC CGG GTT ATC AC-3′. Linkers *Spe*I-C3 and *Sna*BI-C3 created the codons for the C3 epitope in-frame to the codons for Hib porin. The ligation mixtures were used to transform *E. coli* strain DH5α with selection for ampicillin resistance. Plasmids from transformants were isolated, mapped, and sequenced. Plasmids pFFA03 and pFFA04 were digested to completion with *Pvu*II and *Mlu*I; the isolated mutagenized fragment was ligated to the 11.6-kb *Pvu*II-*Mlu*I DNA from pEJH39-1-35.

DNA Sequence Determination

For dideoxy sequencing (15), the T7 sequencing kit (Pharmacia Biotech Inc.) was used. One oligonucleotide was adequate for all DNA sequencing across regions encoding amino acids corresponding to loop 3 and loop 4: 5′-T(584) GAA GTA AAA CTT GGT CGT (603)G-3′; the numbers correspond to the published sequence of the Hib porin gene (5).

Preparation of Outer Membrane Vesicles, SDS-PAGE, and Immunoblotting

Outer membrane vesicles were obtained by treatment of cells with Tris-lysozyme-EDTA (19). Samples of vesicles containing outer membrane proteins were suspended in electrophoresis sample buffer with 2% (w/v) SDS, heated for 5 min at 100 °C, and run on 10% (w/v) polyacrylamide gels. For immunoblotting, outer membrane proteins were subjected to SDS-PAGE, transferred to nitrocellulose paper (Schleicher & Schuell), and probed with monoclonal antibodies or polyclonal antibodies (12) that were diluted 1/2000.

Flow Cytometry

Bacteria from mid-log phase cultures were washed in PBS and suspended in PBS to 2×10^9 cells·ml^{-1}. Affinity-purified anti-Hib porin mouse mAbs (14) or anti-peptide (C3 epitope of poliovirus) rabbit hyperimmune serum (18) at 1/100 dilution was mixed with 2×10^8 cells and incubated at room temperature for 1 h. Bacteria were pelleted, washed, and incubated at room temperature for 1 h with anti-mouse or anti-rabbit immunoglobulins conjugated to fluorescein. Bacteria were diluted ten-fold in PBS and analyzed for green fluorescence intensity using a FACScan flow cytometer (Becton Dickinson) with LysisII software. For each sample, 10^4 cells were analyzed.

Lipid Bilayer Experiments

Planar bilayer studies were executed as described previously (20) but with the following change in instrumentation: an Axopatch-1D amplifier (Axon Instruments) was used to measure the ionic current across the membrane.

RESULTS

Molecular Model of Hib Porin—Our proposed model for the secondary structure of Hib porin (12) is consistent with the consensus fold that is derived from five high resolution x-ray structures of nonspecific bacterial porins. We therefore explored possibilities to map the sequence of Hib porin onto the homology-derived scaffold of these structures. Using Hib porin as a target for the FASTA sequence alignment program, only one significantly scoring sequence homolog was found in SWISSPROT, identifying OmpF from *E. coli* as the closest currently known sequence relative. Hib porin and OmpF are 341 and 340 amino acids, respectively; identities are found at 58 amino acids (17% of the sequence), and there is no extended clustering of amino acid identities. This initial alignment was improved as follows. (i) A family alignment of structurally known porins (*R. capsulatus* porin and *E. coli* OmpF and PhoE) was constructed based on their three-dimensionally equivalent residues and using SUPERIMPOSE (21). (ii) The sequences of three other Hi porins were included and the alignment modified so as to place insertions, deletions, and other variable regions of those sequences primarily within loops and turns, while maximizing the number of identities with OmpF and with other members of the structure-based family alignment. A similar strategy to that described here was successfully employed to identify a molecular replacement model, thereby solving the x-ray structure of porin from *Paracoccus denitrificans* (22). Atomic coordinates of OmpF (10) were obtained from the Protein Data Bank (Brookhaven National Laboratory, Upton, NY), entry code 1OMF. Using the Swiss-Model Server (23)[2] and the alignment shown in Fig. 1, a homology model for porin of Hib was generated (Fig. 2). The model maps the sequence of Hib porin onto the structural scaffold of the porin family. This model is not meant to represent the detailed conformation of the loops but rather to indicate the length and position of loops within the overall β-barrel framework. Highlighted in the stereo figure is the modeled Cα backbone of sequences that are proposed to form loop 3. The length of loop 3 for Hib porin is

[2] The Swiss-Model server is accessible on the World-wide Web (URL: http://expasy.hcuge.ch).

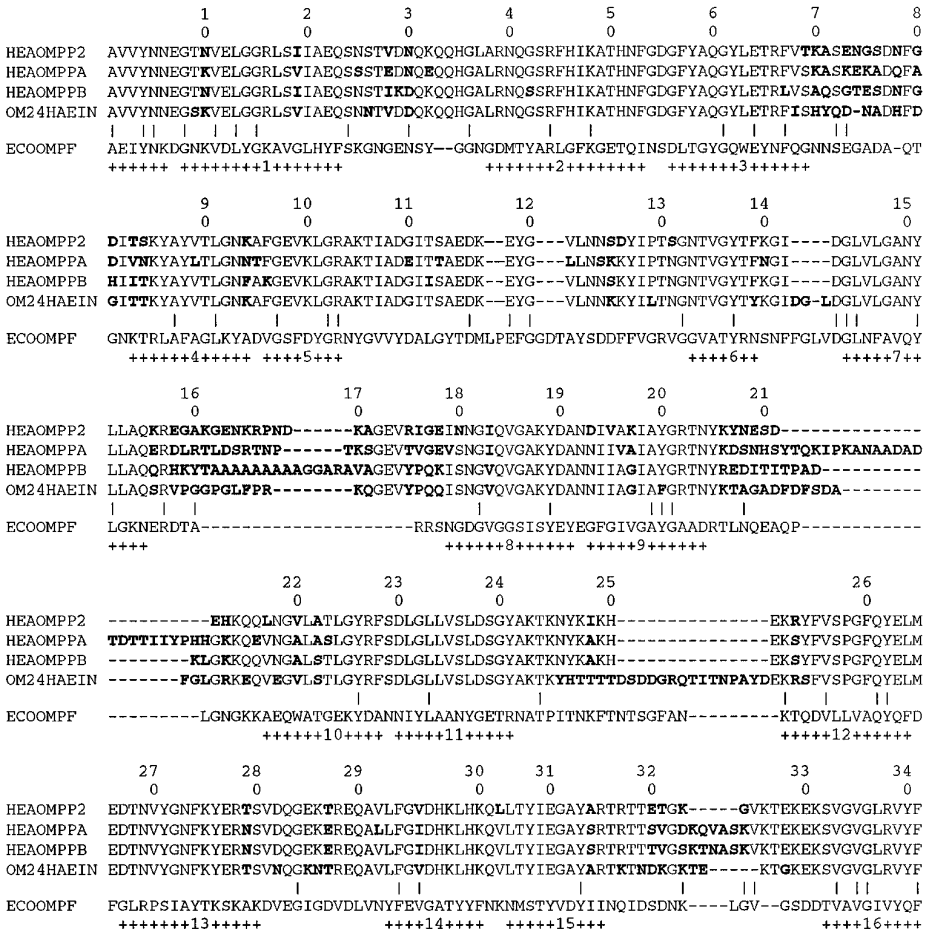

FIG. 1. **Alignment of amino acid sequences of *Haemophilus* and *E. coli* porins.** Initial alignment of OmpF (*bottom sequence*) and Hib porin (*top sequence*) was obtained using FASTA. The *numbers* above the amino acids denote their position in mature Hib porin. Three representative Hi porin sequences are shown below the Hib porin sequence. Residues not conserved among *Haemophilus* porins are shown in *bold*. *Bars* indicate identities between Hib porin and OmpF. Each of the β strands known for OmpF is indicated by +++ symbols on the last line. References for the sequences shown are as follows; HEAOMPP2 is from Ref. 5, HEAOMPPA and HEAOMPPB are from Ref. 26, OM24HAEIN is from Ref. 25, and ECOOMPF is from Ref. 33.

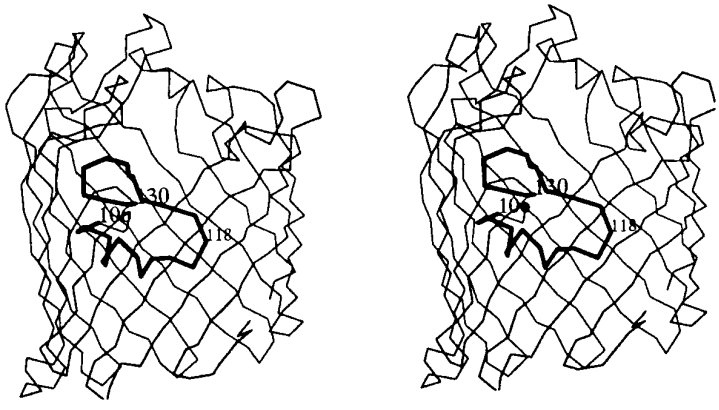

FIG. 2. **Cα trace (stereo) of Hib porin as obtained by homology modeling using the Swiss-Model server and manually improved.** A possible conformation of the Cα backbone in the region of loop 3 is highlighted.

apparently shorter than the length of loop 3 for OmpF of *E. coli*. While there is currently no high resolution structure for Hib porin and therefore no direct evidence for the position of loop 3 within the pore, observations on the differences in the lengths of loops 3 are in agreement with the measured molecular mass exclusion limits for OmpF (600 Da) and Hib porin (1400 Da). The molecular model predicts that deletions of 6 amino acids or of 12 amino acids in loop 3 of Hib porin might alter properties of ion conductivity. The experiments that follow were designed in part to test this prediction.

A Nontypeable Hi Strain Deleted for Its Porin Gene—Plasmid pRS21 was constructed by replacing the sequences coding for the mature form of Hib porin in pEJH39-1-35 with a kanamycin resistance cassette. The kanamycin resistance cassette in this construct was flanked by sequences upstream and downstream of the Hib *ompP2* gene. A *Bam*HI fragment derived from pRS21 and containing the kanamycin resistance cassette was used to transform nontypeable Hi; selection was for kanamycin resistance. Hi strain RSFA21, a representative transformant, was subjected to SDS-PAGE analysis and Western blotting of outer membrane preparations. Gain of kanamycin resistance coincided with loss of porin (Fig. 4, *lane 4*). Analyses by Southern blotting (data not shown) confirmed the replacement of the chromosomal copy of the Hi porin gene with the kanamycin resistance cassette. The Hib *ompP2* probe hybridized to a chromosomal DNA fragment from wild type Hi strain KW20 but not to any chromosomal DNA fragment from the porin deletion strain. Conversely, the kanamycin resistance gene probe hybridized to a chromosomal DNA fragment from the porin deletion Hi strain RSFA21 but not to any chromosomal DNA fragment from the wild type Hi strain.

Mutations in Loops 3 and 4 of Hib Porin—We chose to

```
A   113 114                                                          131 132
    ser ala glu asp lys glu tyr gly val leu asn asn ser asp tyr ile pro thr ser gly
630-agt gca gaa gat aaa gaa tat ggc gtt ctc aac aat agt gac tat att cct act agt ggt-689

B   157 158                                                          175 176
    arg glu gly ala lys gly glu asn lys arg pro asn asp lys ala gly glu val arg ile
762-cgt gag ggt gca aaa ggt gaa aat aag cgg cct aat gat aag gct ggt gaa gta acg tat-820

C        116 117         126 127                          113 114         129 130
         asp lys GLU LEU asp tyr                          ser ala GLU LEU pro thr
pRS05 639-gat aaa GAG CTC gac tat-674          pRS06  630 agt gca GAG CTC cct act-683
                  SacI                                             SacI

         160 161         170 171                          157 158         173 174
         ala lys GLU LEU lys ala                          arg glu GLU LEU glu val
pRS07 771-gca aaa GAG CTC aag  gct-806          pRS08  762-cgt gag GAG CTC gaa gta-815
                  SacI                                             SacI

D        129 130                                                      131 132
         pro thr SER ASP ASN PRO ALA SER THR THR ASN LYS ASP LYS THR ser gly
pRS03 678-cct aCT AGT GAT ACC CCG GCG TCG ACC ACT AAC AAG GAT AAG Act agt ggt
                                    SalI

         173 174                                                175 176
         glu val ARG ASP ASN PRO ALA SER THR THR ASN LYS ASP LYS arg ile
pRS04 810-gaa gta cGT GAT AAC CCG GCG TCG ACC ACT AAC AAG GAT AAG Cgt ata
                                    SalI
```

FIG. 3. **Sequences of wild type and mutant Hib porins.** *A*, wild type sequences of loop 3. *B*, wild type sequences of loop 4. *C*, plasmids carrying deletions in *ompP2*. pRS05, 6-amino acid deletion in loop 3; pRS06, 12-amino acid deletion in loop 3; pRS07, 6-amino acid deletion in loop 4; pRS08, 12-amino acid deletion in loop 4. *D*, plasmids carrying insertions in *ompP2*. pRS03, C3 epitope insertion in loop 3; pRS04, C3 epitope insertion in loop 4. The first nucleotide of each sequence is preceded by a *number* indicating its position in the published sequence of the Hib porin gene (5); the position of the last nucleotide is also indicated. The wild type Hib porin nucleotide sequences are shown in *lowercase letters*; *uppercase letters* correspond to the additional sequences of the mutations. The corresponding amino acid sequences are indicated above the nucleotide sequence; the *numbers* above the amino acids denote their position in mature Hib porin. The amino acid sequence of the C3 epitope is in *italics*.

introduce deletions and insertions into the proposed loop 3 and the proposed loop 4 of Hib porin. The amino acids deleted were those that correspond to epitopes (12) recognized by the anti-Hib porin mAbs POR.1 (loop 3) and POR.4 (loop 4). The gene sequences of the mutations created by site-directed mutagenesis and the resulting mutant amino acid sequences of Hib porins are shown in Fig. 3. Since all four oligonucleotides used for construction of the deletions contained 6 nucleotides that introduced a *Sac*I site, each deletion protein gained 2 additional amino acids, Glu and Leu. For example, to create a net deletion of 6 amino acids in loop 3 as encoded by pRS05, 8 amino acids were removed; they were replaced by 2 amino acids, Glu and Leu. The ends of the linkers *Spe*I-C3 and *Sna*BI-C3 were compatible for insertion at *Spe*I and *Sna*BI restriction sites, respectively. There were net insertions of 13 amino acids and 12 amino acids in the loop 3-C3 and loop 4-C3 mutant proteins.

Plasmids containing the cloned Hib porin gene (pEJH39-1-35) or containing deletions and insertions in Hib *ompP2* (pRS03 to pRS08) were used to transform the porin deletion Hi strain RSFA21. Proteins expressed in the transformants (RS01 and RS03 to RS08) were initially detected by SDS-PAGE of total cell lysates. To determine the cellular location of the mutant proteins, outer membrane vesicles were prepared, run on polyacrylamide gels and stained with Coomassie Blue (Fig. 4). Deletions in loop 3 (Fig. 4, *lanes 6* and *7*) or C3 epitope insertion in loop 3 of Hib porin (Fig. 4, *lane 10*) altered the mobility of the mutant porins when compared with the migration of wild type Hib porin. Deletions in loop 4 (Fig. 4, *lanes 8* and *9*) or C3 insertion in loop 4 (Fig. 4, *lane 11*) did not alter the mobility of the mutant porins when compared with the wild type Hib porin.

Immunoblotting of Mutant Porins—Three anti-Hib porin mAbs (POR.1, POR.4, and POR.6; Ref. 14) and a polyclonal antibody against the C3 epitope of poliovirus designated 928 (18) were used to analyze mutant proteins (data not shown). mAb POR.1 did not react with mutant Hib porins from strains RS05 and RS06, and mAb POR.4 did not react with mutant Hib porins from strains RS07 and RS08. Genetic deletions of sequences coding for the epitopes recognized by mAbs POR.1 and POR.4 therefore abolished the reactivities of these mAbs to the corresponding mutant Hib porins. Because sequences coding for the epitope recognized by mAb POR.6 were present in the genetic constructs pRS05, pRS06, pRS07, pRS08, pRS03, and pRS04, mAb POR.6 reacted with all the mutant Hib porins. Anti-C3 polyclonal antibodies reacted only with mutant Hib porins from strains RS03 and RS04. Only the proteins from the

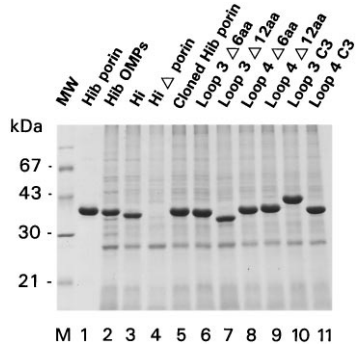

FIG. 4. **Outer membrane proteins of strains expressing mutant Hib porins.** Protein samples were resolved by SDS-PAGE (10% gel) and stained with Coomassie Blue. *Lane M*, 7.5 μg of molecular weight markers; *lane 1*, 2.5 μg of FPLC-purified Hib porin (14); *lanes 2–11*, variable amounts (1.5–7.5 μg) of outer membrane proteins from Hib strains were loaded, the amounts having been adjusted to obtain comparable levels of staining for the 28-kDa protein that is common to all preparations.

mutant strains producing Hib porins with C3 epitope insertions showed reactivity to the anti-C3 antibodies.

Flow Cytometric Analyses—Anti-Hib porin mAb POR.6, known to bind to the surface-exposed epitope ^{318}TTETGKGV^{325} in Hib porin (14), was used to stain intact bacterial cells of strains expressing the mutant porins (data not shown). mAb POR.6 recognized all the mutant Hi porins (RS03 to RS08) at the cell surface and with similar signals of relative fluorescence intensities to strains harboring wild type porins. Such experiments confirm the outer membrane localization and orientation of the proposed loop 8 in all mutant porins. mAb POR.4, reactive against the sequence ^{161}GENKRPNDKAG^{172} that is known to be surface-exposed in loop 4, was also tested in flow cytometry *versus* Hi strains expressing mutant porins. Predictably, mAb POR.4 did not react with Hi strains RS07 and RS08 because the antibody-reactive sequences in the mutant porins had been deleted. mAb POR.4 displayed similar signals of fluorescence intensities against Hi strains RS05 and RS06 as with Hib strain DL42. Deletions in loop 3 of the mutant porins apparently did not perturb the recognition by mAbs of unmodified loop 4. When anti-C3 polyclonal antibodies were used, the C3 epitope in the mutant Hib porin expressed in strain RS04 was detected by flow cytometry but not the C3 epitope in the mutant Hib porin expressed in strain RS03.

Reconstitution into Planar Lipid Bilayers—Wild type Hib porin and mutant *Haemophilus* porins engineered with deletions in the proposed loop 3 and the proposed loop 4 were purified to homogeneity by detergent extraction of outer membranes followed by fast protein liquid chromatography on Q-Sepharose in the presence of Zwittergent Z-3,14 (14). Each preparation of purified porin was tested for its ability to form channels in planar lipid bilayers. The conductances of over 200 channel insertion steps were measured for those porin species that formed stable pores, and the data are presented in the form of histograms (Fig. 5). Wild type Hib porin showed a relatively narrow distribution of conductance steps similar to what we have previously reported (20); approximately 40% of the channels showed conductances between 0.80 and 0.95 nS with 0.85 nS as the most frequently measured channel conductance (Fig. 5*A*). Mutant *Haemophilus* porin possessing a 6-amino acid deletion in loop 3 formed channels that displayed a broad distribution of conductance steps (Fig. 5*B*); calculation of the geometric mean indicated an average single channel conductance of 0.81 nS. Mutant *Haemophilus* porin possessing a 12-amino acid deletion in loop 3 formed pores that exhibited excessive levels of electrical noise due to their instability; therefore no histogram is presented. Interestingly, mutant *Haemophilus* porins having 6 or 12 amino acid deletions in loop 4 showed a distribution of conductance steps that was shifted toward higher conductance values when compared with the wild type Hib porin (Fig. 5, *C* and *D*). Deletion of 6 amino acids from loop 4 resulted in channels that showed a wide distribution of conductance steps; calculation of the geometric mean provided an average single channel conductance of 1.15 nS. *Haemophilus* porin deleted for 12 amino acids in loop 4 showed approximately 40% of conductance steps in the 1.00 to 1.10 nS range with 1.05 nS as the most frequently measured channel conductance.

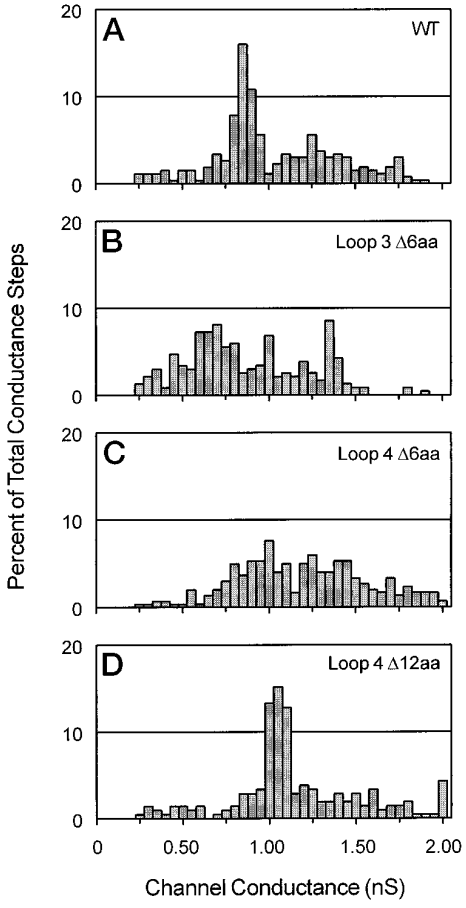

FIG. 5. **Comparison of channel conductances as measured in planar bilayers for porins purified from four Hi strains: RS01 (*panel A*), RS05 (*panel B*), RS07 (*panel C*), and RS08 (*panel D*).** Captions on the right indicate sample characteristics. The different porins were diluted with 50 mM Tris-HCl (pH 8.0) to 5 ng·μl^{-1}. Approximately 1 μl of this material was added to the Teflon chamber containing 5 ml of 1 M KCl such that the final porin concentration in the chamber was 1 ng·ml^{-1}. The total number of conductance steps analyzed was as follows: *panel A*, 269; *panel B* 235; *panel C*, 303; *panel D*, 209.

DISCUSSION

Multiple sequences in a protein family reflect the history of the evolutionary process of mutation and selection. Despite low sequence identity, structurally known proteins of the porin family superimpose surprisingly well. This indicates the importance of structural constraints given by the interaction with the lipid bilayer, the rotational pseudo-symmetry of the closed 16-stranded barrel of the nonspecific porins, and the absence of a functional requirement for molecular rearrangements such as those found for many enzymes. In the present situation, homology modeling appears to be an ideal route to obtain a structural hypothesis for another family member. The success of homology modeling depends critically on the correctness of the sequence alignment used. Fortunately, in the case of the porin family, a highly accurate structure-based family alignment can be constructed and used as a framework for alignment of further family members. The availability of four different Hi porin sequences increased the reliability of the alignment. We are therefore confident that the maximum sequence shift error does not exceed two residues for the major part of the alignment. The homology-derived structural model attains the accuracy required for mapping of experimental results to structural features.

Our studies were designed to create genetically engineered Hib porins that were predicted to display altered phenotypes. One of our prerequisites was a *Haemophilus* strain that no longer expressed wild type porin. The porin-minus Hib strain DL42/2F4$^-$ (24), generated by insertional inactivation of *ompP2* with an out-of-frame *Eco*RI linker, is not stable. Revertants that we isolated by their rapid growth on chocolate plates were analyzed by SDS-PAGE; staining with Coomassie Blue demonstrated a major outer membrane protein. By immunoblotting these proteins were shown to be related but not iden-

tical to wild type Hib porin.

To create a stable mutation in *ompP2*, we chose to remove 98% of the porin coding sequences from the chromosome of Hi strain KW20. Homology between Hi and Hib *ompP2* sequences is approximately 75–85% (25, 26). Since the kanamycin resistance cassette in pRS21 is flanked by sequences upstream and downstream of Hib *ompP2*, the genetic relatedness between Hi and Hib was sufficient to give rise to porin deletion strains by homologous recombination. Hi strain RSFA21 was ideal for the expression and characterization of mutant Hib porins.

The signals of relative fluorescence intensity by two mAbs (POR.4 and POR.6) against intact Hi cells harboring mutant porins were compared with the signals displayed by intact cells with wild type porins. Because flow cytometry showed the staining properties to be unchanged, we conclude that all mutant proteins were localized to the outer membrane. Deletions or C3 epitope insertions in our selected regions of Hib porin apparently did not affect the targeting and outer membrane assembly of mutant proteins. That 13 additional amino acids (C3 epitope plus Ser and Thr) can be accommodated between residues 115–133 in Hib porin supports our proposal that this region in Hib porin forms a loop. By flow cytometry, the C3 epitope inserted in loop 4 of Hib porin was surface-exposed, a result that extends our earlier work (12, 14). However, the C3 epitope inserted in the proposed loop 3 of Hib porin was not surface-exposed, in accordance with our molecular modeling of loop 3's folding back into the β barrel.

In all known porin structures, loop 3 traces a path along the inside of the barrel wall creating a constriction site of typically 8 Å by 10 Å. Misra and Benson (27) isolated several mutant strains of *E. coli*, which lacked a functional *lamB* gene that encodes a maltodextrin-specific channel and yet were capable of growth on maltodextrins as sole carbon source. Some of the mutant strains expressed altered OmpF or OmpC proteins with deletions in loop 3 of 6 (Leu^{109}–Met^{114}) or 8 (Trp^{103}–Phe^{110}) amino acids, respectively. Since the wild type OmpF and OmpC pores are too small to accommodate large maltodextrins, it was proposed that the deletions in loop 3 resulted in larger channel diameters for OmpF and OmpC. Consequently, if loop 3 of Hib porin were folded into the lumen of the pore, we would expect that deletions in this loop might affect the channel constriction and give rise to altered single channel conductance. While deletion of 6 amino acids from loop 3 of Hib porin affected channel conductance, it resulted not in an increased channel conductance but in a wider distribution of conductance steps (Fig. 5*B*). One possible explanation for such a wider distribution of conductance steps is that the deletion in loop 3 increased this loop's conformational flexibility such that in distinct channels this internal loop was positioned differently. In support of this possibility, analysis (28) of the Δ109–114 OmpF mutant at atomic resolution indicated that residues 117–123 of loop 3 were highly disordered compared with the wild type OmpF.

Hib porin with a deletion of 12 amino acids from loop 3 was purified, reconstituted into planar bilayers, and showed channels that were noisy and unstable. The x-ray structure of *E. coli* OmpF porin indicated that by its interaction with the barrel wall, loop 3 contributes to the stability of the barrel and perhaps its shape (10). It is likely that deletion of 12 amino acids from loop 3 of Hib porin was a radical change to the structure of this loop; this mutant *Haemophilus* porin could be purified but was no longer stable *in vitro*.

Surprisingly, deletion of 6 or 12 amino acids from loop 4 of Hib porin resulted in an increased single channel conductance (1.15 and 1.05 nS, respectively) compared with wild type Hib porin (0.85 nS). Single channel conductance is dependent on pore dimensions as well as the charge distribution within the pore. The increased channel conductances thus reflect altered channel electrostatics that increase ion flow and/or they reflect larger channel dimensions. For the following reasons, we propose that the deletions in loop 4 enlarge the Hib pore. (i) We previously implicated loop 4 of Hib porin in the changes in channel conductance that occur during voltage gating and suggested that this loop was capable of influencing pore size (20). (ii) In *E. coli* OmpF several external loops including loop 4 are inclined toward the central axis of the pore and they significantly narrow the channel entrance to a diameter as small as 11 Å (10). Loop 4 of Hib porin was predicted (Fig. 1) to be much longer than the corresponding loop in OmpF (23 *versus* 8 amino acids, respectively). If loop 4 of Hib porin were oriented toward the central axis of the pore, it is feasible that deletions in this long loop would widen the pore entrance and increase the single channel conductance. Furthermore, it has been suggested that the presence of a relatively large loop 4 (22 amino acids) in *E. coli* OmpC partly accounts for OmpC's forming smaller pores than OmpF (6). Accordingly, Vakharia and Misra (29) isolated a strain of *E. coli* expressing OmpC deleted for 22 amino acids (P156-G177) in loop 4 and showed that this deletion increased OmpC-mediated solute diffusion.

Definitive explanations for the ion conductivities of wild type and mutant Hib porins may ultimately be forthcoming from high resolution structural information. We recently succeeded in obtaining crystals of Hib porin, and, upon subjecting these crystals to synchrotron irradiation, they diffracted to near 4 Å. Our continuing efforts are directed to improving the qualities and diffraction properties of Hib porin crystals, with the objective of solving the structure of Hib porin at atomic resolution.

Acknowledgments—Synthesis of oligonucleotides was performed at the Sheldon Biotechnology Center (SBC), McGill University (SBC is supported by MRC Maintenance Grant MT-11107 and NSERC Infrastructure Grant INF 0103603). *Haemophilus* strains KW20, DB117, and DL42 were provided by Dr. R. Redfield and Hi strain DL42/2F4$^-$ and plasmid pEJH39-1-35 by Dr. E. J. Hansen. We appreciate helpful discussions with Dr. G. Barcak on genetics of *Haemophilus*, the availability of apparatus for planar bilayer measurements in the laboratory of Dr. R. Laprade (Université de Montréal), and a critical reading of the manuscript by G. S. Moeck.

REFERENCES

1. Benz, R. (1994) in *Bacterial Cell Wall* (Ghuysen, J.-M., and Hakenbeck, R., eds) pp. 397–423, Elsevier Science Publishers, Amsterdam
2. Nikaido, H., and Vaara, M. (1985) *Microbiol. Rev.* **49,** 1–32
3. Jeanteur, D., Lakey, J. H., and Pattus, F. (1994) in *Bacterial Cell Wall* (Ghuysen, J.-M., and Hakenbeck, R., eds) pp. 363–380, Elsevier Science Publishers, Amsterdam
4. Hansen, E. J., Hasemann, C., Clausell, A., Capra, J. D., Orth, K., Moomaw, C. R., Slaughter, C. A., Latimer, J. L., and Miller, E. E. (1989) *Infect. Immun.* **57,** 1100–1107
5. Munson, R., Jr., and Tolan, R. W., Jr. (1989) *Infect. Immun.* **57,** 88–94
6. Nikaido, H. (1992) *Mol. Microbiol.* **6,** 435–42
7. Vachon, V., Lyew, D. J., and Coulton, J. W. (1985) *J. Bacteriol.* **162,** 918–924
8. Weiss, M. S., Abele, U., Weckesser, J., Welte, W., Schiltz, E., and Schulz, G. E. (1991) *Science* **254,** 1627–1630
9. Kreusch, A., Neubuser, A., Schiltz, E., Weckesser, J., and Schulz, G. E. (1994) *Protein Sci.* **3,** 58–63
10. Cowan, S. W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R. A., Jansonius, J. N., and Rosenbusch, J. P. (1992) *Nature* **358,** 727–733
11. Schirmer, T., Keller, T. A., Wang, Y.-F., and Rosenbusch, J. P. (1995) *Science* **267,** 512–514
12. Srikumar, R., Dahan, D., Gras, M. F., Ratcliffe, M. J. H., van Alphen, L., and Coulton, J. W. (1992) *J. Bacteriol.* **174,** 4007–4016
13. Hansen, E. J., Pelzel, S. E., Orth, K., Moomaw, C. R., Radoff, J. D., and Slaughter, C. A. (1989) *Infect. Immun.* **57,** 3270–3275
14. Srikumar, R., Chin, A. C., Vachon, V., Richardson, C. D., Ratcliffe, M. J. H., Saarinen, L., Käyhty, H., Mäkelä, P. H., and Coulton, J. W. (1992) *Mol. Microbiol.* **6,** 665–676
15. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
16. Barcak, G. J., Chandler, M. S., Redfield, R. J., and Tomb, J.-F. (1991) *Methods Enzymol.* **204,** 321–342
17. Charbit, A. J., Ronco, J., Michel, V., Werts, C., and Hofnung, M. (1991) *J. Bacteriol.* **173,** 262–275

18. Moeck, G. S., Fazly Bazzaz, B. S., Gras, M. F., Ravi, T. S., Ratcliffe, M. J. H., and Coulton, J. W. (1994) *J. Bacteriol.* **176,** 4250–4259
19. Hantke, K. (1981) *Mol. Gen. Genet.* **182,** 288–292
20. Dahan, D., Vachon, V., Laprade, R., and Coulton, J. W. (1994) *Biochim. Biophys. Acta* **1189,** 204–211
21. Diederichs, K. (1995) *Proteins Struct. Funct. Genet* **23,** 187–195
22. Hirsch, A., Breed, J., Saxena, K., Richter, O.-M. H., Ludwig, B., Diederichs, K., and Welte, W. (1997) *FEBS Lett.* **404,** 208–210
23. Peitsch, M. C. (1995) *Bio/Technology* **13,** 658–660
24. Cope, L. D., Pelzel, S. E., Latimer, J. L., and Hansen, E. J. (1990) *Infect. Immun.* **58,** 3312–3318
25. Duim, B., van Alphen, L., Eijk, P., Jansen, H. M., and Dankert, J. (1994) *Mol. Microbiol.* **11,** 1181–1189
26. Sikkema, D. J., and Murphy, T. F. (1992) *Infect. Immun.* **60,** 5204–5211
27. Misra, R., and Benson, S. A. (1988) *J. Bacteriol.* **170,** 528–533
28. Lou, K.-L., Saint, N., Prilipov, A., Rummel, G., Benson, S. A., Rosenbusch, J. P., and Schirmer, T. (1996) *J. Biol. Chem.* **271,** 20669–20675
29. Vakharia, H., and Misra, R. (1996) *Mol. Microbiol.* **19,** 881–889
30. Alexander, H., and Leidy, G. (1951) *J. Exp. Med.* **93,** 345–359
31. Setlow, J. K., Boling, M. E., Beattie, K. L., and Kimball, R. F. (1972) *J. Mol. Biol.* **68,** 361–378
32. Vieira, J., and Messing, J. (1982) *Gene* (*Amst.*) **19,** 259–268
33. Inokuchi, K., Mutoh, N., Matsuyama, S.-I., and Mizushima S. (1982) *Nucleic Acids Res.* **10,** 6957–6968