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Pseudomonas aeruginosa Outer Membrane Lipoprotein I Gene: Molecular Cloning, Sequence, and Expression in *Escherichia coli*

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Lipoprotein I (OprI) is one of the major proteins of the outer membrane of *Pseudomonas aeruginosa*. Like porin protein F (OprF), it is a vaccine candidate because it antigenically cross-reacts with all serotype strains of the International Antigenic Typing Scheme. Since lipoprotein I was expressed in *Escherichia coli* under the control of its own promoter, we were able to isolate the gene by screening a λ EMBL3 phage library with a mouse monoclonal antibody directed against lipoprotein I. The monocistronic OprI mRNA encodes a precursor protein of 83 amino acid residues including a signal peptide of 19 residues. The mature protein has a molecular weight of 6,950, not including bound glycerol and lipid. Although the amino acid sequences of protein I of *P. aeruginosa* and Braun's lipoprotein of *E. coli* differ considerably (only 30.1% identical amino acid residues), the sequences at the signal peptidase cleavage site and at the C terminus, which is the attachment site to peptidoglycan in *E. coli*, are identical. Using lipoprotein I expressed in *E. coli*, it can now be tested whether this protein alone, without *P. aeruginosa* lipopolysaccharide contaminations, has a protective effect against *P. aeruginosa* infections.

Pseudomonas aeruginosa continues to be a relevant pathogen for humans because of its lack of susceptibility to antibiotic treatment. This property results from its high intrinsic, as well as emerging, resistance to antibiotics (20). Nosocomial pneumonias, septicemias in patients with burn wounds, hospital-acquired urinary tract infections, and eye and ear infections are frequently caused by *P. aeruginosa* (42).

Much effort has therefore been invested in the design of vaccines which can protect high-risk groups from *P. aeruginosa* infections. One group of potential vaccine candidates is based on polysaccharide components that *P. aeruginosa* presents to its host (9, 40, 50). The outer membrane proteins are a second group because there is a limited number of abundant proteins, three of which, porin protein F (OprF), lipoprotein I (OprI), and protein H2 (OprH2), cross-react immunologically between the different serotypes of *P. aeruginosa* (35). Furthermore, protection of mice against *P. aeruginosa* was obtained after administering either a mixture of outer membrane proteins (46) or a purified OprF preparation (18, 29). Recently the gene encoding porin protein F (OprF) has been cloned and characterized in detail (13, 49).

In the present study we report the cloning and characterization of the gene coding for *P. aeruginosa* lipoprotein I (OprI). Its counterpart in *Escherichia coli*, Braun's lipoprotein, has the probable function of a physical link between the outer membrane and the peptidoglycan layer (16). Both proteins are small outer membrane proteins containing covalently bound fatty acids (21, 32), but they differ considerably in their amino acid compositions and their fatty acid contents (32). Here we show for *P. aeruginosa* lipoprotein I that the deduced amino acid sequence of 83 amino acid residues (including the leader peptide) differs considerably from the sequence of Braun's lipoprotein. Some important features, however, are identical, like the signal peptidase recognition sequence and the two last C-terminal residues, which in *E. coli* provide the link to the peptidoglycan layer.

The cloning and expression of *P. aeruginosa* outer membrane lipoprotein I (OprI) in *E. coli* will be the prerequisite for testing its immunogenic and protective capacities against *P. aeruginosa* infections.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. aeruginosa* serotype 12 (International Antigenic Typing Scheme; ATCC 33359) was a clinical isolate from the tracheal secretions of a pneumonia patient. *P. aeruginosa* serotype 6 was obtained from the American Type Culture Collection, Rockville, Md. (ATCC 33354). *E. coli* NM539 (*supF hsdR* [P2 *cox*3]) was used as host (15) for plating of the genomic library, and *E. coli* JM109 [*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* λ^- Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q} ZΔM15)] (53) was used for subcloning in plasmids. *P. aeruginosa* and *E. coli* JM109 were grown in LB broth. Lambda phage was grown on Trypticase agar plates in Trypticase top agarose (BBL Microbiology Systems, Cockeysville, Md.). For large-scale phage preparations, *E. coli* NM539 cells were grown in NZ medium (27).

Isolation of monoclonal antibodies. The isolation of monoclonal antibodies has been described recently (28). Outer membrane proteins were extracted from *P. aeruginosa* serotype 12 cells by the method of Mizuno and Kageyama (33) and used for immunization of mice.

Monoclonal antibodies were produced by standard methods. One of these monoclonal antibodies, 6A4, was specific for lipoprotein I and was used in this study.

Screening of a genomic library of *P. aeruginosa* DNA for lipoprotein I sequences. The construction of the *P. aeruginosa* (serotype 6) genomic library in λ EMBL3 phage has been described previously (13).

Recombinant phage were plated at a density of 400 PFU per 9-cm petri dish. Immunodetection of *P. aeruginosa*

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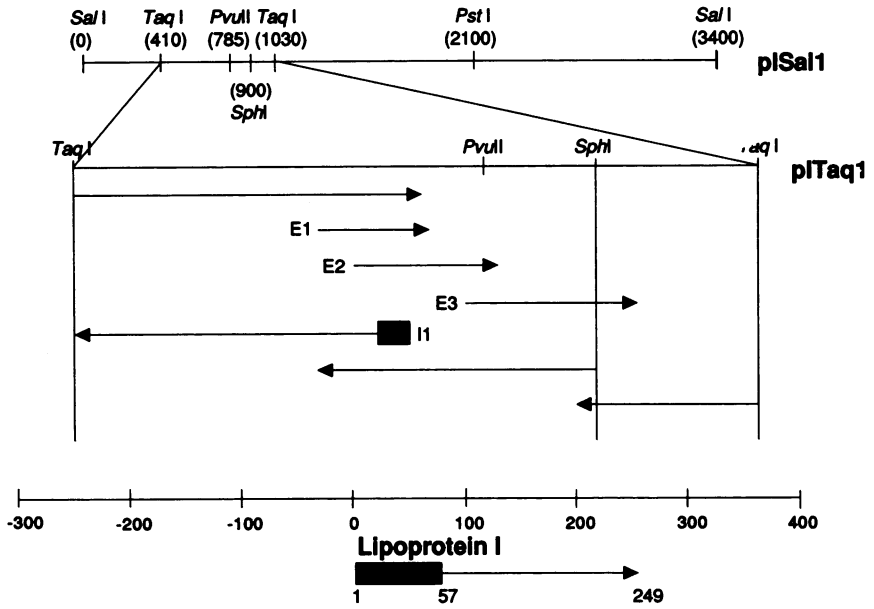

FIG. 1. Physical map of the 3.4-kb *Sal*I fragment (pISal1) containing the *P. aeruginosa* lipoprotein I gene. Sequencing strategy used for subclone pITaq1, the smallest subclone that contains the complete gene; exonuclease III deletion clones of pITaq1 (E1, E2, E3); and an oligonucleotide primer (black square, I1) for sequence analysis. The protein-coding region is indicated at the bottom by a horizontal arrow; the signal peptide-coding region is drawn as a solid bar.

lipoprotein I-positive plaques was carried out by a modification of the method of Young and Davis (54). Plaques were lifted on nitrocellulose filters (Schleicher and Schüll, Dassel, Federal Republic of Germany) for 6 h at 37°C (27). Filters were then blocked with 20% newborn calf serum (GIBCO, Eggenstein, Federal Republic of Germany) in Tris-buffered saline (TBS; 50 mM Tris hydrochloride, 150 mM NaCl, pH 8.0) for 4 h at room temperature. Filters were then incubated overnight with 20% newborn calf serum in TBS containing 1% (vol/vol) ascitic fluid with anti-lipoprotein I antibody 6A4. Filters were washed at room temperature for 5 min in TBS, for 15 min in TBS containing 0.1% (vol/vol) Nonidet P-40 (Sigma, Deisenhofen, Federal Republic of Germany), and again for 5 min in TBS. The bound antibodies were reacted with a 1:1,000 dilution of alkaline phosphatase-coupled rabbit anti-mouse antibodies (Dakopatts, Hamburg, Federal Republic of Germany) in 20% newborn calf serum in TBS for 2 h at room temperature. After washing as described above, positive plaques could be made visible by an alkaline phosphatase color test (2) using 5-bromo-4-chloro-3-indolylphosphate (Boehringer, Mannheim, Federal Republic of Germany) and Nitrotetrazolium blue dye (Sigma). Positive plaques were picked, and DNA was prepared by standard methods (27).

Subcloning procedures. Subclones of the positive λ EMBL3 clone were constructed in the plasmids pBR322 (3) and pUC19 (53). For the identification of lipoprotein I-producing transformants, a colony immunoscreening procedure was used (31). Transformants were streaked out in duplicate on nitrocellulose filters that had been placed on top of LB plates with ampicillin. To lyse bacteria, one of the duplicate filters was placed three times for 2 min each on a Whatman 3MM (Maidstone, Great Britain) paper saturated with a lysozyme solution (5 mg/ml in H_2O), then three times for 2 min each on 0.5% *N,N*-dimethyl-dodecyl-amine-*N*-oxide–10 mM Tris hydrochloride (pH 8.0). The filters with the lysed colonies were then treated in the same way as the filters after plaque lifting. Times for blocking and reaction with the first or second antibody could be reduced to 1 h.

Nucleotide sequence analysis. DNA of different subclones was sequenced by the chain termination method of Sanger et al. (43) as modified for supercoiled plasmids (5). The insert of the clone pITaq1 was shortened from one end by the combined action of exonucleases III and VII (53) and sequenced with standard M13 primers. One specific oligodeoxynucleotide primer, I1 (see Fig. 1 and below), was constructed in order to complete the sequence of the second strand.

Mapping of the transcription start site. RNA was extracted from *P. aeruginosa* cells by the hot phenol method (1). For Northern (RNA) blots, RNA was denatured by glyoxal treatment, separated on agarose gels, and blotted as described by Thomas (44). The 5′ end of the lipoprotein I mRNA was mapped by the S1 protection method (47) as described previously for the porin F mRNA (13). Alternatively, a primer extension reaction using reverse transcriptase was carried out (12). Either 20 or 40 μg of total *P. aeruginosa* RNA was used. cDNA synthesis was primed with the oligodeoxynucleotide I1 (5′-CAGAACAGCAGC CAGAG-3′), which is complementary to the OprI DNA sequence between bases 32 and 48 close to the 5′ end of the coding region (see Fig. 1 and 2) and stopped at the 5′ end of the RNA. Radioactivity was incorporated into the cDNA by the addition of 10 μCi of [α-^{32}P]dCTP (Amersham, Brunswick, Federal Republic of Germany). The RNA was hydrolyzed by alkali treatment, and after neutralization, the cDNA was analyzed on a sequencing gel together with a Sanger dideoxy sequencing reaction on plasmid DNA (0.9-kilobase [kb] *Sph*I-*Sal*I fragment) using the same primer.

Preparation, electrophoresis, and immunoblotting of bacterial membranes. *E. coli* cells, carrying plasmids coding for lipoprotein I, and *P. aeruginosa* cells were broken and separated into a "soluble fraction" and a "membrane fraction" by an adaptation of the method of Mizuno and

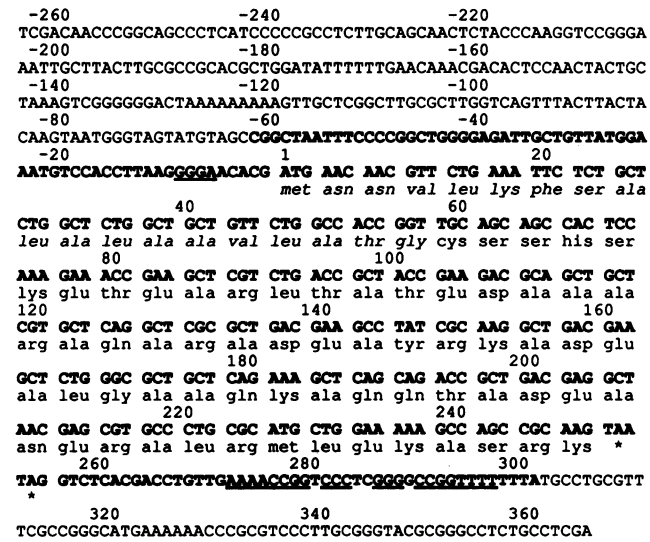

FIG. 2. Nucleotide sequence of the *P. aeruginosa* lipoprotein I gene. The transcribed region is indicated by boldface letters. The putative Shine-Dalgarno sequence, GGGA, and the putative rho-independent terminator region are doubly underlined. The signal peptide is indicated by italics.

Kageyama (33). Cells (400 ml) were harvested at late log phase by centrifugation at 4,000 × *g* for 10 min at 4°C, washed once in buffer I (50 mM glucose, 25 mM Tris hydrochloride, 10 mM EDTA, pH 8.0), and suspended in 30 ml of the same buffer. The cells were then broken by two passages through a French press (Aminco, SLM Instruments Inc., Urbana, Ill.). The French press homogenate was centrifuged for 60 min at 100,000 × *g* and 4°C, and the supernatant was kept as the soluble fraction. The precipitate was suspended in 12 ml of buffer I, 1% (vol/vol) Triton X-100 was added, and the membrane fraction was solubilized by shaking for 60 min at 37°C. Insoluble material was precipitated by 10 min of centrifugation at 12,000 rpm in Eppendorf tubes in a Sigma centrifuge.

The proteins in the soluble and membrane fractions were separated by electrophoresis on 15% polyacrylamide gels (14). After electrophoresis, proteins were stained with Coomassie blue R250 (Serva, Westbury, N.Y.). Alternatively, for immunoblotting (45), proteins were electrophoretically transferred to nitrocellulose membranes (Sartorius, Göttingen, Federal Republic of Germany) in a semidry blotting apparatus (Orpegen, Heidelberg, Federal Republic of Germany) (26). The immunodetection of lipoprotein I bands was done with the 6A4 monoclonal antibodies and an alkaline phosphatase color reaction as described above for the colony screening procedure.

Computer analysis. Analysis of DNA sequences was carried out on a Micro VAX computer (Digital Equipment Corp.) with the SEQED, MAP, and TERMINATOR (4) programs of the University of Wisconsin Genetics Computer Group program package version 4 and the RELATE (10) program of the Protein Identification Resource (17) program package.

RESULTS

Mouse monoclonal antibodies to *P. aeruginosa* outer membrane proteins were raised as described (28). One of them, antibody 6A4, was specific for *P. aeruginosa* lipoprotein I (OprI) and did not cross-react with *E. coli* (results not shown; compare reference 19). It was used in the present study to identify the gene in a λ EMBL3 library. The same antibody has shown protective activity against *P. aeruginosa* infection in a mouse model (A. Eckhardt, M. M. Heiss, W. Ehret, W. Permanetter, and B.-U. von Specht, manuscript in preparation). Furthermore, its primary structure has been determined by cDNA cloning and DNA sequence analysis (28) with the aim of expression of the recombinant protein in different systems.

Isolation of λ clones that express *P. aeruginosa* lipoprotein I. Previous experience with the cloning of *P. aeruginosa* porin protein F had shown that this protein is expressed in *E. coli* under the control of the *P. aeruginosa* promoter (13, 49). Since lipoprotein I (OprI) is also an abundant outer membrane protein (32), it was assumed that it might also be expressed in *E. coli* under the control of its own promoter, thus allowing the screening of the genomic library of *P. aeruginosa* serotype 6 DNA in λ EMBL3 phage directly with the protein I antibody 6A4.

Approximately 15 genome equivalents (3,600 phage) were plated. Plaques were lifted on nitrocellulose membranes for 6 h at 37°C to accumulate enough gene product. Filters were blocked and then reacted with the monoclonal antibody 6A4 specific for protein I. The bound antibodies were visualized with an alkaline phosphatase color test.

Fourteen positive signals were obtained, six positively reacting phage, λI1 to λI6, were selected and isolated, and DNA was prepared from liquid cultures.

First we tested whether the positively reacting phage expressed a protein of the correct size. For a control, protein I was isolated from a liquid culture of *P. aeruginosa* according to Mizuno and Kageyama (32). Plate lysates of four positively reacting phage were prepared (27), separated by polyacrylamide gel electrophoresis, electrophoretically blotted, and reacted with the lipoprotein I antibody. The protein expressed in all four of the λ EMBL3 lysates had the same apparent molecular weight as the authentic protein I of *P. aeruginosa* (for the lysate preparation of phage λI5, see Fig. 4, lanes 1; the other results are not shown). These results demonstrated that the positive λ clones all contained the complete lipoprotein I gene.

Subcloning of the *P. aeruginosa* lipoprotein I gene. Since no amino acid sequence information about *P. aeruginosa* lipoprotein I was available which would have allowed the construction and use of specific oligodeoxynucleotide probes, it was necessary to continue the immunoscreening procedure for subclones of the recombinant λ EMBL3 phage containing the protein I gene.

The first approach taken was to subclone *Sal*I fragments of the phage λI5 into pBR322. This vector was chosen because it has a lower copy number than the pUC vectors, and the possible toxic effect of the *P. aeruginosa* lipoprotein I on *E. coli* should have been reduced. Cleavage of the λI5 DNA with *Sal*I resulted in fragments of 8.0, 3.7, 3.4, 3.0, 1.5, and 0.6 kb plus the λ arms. One pBR322 subclone, pISal1, that reacted positively contained the 3.4-kb *Sal*I fragment of λI5. No adverse effect on *E. coli* transformed with this plasmid was observed; therefore, further subcloning was done in pUC19 (see Fig. 1). The 3.4-kb fragment was divided into 2.1- and 1.3-kb *Sal*I-*Pst*I pUC19 subclones, the larger of which still reacted positively with the antibody. This fragment could be further divided into a positively reacting *Sph*I-*Sal*I fragment of 0.9 kb and an *Sph*I-*Pst*I fragment of 1.2 kb. Finally, a 625-base-pair *Taq*I fragment could be

subcloned that overlaps the *Sph*I site and contains the whole gene (subclone pITaq1).

DNA sequence analysis. From the amino acid composition data of Mizuno and Kageyama (32) it was known that the *P. aeruginosa* lipoprotein I contains no proline, valine, isoleucine, phenylalanine, or tryptophan. Therefore it was easy to identify potential protein I coding sequences. The 0.9-kb *Sph*I-*Sal*I fragment was sequenced from both sides. The sequence next to the *Sph*I site was devoid of codons for the five amino acids not present in protein I. The open reading frame starts 228 nucleotides away from the *Sph*I site and contains the major N-terminal portion of the protein I gene. Sequence analysis of the adjacent *Sph*I-*Pst*I fragment cloned in pUC19 showed that the seven C-terminal amino acid residues are encoded in this fragment. The complete sequence of the lipoprotein I gene (Fig. 2) is contained in the 625-base-pair *Taq*I subclone pITaq1, both strands of which were sequenced (Fig. 1). The sequence was assembled from the sequences of several subclones as well as three exonuclease III deletion clones. The last part of the sequence of the second strand was confirmed using the primer extension oligonucleotide I1 (see below and Fig. 1).

The open reading frame most likely codes for lipoprotein I for the following reasons. The polypeptide encoded is small; the N terminus contains a typical procaryotic signal sequence for transport across the cytoplasmic membrane with a positively charged lysine residue close to the initiator methionine and a central portion of nine hydrophobic amino acid residues (39). Two lines of evidence indicate that the signal sequence has a length of 19 amino acid residues and that the N-terminal amino acid residue of the mature protein I is the cysteine in position 20. First, the cysteine is contained in the sequence Gly-Cys-Ser-Ser, which is identical in the *E. coli* lipoprotein, the cysteine being the N-terminal amino acid residue (24). This residue is also the one modified with glycerol and fatty acid residues in *E. coli* (21). In *P. aeruginosa* the amino acid composition analysis had exhibited a peak, X, which was thought to possibly be glyceryl cysteine (32); this would correspond to cysteine also being the N-terminal amino acid in the mature *P. aeruginosa* lipoprotein. Furthermore, the open reading frame yielded a sequence with a highly nonrandom amino acid composition for the mature protein of $Ala_{18}Arg_7Asn_1Asp_4Cys_1Gln_4Glu_8Gly_1His_1Leu_4Lys_5Met_1Ser_4Thr_4Tyr_1$, which is in complete agreement with the published amino acid composition. The molecular weight of the unmodified mature protein of 64 amino acid residues was calculated to be 6,950.

Transcription initiation and termination sites. The transcriptional start site of the *P. aeruginosa* lipoprotein I gene was mapped by primer extension with reverse transcriptase. The primer was the oligodeoxynucleotide II, which is complementary to the sequence between bases 32 and 48 of the lipoprotein I gene (see Fig. 2). The template was *P. aeruginosa* RNA (20 or 40 μg). The product of this reaction was analyzed on a sequencing gel, together with four sequencing reactions of the clone pITaq1, with the same primer. The cDNA comigrated with a G in position −62 from the initiator methionine codon (Fig. 3B), which would correspond to C-62 being the starting nucleotide. There was also a neighboring minor band that migrated with the C in position −61.

The results of the primer extension analysis were checked by an S1 protection experiment. A 373-base-pair *Taq*I-*Pvu*II fragment which was 5′-end labeled with [γ-^{32}P]ATP at the *Pvu*II site was generated from pITaq1 (see Fig. 1). This fragment was hybridized to *P. aeruginosa* RNA at 50 and 55°C in 80% formamide–0.4 M NaCl–40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)]–1 mM EDTA (pH 6.5). A fragment of 171 ± 3 bases was the main protected fragment (Fig. 3A). The transcriptional start site was calculated at position −59 ± 3 as counted from the initiator methionine. This result agrees well with the primer extension experiment.

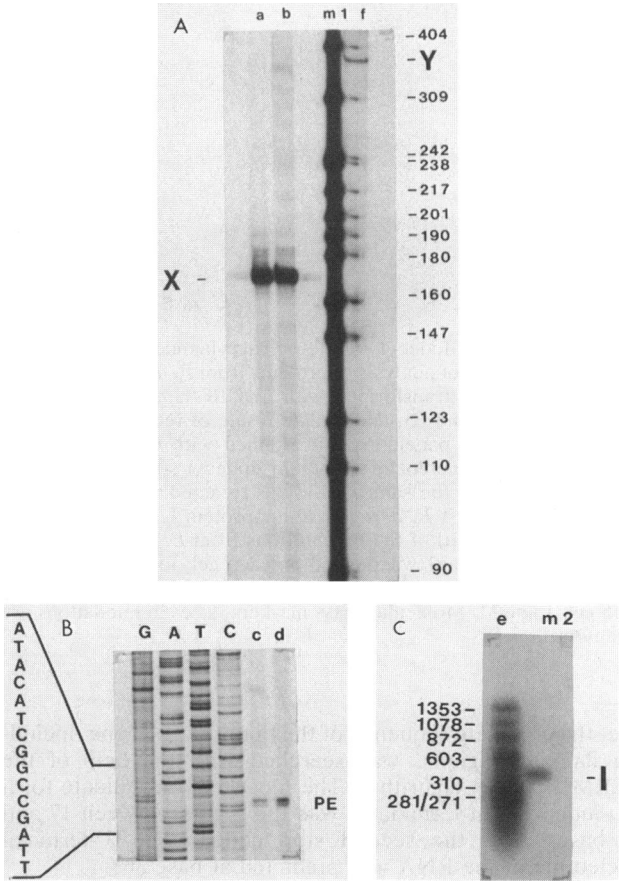

FIG. 3. (A) Mapping of the transcription start site by S1 nuclease protection. Lanes a and b, Reaction mixtures after hybridization at 50 and 55°C, respectively, and S1 nuclease digestion (X indicates the main protected fragment of 171 ± 3 bases, and Y marks the position of the signal from the 373-base-pair *Taq*I-*Pvu*II fragment); lane ml, size marker (pBR322 cut with *Hpa*II); lane f, sample of the 373-base-pair *Taq*I-*Pvu*II fragment. (B) Primer extension analysis. Lanes c and d, Primer extension reactions of *P. aeruginosa* RNA (20 and 40 μg, respectively) primed with the N-terminal lipoprotein I oligonucleotide probe. PE indicates the primer extension products. For the sequencing reactions in the first four lanes, the same oligonucleotide probe was used as sequencing primer. (C) Northern blot of *P. aeruginosa* RNA. Lane e, Hybridization of electrophoretically separated *P. aeruginosa* RNA to the N-terminal lipoprotein I-specific oligonucleotide probe; lane m2, size marker (φX174 DNA cut with *Hae*III). I, Lipoprotein I RNA signal.

With the knowledge of the transcriptional start site, the upstream sequences were searched for a similarity to *E. coli* promoter consensus sequences. In the −35 region, the sequence TTGGTC is homologous to the *E. coli* consensus sequence TTGaca; in the −10 region, the sequence TAG TAT is homologous to the *E. coli* consensus sequence TAtaaT (30). Thus the promoter elements of the lipoprotein I gene of *P. aeruginosa* contain all six highly conserved bases of the two *E. coli* consensus hexamer elements plus one of the weakly conserved bases.

To determine the most likely transcription termination

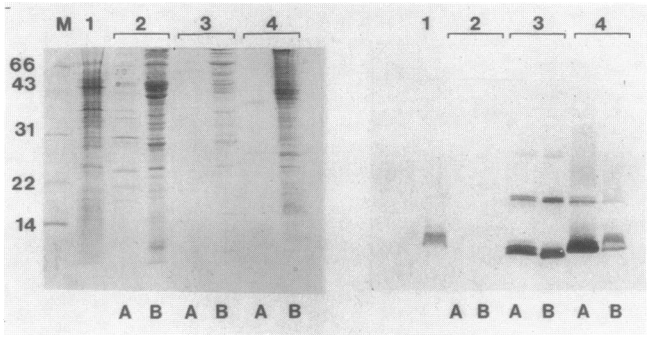

FIG. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblot analysis of proteins from *P. aeruginosa* (lanes 4A and B), *E. coli* transformed with pUC18 (lanes 2A and B) or pITaq1 (lanes 3A and B), and a plate lysate of recombinant phage λI5 (lanes 1). Left panel, Proteins stained with Coomassie blue. Right panel, Immunoblot of a sodium dodecyl sulfate-polyacrylamide gel run under the same conditions, reacted with monoclonal antibody 6A4 against *P. aeruginosa* lipoprotein I. Lanes A, Membrane fractions: 25 μl of the preparations from *E. coli* transformed with pUC18 or pITaq1 was loaded on each gel; for *P. aeruginosa*, 1.5 μl was loaded. Lanes B, Soluble fractions: 25 μl was loaded on each gel. Lane M, Molecular mass markers; sizes in kilodaltons are given on the left.

site, the complete sequence of the lipoprotein I gene, including flanking regions, was searched with the help of the TERMINATOR algorithm. The most likely candidate for a rho-independent terminator was the region between 17 and 42 bases after the second stop codon. The 3′-terminal nucleotide of the RNA was predicted at base 301.

The results of 5′-end mapping experiments, together with the prediction of the 3′ end, yielded a length of 363 bases for the lipoprotein I mRNA. As a control, a Northern blot of *P. aeruginosa* RNA was hybridized with the ^{32}P-labeled oligodeoxynucleotide I1, which was also used for the primer extension reaction (Fig. 3C). Only one signal was obtained, which corresponds to an RNA of 400 ± 50 bases. This supports the previous results.

Expression of the *P. aeruginosa* lipoprotein I in *E. coli*. The proteins which were expressed in *E. coli* cells harboring the plasmid pITaq1 were analyzed for lipoprotein I by polyacrylamide gel electrophoresis and immunoblotting (Fig. 4). As a negative control, *E. coli* cells transformed with pUC18, and as a positive control, *P. aeruginosa* cells were grown. A membrane fraction and a soluble fraction were obtained from each of the three bacterial cultures as described. For the *E. coli*(pITaq1) preparation, the immunoblot shows a strong reaction of the lipoprotein I antibody with a band of the correct size. No signal could be detected in the case of *E. coli*(pUC18) (control plasmid). Both membrane and soluble fractions contained lipoprotein I. In the case of lipoprotein I expressed in *E. coli*, a 2.5-fold larger sample of the membrane fraction was loaded; therefore, the larger part of protein I is contained in the soluble fraction. This was different for the preparations form *P. aeruginosa* cells, where only a very small portion of the membrane fraction was loaded. Therefore the major part of the lipoprotein I was observed in the membrane fraction. The phage lysate from clone λI5 (Fig. 4, lanes 1) also contained material that reacted with the protein I antibody. The bands of higher molecular weight that reacted with antilipoprotein antibody in preparations from *P. aeruginosa* and *E. coli*(pITaq1) possibly correspond to multimeric forms of lipoprotein I.

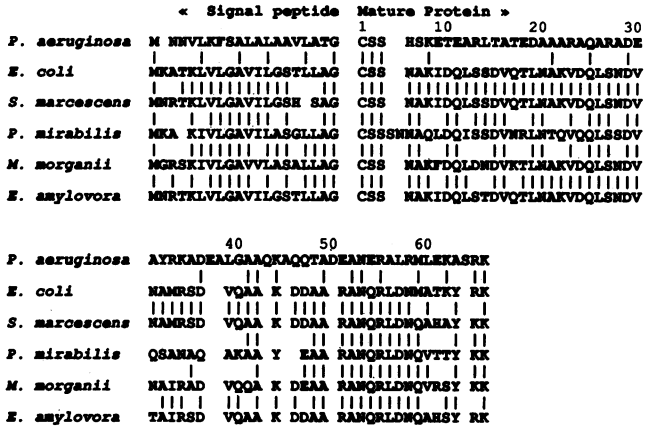

FIG. 5. Homology of *P. aeruginosa* lipoprotein I to the lipoproteins of the enterobacteria *E. coli* (36), *S. marcescens* (37), *P. mirabilis* (7), *M. morganii* (22), and *E. amylovora* (51). The numbering starts with the N-terminal cysteine residues of the mature proteins. Only identical amino acids are connected by vertical lines.

This would be analogous to the case of the *E. coli* lipoprotein, where trimeric forms were observed (8).

Relationship of *P. aeruginosa* lipoprotein I to the lipoproteins of other bacteria. The Max Planck Institute Protein Sequence Database (MIPSX; release 10.0, Nov. 1988; F. Pfeiffer, MPI für Biochemie, Martinsried, Federal Republic of Germany) contains nine bacterial lipoprotein sequences, most of them derived from nucleic acid sequences: the sequence of Braun's lipoprotein of *E. coli* (36), two more lipoproteins of the same species (6, 52), two lipoproteins from *Haemophilus influenzae* (11), and the lipoproteins of *Erwinia amylovora* (51), *Morganella morganii* (22), *Proteus mirabilis* (7), and *Serratia marcescens* (37). A comparison of each of these sequences with each other and with the *P. aeruginosa* lipoprotein I sequence with the RELATE program resulted in a table of standard deviation scores which were taken as a measure of homology. There is a central group of the enterobacterial lipoproteins of *E. coli*, *E. amylovora*, *M. morganii*, *S. marcescens*, and *P. mirabilis* with standard deviation scores between 10.7 and 24.1. To this group, but not to any of the other lipoproteins, lipoprotein I of *P. aeruginosa* is loosely associated with standard deviation scores between 2.1 and 7.6 (average 5.7). Figure 5 shows the alignment of the enterobacterial lipoproteins and *P. aeruginosa* lipoprotein I.

DISCUSSION

Lipoprotein I (OprI), like porin protein F (OprF), belongs to the group of outer membrane proteins of *P. aeruginosa* which are immunologically cross-reactive in all the International Antigenic Typing Scheme serotypes (35). Its counterpart in *E. coli*, Braun's lipoprotein, has been the object of various studies because of its unusual posttranslational modification (21) and processing by a separate, globomycin-sensitive type of prolipoprotein signal peptidase (23). From amino acid composition analysis (32) and immunological data (19) it is known that the lipoproteins of *E. coli* and *P. aeruginosa* cannot be closely related. We decided to clone the *P. aeruginosa* lipoprotein I to investigate its use as a vaccine component and furthermore to put the gene into an *E. coli* background so as to learn more about lipoprotein modification and processing.

The gene for lipoprotein I was identified in a λ EMBL3 genomic library of *P. aeruginosa* DNA and subcloned into pBR322 and pUC19, using the mouse monoclonal antibody 6A4 for the different immunoscreening procedures. The open reading frame that was found encoded a protein of 64 amino acid residues that had exactly the published amino acid composition, preceded by a normal signal peptide of 19 amino acid residues. This provided proof that our antibody recognized lipoprotein I as it was characterized by Mizuno and Kageyama (32).

The G+C content of the lipoprotein I gene (59.4%) was similar to the G+C content of the porin F gene (60.1%) and lower than the average G+C content of the *P. aeruginosa* genome of 67% (38). This approaches the situation of the pilin genes, which have the lowest G+C content of the known *P. aeruginosa* genes (48). In the case of the lipoprotein I gene, the main contribution to the low G+C content seems to be the high incidence of GCT codons for alanine (18 of 23 codons). If all the alanine codons were changed according to the average distribution of alanine codons in the known *P. aeruginosa* genes (48), the G+C content would rise to 65.9%. It remains surprising that *P. aeruginosa* uses such a large number of rare codons in a gene that is highly expressed, whereas the *E. coli* lipoprotein gene is devoid of a number of rare codons (25).

The results of the transcriptional mapping experiments of the lipoprotein I gene using primer extension, S1 mapping, TERMINATOR, and Northern blot analysis were comparable to the results that were obtained previously for the porin F gene (13). Both mRNAs are obviously monocistronic. The 5′-untranslated sequences (62 bases for lipoprotein I and 58 bases for porin F) and the 3′-untranslated sequences (46 bases for lipoprotein I and 47 bases for porin F) are similar. The 5′ leader sequence of lipoprotein I contains two more ATG codons, which are in the same reading frame and potentially code for short peptides of 10 or 8 amino acid residues. This may, as in the similar case of porin F, enhance transcription initiation of the highly expressed gene, as has been suggested before (34, 41).

Upstream of the transcriptional start site of the lipoprotein I gene, promoter elements are found which resemble the consensus sequences of strong *E. coli* promoters. The −35 element is TTGGTC for the lipoprotein I gene and TTGTCT for the porin F gene (13), as compared to the *E. coli* consensus sequence of TTGaca. The −10 element is TAG TAT for the lipoprotein I gene and TAAACT for the porin F gene, as compared to the *E. coli* consensus TAtaaT. As a consequence of these rather strong similarities, both genes are expressed under the control of the *P. aeruginosa* promoters in *E. coli*, which allowed us in the present study to identify the gene by immunoscreening without the use of an expression library. One important difference is that expression of lipoprotein I, even from high-copy plasmids like pUC19, did not lead to any observable toxic effects on *E. coli*, whereas porin F expression was rather toxic for *E. coli* (13, 49).

From the amino acid composition data of the *P. aeruginosa* lipoprotein I (32) and the sequence of the *E. coli* lipoprotein (36), no strong homology between both lipoproteins was expected. On the other hand, as described in Results, the conserved nature of the functionally important sites allowed the identification of the N terminus of the mature protein. Further investigation of the homology between known lipoprotein sequences placed the sequences of different members of the family *Enterobacteriaceae* in a group of high homology (see Fig. 5) and the *P. aeruginosa* lipoprotein I sequence in a position of significantly lower homology, with mainly the functional sites being conserved. This reflects the larger phylogenetic distance between the *Enterobacteriaceae* and pseudomonads. Addition of more lipoprotein sequences to the known ones may add some useful information for evolutionary studies.

The most important function of the lipoprotein in *E. coli* is to link the outer membrane to the peptidoglycan layer, with the N-terminal, lipid-carrying part inserted into the outer membrane and the C terminus bound covalently to the peptidoglycan. *lpp* mutants of *E. coli* can survive, but their outer membrane fails to invaginate normally during cell division, leading to the formation of large blebs. As a consequence, cells are less viable and more sensitive to some antibiotics (16). Complementation of these strains with plasmids bearing the *P. aeruginosa* lipoprotein I gene will show to what extent this gene can replace the *E. coli* lipoprotein gene. For these experiments it is important to provide plasmids that allow sufficient expression of the heterologous gene. The similar organization of the amino acid sequences of both lipoproteins strongly suggests a similar way of modification as well as a similar function.

To investigate the expression of *P. aeruginosa* lipoprotein I in *E. coli* more closely, it will be important to test which of the posttranslational modification steps, such as addition of glycerol, acylation of glycerol, cleavage by signal peptidase II, and acylation of the N terminus, are carried out in *E. coli*. Especially interesting will be the question of how many fatty acid residues are added, since the *E. coli* lipoprotein contains three residues (21) and the *P. aeruginosa* lipoprotein I contains only one (32). The amount of processing will determine the structure of the material which is used for immunization experiments; inversely, the structure of the material obtained might be influenced by changing the level of expression.

The use of recombinant *P. aeruginosa* lipoprotein I for mouse protection experiments will permit testing of whether this antigen is protective without any contamination of *P. aeruginosa* lipopolysaccharide. This is currently under investigation, as well as vaccination studies using preparations of recombinant porin F. Since both antigens are cross-reactive between the different International Antigenic Typing Scheme serotypes (35), cross-protectivity of recombinant outer membrane proteins from one strain towards challenge with another strain might be possible.

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