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Outer Membrane Protein H1 of *Pseudomonas aeruginosa*: Purification of the Protein and Cloning and Nucleotide Sequence of the Gene

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Overexpression of the divalent cation-regulated outer membrane protein H1 of *Pseudomonas aeruginosa* is associated with resistance to polymyxin B, aminoglycosides, and EDTA. Protein H1 is believed to act by replacing divalent cations at binding sites on lipopolysaccharide, thereby preventing disruption of the sites and subsequent self-promoted uptake of the antibiotics. Protein H1 purified by two cycles of anion-exchange chromatography was apparently associated with lipopolysaccharide. Lipopolysaccharide-free protein H1 was purified in high yield by preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis and was subjected to N-terminal amino acid sequencing. Complementary oligodeoxyribonucleotides were used to clone the structural gene for protein H1, *oprH*, into *Escherichia coli*. Successful cloning was confirmed by nucleotide sequence analysis. Southern hybridization suggested that *oprH* was present as a single-copy gene in *P. aeruginosa*. The deduced amino acid sequence revealed that H1 was a slightly basic polypeptide of 178 residues, with a leader sequence typical of an exported procaryotic protein. It had little similarity, however, to other bacterial surface proteins for which sequence data were available. No expression of protein H1, from its own or the *lac* promoter, was detected in *E. coli*. We concluded that, as for some other regulated *Pseudomonas* genes, expression of *oprH*, at least under some conditions, is blocked in *E. coli*.

The outer membranes of gram-negative bacteria function as permeability barriers between the external environment and the interior of the cell (26). Antibiotics are known to cross the outer membrane by diffusion through porins, if the drugs are small hydrophilic molecules, or (in wild types of certain species and mutants of others) by diffusion through the lipopolysaccharide (LPS)-phospholipid bilayer, if the drugs are hydrophobic (10). A third pathway, self-promoted uptake (10, 13, 24), has been described in *Pseudomonas aeruginosa* and may operate in other gram-negative species. The self-promoted pathway is apparently utilized by various polycationic antibiotics (e.g., polymyxin B and aminoglycosides) whose initial interaction with the cell is with LPS (26). LPS is a major constituent of gram-negative outer membranes and is stabilized by interactions between divalent cations (such as Mg^{2+}) and negatively charged groups in the lipid A and core regions of the LPS (26). These interactions can be disrupted by competitive displacement of divalent cations by polycations (e.g., polymyxin B) or by removal of divalent cations by chelators (e.g., EDTA). It has been proposed that this interaction with LPS is requisite for subsequent penetration of these antibiotics across the outer membrane (21). Because polycationic antibiotics are proposed to promote their own uptake across the outer membrane, this uptake has been termed self-promoted uptake. Several different types of experimental approaches have been used to test this hypothesis (13, 24, 25). Chapman and Georgopapadakou (4) have recently suggested that the quinolone antibiotic fleroxacin, which may act as a chelator of divalent cations, also passes across the outer membrane of *Escherichia coli* by the self-promoted uptake pathway.

The existence of the self-promoted uptake pathway was first postulated to explain the properties of *P. aeruginosa* cells that overproduced an outer membrane protein, H1 (24). Protein H1 is induced up to 24-fold under conditions of divalent cation depletion (24, 25). Under these conditions, it is the major protein of the outer membrane and probably of the entire cell (24). Protein H1 is also overproduced constitutively by the mutants H181 and H185 (24). Protein H1-overproducing cells, whether mutants or wild-type cells grown under inducing conditions, were found to be cross-resistant to polymyxin B, gentamicin and other aminoglycosides, and EDTA (13, 24). These cells also had altered kinetics of uptake of streptomycin (13) and decreased concentrations of Mg^{2+} in their cell envelopes (24). They were not, however, altered in susceptibility to other antibiotics or in the hydrophilic antibiotic permeation pathway across the outer membrane (25). It was therefore proposed that protein H1 would block self-promoted uptake by binding LPS at sites normally occupied by divalent cations. A protein stably anchored in the membrane would not be displaced readily by polycations and would thus block the penetration of the outer membrane by these compounds (10). Similarly, protein H1 would block the ability of EDTA to cause disruption of LPS-divalent cation interactions by chelation. If these interpretations are correct, the resistance properties of H1-overproducing cells suggested that self-promoted uptake was relevant to the lethal action of polymyxin B, aminoglycosides, and EDTA. These findings were of considerable interest in view of the medical importance and high intrinsic antibiotic resistance of *P. aeruginosa* (41).

We were interested in determining more precisely the general properties of protein H1 to see if these were consistent with its proposed role in blocking antibiotic uptake. In this study, protein H1 was purified and subjected to N-terminal amino acid sequencing, and the sequence data were used to design complementary oligonucleotides as probes for cloning the structural gene for H1, *oprH*. The gene (and protein) sequences of *oprH* and studies of its expression in

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E. coli are presented. In addition, we present some preliminary data on protein H1-LPS interaction.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. aeruginosa* prototroph PAO1 (24) was maintained on proteose peptone no. 2 agar (Difco Laboratories, Detroit, Mich.). *E. coli* JM101 [Δ(*lac pro*) *supE thi* F′ (*traD36 proAB lacI*^{q}ZΔM15)] was maintained on M9-glucose agar (20) containing 5 μg of thiamine hydrochloride per ml. *E. coli* DH5αF′ [F′ *endA1 recA1 hsdR17* ($r^- m^+$) *supE44 thi-1* λ^- *gyrA relA1* φ80d *lacZ*ΔM15 Δ(*lacZYA-argF*)U169] (Bethesda Research Laboratories, Inc., Burlington, Ontario, Canada) was maintained on LB agar (20). *E. coli* strains containing plasmids pUC18, pUC9, pUC8 (27, 38), pTZ18R, and pTZ18U (United States Biochemical Corp., Cleveland, Ohio) were grown in the presence of ampicillin sodium at 50 μg/ml (in LB) or 20 μg/ml (in M9-glucose).

SDS-PAGE. Analysis of protein profiles was performed as described previously (11) by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 14% polyacrylamide gels containing 85 mM NaCl to permit separation of proteins H1 and H2. In some experiments, samples were pretreated in 2% trichloroacetic acid and neutralized with Tris before solubilization in SDS and electrophoresis. Gels for analysis of LPS contained 15% polyacrylamide and 6 M urea and were silver stained for LPS by the method of Tsai and Frasch (37).

Purification of protein H1. A polymyxin-resistant, H1-overproducing mutant of PAO1, strain H181 (24), was grown in 1.5-liter cultures in BM2 succinate medium (5). Cultures were shaken vigorously at 37°C. Cells were harvested at an A_{600} of 0.7 to 1.0. They were treated with DNase I, broken in a French pressure cell (American Instrument Co., Silver Spring, Md.), and fractionated by sucrose gradient sedimentation as described previously (11). The outer membranes were solubilized twice in Triton X-100–Tris hydrochloride (pH 8.0) and twice in Triton X-100–Tris hydrochloride (pH 8.0)–EDTA as described previously (12). The fraction that was insoluble in Triton X-100–Tris hydrochloride but soluble in Triton X-100–Tris hydrochloride–EDTA was used as the starting material for purification by either anion-exchange chromatography or preparative SDS-PAGE.

Detergent-solubilized H181 outer membranes were fractionated on a DEAE-Sephacel (Pharmacia, Baie D'Urte, Quebec, Canada) column with 0.1% Triton X-100–20 mM Tris hydrochloride (pH 7.0)–10 mM EDTA as the running buffer. Proteins were eluted with a 0 to 0.3 M NaCl gradient in running buffer. The fractions rich in protein H1 were pooled, dialyzed against buffer, concentrated by dialysis against polyethylene glycol 20000, and applied to a second DEAE-Sephacel column, this time with 2% (rather than 0.1%) Triton X-100 in the column buffer. The early part of the void volume contained apparently pure H1, as judged by SDS-PAGE.

Detergent-solubilized outer membranes were also fractionated on 1.5-mm-thick SDS-polyacrylamide gels. Protein H1 bands were excised and electroeluted essentially as described by Parr et al. (29) except that glycine and methanol were omitted from the electroelution buffer.

Determination of N-terminal amino acid sequence and amino acid composition of H1. Protein H1 purified by SDS-PAGE was dialyzed extensively against 0.1% SDS in deionized water and subjected to N-terminal amino acid sequencing by S. Kielland (University of Victoria, Victoria, British Columbia, Canada). A spinning-up sequenator (model 890-C; Beckman Instruments, Inc., Palo Alto, Calif.) was used. Pure protein H1 for amino acid analysis was dialyzed extensively against deionized water and lyophilized. Amino acid analysis was also performed by S. Kielland by hydrolysis in 6 M HCl at 110°C for 24, 48, and 72 h, followed by application to a Beckman 119-CL automated amino acid analyzer.

Antiserum production, Western blotting (immunoblotting), and cross-linking. Polyclonal rabbit antiserum specific for Protein H1 was produced and purified as described previously (15). Western blotting was performed as described previously (22), using a current of 10 mA for transfer. Blots were developed as described previously (22), using anti-H1 serum at a 1/25 dilution. Cross-linking was performed on outer membranes as described by Reithmeier and Bragg (34), using diothiobis(succinimidyl propionate) (Pierce Chemical Co., Rockford, Ill.) or glutaraldehyde. Products were analyzed by one-dimensional SDS-PAGE and Western blotting. Whole-cell protein preparations for Western blotting were made as described previously (24), using overnight cultures.

Oligonucleotide synthesis. Oligodeoxyribonucleotides were synthesized on a 380A/B DNA synthesizer (Applied Biosystems, Foster City, Calif.) by T. Atkinson (University of British Columbia, Vancouver, British Columbia, Canada). Crude oligonucleotides were purified as described by Atkinson and Smith (1). The N-terminal amino acid sequence of protein H1 (see below) was used in the design of complementary oligodeoxyribonucleotides that were used as probes to identify the structural gene for protein H1, *oprH*. The codon usage established for two *P. aeruginosa* genes (9, 32) was used to limit the number of distinct oligonucleotide molecules in each pool. Oligonucleotides 1 consisted of 5′-AA^C_TAA^C_TATCCA^A_GAA$^{AACC}_{GTGG}$AA-3′, a mixture of 128 20-base-long molecules corresponding to the amino acid sequence NNIQKSK. Nucleotides A and T in positions 9 and 18 were found at <7.5% frequency in the two *P. aeruginosa* genes (9, 32) and were not included. Oligonucleotides 2, 5′-AACTTCGT^C_GGGCCT^C_GAC^C_GTGGGGCGA-3′, corresponded to a separate sequence of amino acids NFVGLT WGE. Oligonucleotides 2 were made longer (26 bases) and limited to eight oligonucleotide molecules, with all codons occurring in <10% of residues in the *P. aeruginosa* sequences eliminated (see references 2 and 18 for discussions of oligonucleotide design).

DNA techniques. All DNA techniques were carried out as described by Maniatis et al. (20) except for chromosomal DNA isolation (8), Southern blotting using Zeta-probe cationic nylon membrane (Bio-Rad Laboratories, Richmond, Calif.), ^{32}P end labeling of oligonucleotides (40), random hexamer ^{32}P labeling of double-stranded DNA (6) and subsequent purification by Elutip (Schleicher & Schuell, Inc., Keene, N.H.), fragment isolation and size fractionation on agarose gels with use of DEAE membranes (NA45; Schleicher & Schuell), quick-lysis plasmid preparation (14), and slot lysis agarose gel electrophoresis (35). Restriction and modification enzymes were obtained from various manufacturers and were used as recommended. Incorporation of [γ-^{32}P]ATP into oligonucleotides was measured by subjecting a small portion of the labeling mixture to descending chromatography on a strip of DE81 paper (Whatman, Inc., Clifton, N.J.) in 0.3 M ammonium formate (pH 8.0) and then dividing the strip horizontally and measuring Cerenkov radiation in a scintillation counter. Clones with inserts in pUC or pTZ vectors were distinguished from clones containing vector alone by using X-Gal (5-bromo-4-chloro-3-indoyl-

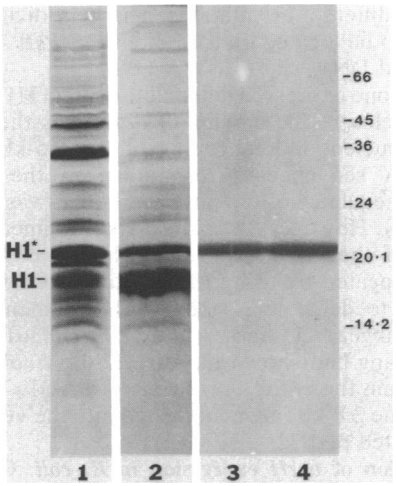

FIG. 1. Coomassie blue-stained SDS–14% polyacrylamide gel electrophoretogram of the stages in purification of protein H1. Lanes: 1, outer membrane of strain H181 (40 μg of protein); 2, Triton X-100–Tris hydrochloride-insoluble, Triton X-100–Tris hydrochloride–EDTA-soluble H181 outer membrane fraction (40 μg of protein); 3 and 4, protein H1 purified by preparative SDS-PAGE (12.5 μg of protein). The samples in lanes 1 to 3 were solubilized in SDS at 100°C; the sample in lane 4 was solubilized at 22°C. The heat-modified (H1*) and heat-unmodified (H1) bands of protein H1 are indicated on the left; running positions of molecular weight standards (in thousands) are shown on the right.

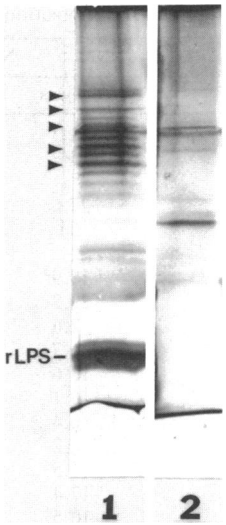

FIG. 2. SDS–15% polyacrylamide–urea gel electrophoretogram of purified protein H1 samples, silver stained for LPS. No protease treatment was included. Lanes: 1, protein H1 purified by ion-exchange chromatography (6 μg of protein); 2, protein H1 purified by preparative SDS-PAGE (12.5 μg of protein). Rough core LPS (rLPS) and O-antigen-containing LPS (arrows) are indicated on the left.

β-D-galactopyranoside; Clontech Laboratories, Palo Alto, Calif.).

DNA sequencing. Single-stranded DNA isolation and dideoxy-DNA sequencing reactions were performed as recommended by United States Biochemical Corp., using [^{35}S]dATP and Sequenase. Separation of products was performed on 6% polyacrylamide–7 M urea–Tris borate–EDTA buffer gradient gels (3), using either a Poker Face apparatus (Hoefer Scientific Instruments, San Francisco, Calif.) maintained at 65°C or a model S2 apparatus (Bethesda Research Laboratories).

DNA and protein sequence analyses. Sequences were analyzed with the aid of a computer program, SEQNCE version 2.2 (Delaney Software Ltd., Vancouver, British Columbia, Canada). The amino acid sequence of the mature protein was compared with those in a data bank of 7,396 sequences by using the FASTA program (30).

RESULTS

Purification and properties of outer membrane protein H1. Outer membranes prepared by sucrose gradient sedimentation from the protein H1-overproducing mutant of *P. aeruginosa* PAO1, H181 (25), contain H1 as the major protein (25; Fig. 1, lane 1). When solubilized at 100°C in SDS, protein H1 gives two bands on SDS-polyacrylamide gels: a heat-modified band (H1*) of apparent molecular weight 21,000 and a residual heat-unmodified band (H1) of 18,000 (11; Fig. 1). When the protein is solubilized at 22°C, only the heat-unmodified form appears (11). When the sample was pretreated with trichloroacetic acid before heating at 100°C in SDS, virtually all of the protein H1 appeared in the H1* position. The H181 outer membrane was solubilized in Triton X-100–EDTA to give a fraction enriched in protein H1 (Fig. 1, lane 2). This fraction was then further purified by preparative SDS-PAGE or by ion-exchange chromatography in DEAE-Sephacel. When the heat-modified (H1*) band was excised from the preparative SDS-polyacrylamide gel, the pure protein ran in the H1* position even when solubilized at 22°C, which indicated that the protein did not renature during the procedure (lanes 3 and 4). By contrast, the protein purified by ion-exchange chromatography was not denatured (data not shown). However, the yield of pure protein from SDS-PAGE (>50%) was more than 10-fold higher than the yield from chromatography.

The LPS compositions of the purified protein H1 samples are shown in Fig. 2. The protein purified by SDS-PAGE contained no detectable LPS (lane 2), but protein purified by chromatography contained substantial amounts of LPS (lane 1). The molar ratio of LPS to protein in the protein H1 purified by chromatography was estimated, by comparison with pure LPS run on the same gel, to be at least 1:1 (data not shown). The LPS contaminating the pure protein H1 contained a higher proportion of molecules with O side chains than of molecules lacking O side chains than did the bulk LPS of the outer membranes. These results suggested that protein H1 is associated with smooth LPS in outer membranes and that this association is disrupted by SDS-PAGE. Alternatively, the LPS may have copurified with H1 in the absence of any association. However, it should be noted that the bulk of LPS eluted from the column in fractions well separated from the fractions containing protein H1. In an attempt to resolve this question, purified PAO1 LPS was sonicated with pure, denatured H1, and the mixture was analyzed on SDS-PAGE to look for renaturation. No protein was observed in the renatured position (apparent molecular weight, 18,000). No cross-linking of H1 with LPS (or any other membrane constituent) could be observed by using either diothiobis(succinimidyl propionate) or glutaraldehyde (data not shown).

Protein H1 purified by SDS-PAGE (H1* band; Fig. 1, lanes 3 and 4) was used for N-terminal amino acid sequencing and amino acid analysis because of the high yield obtained with that method and the absence of LPS in the

TABLE 1. Amino acid composition of protein H1

Amino acid (1-letter code)	No. of residues	
	Analysis[a]	Sequence[b]
Alanine (A)	11.3	11
Arginine (R)	5.2	7
Asparagine (N)		17
Aspartate (D)	27.6[c]	10
Cysteine (C)	0	0
Glutamate (E)		7
Glutamine (Q)	18.9[d]	10
Glycine (G)	29.2	23
Histidine (H)	2.6	2
Isoleucine (I)	6.7	7
Leucine (L)	18.6	20
Lysine (K)	10.1	11
Methionine (M)	0.4	1
Phenylalanine (F)	6.2	7
Proline (P)	1.5	3
Serine (S)	17.4	16
Threonine (T)	10.5	11
Tryptophan (W)	1	2
Tyrosine (Y)	5.5	9
Valine (V)	5.5	4
Total	178.2	178

[a] Amino acid composition according to analyses performed on purified H1. The protein was assumed to contain 178 residues. Cysteine and tryptophan were not determined; for the purpose of calculation, the number of cysteine residues was assumed to be zero, and the number of tryptophan residues was assumed to be one (since one tryptophan residue was found in the N-terminal amino acid sequence). Numbers are median values of three separate assays rounded off to one decimal point.

[b] Amino acid composition according the sequence of the mature protein, derived from the nucleotide sequence (Fig. 3).

[c] Asparagine and aspartate residues combined.

[d] Glutamate and glutamine residues combined.

product. The sequence of the first 22 N-terminal amino acids was determined to be NH_2-ADNFVGLTWGETSNNIQK SKSL. The results of amino acid analyses of purified protein H1 are shown in Table 1.

Molecular cloning of *oprH*. We were unable to pick out the structural gene, *oprH*, for protein H1 by screening, with two radiolabeled oligonucleotides complementary to the N-terminal amino acid sequence (see above), a *P. aeruginosa* PAO1 gene library in the cosmid vector pLAFR1 (8). We therefore probed Southern blots of PAO1 chromosomal DNA that had been digested with various restriction endonucleases, singly and in combinations, with the oligonucleotides. This procedure allowed the identification of restriction fragments possessing DNA sequences complementary to the 5′ end of the *oprH* gene. In no case did both oligonucleotides 1 and 2 hybridize with more than one chromosomal fragment, which indicated that *oprH* was probably present as a single-copy gene (data not shown).

A 1.3-kilobase (kb) *Pst*I chromosomal fragment, large enough to contain the gene (estimated as 550 to 600 base pairs), hybridized strongly with both oligonucleotide probes on Southern blots. *Pst*I-digested chromosomal DNA was size fractionated, and the fraction around 1.3 kb was shown to contain the correct fragment by Southern blotting. This fraction was then ligated into *Pst*I-digested, dephosphorylated pUC18, and the products were transformed into *E. coli* JM101. Several hundred transformants were screened by colony filter hybridization, and positive clones were analyzed by agarose gel electrophoresis and Southern blotting of quick plasmid preparations. Two clones (plasmid designations pGB1 and pGB2) were found to contain the correct fragment in different orientations. The hybridizing sequence was found to be located on a 0.5-kb *Sma*I-*Pst*I subfragment of the cloned DNA.

Neither clone expressed detectable protein H1 on Western blots (see below). The cloning of *oprH* was therefore confirmed by nucleotide sequencing of the 0.5-kb *Sma*I-*Pst*I fragment. A sequence corresponding to the N-terminal amino acid sequence of the purified protein was found (Fig. 3 and below). However, the cloned *Pst*I fragment contained only part of the *oprH* gene. The cloning procedure was therefore repeated by using the 0.5-kb *Sma*I-*Pst*I fragment as a probe to detect a 2.8-kb *Eco*RI fragment in a size-restricted subgenomic library in *E. coli* DH5αF′. Chromosomal mapping had shown that the 2.8-kb *Eco*RI fragment should contain the entire *oprH* gene of PAO1. The plasmid containing the 2.8-kb fragment ligated into the vector pUC18 was designated pGB22.

Investigation of *oprH* expression in *E. coli*. Clones were tested for expression of protein H1 or truncated forms of protein H1 by SDS-PAGE and Western blotting of cell lysates and probing of blots with an H1-specific polyclonal antiserum. Lysates were made from cells grown in either LB broth with the p*lac* inducer isopropylthiogalactoside or in M9-glucose medium (with appropriate supplements) deficient in divalent cations (50 μm Mg^{2+} and 20 μM Ca^{2+}). The former medium induces expression from the *lac* promoter of pUC and pTZ vectors, and the latter was designed to induce expression from the promoter of *oprH* if possible. No expression was detected in either clone containing the 1.3-kb *Pst*I fragment in pUC18 (pGB1 and pGB2). The 1.3-kb fragment was cloned into both orientations of pUC9 (to give pGB3 and pGB4) and pUC8 (to give pGB5 and pGB6) to give fusion of the cloned DNA to the *lacZ′* gene in all three reading frames. The 0.5-kb *Sma*I fragment of pGB2 was subcloned into pTZ18R in both orientations (to give pGB11 and pGB12). No expression of products cross-reactive with protein H1 was observed in any of these clones despite the fact that in pGB11, *oprH* was connected to *lacZ′* by a 90-base-pair stretch of open reading frame (Fig. 3 and below).

When the entire *oprH* gene was cloned into pUC18 (pGB22), there was still no detectable expression of any polypeptides cross-reacting with protein H1 (data not shown). In pGB22, however, p*lac* and *oprH* were in opposite orientations, and no clones were obtained in which they were in the same orientation.

Nucleotide sequence analysis of *oprH*. Nucleotide sequencing of the DNA of the *oprH* region required both subcloning and design of two new oligonucleotides (based on preliminary sequence information) for use as primers. The nucleotide sequence from the *Sma*I site to close to a *Kpn*I site downstream of *oprH* was completed on both strands (Fig. 3). At position 91 there was an ATG codon that signaled the start of the *oprH* coding region. There was the coding sequence for 21 amino acid residues between the ATG and position 157, which was the start of the coding region for the mature protein, according to the predetermined N-terminal amino acid sequence. These 21 residues had the typical characteristics of a procaryotic signal sequence (33). From position 157, the open reading frame continued for a further 534 nucleotides, corresponding to 178 amino acid residues, before a nonsense codon TAA was reached. The first 22 residues of the amino acid sequence matched that determined by N-terminal sequencing of pure H1. The amino acid composition of the 178-residue polypeptide was virtually identical to that determined for purified protein H1 (Table 1)

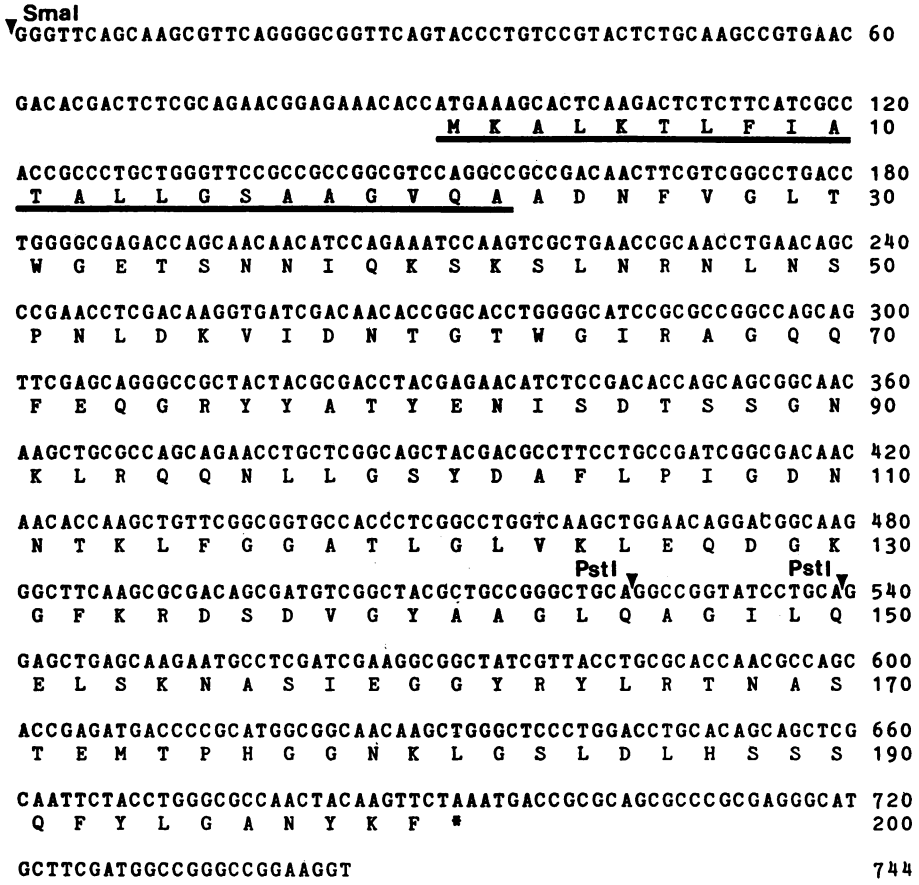

FIG. 3. Nucleotide sequence (upper line) of the *oprH* region and derived amino acid sequence (lower line) of protein H1. The presumed direction of transcription is from left to right. The putative leader (signal) sequence of the protein is underlined. Restriction sites are as indicated. Nucleotide 1 is in the middle of the *Sma*I site; amino acid 1 is the first residue of nascent protein H1. *, Stop codon.

The protein is expected to be slightly basic, since it contained 18 basic residues, 2 histidines, and 17 acidic residues. The positive charges were distributed fairly evenly over the protein except for a cluster around amino acid residue 132 (Fig. 3).

Computer-aided analysis of the amino acid sequence of the mature protein indicated that the protein was likely to form only 10% helical secondary structure (7) and had potential membrane-spanning hydrophobic regions (17). When the amino acid sequence was compared with those in a sequence data bank by using the FASTA program, there were no striking similarities to sequences of other outer membrane proteins whose sequences are known. The highest similarity score was found with hemolysin A of *E. coli*, which had 26.3% identity with *oprH* over a stretch of 95 amino acid residues (not shown).

The entire nucleotide sequence shown in Fig. 3 consisted of 63.0 mol% G+C, close to the value for the *P. aeruginosa* genome of 67.2% (28). The codon usage was very similar to that of other chromosomal genes of *P. aeruginosa* (excluding pilin genes; 39). The 90 base pairs upstream of the ATG start codon contained no sequences resembling the consensus −35 and −10 sequences of *E. coli* promoters (36). It was noted above that no expression of any polypeptide cross-reactive with H1 was detected in cells carrying plasmid pGB11, in which 90 upstream base pairs of open reading frame and the 5′ four-fifths of *oprH* were fused in frame to the *lacZ′* gene on pTZ18R. Between positions 148 and 179 (Fig. 3) there was the potential for stem-loop secondary structure formation in the RNA transcript, indicating a possible site for termination of transcription and consequently a potential attenuator mechanism. Sequence analysis showed that the *Pst*I site found in the middle of *oprH* was actually two *Pst*I sites 15 base pairs apart (Fig. 3).

DISCUSSION

We report here the purification to apparent homogeneity of outer membrane protein H1 of *P. aeruginosa*. The protein has been implicated in antibiotic resistance; specifically, its overproduction appears to block self-promoted uptake of polycationic antibodies and EDTA-Tris across the outer membrane (10, 13, 24). Protein H1 purified by ion-exchange chromatography was contaminated with at least an equal molar amount of LPS that contained a high proportion of O-antigen-containing molecules, which suggested an association between the two (Fig. 2). However, we were unable to confirm this association by cross-linking studies. In any case, proof of H1-LPS association per se in the outer membrane would be of little importance, since any outer membrane protein is likely to be associated with LPS. We need the tools to demonstrate specific interactions between positively charged residues (e.g., lysyl) in H1 and negatively charged groups (e.g., phosphoryl) in LPS. We were unable to test H1-LPS interactions by using dansyl-polymyxin, a fluorescent probe of polycation-binding sites on LPS (21),

because of the effect of detergent on the probe. However, certain mutations in PAO1 LPS have been shown to abolish protein H1-mediated resistance to polymyxin B (A. Bell, and R. E. W. Hancock, unpublished data). Molecular cloning of the gene for protein H1 should allow us to construct protein H1-deficient or altered mutants that can confirm the role of H1 in antibiotic resistance and indicate which parts of the molecule are important in binding to LPS.

Protein H1 purified by preparative SDS-PAGE was obtained in high yield and lacked LPS contamination (Fig. 2). The absence of LPS was also observed for other proteins purified by the same method (29), although other workers (31) have reported that some protein bands on SDS-polyacrylamide gels are complexes of protein and LPS. N-terminal amino acid sequencing of the protein purified in this manner allowed us to design complementary oligonucleotides that were used to identify and clone a chromosomal DNA fragment containing *oprH* DNA. There appeared to be a block on expression of *oprH* in *E. coli*, perhaps because the promoter of *oprH*, like some other regulated *Pseudomonas* promoters (23), is nonfunctional in *E. coli* or is not present on the cloned fragment. No β-galactosidase–H1 fusion products were detected when four-fifths of *oprH* plus 90 upstream base pairs were in frame with the *lacZ'* gene of pUC18 and under control of the *lac* promoter. This raises the possibility of some regulatory signal on the cloned DNA. An inverted repeat structure that straddled part of the *oprH* gene encoding the amino terminus of the protein was identified. Nevertheless, we are undertaking cloning studies in expression vectors before ruling out the possibility of expression of *oprH* in *E. coli*.

Nucleotide sequence data indicated that protein H1 was a slightly basic polypeptide of 178 residues, with a leader sequence typical of exported procaryotic proteins. The sequence obtained matched well with the N-terminal sequence and amino acid composition determined for the pure protein. The predicted secondary structure had little helical content, as also observed for many other outer membrane proteins (26). However, comparison of the protein H1 sequence with that of other sequenced proteins shed little light on possible functions. Furthermore, most outer membrane proteins so far characterized are acidic (19). Our working hypothesis is that the function of H1 is to stabilize the polyanionic cell surface LPS in conditions of divalent cation depletion, which would be in agreement with its basic nature. Such conditions would probably not occur in the human body (15) but may be significant in aqueous environments. Other factors, such as growth temperature (16), have been shown to affect H1 expression to some degree, but it is not known how these act. Further light will no doubt be shed on the topic of function when a protein H1-deficient mutant has been constructed and characterized. We are now undertaking such a study.

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