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Altered Channel Properties of Porins from *Haemophilus influenzae*: Isolates from Cystic Fibrosis Patients

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Abstract. Changes in amino-acid sequence of the unique pore-forming protein of *H. influenzae* (OmpP2; porin) have been associated with increased antimicrobial resistance in *H. influenzae* strains isolated from cystic fibrosis patients. From patients who were subjected to long-term antimicrobial therapy, *H. influenzae* strains 67d and 69a (patient 27) and strains 77a and 77f (patient 30) were isolated. Strains 67d and 77a were previously shown to have elevated values for minimal inhibitory concentrations of antibiotics compared to strains 69a and 77f. Porins were extracted from all four *H. influenzae* strains by detergent treatment and purified to homogeneity by ion exchange chromatography. By reconstitution of the clinical Hi porins into planar lipid bilayers, single-channel conductance, ionic selectivity, and voltage-gating characteristics were assessed. Porins 77a and 77f displayed similar single-channel conductance and ionic selectivity. Current-voltage relationships were determined for the different porins: porin 77f displayed substantial voltage gating at both positive and negative polarity; porin 77a gated at negative polarity only. Porins 67d and 69a showed substantial differences in their pore-forming properties: the single-channel conductance of porin 69a was significantly increased (1.05 nS) relative to porin 67d (0.73 nS). Porin 67d was twice as permeable to cations as porin 69a, and at both positive and negative polarities the extent of voltage gating was greater for porin 67d relative to porin 69a. Expression of the porins in an isogenic, porin-deleted *H. influenzae* background allowed for assessment of the contribution of each porin to the minimum inhibitory concentrations of various antimicrobial compounds. Porin 67d was found to have a decreased susceptibility to the antimicrobials novobiocin and streptomycin. This decreased susceptibility of porin 67d to novobiocin and streptomycin correlates with its decrease in single-channel conductance.

Key words: *Haemophilus influenzae* — Cystic fibrosis — Porin — Ion channel — Antibiotic resistance

Introduction

Haemophilus influenzae (Hi), a small non-motile Gram-negative bacterium, is a commensal of the upper respiratory tract. In non-vaccinated individuals, encapsulated strains of Hi, in particular serotype b, may cause serious disease such as meningitis, epiglottitis, and cellulitis (Peltola, 2000). Non-encapsulated strains of Hi, despite being commensals, are frequent causes of less serious diseases in the respiratory tract, including otitis media, sinusitis, and pneumonia (van Alphen, 1992).

Hi strains that cause infection in CF patients are capable of persistent infection despite prolonged antibiotic treatment with β-lactam antibiotics. Various hypotheses have been proposed for the persistence of Hi strains in these patients, including antigenic drift of major outer membrane (OM) proteins (Duim et al., 1994, 1996; Groeneveld et al., 1989), shielding of bacteria from the bactericidal effects of antibiotics and from specific antibodies by penetration of bacterium between lung epithelial cells (van Schilfgaarde et al., 1999), and reduced permeability of the OM to antibiotics as a result of changes in porin properties (Regelink et al., 1999).

The structures of numerous bacterial porins when determined by X-ray crystallography (Weiss

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Abbreviations: The abbreviations used are: Hi, *H. influenzae; Hib, H. influenzae type B;* CF, cystic fibrosis; OM, outer membrane; PLB, planar lipid bilayer; nS, nanosiemens (s); V_r, reversal potential; V_c, critical voltage; MIC, minimal inhibitory concentration.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant Characteristics	Source or reference
E. coli strains		
XL-1	*recA*1 *endA*1 *gyrA*96. *thi-1 hsdR*17 *supE*44 *relA*1 *lac* [F' *proAB lac*1^q ZΔM15 Tn*10*(Tet^r)]^c	Stratagene
GM48	F$^-$ *dam-3 dcm-6 thr-1 leuB6 ara-14 tonA31 lacY*1 *tsx-78 glnV44 galK2 galT22 thi-1*	(Marinus, 1973)
Haemophilus strains		
ATCC9795	Wild-type Hib subtype 1H *ompP2*$^+$	(Vachon *et al.*, 1985)
DB117	KW20 *rec-1*	(Setlow *et al.*, 1972)
RSFA21	KW20 Δ*ompP2 kan*^{r}	(Srikumar *et al.*, 1997)
27-67d	Hi clinical strain expressing porin 67d	(Moller *et al.*, 1995)
27-69a	Hi clinical strain expressing porin 69a	(Moller *et al.*, 1995)
30-77a	Hi clinical strain expressing porin 77a	(Moller *et al.*, 1995)
30-77f	Hi clinical strain expressing porin 77f	(Moller *et al.*, 1995)
Plasmids		
pFFA02	pBluescript SK(−) ω (*Pvu*II::*Pvu*II-*Ssp*I pEJH39-1-35 (1-kb sequences coding for mature Hib porin)	(Srikumar *et al.*, 1997)
pMA01	pEJH39-1-35 (*Mlu*I site in *tet*^{r} eliminated)	(Arbing *et al.*, 2001)
pMA22	pFFA02 containing the coding sequence of *H. influenzae* porin 77a (GenBank AF052548)	This study
pMA23	pFFA02 containing the coding sequence of *H. influenzae* porin 77f (GenBank AF052551)	This study
pMA24	pFFA02 containing the coding sequence of *H. influenzae* porin 67d (GenBank AF052541)	This study
pMA25	pFFA02 containing the coding sequence of *H. influenzae* porin 69a (GenBank AF052542)	This study
pMA27	pFFA02 containing the coding sequence of *H. influenzae* porin 69a (GenBank AF052542)	This study
pMA32	pMA01 carrying porin 77a (GenBank AF052548)	This study
pMA33	pMA01 carrying porin 77f (GenBank AF052551)	This study
pMA34	pMA01 carrying porin 67d (GenBank AF052541)	This study
pMA35	pMA01 carrying porin 69a (GenBank AF052542)	This study

et al., 1991; Cowan et al., 1992; Kreusch & Schulz, 1994; Hirsch et al., 1997; Dutzler et al., 1999; Zeth et al., 2000) reveal a common fold. The tertiary structure of porin is a hollow sixteen-stranded β-barrel. The β-strands span the membrane in an anti-parallel fashion and are connected on the extracellular side of the membrane by long loops and on the periplasmic face of the membranes by short turns. One of the loops (loop 3) is folded back into the barrel to constrict the channel. This constriction serves two functions: first, it restricts the diffusion of large solutes through the channel and establishes the molecular mass exclusion limit; second, it generates a charged constriction zone. The charged constriction zone limits the passage of solutes to those that are polar and that can orient themselves within the transverse electrical field.

In addition to the molecular mass exclusion limit, porins display other properties, including single-channel conductance, ion selectivity, and voltage gating (Schirmer, 1998). Voltage gating of porins occurs when porin proteins in PLB experiments are subjected to membrane potentials that exceed a critical threshold voltage. Above this critical voltage (V_c), ion conductance through the pore becomes nonlinear and the flux of ions through the channel is restricted, apparently in response to conformational changes that occur in the channel (Schulz, 1996). Critical voltages differ considerably between porin species. *E. coli* porins close above critical voltages of 100 mV (Phale et al., 2001), whereas some Hib porin subtypes have critical voltages as low as 50 mV (Dahan et al., 1994). The physiological relevance of voltage gating has yet to be demonstrated, but it may be a mechanism by which OM permeability is modulated.

In the present study, we examined the electrophysiological properties of porins from CF patient-derived Hi strains that differ in their susceptibility to β-lactam antibiotics. The goal of this study was to determine the contribution of Hi porins to antibiotic resistance.

Materials and Methods

Bacterial Strains, Plasmids, and Media

Strains and plasmids used in this study are listed in Table 1. Briefly, Hi clinical strains were isolated from sputum samples from two CF patients during a long-term study on persistent Hi infection (Moller

et al., 1995). Clinical Hi strains were isolated from patients throughout the duration of the study although they were not necessarily recovered from consecutive sputum samples. Strains 77a and 77f isolated from patient 30 are genotypically identical by random amplified polymorphic DNA (RAPD) analysis (van Belkum et al., 1994), but they show variability in their outer membrane protein (OMP) profiles. Strains 67d and 69a isolated from patient 27 are genotypically (van Belkum et al., 1994) and phenotypically distinct by analyses of RAPD patterns and OMP profiles. Strains 77a and 67d had increased minimum inhibitory concentration (MIC) values for the antibiotics ampicillin, penicillin, cephalothin, and chloramphenicol relative to strains 77f and 69a (Regelink et al., 1999).

Haemophilus was grown on chocolate agar plates (36 g/1 GC base), hemoglobin (10 g/1), and IsoVitox supplements (20 ml/1) at 37°C with 5% CO_2. Liquid cultures of Hi were grown in brain heart infusion broth supplemented with hemin (10 mg/1) and NAD^+ (10 mg/1). Medium for *E. coli* strains has been previously described (Arbing, Hanrahan & Coulton, 2001). Antibiotic concentrations for selection of chromosomal and plasmid markers after transformation of Hi were 20 mg/1 of kanamycin, and 10mg/1 of tetracycline. Media were purchased from Oxoid and antibiotics, from Sigma.

Molecular Biology Techniques

Restriction endonucleases (New England BioLabs) and T4 DNA ligase (Invitrogen) were used in accordance with the manufacturers' guidelines. *E. coli* strain DH5α or Hi strain DB117 was used for isolation of plasmid DNA using plasmid maxi or mini prep kits from QIAGEN. *E. coli* strain GM48 was used to obtain non-methylated DNA for restriction enzyme digestions with methylation-sensitive enzymes. DNA was extracted from agarose gels using the QIAGEN gel purification kit. *E. coli* cells were made competent for transformation with $CaCl_2$ (Sambrook, Fritsch & Maniatis, 1989). Hi strains were made competent for DNA uptake using $CaCl_2$ by the method of Barcak et al. (Barcak et al., 1991).

Cloning of Hi Porins

Porin genes from Hi clinical strains 27-67d (GenBank accession #AF052541), 27-69a (GenBank accession #AF052542), 30-77a (GenBank accession #AF052548), and 30-77f (GenBank accession #AF052551) were amplified by PCR using the primers 5′-ATTAATCGTTGGTGCATTCG and 5′-GAAGTAAACGCGTAAACCTACAC. The l.0-kb fragments were restriction enzyme-digested with *Pvu*II and *Mlu*I and ligated into pFFA02 to make plasmids pMA22, pMA 23 and pMA24. The *ompP2* gene for *H. influenzae* strains 27-69a lacks the *Pvu*II site but is homologous to the 5′-region of pMA22 upstream of a *Bcl*I site. The porin gene from *H. influenzae* strains 27-69a was PCR-amplified with the same primers and ligated into the PCR cloning vector pTrueBlue-PvuII (GenomicsOne), following the manufacturer's recommendations to give pMA27. pMA27 and pMA22 were transformed into *E. coli* host strain GM48 to overcome *dam* methylation sensitivity of *Bcl*I restriction endonuclease and the plasmids were isolated using a QIAGEN miniprep kit. Both plasmids were digested with the enzymes *Bcl*I and *Mlu*I. The 1.0-kb fragment from pMA27 was ligated to the 2.5-kb fragment of pMA22 and transformed into *E. coli* strain XL-1 to give pMA25. The 1.0-kb *Pvu*II-*Mlu*I fragments from pMA22 to pMA25 were isolated by restriction enzyme digestion and ligated to the 11.6-kb *Pvu*II-*Mlu*I fragment from pMA01. Competent Hi host strain DB117 was transformed with the ligation mix, selecting for tetracycline resistance. Recombinant plasmids containing the genes for the cloned porins (pMA32 to pMA35) were isolated and transformed into porin-deleted Hi strain RSFA21, with selection for tetracycline and kanamycin.

Protein Purification

The porins were detergent-extracted from outer membranes as previously described (Srikumar et al., 1992) and purified to homogeneity by fast protein liquid chromatography on Q-Sepharose media with the detergent Zwittergent Z-3,14 (Calbiochem). The qualities of the protein preparations were assessed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) followed by staining with Coomassie blue.

Planar Lipid Bilayer Assays

Planar lipid bilayer assays were performed as described previously (Arbing et al., 2000), using a modification of the Mueller and Rudin technique with a bilayer chamber from Warner Instruments, Hamden, CT. Bilayers were formed from a solution of monoolein (Sigma) at a concentration of 25 mg/ml in *n*-decane with KCl as the electrolyte. Bilayer integrity was monitored visually and by measuring the membrane capacitance. Upon stabilization of the bilayer, protein was added to the *cis* side of the bilayer chamber to a final concentration of 1 ng/ml. Electrical measurements and voltages were applied using an Axopatch 200B amplifier and data were acquired with the Axoscope software program (Axon Instruments, Union City, CA).

Single-channel data were organized into histograms and average single-channel conductance was determined by calculating the geometric mean from the histograms. Time constants (τ) of channel closure were calculated by least-squares fits of single exponentials to macroscopic current-decay curves using the program Origin 6.1 (Microcal; Northampton, MA).

Ionic selectivity measurements were performed by adding porin samples to the *cis* side of the bilayer with 100 mM KCl as the electrolyte. After the membrane had stabilized with respect to porin insertion (approximately 30–150 porin monomers inserted), an ionic gradient was established by the addition of concentrated KCl to the *cis* side of the chamber, bringing the KCl concentration on the *cis* side of the chamber to 500 mM. Reversal potentials (V_r) were determined from polynomial fits of the I/V data under asymmetrical electrolyte conditions. Permeability ratios (P_K/P_{Cl}) were calculated with the Goldman-Hodgkin-Katz equation (Hille, 1992).

Orientation of Porin in Artificial Membranes

Liposomes were made from asolectin (Sigma) by sonicating 40 ml of a 10 mg/ml solution of asolectin in 5 mM Tris-HCl pH 8.0 for 5 cycles of 45 sec with intervals of 45 sec between sonications. The solution was kept on ice during the procedure. The resulting liposome preparation was dialyzed against three changes of distilled water (4 hr per change). Liposomes were then filtered through a 0.45-μm filter and stored at 4°C. To incorporate porin into the liposomes, 100 μg of purified porin at 0.5 mg/ml in 50 mM Tris pH 8.0, 200 mM NaCl, 0.1% Zwittergent Z-3,14 was incubated with 400 μl of liposomes for 3 hr at room temperature. Proteoliposomes were diluted to 10 ml with phosphate buffered saline (PBS), collected by ultracentrifugation at 100,000× *g* for 60 min, washed twice with 10 ml of PBS, resuspended in 100 μl of PBS and dialyzed overnight against PBS.

Proteoliposomes containing reconstituted porin were subjected to proteolysis by incubation at 37°C with 1 μg of sequencing-grade chemically modified trypsin (Roche Diagnostics). Samples containing 2 μg of protein were removed at 1, 2, and 8 hr and proteolysis stopped with the addition of SDS-PAGE sample buffer and

heating at 100°C for 5 min. Detergent-solubilized (Zwittergent 3-14) Hib porin was trypsinized as a positive control for enzymatic digestion; proteoliposomes incubated with trypsin in the presence of the protease inhibitor Complete (Roche Diagnostics) acted as a negative control. Samples containing 2 μg of porin were electrophoresed on a 12% SDS-PAGE gel and silver stained by the procedure of Morrissey (Morrissey, 1981).

Antibiotic Susceptibility Testing

Hi strain RSFA21 transformed with plasmid pMA01 or pMA32 through pMA35 were tested for their sensitivity to various antibiotics including β-lactams (ampicillin, penicillin, and cephalothin) and non-β-lactams (chloramphenicol, streptomycin, and novobiocin). Bacteria were grown overnight on chocolate agar and a suspension containing 2×10^8 to 8×10^8 CFU/ml was prepared in a 0.9% saline solution by taking colonies directly from the plates. Following the recommendations of the National Committee for Clinical Laboratory Standards (2000), 10 μl was transferred to 10 ml of Haemophilus Test Medium (HTM) broth (Becton-Dickinson) to prepare the inoculum for a broth microdilution assay. Serial twofold dilutions of antimicrobial agents in HTM (50 μl) were made in the wells of a sterile microtiter plate. Antibiotic concentrations were in the following ranges: ampicillin, 0.03–16.0 μg/ml; penicillin, 0.006–3.0 μg/ml; cephalothin, 0.125–64.0 μg/ml; chloramphenicol, 0.06–32.0 μg/ml; streptomycin, 0.15–10.0 μg/ml; and novobiocin, 0.015–1.0 μg/ml. Control wells contained HTM without antibiotic. HTM containing bacterial suspensions of 1×10^5 to 4×10^5 CFU/ml were added to each well and the plates were incubated for 20–24 hr at 37°C. All experiments were done in triplicate and the lowest antibiotic concentration to inhibit growth was defined as the MIC. *H. influenzae* strains ATCC 49247 and ATCC 49766 were used as controls.

Results

Porin Purification

Porins were purified to homogeneity from the clinical Hi strains. Qualities of the protein preparations were assessed by SDS-PAGE followed by staining with Coomassie blue (Fig. 1). Porins from clinical Hi strains (Fig. 1, lanes *2* to *5*) migrated with an electrophoretic mobility of approximately 38 kDa, similar to porin from our reference Hib strain ATCC 9795 (Fig. 1, lane *1*).

Single-Channel Conductances

Each porin was reconstituted into PLBs and its pore-forming properties were evaluated. All channels formed stable pores. The single-channel conductances of approximately 200 to 350 channels were measured and organized into histograms to illustrate the differences in single-channel conductances (Fig. 2). The histograms for the different clinical Hi porins all show bimodal distribution of conductance steps (Fig. 2, panels *B*–E), in contrast with the unimodal distribution typically observed with wild-type Hib porin (Fig. 2, panel *A*). Mean conductance values were determined for the entire population of conductance increments (Table 2). Porins 77a and 77f have similar single-channel conductances with values of 0.87 ± 0.38 nS and 0.92 ± 0.38 nS, respectively. These values are not significantly different and most likely reflect similarity in structure for these two porins that have only four differences between their amino-acid sequences. Two of these substitutions occur in loop 4 and two in loop 6 (Fig. 3). The single-channel conductance values for porins 67d and 69a were markedly different: 0.73 ± 0.37 nS and 1.05 ± 0.42 nS, respectively.

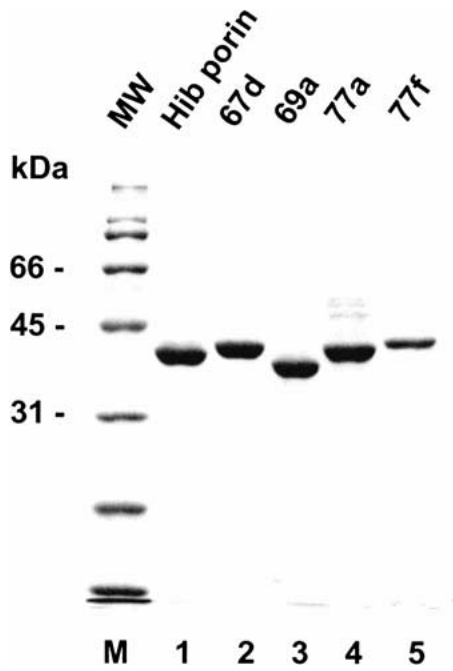

Fig. 1. Purified clinical Hi porins. Purified protein samples were resolved by SDS-PAGE (10% gel) and stained with Coomassie blue. Lane *M*, approximately 3 μg broad-range prestained molecular weight markers; lane *1*, 2.5 μg of FPLC-purified wild-type Hib porin from strain ATCC 9795; lanes *2* through *5*, 2 to 4 μg of FPLC-purified porins 67d, 69a, 77a, and 77f.

Ion Selectivity

The ion selectivity of the porins was calculated by determining the reversal potential (V_r) for each porin under asymmetrical salt conditions. The value of V_r was used to solve the Goldman-Hodgkin-Katz equation and to determine the permeability ratio of cations to anions (P_c/P_a) for each channel. Porins 77a and 77f had almost identical values for the equilibrium permeability ratio P_K/P_{Cl}: 1.05 ± 0.02 for 77a and 1.09 ± 0.09 for 77f. The cation selectivity of porin 67d was increased relative to porins 77a and 77f, with a P_K/P_{Cl} value of 2.09 ± 0.10. The value for porin 69a was 1.14 ± 0.03. The differences between 67d and 69a were more pronounced than those between 77a and 77f, with 67d having an almost two-

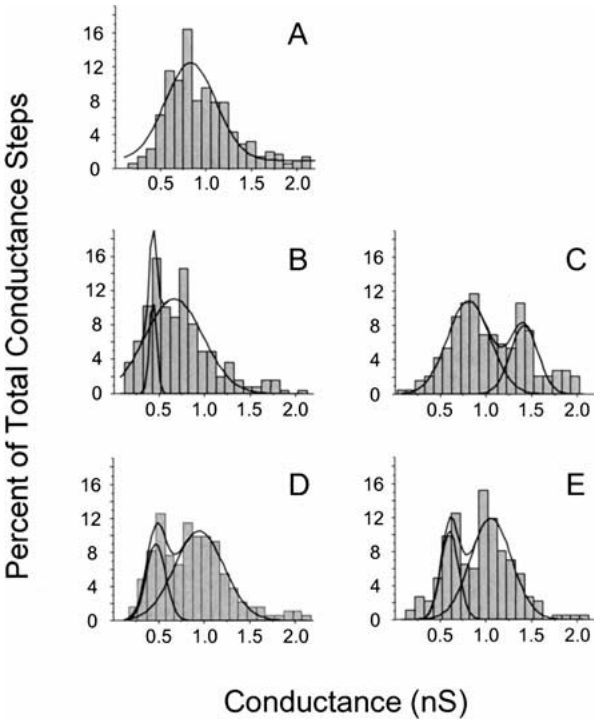

Fig. 2. Single-channel conductances measured in planar lipid bilayers for wild-type Hib porin and the four clinical Hi porins: wild-type Hib porin (panel *A*), porin 77a (panel *B*), porin 77f (panel *C*), porin 67d (panel *D*), and porin 69a (panel *E*). The porins were diluted with 10 mM Tris pH 8.0 to 1 ng/μl, and approximately 5 μl of this material were added to the *cis* side of the bilayer chamber so that the final porin concentration was approximately 1 ng/ml. The electrolyte solution was 1 M KCl and a holding potential of +10 mV was used. The total number of conductance steps analyzed was as follows: panel *A*, 249; panel *B*, 183; panel *C*, 349; panel *D*, 247; panel *E*, 189. The distribution of each mode, as well as the bimodal distribution, are indicated by the solid lines and were fit to the histograms using MicroCal Origin 6.1.

fold increase in selectivity for cations over anions in comparison with 69a.

Current-voltage Relationships

To determine the critical voltage (V_c) and the extent of voltage-dependent gating for the porins, we added each porin to the *cis* side of the bilayer chamber and waited until porin insertions had stabilized with 25 to 75 porin molecules inserted in the bilayer. V_c was determined by stepping the voltage from a holding potential of 10 mV to higher test potentials in 10-mV increments. Measurements were made at both positive and negative polarities; the data were normalized and displayed as current-voltage curves with standard deviation (Fig. 4). Compared to wild-type Hib porin (Fig. 4, panel *A*), the clinical porins had different current-voltage relationships and a reduced V_c. Wild-type porin was determined to have a V_c of +/−75 mV and current decay at 80 mV was to approximately 90 to 95% of the instantaneous current (Arbing et al., 2000; Dahan et al., 1994). Porin 77a gated at only negative potentials with a V_c of −20 mV (Fig. 4, panel *B*) while porin 77f had a V_c of +20 mV/−40 mV (Fig. 4, panel *C*). Steady-state current for porin 77a at −80 mV was 64.6 ± 5.3% of the instantaneous current. For porin 77f at potentials of +/−80 mV, steady-state currents were 59.1 ± 6.3% and 67.0 ± 2.4% of the instantaneous current. Porin 67d had a V_c of +40/−30 mV (Fig. 4, panel *D*) and steady-state currents at potentials of +/−80 mV were 60.6 ± 5.3% and 72.7 ± 9.3% of the instantaneous current, respectively. The critical voltage for porin 69a was +/−40 mV (Fig. 4, panel *E*) and current decayed at +/−80 mV to steady-state levels that were 74.1 ± 2.6% and 89.5 ± 0.2% of the initial currents, respectively.

Time constants (τ) for pore closure were obtained by fitting single exponentials to the macroscopic current-relaxation curves. Our previous study (Arbing et al., 2000) determined that the time constant for closure of Hib porin was 103.3 ± 41.6 sec at +80 mV. The clinical porins were found to have τ values that were similar to each other and to the reference porin (Table 2). Some of the porins displayed faster inactivation than wild-type porin, but none exhibited the faster time constant (2.8 ± 0.9 sec at +80 mV) observed in our studies on succinylated porin (Arbing, Hanrahan & Coulton, 2000).

Asymetric Insertion of Porin into Artificial Bilayers

Previous analyses of electrophysiological properties of Hib porins have indicated that they insert into artificial membranes (PLB experiments) in a unidirectional manner (Dahan et al., 1994). To correlate the voltage sensitivities of porin with the orientation of porin in the OM, we needed to determine the orientation of Hi porins in artificial membranes. We made liposomes of asolectin and reconstituted Hib porin. Detergent-solubilized Hib porin subjected to trypsinolysis is proteolytically cleaved in extracellular loops 1 and 4 (Srikumar et al., 1992) to yield fragments with masses of 14 to 19.5 kDa. Detergent-solubilized Hib porin reconstituted into liposomes in the outside-out orientation is predicted to yield a similar pattern of fragments, with loops 1 and 4 accessible to trypsin. Loops of porin reconstituted in the outside-in orientation would be inaccessible to proteolytic cleavage and SDS-PAGE analysis would reveal full-length Hib porin. Detergent-solubilized porin and liposome-reconstituted porin were each subjected to trypsinolysis and their SDS-PAGE analyses disclosed the same cleavage products (Fig. 5). Hib porin reconstituted in liposomes was proteolytically cleaved to generate peptides with molecular masses between 17.1 and 19.5 kDa, consistent with proteolytic cleavage in loop 4 (Srikumar et al., 1992). Prolonged incubation with trypsin (8 hr) resulted in

Table 2. Electrophysiological characteristics of Hi porins

Protein	Conductance ± SD (nS)	P_K/P_{Cl}	V_c (mV)		Time constants (τ) (sec)	
			+	−	+ 80mV	−80 mV
Wild-type Hib porin	0.85 ± 0.4[a]	1.6[b]	75[a]	75[a]	103.3 ± 41.6[a]	-
77a	0.87 ± 0.38	1.05 ± 0.02	-	20	-	71.8 ± 8.3
77f	0.92 ± 0.38	1.09 ± 0.09	20	40	126.2 ± 10.0	32.0 ± 11.1
67d	0.73 ± 0.37	2.09 ± 0.10	40	30	70.5 ± 8.3	227.2 ± 109.4
69a	1.05 ± 0.42	1.14 ± 0.03	40	40	138.4 ± 28.5	-

[a] from Arbing *et al*., 2000. [b] from Vachon *et al*., 1986.

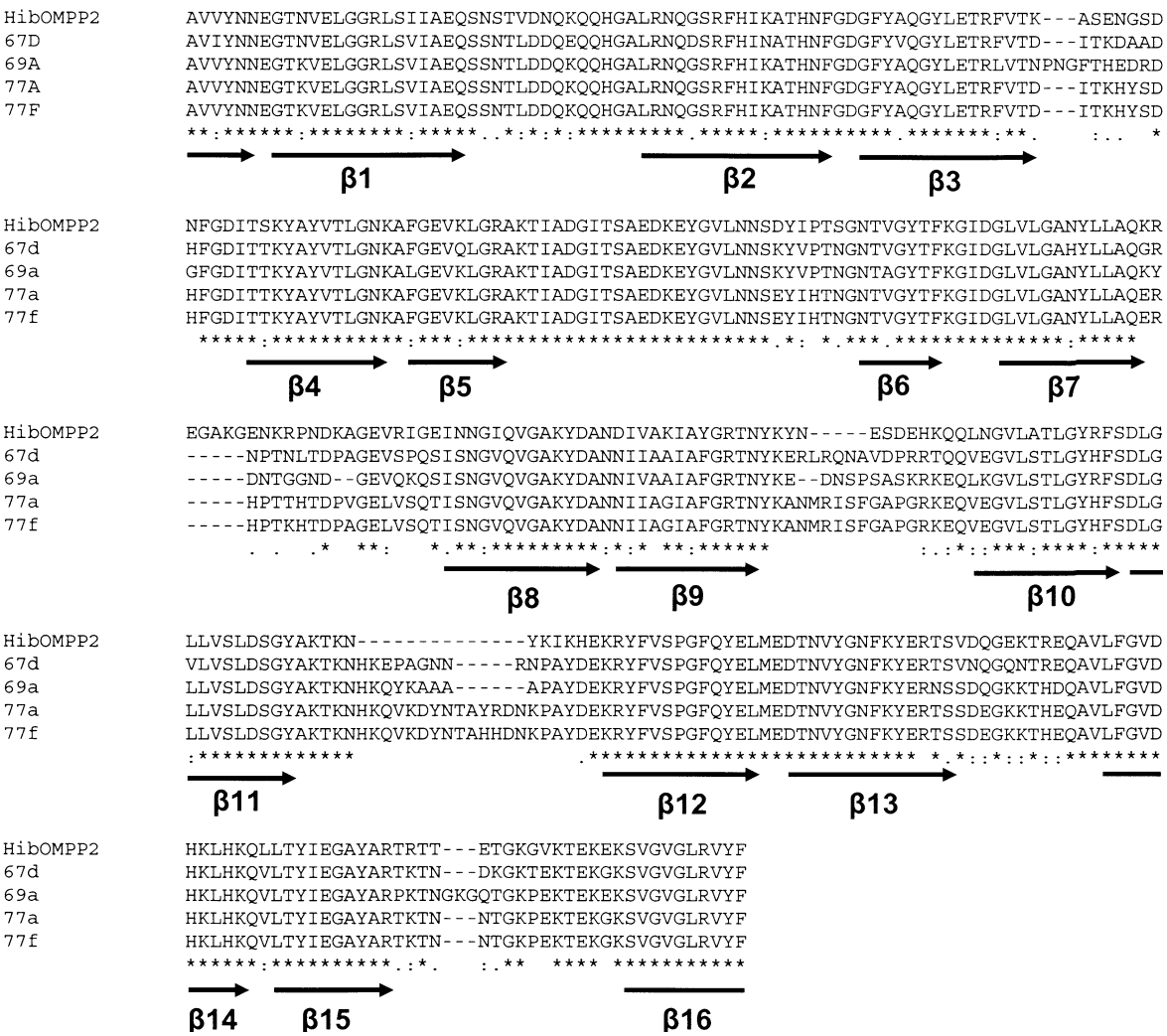

Fig. 3. Alignment of amino-acid sequences of wild-type Hib porin and clinical Hi porins isolated from CF patients. The OmpP2 amino-acid sequences were deduced from the DNA sequences. The Hib porin sequence is that of Hib strain ATCC 9795. The position of β-strands (indicated by arrows) is deduced from our homology model (Srikumar et al., 1997) of Hib porin. Our model incorporates data from the atomic resolution structures of three bacterial porins and has been experimentally verified by flow cytometry assays (Srikumar et al., 1992, 1997) of whole bacterial cells mapping monoclonal antibody epitopes and analysis (Regelink et al., 1999) of antigenic drift of Hi porin sequences. Amino acids are given as standard one-letter code.

complete proteolysis of Hib porin, as evidenced by its absence at 38 kDa. However, the fragments were not well resolved, perhaps a result of their association with lipids from asolectin liposomes. We conclude that porin, which is reconstituted into liposomal membranes in the outside-out orientation, would

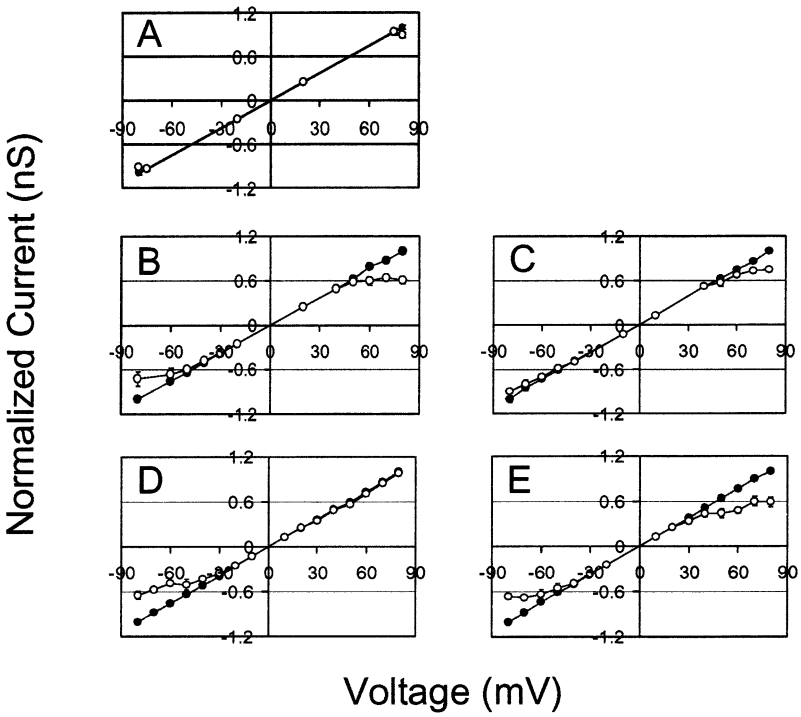

Fig. 4. Current-voltage relationships of planar lipid bilayers containing porin from Hib or Hi clinical porins: wild-type Hib porin (panel *A*), porin 77a (panel *B*), porin 77f (panel *C*), porin 67d (panel *D*), and porin 69a (panel *E*). Filled circles represent the instantaneous current through membranes, while open circles represent steady-state current reached one to two minutes after application of the test holding potential. Each symbol represents the values (±SD) from 3 to 10 experiments. Error bars may lie within the thickness of the symbols. Porin samples were added to the *cis* side of the chamber after bilayer formation to allow porin insertions from only one side of the chamber.

have the same orientation in other artificial bilayers including PLBs.

Antibiotic Susceptibilities of an Hi Strain Expressing Recombinant Porins

To determine the specific contribution of each clinical Hi porin to the MICs for different antibiotics, we cloned the four *ompP2* genes into pMA01 and expressed the porins in an isogenic background, Hi strain RSFA21 from which the *ompP2* gene is deleted. MICs were determined by the broth-microdilution assay and revealed no differences in the MIC values for the following antibiotics: ampicillin (0.25 μg/ml), penicillin (0.75 μg/ml), cephalothin (2.0 μg/ml), and chloramphenicol (1.0 μg/ml). Differences in susceptibility were found for novobiocin and streptomycin. Hi strain RSFA21 expressing three of the clinical porins (77a, 77f, and 67d) had a higher MIC (10 μg/ml) for streptomycin than did Hi strain RSFA21 expressing wild-type Hib porin and porin 69a (5 μg/ml). Hi strain RSFA21 expressing porin 67d had an increase in MIC for novobiocin (0.5 μg/ml) when compared to wild-type Hib porin and the three other clinical Hi porins (0.125 μg/ml) expressed in the same genetic background.

Discussion

This study assesses changes in the properties of porins isolated from CF patient-derived Hi strains and correlates changes in electrophysiological properties with antibiotic susceptibility. We investigated the electrophysiological properties of porins of CF patient-derived Hi strains and included measurements of ionic selectivity and voltage gating in addition to single channel conductance. Additionally, we expressed the clinical porins in an isogenic background (deleted for *ompP2*) to determine the contribution of each porin to the MIC. Taken together, these data establish single-channel conductance as the critical porin property in altering the OM permeability of Hi clinical isolates.

Sequence alignments of Hi porins from CF patients show extensive diversity in their loop regions, while sequences corresponding to membrane spanning regions of Hi porins are highly conserved. Extensive variation in loops 2 and 4–8 has been described (Regelink et al., 1999) for CF patient-derived Hi strains, while variation in loops 5 and 6 is characteristic of Hi porin antigenic variants (Duim et al., 1996; Vogel et al., 1996). Alignment of the sequences of the clinical porins in the present study with our model porin from Hib reveals numerous differences in the loop regions (Fig. 3). Variation occurs primarily in loops 4, 5, and 6, and these changes are probably due to antigenic variation in response to immunological pressure of the host. Regelink et al. (1999) proposed that changes in antibiotic susceptibility are attributable to amino acid-sequence diversity in loops 1 and 3. One strain (70b) was identified as being more resistant than a genotypically identical strain (70f), and it had a decreased

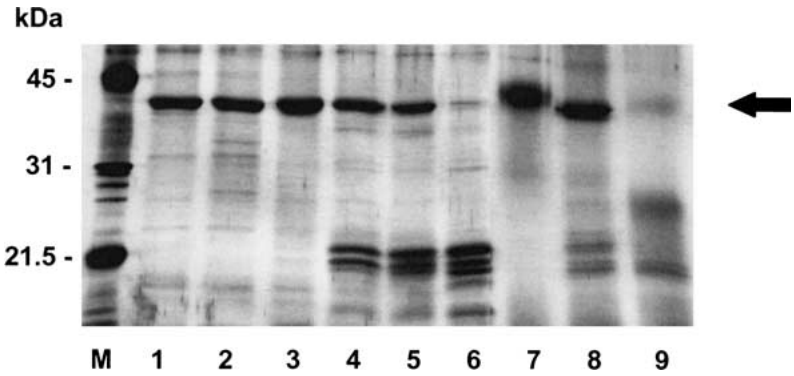

Fig. 5. Determination of orientation of Hib porin in artificial bilayers. Hib porin reconstituted into liposomes and detergent-solubilized Hib porin were subjected to proteolysis with trypsin for 0 to 8 hr. Samples were electrophoresed on 12% SDS-PAGE and silver-stained. All lanes contain 2 μg of protein. Lanes *1* through *6* contain detergent-solubilized protein and lanes *7* through *9*, liposome-reconstituted protein. Lanes *1* and *2* contain porin in the absence of trypsin at 0 and 8 hr, respectively. Lane *3* contains: porin, trypsin, and protease inhibitor at 8 hr. Lanes *4* through *6* contain porin and trypsin at 1, 2, and 8 hr, respectively. Lane *7* contains reconstituted porin at 8 hr. Lanes *8* and *9* contain reconstituted porin and trypsin at 1 and 8 hr, respectively. Full-length porin is labeled with the black arrow.

single-channel conductance relative to 70f. Porin 70b has amino-acid substitutions Lys^{107} Glu and Arg^{131} His in loop 3 and two additional charged residues in loop 1, a glutamate and an arginine, compared to porin isolate 70f. A conclusion from their study was that the fourfold difference in MICs was associated with porin variation (Regelink et al., 1999). The clinical Hi porins that we examined had no differences in loop 3 sequences between the pairs themselves, but porin 67d has the substitutions Lys^{22} Glu in loop 1 and Gly^{22} Asp in β-strand 2, compared to porin 69a. Noteworthy is that the differences between porins 77a and 77f are in loops 4 and 6 (Fig. 3).

Electrophysiological Properties of Hi Porins

When the clinical Hi porins were reconstituted into PLBs to measure single-channel conductance, the resulting histograms revealed (Fig. 2) a bimodal distribution for each porin population. This bimodal distribution is consistent with our earlier report (Regelink et al., 1999) on Hi porins from clinical strains. As evaluated by SDS-PAGE, our preparations are of quality similar to those of our previous study. The cause of the bimodal distribution is not known but, as previously postulated (Regelink et al., 1999), there may be two channel populations as a result of distinct conformational states of the protein, or there may be populations of distinct subunit associations.

Porins 77a and 77f from patient 30 display similar single-channel conductances: 0.87 ± 0.38 nS and 0.92 ± 0.38 nS, respectively. This suggests that the four amino-acid differences do not contribute to reorientation of the extracellular loops and thus to single-channel conductances. These values are only slightly increased relative to Hib porin: 0.85 ± 0.40 nS. The single-channel conductance values for 67d and 69a differ substantially, 0.73 ± 0.37 nS and 1.05 ± 0.42 nS, respectively. Extensive variation in the porin sequences leads to significant differences in the single-channel conductances of porins 67d and 69a. The loop 3 sequence is identical in both porins; however, porin 67d has slightly longer lengths of loops 4, 5, and 6 and shorter lengths of loops 2 and 8. The smaller single-channel conductance of the porin from the more resistant isolate, 67d, is consistent with the smaller single-channel conductance of the resistant strain (70b, 0.83 nS) versus the susceptible strain (70f, 1.2 nS) from the previous study (Regelink et al., 1999).

Measurements of the ionic selectivity of the clinical Hi porins identified the following differences. Porins 77a and 77f had P_K/P_{Cl} of 1.05 ± 0.02 and 1.09 ± 0.09, respectively, values that represent virtually neutral selectivity. These values are less than that of wild-type Hib porin which has a P_K/P_{Cl} of 1.6 (Vachon, Laptade & Coulton, 1986). The similar ionic selectivity of these two porins is not surprising, as the differences between the two porins occur in the extracellular loops, and the net charge (+3) of the proteins is the same. The ionic selectivity of porins 67d and 69a show a greater difference than that observed between porins 77a and 77f. Porin 67d has a P_K/P_{Cl} of 2.09 ± 0.10, while that of 69a is 1.14 ± 0.03. The results are in agreement with the change in net charge of the porins (67d, −1; 69a, +6). In summary, our observed changes in ionic selectivity were too small to influence the MIC profiles for these clinical Hi porins.

The amino-acid substitutions between the clinical Hi porins occurs primarily in the extracellular loops, although the pore of 67d would be expected to have less positive charge than 69a, as displayed by the mutations Lys^{10}Gln and Lys^{48}Gln, both of which are located in the pore of our Hib porin homology model (Srikumar et al., 1997). While these residues are located within the pore, they are not part of the positively charged cluster of amino acids that oppose the constriction loop. Mutagenesis studies on the porins PhoE (Bauer et al., 1989) and OmpF (Saint et al., 1996) of *E. coli*, and porin from *P. denitrificans* (Saxena et al., 1999) revealed that the mutations that most strongly influence ionic selectivity are those that contribute to the charged electrical field of the con-

striction site. The loss of peripheral charges in 67d adequately explains the modest increase in cation selectivity.

The clinical porins displayed altered voltage-gating characteristics. Wild-type Hib porin has a critical voltage of $+/-75$ mV (Arbing et al., 2000). The porins had decreased critical voltages for gating with V_c between 20 to 40 mV at both positive and negative polarity. With the exception of 77a, which gated at negative potentials only, all porins gated at both negative and positive polarities. The current decay at $+/-80$ mV differed between the clinical Hi porins, and the maximum decay was 40% of the instantaneous current (Fig. 4).

Oriented Insertion of Porin into Artificial Membranes

Asymmetry of gating has been observed previously for Hi porins. Dahan et al. (1994) suggested that asymmetric gating results from an oriented insertion of porin molecules within the lipid bilayer. Porin has an asymmetric structure, therefore it probably inserts into the lipid bilayer in a unidirectional manner. Proteolysis of detergent-solubilized porin and liposome-reconstituted porin confirmed this prediction and revealed that porin reconstitutes in an outside-out orientation. This should be favored energetically because the hydrophilic extracellular loops are not translocated through the liposome bilayer.

Knowing the orientation of porin in the lipid bilayer allows us to interpret polarity-dependent gating in terms of voltages that may exist across the bacterial OM. In our experiments, we apply the voltage in the *cis* chamber; this corresponds to the exterior of the cell, as porin reconstitutes in the outside-out orientation. Application of positive voltages represents a negative periplasmic potential and applied negative potentials represent positive periplasmic potentials. Under what conditions might we expect a potential to exist across the OM? Negative periplasmic potentials of up to 100 mV have been determined to lie across the OM of *E. coli* under conditions of low ionic strength (Sen, Hellman & Nikaido, 1988). Whichever conditions apply, there is no clear consensus between our clinical Hi porins. At polarities at which the periplasm would be negative, three porins (67d, 69a, and 77f) show substantial gating, while at potentials that result in a positive periplasm, three porins (67d, 77a, and 77f) also demonstrate substantial channel closure. Voltage gating of porins may play a role in mediating OM permeability since V_c is well within the range measured by Sen et al. (1988). A model system that measures diffusion across the OM in isogenic strains expressing voltage-sensitive or voltage-insensitive porins and under conditions that exist in the CF lung may elucidate a role for voltage gating in vivo.

Table 3. MICs of Hi strain RSFA21 expressing clinical Hi porin genes

Antibiotic (M_r)	MIC (μg/ml)				
	Wild-type porin Hib	77a	77f	67d	69a
Novobiocin (635)	0.125	0.125	0.125	0.5	0.125
Streptomycin (1457)	5	10	10	10	5

Contribution of Porin to OM Permeability

The expression of clinical Hi porins in an isogenic background allows for assessment of the specific contribution of porin to the OM permeability barrier. One method to assess OM permeability is to determine the MICs of a panel of antimicrobial agents. When the MICs were determined for three β-lactams, no difference was found between the host strain RSFA21 expressing wild-type porin or expressing the clinical Hi porins. This is at variance with the report (Regelink et al., 1999) that there are marked differences in the sensitivities of these strains to ampicillin, penicillin, cephalothin, and chloramphenicol. Thus, the large channel of the Hi porins does not constitute an effective barrier to the entry of small molecular weight antibiotics into the cell. Differences in MICs were observed when we tested larger antibiotics, novobiocin and streptomycin (Table 3). Porin 67d had the highest MIC for novobiocin and streptomycin, indicating that the smaller pore size of this porin, as measured in single-channel conductance experiments, indeed results in increased resistance to the influx of these compounds. Porins 77a and 77f also had increased MICs for streptomycin despite a slightly increased single-channel conductance when compared to wild-type porin. Porin 69a, which had the largest single-channel conductance, consistently had the lowest MIC values. Our data demonstrate that the large channel of Hi porins does not hinder the influx of small antibiotics across the OM.

Alterations in penicillin-binding proteins (Clairoux et al., 1992) and the limited diffusion of antibiotics by the viscous nature of CF lung sputum (Regelink et al., 1999) have been proposed as Hi resistance mechanisms. A functional multidrug efflux pump that contributes to resistance to a variety of compounds including dyes and erythromycin has recently been demonstrated (Sanchez et al., 1997). We have characterized porins from Hi strains that cause persistent lung infection in CF patients despite long-term antimicrobial therapy with β-lactam antibiotics. PLB analysis demonstrated that one of the porins (67d) displayed a reduced single-channel conductance that would contribute to a decreased OM permeability. When expressed in an isogenic background,

this porin had an increased MIC for larger antimicrobial compounds relative to the other porins. We did not observe the fourfold differences in MICs for β-lactams that were observed in the previous study on Hi clinical strains expressing these same porins (Regelink et al., 1999), thus, a porin-mediated decrease in OM permeability is but one factor in Hi antibiotic resistance. Our results confirm that the OM of *H. influenzae* is an ineffective barrier to the penetration of β-lactam antibiotics (Coulton, Mason & Dorrance, 1983). We have now demonstrated that resistance to larger antimicrobials can be obtained through altered porin channel properties.

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