JOURNAL OF BACTERIOLOGY, June 1988 p. 2784–2789
0021-9193/88/062784-06$02.00/0

Vol. 170, No. 6

Nucleotide Sequence and Expression in *Escherichia coli* of the *Pseudomonas aeruginosa lasA* Gene

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Received 29 December 1987/Accepted 16 March 1988

***Pseudomonas aeruginosa* PAO-E64 is a mutant which produces parental levels of elastase antigen but has no elastolytic activity at 37°C. The lesion (*lasA1*) in PAO-E64 is not a mutation in the structural gene for *P. aeruginosa* elastase (P. A. Schad, R. A. Bever, T. I. Nicas, F. Leduce, L. F. Hanne, and B. H. Iglewski, J. Bacteriol. 169:2691-2696, 1987). A 1.7-kilobase segment of DNA that complements the *lasA1* lesion was sequenced. Computer analysis of the DNA sequence showed that it contained an open reading frame which encoded a 41,111-dalton protein. The *lasA* gene was expressed under an inducible PT-7 promoter, and a 40,000-dalton protein was detected in *Escherichia coli* lysates. The *lasA* protein was localized in the outer membrane fraction of *E. coli*. This *lasA* protein produced in *E. coli* activated the extracellular elastase produced by the *P. aeruginosa* mutant, PAO-E64.**

Pseudomonas aeruginosa produces two extracellular proteases believed to be involved in virulence. Elastase, the most active protease (22, 42), is produced by 75 to 95% of *P. aeruginosa* strains tested (32). This protease inactivates a variety of biologically active compounds including immunoglobulin G, complement, elastin, collagen, and α_1-proteinase inhibitor (8, 31, 33, 39). In addition to the in vitro inactivation of these compounds, there is evidence that elastase plays a role in the pathogenesis of *P. aeruginosa* infections (18, 23, 27, 32, 34).

Although elastase is recognized as an important factor in the pathogenesis of *P. aeruginosa* infections, little is known about its regulation, processing, and secretion. The elastase found in the periplasm of *P. aeruginosa* is elastolytically inactive (9, 22). Presumably, elastase is activated upon transport across the outer membrane (9, 22). The inactive periplasmic elastase is slightly larger than the excreted (active) form by approximately 500 to 1,000 daltons (Da) (24). Previously, Ohman et al. (35) reported the isolation of a *P. aeruginosa* PAO1 mutant (PAO-E64) that produced parental levels of elastase antigen and was proteolytic but devoid of elastolytic activity at 37°C (35). The mutation in PAO-E64, *lasA1*, was mapped at 75 min on the *P. aeruginosa* PAO1 chromosome (19). It was previously shown that the *lasA* gene is not the elastase structural gene (38). However, the *lasA* gene product may be involved in processing or secretion of elastase (11, 12, 38). In this study, we have analyzed the DNA sequence of the *lasA* gene, its expression and location in *Escherichia coli*, and its possible role in elastase processing and activation.

MATERIALS AND METHODS

Bacterial strains, plasmids, bacteriophage, and growth conditions. Bacterial strains, plasmids, and phage are shown in Table 1. *E. coli* and *P. aeruginosa* were grown in LB broth containing 1% bacto-tryptone, 0.5% yeast extract (Difco Laboratories, Detroit, Mich.), and 1% NaCl (pH 7.2) at 37°C unless stated otherwise. M9 minimal salts are described by Maniatis et al. (29). Antibiotics used for *E. coli* were ampicillin (100 μg/ml), kanamycin (50 μg/ml), chloramphenicol (100 μg/ml), and tetracycline (25 μg/ml) (Sigma Chemical Co., St. Louis, Mo.).

Gel electrophoresis. Agarose gel electrophoresis was carried out as described by Maniatis et al. (29), using Tris-borate-EDTA buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli (26). Stacking gels were 5% acrylamide with 10% separating gels (acrylamide-bis, 30:1). SDS-PAGE reagents were purchased from Bio-Rad Laboratories (Richmond, Calif.). The ^{14}C-methylated protein standards were from Amersham Corp. (Arlington Heights, Ill.).

DNA purification and subcloning. DNA was purified from *E. coli* as described by Holmes and Quigley for small volumes (17). Large-scale preparations of plasmid DNA were described previously (38). DNA fragments used in subcloning were purified by agarose gel electrophoresis and eluted on an IBI Electroeluter as recommended by the manufacturer (International Biotechnologies Inc. [IBI], New Haven, Conn.). Deletion subclones were generated by digestion with the appropriate restriction enzymes. Blunt-end ligation of incompatible ends was done by using Klenow fragment followed by ligation with T4 DNA ligase. Restriction enzymes were purchased from Boehringer Mannheim Biochemicals (Indianapolis, Ind.), Bethesda Research Laboratories (Gaithersburg, Md.), IBI, New England BioLabs, Inc., (Beverly, Mass.), and Promega Biotec (Madison, Wis.). T4 DNA ligase and Klenow fragment were from Bethesda Research Laboratories and Boehringer Mannheim Biochemicals.

DNA sequencing. A modification of the Sanger dideoxy-DNA sequencing method was used for clones prepared in the M13 vectors (37, 43). This modification was the substitution of dGTP with 7-deaza-dGTP (1, 30). Subclones for sequencing were generated on the basis of the restriction map shown in Fig. 1A. Additional overlapping clones were generated by the method of Dale as described in the IBI cycloning kit (6). Deoxynucleotides and dideoxynucleotides were purchased from Pharmacia, Inc., (Piscataway, N.J.). The nucleotide 7-deaza-dGTP was purchased from Boehringer Mannheim Biochemicals. The 17-mer primer was from New England BioLabs. The isotope [α-^{35}S]dCTP (1,000

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TABLE 1. Bacterial strains, phages, and plasmids

Strain, phage, or plasmid	Genotype or phenotype	Source or reference
Strains		
E. coli		
HB101	General cloning host	2
JM107	General host for M13	43
K38	Host for PT7 expression system HfrC (λ)	36
P. aeruginosa		
PAO1	Prototroph	16
PAO-E64	*lasA1* mutant of PAO1	35
Phage M13mp18/19	Single-stranded DNA-sequencing vectors	43
Plasmids		
pT7-7	T7 RNA promoter vector	41
pGM1-2	Inducible T7 RNA by polymerase	41
pLAFR	IncP1, Tc^r, λ cos^+	10
pHN3	25-kb *Eco*RI insert in pLAFR, *lasA1*	38
pRB1822	pPS1875 2.2-kb *Sal*I-*Hin*dIII fragment ligated to pUC18, *lasA1*	38
pPS1816	*Sma*I deletion of pRB1822	This study
pPS1816-1	pPS1816 inserted into *Eco*RI site of pLAFR	This study
pPS1916	*Sma*I-*Hin*dIII fragment of pPS1816 in pUC19	This study
pT7-17	*Sma*I-*Hin*dIII fragment of pPS1816 in pT7-7	This study

Ci/mM) was from Amersham Corp. Sequence data were analyzed with IBI sequencing software and the University of Wisconsin Genetics Computer Group software (version 5.0) (7). The codon usage algorithm of Gribskov et al. (13) was used with a codon usage table compiled from published *P. aeruginosa* sequence data (S. West and B. Iglewski, manuscript in preparation).

Promoter studies of *lasA* and expression in *E. coli*. The promoterless chloramphenicol acetyltransferase gene block cartridge (CAT) (Pharmacia, Inc.) was inserted into the *Xho*I site of pRB1822, pPS1816, and pPS1916, and the orientation of the gene cartridge was determined by restriction analysis (4).

The T7 RNA polymerase-promoter expression system (pT7-7) was used to express the *lasA* gene in *E. coli* K38 (41). The 1.7-kilobase (kb) *Sma*I-*Hin*dIII fragment of pPS1816 was inserted into the *Sma*I-*Hin*dIII sites of pT7-7 (pT7-17). The proteins expressed were labeled by using a modification of the method described by Tabor and Richardson (personal communication). Briefly, cells containing both plasmids (pGP1-2 and pT7-17) were grown in LB medium with appropriate antibiotics to an A_{595} of 0.5. The cells (0.2 ml) were washed in M9 salts and suspended in M9 salts containing 0.01% of 19 of the 20 amino acids (minus leucine) supplemented with 20 μg of thiamine per ml. The cells were incubated for 2 h at 30°C to deplete free leucine, followed by

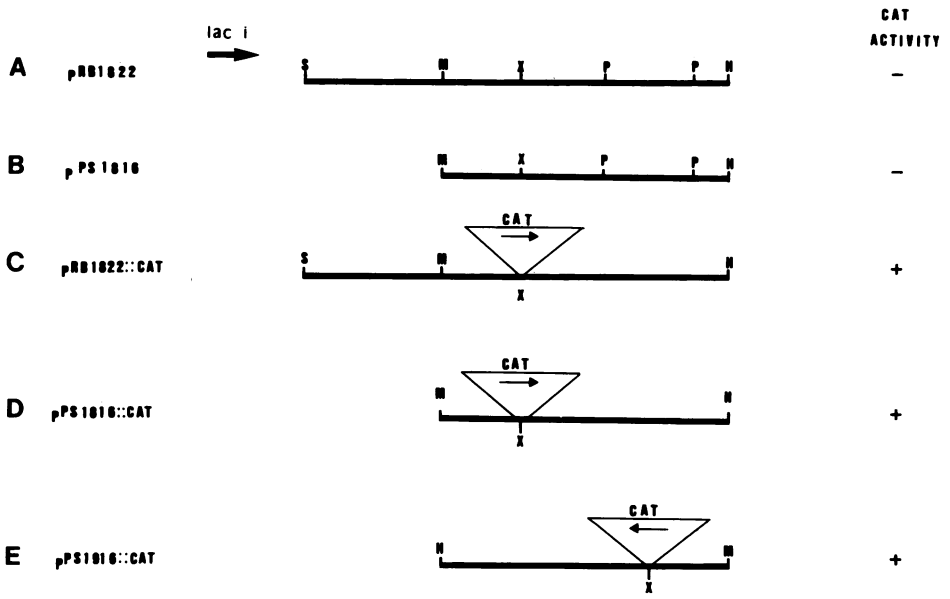

FIG. 1. Restriction endonuclease map of two *lasA*-containing plasmids showing direction of lactose promoter (lac i) present in all recombinant plasmids (vector sequences not shown). (A) pRB1822. (B) pPS1816 *Sma*I deletion of pRB1822. (C) Insertion of CAT cartridge into pRB1822, showing direction of CAT insertion. (D) Insertion of CAT into pPS1816. (E) Insertion of CAT into pPS1916. Symbols: H, *Hin*dIII; M, *Sma*I; P, *Pst*I; S, *Sal*I; X, *Xho*I.

an additional incubation at 42°C for 15 min. Rifampin (200 μg/ml, final concentration) was added, and the cells were incubated an additional 10 min at 42°C. The temperature was shifted to 30°C for 20 min, and after addition of 10 μCi of [^{3}H]leucine (50 Ci/mM) (Amersham), the cells were incubated for an additional 15 min at 30°C. Cells were pelleted and suspended in 75 μl of cracking buffer (60 mM Tris hydrochloride [pH 6.8], 1% SDS, 1% 2-mercaptoethanol, 10% glycerol, and 0.01% bromophenol blue). The samples were heated for 5 min at 100°C, and 25 μl was loaded onto a 10% SDS-PAGE gel.

***E. coli* fractionation.** *E. coli* K38 cells (15 ml) were grown and the T7 RNA polymerase was induced as described above. The labeling was done at 37°C with 200 μCi of [^{3}H]leucine (50 Ci/mM) for 45 min. Labeled cells were fractionated by a modification of the method of Koshland and Botstein (25). Cells were washed twice in M9 salts and suspended in 0.5 ml of sucrose buffer (20% sucrose, 10 mM Tris [pH 7.5]) to which 5 μl of 0.5 M EDTA (pH 8.0) was added. Following incubation on ice for 10 min, the cells were centrifuged and the pellets were suspended in 0.5 ml of cold distilled H_2O and incubated on ice for 10 min. Cells were centrifuged, and the supernatants (periplasmic fractions) were kept. The pellets were suspended, and the cells were lysed by two passages through a French pressure cell at 15,000 lb/in^2. The cell lysates were centrifuged at 100,000 × *g* for 1 h, and the supernatants (cytoplasmic fractions) were removed. The total membrane pellets were suspended in distilled H_2O, and a sample was stored for further analysis. The remaining membranes were further fractionated into inner and outer membranes on sucrose gradients as described by Hancock and Nikaido (14) as modified by Hindahl and Iglewski (15). All fractions were stored at −20°C until analysis. Protein determination was by the method of Lowry et al. (28), with bovine serum albumin as a standard. Trichloroacetic acid precipitation of labeled fractions was done as described by Chung and Collier (3).

Elastase assay and elastase activation. Cultures were grown overnight at 37°C in Peptone Trypticase Soy broth (35). Elastase activity was measured by using Elastin Congo red (Sigma) as previously described (38). Activation studies were carried out by adding 100 μg of *E. coli* membrane fractions to 250 μl of culture supernatants from PAO1 and PAO-E64. Following incubation for 15 min at 37°C, these samples were assayed for elastolytic activity.

RESULTS

Subcloning of pRB1822 and analysis of *lasA* gene orientation. The plasmid pRB1822 (Fig. 1A) contains a 2.2-kb insert subcloned from the plasmid pHN3 (38). Both of these plasmids contain *P. aeruginosa* PAO1 chromosomal DNA which complements the *lasA1* lesion in the mutant PAO-E64 (35). Goldberg and Ohman (11) have reported that insertion into the *Xho*I site inactivates the *lasA* gene. They noted that the *lasA* gene promoter was expressed in *E. coli* and the direction of *lasA* transcription was from *Xho*I toward the *Hin*dIII site (11). We confirmed and extended these observations by inserting the promoterless CAT gene block into the *Xho*I site of pRB1822 which contains 2.2 kb of *P. aeruginosa* DNA, compared with the 4 kb used by Goldberg and Ohman (11). Chloramphenicol resistance was achieved in only one direction (Fig. 1C). To further localize the *lasA* promoter, the *Sma*I-*Hin*dIII fragment of pRB1822 was inserted into pUC18 (pPS1816) and pUC19 (pPS1916) and the CAT gene block was inserted into the *Xho*I site of these plasmids. Chloramphenicol resistance occurred only when CAT was inserted in one direction such that transcription was from *Sma*I towards *Hin*dIII (Fig. 1B, D, and E). These data indicate that a *P. aeruginosa* promoter was contained within approximately a 360-base-pair (bp) region of DNA between the *Sma*I and *Xho*I sites (Fig. 1A).

Sequencing of the *lasA* gene. The 1.7-kb *Sma*I-*Hin*dIII fragment was put into the broad-host-range plasmid pLAFR (pPS1816-1). The plasmid pPS1816-1 complemented the mutation in PAO-E64, indicating that the 1.7-kb *Sma*I-*Hin*dIII fragment contained the *lasA* gene (data not shown). The 1.7-kb fragment of pPS1816 was sequenced and was found to contain 1,712 bp of *Pseudomonas* DNA. Computer analysis of the sequence data showed several possible open reading

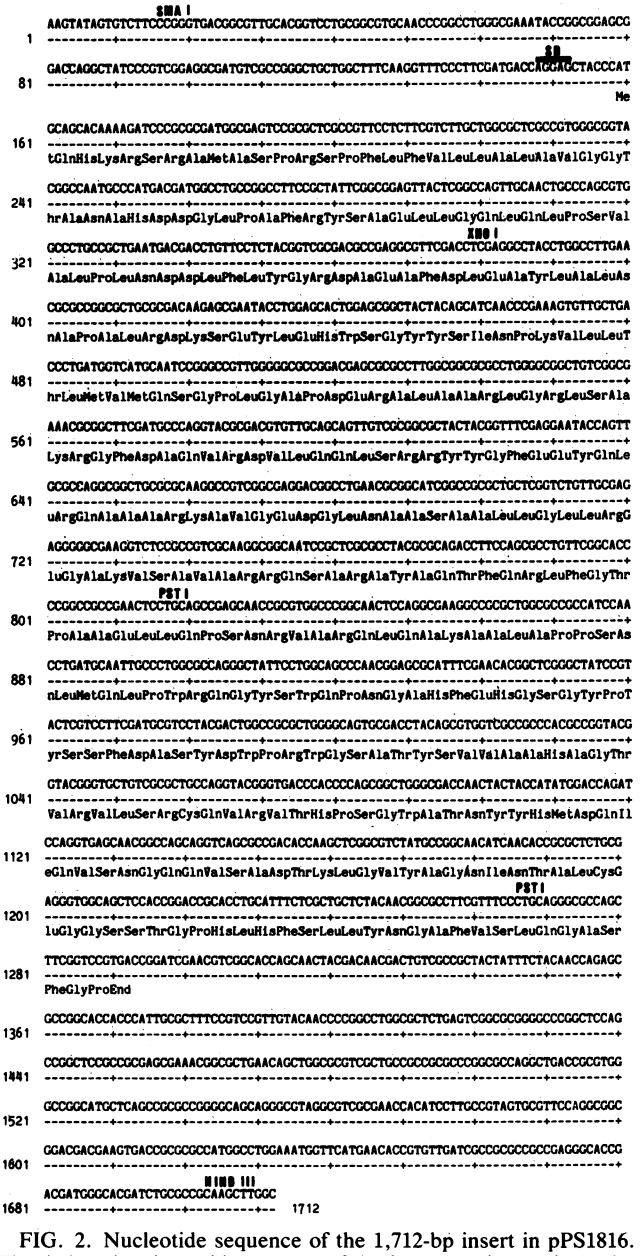

FIG. 2. Nucleotide sequence of the 1,712-bp insert in pPS1816. The deduced amino acid sequence of the *lasA* protein starting at bp 159 is shown below the DNA sequence. The Shine-Dalgarno box (SD) is shown as a broad line. Relevant restriction sites are shown.

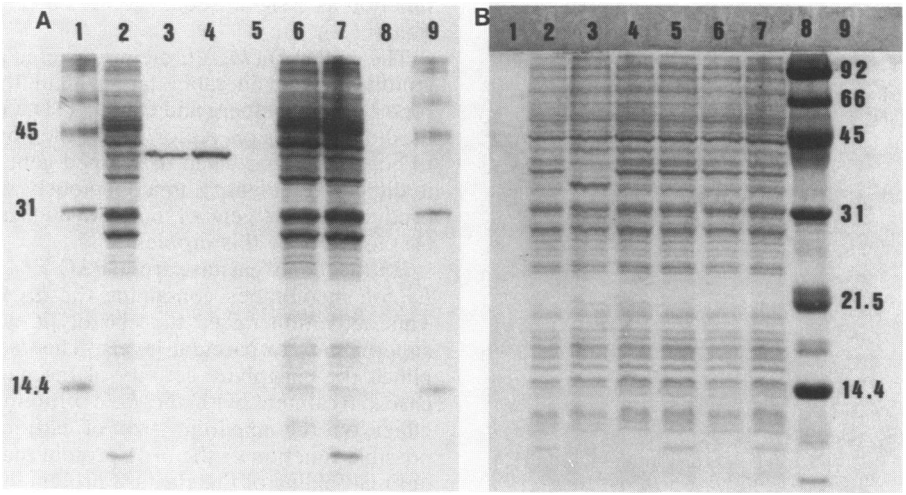

FIG. 3. The *lasA* gene product in *E. coli* after induction of the T7 promoter. (A) Autoradiograph of expressed *lasA* protein. (B) Coomassie blue-stained SDS-PAGE gel. Lanes in both panels: 1 and 9, ^{14}C-methylated protein standards given in kilodaltons; 2, induction of T7 promoter and expression of *lasA* protein without rifampin inhibition of host RNA polymerase; 3, expression of *lasA* protein with rifampin (sample was heated at 100°C for 10 min); 4, *lasA1* without heating; 5, induced pT7-7 vector; 6, *lasA* expression without induction of T7 RNA polymerase and without inhibition of host RNA polymerase by rifampin; 7, pT7 vector alone (same conditions as 6); 8, BioRad PAGE standards.

frames (ORF) (data not shown). When the codon usage algorithm of Gribskov et al. (13) was used, a large ORF with significant codon usage was detected starting at bp 105 and ending at bp 1290. Analysis of the ORF revealed that although the first methionine is encountered at bp 105, codon usage is not statistically significant until bp 160 (data not shown). No ribosome-binding site (40) can be found upstream of bp 105; however, a ribosome-binding site is seen starting at bp 148 with the sequence AGGAG. This binding site is 6 bp upstream from methionine (bp 159). The DNA sequence of the 1.7-kb fragment and the translated product of the ORF starting at bp 159 and ending at bp 1289 is shown in Fig. 2. This ORF encodes for a 41,111-Da protein containing 377 amino acid residues.

Expression of the *lasA* gene product in an *E. coli* expression system. The pT7-7 vector contains the bacteriophage T7 promoter along with an inducible T7 RNA polymerase on a second plasmid (41). Rifampin is used to inhibit the host RNA polymerase. Therefore, DNA inserted into the multicloning sites of the T7 promoter is expressed without concurrent expression of host proteins, allowing one to radiolabel the protein of interest. The 1,712-bp fragment of pPS1816 was cloned into the pT7-7 expression vector (pT7-17), and the expressed proteins were analyzed on SDS-PAGE (Fig. 3). A 40,000-Da protein (the *lasA* protein) was seen when the T7 promoter was induced at 42°C with the addition of rifampin (Fig. 3, lanes 3 and 4). The 40,000-Da *lasA* protein was not heat modifiable (Fig. 3, lanes 3 and 4).

To determine the location of the 40,000-Da *lasA* protein, *E. coli* containing the *lasA* protein was fractionated into periplasmic, cytoplasmic, and membrane fractions. Of the trichloroacetic acid-precipitable radioactivity, 93% was associated with the membrane fraction (Table 2). This total membrane fraction was further fractionated on a sucrose gradient to separate the inner and outer membranes. Of the trichloroacetic acid-precipitable radioactivity, 77% was associated with the outer membrane fraction (Table 2). Analysis of these *E. coli* fractions on SDS-PAGE revealed that the only labeled protein was the 40,000-Da *lasA* protein found in the total membrane fraction and in the outer membrane fraction of induced pT7-17 *E. coli* (Fig. 4).

Activation of PAO-E64 culture supernatants with *lasA* protein. The elastolytic activity of culture supernatants of the parent strain PAO1 and the *lasA1* mutant strain, PAO-E64, were compared in the presence of outer membrane preparations from *E. coli* containing the vector control or the *lasA* protein. PAO-E64 supernatants showed a 36% increase in activity when incubated with outer membranes containing the *lasA* protein (Table 3). Supernatants from the parent strain also showed an increase in elastolytic activity when incubated with membranes containing the *lasA* protein. Membranes from *E. coli* containing only the vector had no significant effect on the elastolytic activity of the culture supernatants. Treatment of either PAO1 or PAO-E64 elastase with *lasA* protein did not alter the apparent molecular weight of these proteins on SDS-PAGE (data not shown).

DISCUSSION

We have previously reported the cloning of two *P. aeruginosa* genes involved in elastase expression (38). One of

TABLE 2. Localization of *lasA* protein in *E. coli*

E. coli fraction containing *lasA*[a]	Without sucrose fractionation		With sucrose fractionation[b]	
	cpm[c]	% Total incorporation	cpm[c]	% Total incorporation
Periplasm	307	1.8	307	1.6
Cytoplasm	823	4.8	823	4.3
Total membranes	16,023	93.4		
Inner membrane			3,353	17.4
Outer membrane			14,772	76.7

[a] *E. coli* containing vector alone incorporated <500 cpm in any fraction.
[b] Membranes were fractionated on a sucrose gradient, and inner and outer membranes were removed.
[c] cpm are based on 10 μg of total protein precipitated on trichloroacetic acid filters after washing. See Materials and Methods for details.

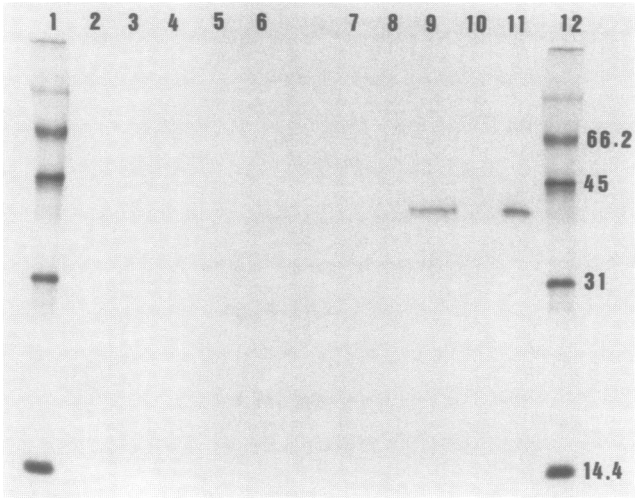

FIG. 4. Autoradiogram of cell fractions of *E. coli* containing the *lasA* protein. Lanes: 1 and 12, ^{14}C-methylated standards given in kilodaltons; 2 to 6, *E. coli* containing the vector, pT7-7 (2, periplasmic; 3, cytoplasm; 4, total membranes; 5, inner membrane; 6, outer membrane); 7 to 11, *E. coli* containing the *lasA* clone, pT7-17 (7, periplasmic; 8, cytoplasm; 9, total membranes; 10, inner membranes; 11, outer membranes).

these genes, *lasA*, complements the mutation in PAO-E64. This mutant produces an extracellular elastase which is the same size as the parent elastase and is proteolytic but is deficient in elastolytic activity at 37°C. We have sequenced and analyzed a 1,712-bp *Sma*I-*Hin*dIII fragment which complements PAO-E64. Analysis showed that this DNA contained a large open reading frame which encoded a 377-amino-acid protein with a predicted molecular weight of 41,111 (Fig. 2). When the *lasA* gene was expressed in *E. coli*, a protein, the *lasA* protein, with an estimated molecular weight of 40,000 was found. Fractionation studies showed that this protein was associated with the outer membrane of *E. coli* (Table 2). Analysis of the deduced amino acid sequence suggested that the amino-terminal region of the *lasA* protein (Fig. 2) is similar to other procaryotic signal sequences (20, 21). The location of the *lasA* protein in *P. aeruginosa* and the ability of its amino-terminal region to function as a signal sequence are currently under investigation.

TABLE 3. Activation of *P. aeruginosa* extracellular elastase with *E. coli* membranes containing the *lasA* protein

E. coli membrane fraction	Elastolytic activity of culture supernatants[a]			
	PAO1		PAO-E64	
	Sp act[b]	% Increase in activity[c]	Sp act[b]	% Increase in activity[c]
None	6.6		4.0	
Vector membranes	7.2	9 %	4.0	0%
lasA membranes	8.4	22.6%	6.3	36%

[a] *E. coli* fractions demonstrated no elastolytic activity when 100 μg of total protein was used and when the fractions were incubated overnight at 37°C with Elastin Congo red as a substrate (see Materials and Methods). Supernatants were adjusted to 10 mg of total extracellular protein per ml.

[b] Elastase specific activity was determined from a standard curve with purified *P. aeruginosa* elastase. These experiments were repeated three times with equivalent results.

[c] Increase in elastolytic activity was determined as the increase in specific activity following preincubation with various membrane fractions as compared with culture supernatants preincubated with buffer alone.

The 40,000-Da *lasA* protein predicted and observed in our study was considerably larger than the 31,000-Da value reported by Goldberg and Ohman (11). Goldberg and Ohman used an expression vector where the host proteins continue to be synthesized when the cloned gene is induced, making it difficult to identify unambiguously the correct product produced by the gene of interest. We used the pT7-7 system (41) to alleviate this problem.

Extracellular elastase from PAO-E64 was activated with *E. coli* membranes containing the *lasA* protein (Table 3). This activation raised the elastolytic activity of PAO-E64 supernatants to parental levels. This *lasA* protein also amplified the elastolytic activity of the parent PAO1. In both cases, treatment with the *lasA* protein had no detectable effect on the apparent size of extracellular elastase. A possible function of the *lasA* protein may be to mediate the optimal folding of the elastase protein into its elastolytically active form. Alternatively, the *lasA* protein may activate elastase by modifying it in some manner not detectable on SDS-PAGE. However, the elastolytic activation of elastase by the *lasA* protein is apparently not absolutely required for excretion of elastase to the external medium because the mutant PAO-E64 produces parental levels of extracellular elastase protein (35).

Kessler and Safrin (24) have shown that elastase found in the periplasm of *P. aeruginosa* is elastolytically inactive and slightly larger (500 Da) than extracellular elastase. Using the same nondenaturing gel system as Kessler and Safrin, we were unable to show a size change of extracellular elastase before or after treatment with lasA protein. However, computer analysis of the deduced amino acid sequence of the *lasA* protein revealed significant homology (score of 39%) with beta-lytic protease of *Myxobacter* spp., a neutral metallo-endopeptidase (5). Although we were unable to demonstrate a proteolytic processing event associated with the *lasA* protein produced in *E. coli*, we cannot exclude the possibility that this protein may have this function in *P. aeruginosa*.

A third possible function of the *lasA* protein has been suggested by Goldberg and Ohman (11). They observed a 47,000-Da elastase precursor associated with the membrane fraction of the *lasA*$^+$ parent and a *lasA* deletion mutant of *P. aeruginosa*. The large elastase precursor was also found in the cytoplasm of the *lasA* mutant but not in the cytoplasm of the *lasA*$^+$ parent strain. Goldberg and Ohman suggested that the *lasA* protein is involved in the efficient secretion of elastase. We are currently examining the potential function of the *lasA* protein in elastase secretion and excretion.

ACKNOWLEDGMENTS

This work was supported in part by Public Health Service grants AI21310 and AI24423 from the National Institutes of Health.

We thank S. Tabor for the pT7-7 expression system and T. I. Nicas for help in reviewing the manuscript.

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