

## Expression in *Escherichia coli* and Function of *Pseudomonas aeruginosa* Outer Membrane Porin Protein F

WENDY A. WOODRUFF,<sup>1</sup> THOMAS R. PARR, JR.,<sup>1</sup> ROBERT E. W. HANCOCK,<sup>1\*</sup> LARRY F. HANNE,<sup>2†</sup>  
THALIA I. NICAS,<sup>2‡</sup> AND BARBARA H. IGLEWSKI<sup>2</sup>

*Department of Microbiology, University of British Columbia, Vancouver, British Columbia, Canada, V6T 1W5,<sup>1</sup> and  
Department of Microbiology and Immunology, Oregon Health Sciences University, Portland, Oregon 97201<sup>2</sup>*

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The gene encoding porin protein F of *Pseudomonas aeruginosa* was cloned onto a cosmid vector into *Escherichia coli*. Protein F was expressed as the predominant outer membrane protein in a porin-deficient *E. coli* background and was clearly visible on one-dimensional sodium dodecyl sulfate-polyacrylamide gels in a porin-sufficient background. The identity of the protein F from the *E. coli* clone and native *P. aeruginosa* protein F was demonstrated by their identical mobilities on sodium dodecyl sulfate-polyacrylamide gel electrophoretograms, 2-mercaptoethanol modifiabilities, and reactivities with monoclonal antibodies specific of two separate epitopes of protein F. In the course of gene subcloning, a 2-kilobase DNA fragment was isolated, with an apparent truncation of the part of the gene encoding the carboxy terminus of protein F. This subclone produced a 24,000-molecular-weight, outer membrane-associated, truncated protein F derivative which was not 2-mercaptoethanol modifiable and which reacted with only one of the two classes of protein F-specific monoclonal antibodies. The 2-kilobase fragment was used in Southern blot hybridizations to construct a restriction map of the cloned and subcloned fragments and to demonstrate with restriction digests of whole *P. aeruginosa* DNA that only one copy of the protein F gene was present in the *P. aeruginosa* chromosome. The protein F produced by the original cosmid clone in a porin-deficient *E. coli* background was purified. To demonstrate retention of porin function after cloning, the protein F from the *E. coli* clone was incorporated into black lipid bilayer membranes. Two major classes of channels were revealed. The predominant class of channels had an average conductance of 0.36 nS in 1 M KCl, whereas larger channels (4 to 7 nS) were seen at a lower frequency. Similar channel sizes were observed for porin protein F purified by the same method from *P. aeruginosa* outer membranes.

The outer membrane of *Pseudomonas aeruginosa*, like those of all other gram-negative bacteria, is a bilayer containing lipopolysaccharide (LPS), phospholipids, and a few major species of proteins. One of the predominant proteins is porin protein F. Protein F has been purified and shown to form large channels with weak ion selectivity in black lipid bilayers. Surprisingly, few of the protein F molecules appeared to form functional channels in this assay (3). These data were consistent with results of other model membrane studies of protein F (14, 15, 33). A role for protein F in the inherent resistance of *P. aeruginosa* to antibiotics was postulated based on results of the black lipid bilayer studies (3), as well as whole cell permeability assays with a protein F-deficient mutant (25). Because it appears that the high intrinsic antibiotic resistance of *P. aeruginosa* results from low permeability of antibiotics through the outer membrane (1), it was proposed that this was due to the functional heterogeneity of protein F, in that a substantial proportion of protein F molecules did not form channels capable of functioning in antibiotic uptake (3, 25, 33). At least two explanations for the proposed functional heterogeneity are possible. It could result from an extrinsic factor, for example, the interaction of different types of LPS molecules in the outer

membrane with protein F (1, 17). Alternatively, functional heterogeneity might be generated during synthesis, assembly, or translocation to the outer membrane of protein F.

To explore the possible extrinsic factors that influence the function of protein F, we decided to put protein F into a new background in which the LPS would be different. The most straightforward approach was to clone the gene for protein F into *Escherichia coli*. The structural genes for several other porins and peptidoglycan-associated proteins have been cloned from nonenterobacterial strains into *E. coli*, including proteins from *Vibrio cholerae* (19, 30) and *Chlamydia trachomatis* (24). However, none of these cloned proteins has been demonstrated to retain porin activity in *E. coli*. In this report we describe the molecular cloning of the structural gene for porin protein F and demonstrate that the cloned gene product is expressed in the *E. coli* outer membrane and that following isolation it is functional as a porin.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** *P. aeruginosa* was used in this study. The *E. coli* K-12 strains used included HB101 (*hsd-20 recA13 ara-14 proA2 lac-41 galK2 mtl-1 xyl-5 supE44*) (18), MM294 (*pro thi endA hsdR*) (18), JF733 (*aroA ilv met his purE41 pro cyc-1 xyl lacY29 cpsL77 tsx-63 ompA ompC*) (9), and TB1 [*ara Δ(lac proAB) thi rpsL/φ80 dlacZΔM15 hsdR*] (20). Plasmids used were pLAFR1 (IncP1 Tc<sup>r</sup> rlx λ *cos*) (10), pCP13 (IncP1 Tc<sup>r</sup> Km<sup>r</sup> rlx λ *cos*) (8), pRK2013 (ColE1 tra<sup>+</sup> Km<sup>r</sup>) (11), pUC8, and pUC9 (ColE1 Ap<sup>r</sup>) (20).

\* Corresponding author.

† Present address: Biology Department, California State University, Chico, CA 95929.

‡ Present address: Department of Microbiology and Immunology, University of Ottawa, Ottawa, Ontario, Canada, K1H 8M5.

**Growth media.** LB medium was 1% (wt/vol) tryptone (Difco Laboratories, Detroit, Mich.), 0.5% yeast extract (Difco), and 0.5% NaCl (pH 7.2). TY medium was 0.8% tryptone, 0.5% yeast extract, and 0.5% NaCl (pH 7.0). For the growth of *E. coli* JF733, the concentration of NaCl was increased to 1.7% (300 mM), to suppress OmpF production, and 0.1% glucose was added to prevent the induction of the LamB porin. Media were solidified with 2.0% agar (Difco). Antibiotics were used in selection media at the following concentrations (in micrograms per milliliter): tetracycline, 25; ampicillin, 50; kanamycin, 25; streptomycin, 200. Recombinant transformants derived from the pUC8 and pUC9 vectors were selected as white colonies on TY agar supplemented with 50 µg of ampicillin per ml and spread with 50 µg of 5-bromo-4-chloro-indoly-β-D-galactoside.

**Monoclonal antibodies.** The production and characterization of monoclonal antibodies MA4-4 and MA5-8 specific for protein F has been described in detail by Mutharia and Hancock (22, 23).

**DNA procedures.** Plasmids were isolated by an alkaline lysis method (18), followed by centrifugation in ethidium bromide-cesium chloride gradients. Small-scale isolation of plasmid DNA was done by using a modification (7) of the rapid boiling method of Holmes and Quigley (16). Restriction endonucleases and T4 ligase were purchased from Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, Md., and reaction conditions were as specified by the manufacturer. Restriction fragments were examined by electrophoresis in 0.7% agarose in 89 mM Tris–89 mM borate–2 mM EDTA buffer. Southern blot hybridization (29) was performed with, as a hybridization probe, a 2.0-kilobase (kb) *SalI-PstI* restriction fragment from plasmid pWW5 (see below) after radiolabeling of this fragment by nick translation with [ $\alpha$ -<sup>32</sup>P]dATP and the Klenow fragment of DNA polymerase I (27). Plasmid DNA was usually introduced into host bacterial strains by the transformation of calcium chloride-treated cells (18). To construct *E. coli* JF733(pHN4), MM294(pHN4) was used as the donor strain in a triparental mating (11) with the conjugative functions of *E. coli* HB101(pRK2013) acting on the *rlx* site of pHN4 for conjugal transfer. Transconjugants were selected as resistant to tetracycline and streptomycin.

**Construction of a *P. aeruginosa* PAO1 clone bank.** The clone bank was constructed as described in detail by Goldberg and Ohman (11). Briefly, PAO1 genomic DNA was partially digested with *EcoRI* and size fractionated by centrifugation through 10 to 40% sucrose gradients. DNA fragments of 20 to 25 kb were ligated into the *EcoRI* site of pLAFR1, a cosmid vector (10). Recombinant molecules were packed in vitro into phage lambda particles and transduced into *E. coli* HB101. The transductants were plated onto L agar containing 10 µg of tetracycline per ml. Colonies were scraped off the plates into broth plus 10% glycerol and stored at –70°C.

**Screening procedure for protein F clones.** Bacterial colonies were transferred from agar plates onto prewashed nitrocellulose filters by contact. The colonies were lysed by placing the filters in an atmosphere saturated with chloroform vapor for 15 min. The filters were removed, allowed to air dry briefly, and then incubated for 1 h at 37°C on a rocking platform with phosphate-buffered saline (PBS) containing 3% bovine serum albumin, 1 µg of DNase per ml, and 40 µg of lysozyme per ml. The filters were rinsed thoroughly with PBS (any residual colony material was scraped off with a gloved finger) and incubated for 2 h at 37°C with monoclonal antibody MA5-8 or MA4-4 diluted in PBS with 1% bovine

serum albumin. After filters were washed three times for 10 min with PBS, they were incubated with a goat-anti-mouse immunoglobulin G-peroxidase conjugate for 1 h at 37°C and then washed again. The blots were developed with 4-chloro-1-naphthol, a histochemical stain for peroxidase (23).

**Outer membrane preparation, electrophoresis, and immunoblotting.** Purified outer membranes were prepared on sucrose gradients (13). Outer membrane proteins were separated by electrophoresis on an 11% polyacrylamide gel (13) after solubilization at 20 or 88°C for 10 min. Following electrophoresis, proteins were visualized by staining with Coomassie brilliant blue R250 (13). For immunoblotting, proteins were electrophoretically transferred to nitrocellulose from the gel (22). The protein F was detected immunologically as described previously (22).

**Black lipid bilayer methods.** The black lipid bilayer methods used in the characterization of the porins have been described in detail elsewhere (3, 4). The lipid used to form the membrane was 1.5% oxidized cholesterol in *n*-decane, a generous gift from R. Benz. All experiments were done at room temperature. The electrical measurements were made by immersing Ag–AgCl electrodes (In Vivo Metric, Hearldsburg, Calif.) into aqueous solutions on either side of a Teflon (E. I. du Pont de Nemours and Co., Inc., Wilmington, Del.) divider which was perforated with a small hole (0.1 to 2 mm<sup>2</sup> in diameter) over which a membrane was painted. Formation of the bilayer was recognized after the membrane turned optically black when viewed with incident light. The fluctuations in current were amplified 10<sup>9</sup>- or 10<sup>10</sup>-fold with preamplifier (X27; Keithley, Cleveland, Ohio) and monitored with a storage oscilloscope (model 5115; Tektronix, Beaverton, Ore.) with a plug-in amplifier (model 5A22; Tektronix). Observations were recorded on a strip chart recorder for further analysis.

**Purification of porins.** *E. coli* OmpF porin was purified by conventional chromatography as described previously (26). Protein F from *P. aeruginosa* and *E. coli* was purified by a modification of the method of Parr et al. (26). This method was used to ensure that residual OmpF porin from strain JF733 did not contaminate the preparations used for functional analysis of the protein F samples. French-pressed outer membranes of either the *P. aeruginosa* or the protein F-containing *E. coli* *ompC* mutant strain JF733(pHN4) (which had been grown in a high salt concentration to suppress the production of the *E. coli* OmpF porin) were Triton X-100–EDTA extracted as described previously (14). The Triton X-100–EDTA-insoluble pellets were solubilized at room temperature in 2% sodium dodecyl sulfate (SDS)–Tris hydrochloride (pH 6.8)–10% glycerol and then electrophoresed on 11% SDS-polyacrylamide gels. The protein F band was electroeluted from the appropriate gel segment as described previously (26), but the electroelution was for only 30 min at 50 V. Longer or stronger electroelution conditions resulted in poorly active protein F preparations.

## RESULTS

**Cloning strategy and identification of cosmid clones.** The cosmid bank of *P. aeruginosa* PAO1 genomic DNA was transfected into *E. coli* HB101, and the resultant strains were plated for single colonies and screened for the production of protein F antigen. Approximately 3,500 colonies were screened, and 5 of these reacted with a protein F-specific monoclonal antibody. One clone containing a plasmid, designated pHN4, was arbitrarily selected for further manipu-

lations. This plasmid was isolated and transformed into the porin-deficient *E. coli* JF733.

**Characterization of F protein in *E. coli* (pHN4).** Outer membranes were isolated from *E. coli* HB101(pHN4) and JF733(pHN4). Electrophoretic profiles of these outer membranes are shown in Fig. 1, lane 2, and Fig. 2, lane 7, respectively. In both strains a protein analogous to protein F from *P. aeruginosa* (Fig. 1, lane 1; Fig. 2, lane 5) was observed that was not observed in *E. coli* HB101 (Fig. 1, lane 3) or *E. coli* JF733 (Fig. 2, lane 3). In strain JF733(pHN4) protein F appeared to be the predominant outer membrane protein (Fig. 2, lane 7).

The new band in the outer membrane profile of *E. coli* HB101(pHN4) [and strain JF733(pHN4); data not shown] was identified as protein F by its interaction with protein F-specific monoclonal antibodies MA4-4 and MA5-8 (Fig. 3A and B, lanes 5). The cloned protein exhibited a small amount of a reactive band of higher apparent molecular weight because of partial heat modification, a phenomenon often observed in protein F in *P. aeruginosa* outer membrane electrophoretograms (13, 14). A presumed proteolytic product was also observed which reacted with MA4-4 but not MA5-8 (Fig. 3B, lane 5). A similar pattern of reactivity has been observed previously with trypsin- and papain-generated fragments of protein F (23).

The response of the cloned protein to 2-mercaptoethanol was also investigated. The cloned protein in *E. coli* HB101(pHN4) and *E. coli* JF733(pHN4) increased in apparent molecular weight after heating in 2-mercaptoethanol in a manner analogous to that for native protein F (13). This phenomenon was most easily observed in *E. coli* JF733 (pHN4) (Fig. 2, lanes 4 and 7) because the 2-mercaptoethanol-reduced protein F band was partly obscured by the Omp C-OmpF porin bands of strain HB101(pHN4). The cloned protein in its reduced form reacted with monoclonal antibody MA5-8 but did not react with MA4-4 (data not shown), as previously observed for native protein F (23).

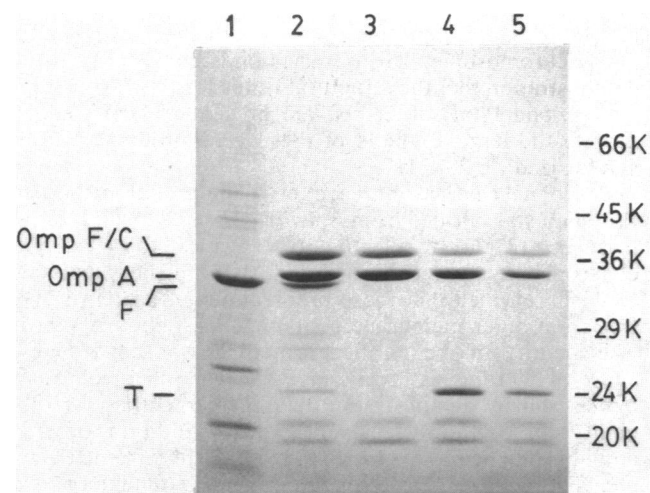


FIG. 1. SDS-polyacrylamide gel electrophoresis profiles of outer membrane preparations. For each sample, 15  $\mu$ g of outer membrane protein was solubilized at 88°C for 10 min without 2-mercaptoethanol. Lane 1, *P. aeruginosa* PAO1; lane 2, *E. coli* HB101(pHN4); lane 3, *E. coli* HB101; lane 4, *E. coli* HB101(pWW1); lane 5, *E. coli* HB101(pWW4). Molecular weight markers (in thousands [K]) are indicated on the right. The position of protein F (F) and the truncated protein F gene product (T) are indicated on the left.

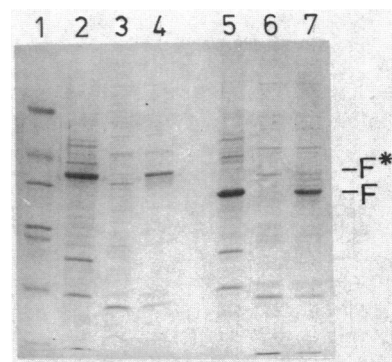


FIG. 2. SDS-polyacrylamide gel electrophoretograms demonstrating the 2-mercaptoethanol modifiability of protein F in *E. coli* and *P. aeruginosa* outer membrane preparations. Samples were treated as described in the legend to Fig. 1, except that 5% 2-mercaptoethanol was added to the samples in lanes 2, 3, and 4 before heating. Lanes 2 and 5, *P. aeruginosa* PAO1; lanes 3 and 6, *E. coli* JF733; lanes 4 and 7, *E. coli* JF733(pHN4). The 2-mercaptoethanol-modified (F\*) and unmodified (F) forms of protein F are indicated. Molecular weight markers (lane 1) were bovine serum albumin (66,000), ovalbumin (45,000), glycerol-3-phosphate dehydrogenase (36,000), carbonic anhydrase (29,000), trypsinogen (24,000), soybean trypsin inhibitor (20,100), and alpha-lactalbumin (14,400).

**Subcloning of pHN4.** Subclones and deletion derivatives of plasmid pHN4 were isolated. The protein F gene was localized on an 11-kb *Eco*RI fragment cloned into pLAFR1. This subclone was designated pWW13 (Fig. 4). Outer membranes isolated from *E. coli* HB101(pWW13) showed a protein with electrophoretic and immunological characteristics identical to those of protein F in *E. coli* HB101(pHN4) (data not shown).

The parent plasmid pHN4 was also digested with *Xho*I and transformed into competent *E. coli* HB101 cells. Positive transformants expressing protein F antigen were detected with monoclonal antibody MA5-8. Outer membranes isolated from one such F-positive subclone containing a plas-

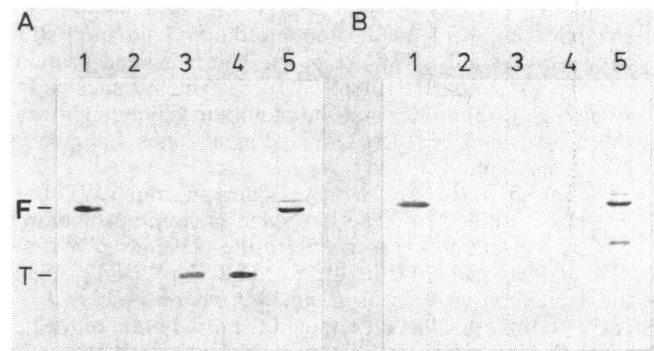


FIG. 3. Western immunoblots of outer membrane proteins of *E. coli* HB101 separated by SDS-polyacrylamide gel electrophoresis. The proteins in panel A were probed with protein F-specific monoclonal antibody MA5-8; those in panel B were probed with monoclonal antibody MA4-4, which recognizes a different epitope of protein F. Lanes 1, *P. aeruginosa* PAO1; lanes 2, *E. coli* HB101(pCP13); lanes 3, *E. coli* HB101(pWW4); lanes 4, *E. coli* HB101(pWW1); lanes 5, *E. coli* HB101(pHN4). The faint upper band in lanes 5 is due to incomplete heat modification of protein F and is often observed in preparations of protein F from *P. aeruginosa* (13). The lower molecular weight band in lane 5 of panel B is presumably a proteolytic product of protein F (22).

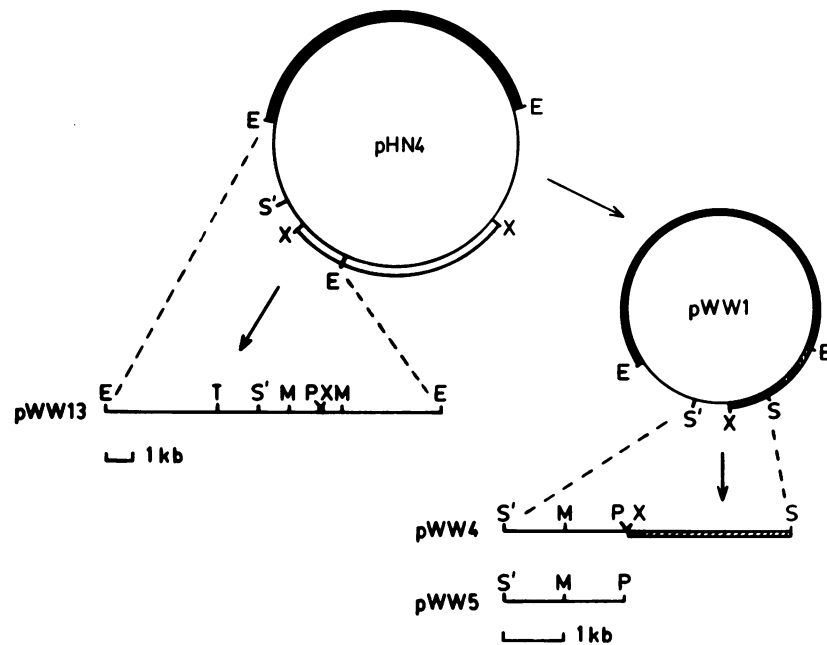


FIG. 4. Restriction maps of the *P. aeruginosa* PAO1 DNA inserts in the recombinant plasmids coding for protein F or the truncated protein F gene product. The thick black line represents vector DNA sequences (shown only for pHN4 and pWW1), the thin black line represents *P. aeruginosa* DNA, and the open box of pHN4 represents the DNA deleted in the construction of pWW1. The cross-hatched region of pWW1 and pWW4 represents DNA which is noncolinear on the *P. aeruginosa* chromosome. The insert sizes and vectors of the recombinant plasmids are as follows: pHN4, insert of approximately 30 kb in pLAFR1; pWW13, insert of 11 kb in pLAFR1; pWW1, insert of approximately 14 kb in pLAFR1; pWW4, insert of 4.7 kb in pCP13; pWW5, insert of 2 kb in pUC8. Plasmid pWW7 had the same insert as pWW5, but the vector used was pUC9 such that the insert was in the reverse orientation. Fragments are aligned such that transcription of the protein F gene is from left to right or counterclockwise. The restriction sites are indicated by one-letter codes as follows: A, *AccI*; E, *EcoRI*; M, *SmaI*; P, *PstI*; S, *Sall*; T, *SstI*; X, *XhoI*. A *Sall* site common to all of the inserts is indicated as S'. For ease of reading, not all restriction sites are shown. A 1.0-kb size reference marker is given for the linear fragments.

mid designated pWW1 (Fig. 4) showed a protein with an apparent molecular weight of 24,000 which reacted with MA5-8, but not with MA4-4 (Fig. 1, lane 4; Fig. 3A and B, lanes 4). This truncated protein F was not 2-mercaptoethanol modifiable (data not shown). Presumably, the region of the gene coding for the carboxy terminus of protein F was deleted when pHN4 was digested with *XhoI* (Fig. 4). A *Sall* fragment of pWW1 was ligated into the *Sall* site of pCP13 to generate plasmid pWW4 (Fig. 4). The truncated protein F product of *E. coli* HB101(pWW4) outer membranes had the same electrophoretic and immunoblot characteristics as those of *E. coli* HB101(pWW1) (Fig. 1, lanes 4 and 5; Fig. 3A, lanes 3 and 4).

A 2.0-kb *Sall-PstI* fragment from plasmid pWW4 was ligated into pUC8 and pUC9 (which have *lac* promoters in opposite orientations) to generate the plasmids pWW5 and pWW7, respectively (Fig. 4). *E. coli* TB1(pWW5) produced the truncated protein F fragment, whereas *E. coli* TB1(pWW7) did not. This determined the orientation of the gene (Fig. 4). The truncated protein produced by pWW5 had an apparent molecular weight of 24,000 and coelectrophoresed with the peptide produced by *E. coli* HB101(pWW1) and HB101(pWW4) (data not shown).

**DNA characterization.** The protein F subclones were mapped with restriction endonucleases. The 2.0-kb *Sall-PstI* fragment from pWW5 was radiolabeled by nick translation and used to probe the restriction digests to facilitate mapping of the larger inserts. Restriction maps of all fragments described above are shown in Fig. 4. The noncolinear regions of the inserts of pWW1 and pWW4 resulting from the ligation of the *XhoI* ends of pHN4 are indicated.

The  $^{32}\text{P}$ -labeled 2.0-kb probe was hybridized to digests of *P. aeruginosa* PAO1 genomic DNA. In digests of the genomic DNA with *BamHI*, *ClaI*, *EcoRI*, *HindIII*, *Sall*, *XbaI*, and *XhoI*, the probe hybridized to a single band (data not shown). From these data it was presumed that there was one copy of the gene for protein F in the *P. aeruginosa* PAO1 chromosome. This observation is consistent with the demonstration that the structural genes for the porin OmpF, OmpC, and PhoE of *E. coli* are present as single copies, despite the large amounts of these gene products that are synthesized (9, 28, 31).

**Functional characterization of protein F produced in *E. coli*.** Protein F was purified from *E. coli* JF733(pHN4) and from *P. aeruginosa* PAO1 by selective solubilization, followed by gel electrophoresis and electroelution. SDS-polyacrylamide gel analysis revealed that these preparations of protein F were free from outer membrane contaminants (data not shown). The identity of the purified protein F was confirmed by Western blotting with protein F-specific monoclonal antibodies and by demonstration of 2-mercaptoethanol modifiability on SDS-polyacrylamide gels. Addition of small amounts (0.6 ng/ml) of electroeluted protein F from either *E. coli* JF733(pHN4) (Fig. 5) or *P. aeruginosa* (data not shown) to the aqueous salt solutions (1 M KCl) bathing a black lipid bilayer membrane resulted in stepwise increases in conductance. By analogy with results of other lipid bilayer experiments, these conductance increments involved the stepwise incorporation of single channel-forming units of protein F into the membrane.

At the lowest concentrations used (0.6 ng/ml) and with the instrumentation set for high sensitivity, incorporation of

small channels was predominantly observed (Fig. 6A and B). The probability histograms of the measured conductance increments (Fig. 6) and the average single-channel conductance in 1 M KCl (0.34 to 0.38 nS) were similar for protein F isolated from either *P. aeruginosa* or the *E. coli* strain harboring the cloned intact protein F gene.

When larger concentrations (7 ng/ml) of the *E. coli*-derived protein F were used and the instrumentation was set for lower sensitivity (under which conditions the channels seen in Fig. 5 would not be measured), larger conductance steps were observed (data not shown). In one series of experiments, approximately 65% of the measured large channels had single channel conductances greater than 4 nS, a magnitude similar to that reported previously (3) for *P. aeruginosa* protein F.

As a control we examined purified, electroeluted *E. coli* OmpF porin. As previously reported (26), an average single-channel conductance of 2.1 nS was observed. Of over 600 measured single-channel events, no events smaller than 0.6 nS (cf Fig. 6) or larger than 4 nS were observed. Similar data were obtained previously for four other *E. coli* porins: OmpC, NmpC, PhoE, protein K (4).

### DISCUSSION

To investigate the influence of other outer membrane components on the function of porin channels, we cloned the structural gene for porin protein F of *P. aeruginosa* into *E. coli*. Members of the family *Enterobacteriaceae* have outer membranes which differ significantly with respect to LPS and protein constituents when compared with the outer membrane of *P. aeruginosa* (1, 9, 17).

Once the structural gene for protein F had been introduced into *E. coli*, it was of interest to investigate the expression of the gene product. Protein F was expressed in large amounts in the outer membrane of *E. coli*. The electrophoretic and immunologic characteristics of the cloned protein were identical to those of protein F in *P. aeruginosa*. This indicates that the signals within the *P. aeruginosa* gene and mRNA transcript for synthesis, assembly, and translocation to the outer membrane are recognized and functional in *E. coli*. It is interesting that in the porin-deficient mutant *E. coli* JF733(pHN4), the amount of protein F in the outer membrane appeared higher than that in the porin-sufficient *E. coli* HB101(pHN4) strain.

Restriction endonuclease mapping of the *P. aeruginosa* insert DNA is presented in Fig. 4. Presumably, the truncated protein F resulted from a deletion of the part of the gene which encoded the carboxy-terminal region of protein F, downstream from the *Xho*I site in plasmids pWW1 and pWW4. This is consistent with the orientation of the gene as

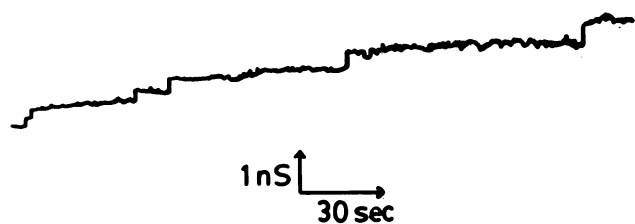


FIG. 5. Conductance increases after addition of 0.6 ng of purified porin protein F from *E. coli* JF733(pHN4) per ml to the aqueous 1 M KCl solutions bathing a lipid bilayer membrane. The applied voltage was 50 mV. The chart recorder tracing started on the left after the membrane had turned black. Each conductance step involved incorporation of a single porin channel into the membrane.

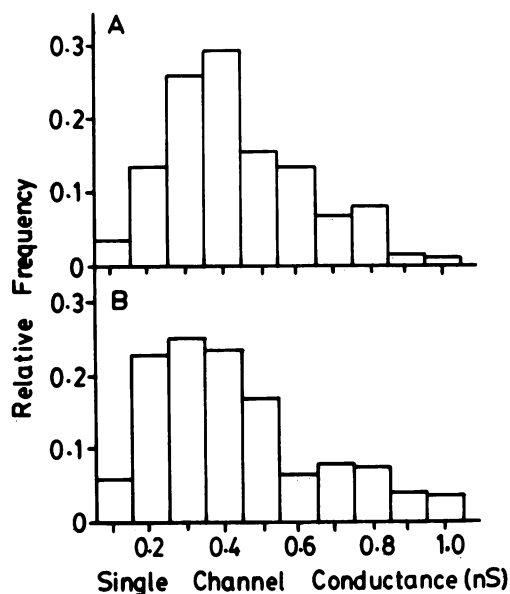


FIG. 6. Histograms of the conductance steps observed after the addition of purified porin protein F from *P. aeruginosa* PAO1 (A) or *E. coli* JF733(pHN4) (B) to the 1 M KCl solutions bathing a lipid bilayer membrane. Individual steps were measured from conductance recordings like that shown in Fig. 5. Based on the shape of the histograms, channels of less than 0.6 nS were chosen to measure the average single-channel conductances of 0.38 nS (*P. aeruginosa* protein F; average of 92 events) and 0.34 nS (*E. coli*-derived protein F; average of 197 events). Presumably, the other peak of conductance increases at about 0.7 to 0.8 nS represents two channels that are entering the membrane simultaneously.

determined by cloning of the pWW5 insert into plasmids pUC8 and pUC9. It is somewhat puzzling that plasmid pWW5 had approximately 150 fewer base pairs (coding capacity approximately 50 amino acids) of the putative protein F structural gene than did plasmids pWW1 and pWW4, because all three plasmids coded for a truncated protein of the same size (i.e., approximately 240 amino acids instead of the approximately 410 amino acids in the native protein F; based on the apparent molecular weights of the fully reduced products [Fig. 1, lane 5; Fig. 2, lane 4]). Nevertheless, a similar phenomenon has been observed previously in studies of the *E. coli* *ompA* gene (5) and was recently reported in a study of the *ompA* gene of *Serratia marcescens* cloned into *E. coli* (6). These researchers concluded that when the carboxy-terminal coding region of the *ompA* gene is deleted, the truncated gene product is proteolytically degraded to a minimum size of approximately 24,000 molecular weight before translocation to the outer membrane. It is possible that a similar degradation of protein F occurred in the *E. coli* clones with a truncation of the structural gene at the carboxy terminus.

Functional studies on electroeluted protein F from the *E. coli* clone (pHN4) and from *P. aeruginosa* revealed extremely similar properties (Fig. 6). However, the demonstration that the predominant channels had small conductances was surprising in light of previous model membrane data attesting to the existence of large protein F channels (3, 24, 25, 33). Furthermore, the large channel size previously observed in vitro was consistent with the ability of pentamethionine to support growth of *P. aeruginosa* (21) but not *E. coli* cells (2), given that the peptidases for

pentamethionine are found internal to the outer membrane (12).

To rationalize the existence of large channels in the *P. aeruginosa* outer membrane with its measured low outer membrane permeability (25) and consequent high intrinsic resistance to antibiotics (25, 32), it had been proposed that protein F is heterogeneous in that only a small proportion (less than 1%) of the approximately 200,000 protein F molecules in the outer membrane form large channels (3, 17). The majority of the available protein F was proposed to be nonfunctional, which is consistent with the poor activity of protein F in model membrane systems (3, 33). However, the data described here suggest that the majority of protein F channels are small (average single-channel conductance in 1 M KCl of 0.36 nS) rather than nonfunctional. Such a small channel may be unable to allow efficient passage of even small organic compounds like amino acids. These small single-channel events could not have been observed by Benz and Hancock (3), who reported the channel size of protein F to be 5.6 nS because at the resolution of the apparatus used to measure the large channels, a 0.36-nS channel could not be detected. In addition, the liposome techniques used in measuring *P. aeruginosa* outer membrane permeability (14, 15) are unable to measure very small channels, and because a substantial number of channels per liposome are required to examine the characteristics of these channels, the largest channel-forming protein present will determine the apparent exclusion limit of any porin preparation.

Therefore, our working hypothesis is that the larger channels are a result of genuine heterogeneity of channel sizes of individual porin F molecules. If this is true it would seem that we have cloned this heterogeneous channel-forming behavior, along with the protein F structural gene, into *E. coli*. Because the background of protein F in *E. coli* (i.e., the sort of molecules it interacts with during export to and insertion into the outer membrane) is different from protein F in *P. aeruginosa*, and because electroeluted and apparently uncontaminated protein F is functional, we feel that any heterogeneity in the function of protein F must be derived from intrinsic factors related to the mode of assembly of active protein F channel-forming units. With the availability of the cloned structural gene of protein F and the other technologies described here, we are now in a position to study these possibilities.

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