Molecular Characterization and Nucleotide Sequence of the *Pseudomonas aeruginosa* Elastase Structural Gene

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The elastase structural gene (*lasB*) from *Pseudomonas aeruginosa* PAO1 has been previously cloned on an 8-kilobase (kb) DNA fragment. The *lasB* gene, cloned in both orientations in pUC18, produced elastase in *Escherichia coli*, indicating that its promoter and translation initiation sites are functional in *E. coli*. Deletion analysis further defined the location of the *lasB* gene to a 3.0-kb *EcoRI-KpnI* fragment (pRB1803). Elastase prepared from *E. coli* TB1(pRB1803) corresponded in molecular weight to mature *P. aeruginosa* extracellular elastase (33,000). The *lasB* gene directed the synthesis of 54- and 50-kilodalton (kDa) proteins in a bacterial cell-free transcription-translation system. The 33-, 50-, and 54-kDa proteins reacted with elastase-specific antiserum. To further characterize the *lasB* gene, the nucleotide sequence of the 3.0-kb *EcoRI-KpnI* fragment was determined. This DNA fragment contained a 1,491-base-pair open reading frame encoding 498 amino acids, corresponding to a predicted molecular weight of 53,600. The deduced amino acid sequence contained a putative signal sequence followed by a large polypeptide which preceded the mature 33-kDa elastase protein. Three zinc ligands and an active site were predicted for the mature elastase on the basis of its amino acid sequence homology with *Bacillus thermoproteolyticus* thermolysin.

Elastase is one of several extracellular enzymes produced by *Pseudomonas aeruginosa* that are thought to be involved in the pathogenesis of *P. aeruginosa* infections (19, 24, 39). The ability of this metalloprotease to degrade elastin and collagen (11, 23, 24) and inactivate human immunoglobulin G (7), serum α_1 -proteinase inhibitor (25, 26), and several complement components (32) emphasizes its potential importance as a virulence factor. The mechanism by which elastase degrades or inactivates such a variety of substrates is not known.

Likewise, little is known about the processing and excretion of elastase. An inactive precursor of elastase (ranging in molecular weight from 33,000 to 47,000) has been isolated from the periplasm of *P. aeruginosa* (8, 9, 16). The inactive precursor could be activated by limited proteolysis or by dissociation from a non-covalently bound inhibitor (8, 16).

A mutant of *P. aeruginosa* PAO1 has been isolated which produces an altered extracellular elastase that is proteolytically active but elastolytically inactive at temperatures above 37°C (28). This mutant, PAO-E64, has a mutation in the *lasA* gene. The LasA protein has been shown to be involved in the final processing of elastase by broadening the substrate specificity of elastase to include elastin (9, 31a).

Knowledge of the structure of the elastase gene and its product would help clarify questions regarding the secretion, processing, and function of elastase. The elastase structural gene (*lasB*) has been isolated and partially characterized (31). In this study, we show the expression of the *lasB* gene in *Escherichia coli* cells and a procaryotic cell-free transcription-translation system, the nucleotide sequence of the *lasB* gene, and its deduced amino acid sequence.

MATERIALS AND METHODS

Bacterial strains, plasmids, and medium. *E. coli* TB1 and JM107 were used as hosts (2, 41). Plasmid pUC18 and bacteriophages M13mp18 and M13mp19 were used as vec-

tors for cloning and nucleotide sequencing (41). *E. coli* was grown in LB broth (21) at 37°C. When appropriate, 100 µg of ampicillin (Sigma Chemical Co., St. Louis, Mo.) per ml was added to the medium.

DNA purification and subcloning. Plasmid and replicative form bacteriophage DNAs were purified by the alkaline lysis technique, followed by cesium chloride-ethidium bromide isopycnic centrifugation (21). DNA fragments used in subcloning were separated by agarose gel electrophoresis and purified on NA45 DEAE-paper (Schleicher & Schuell, Inc., Keene, N.H.) as described by the manufacturer. Procedures for ligation and transformation were performed as described by Maniatis et al. (21). Restriction enzymes, T4 DNA ligase, and Klenow fragment were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md., or Boehringer Mannheim Biochemicals, Indianapolis, Ind.

Gel electrophoresis and immunoblotting. Agarose gel electrophoresis was performed as described by Maniatis et al. (21), with Tris-borate-EDTA buffer. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (18). Stacking gels were 5% acrylamide with a 12% separating gel. SDS-PAGE reagents and molecular weight standards were purchased from Bio-Rad Laboratories, Richmond, Calif.

Protein transfer and immunoblotting were as described by Hindahl and Iglewski (12). Polyclonal anti-pseudomonas elastase serum was prepared in rabbits as previously described (28) and was used at a 1:1,000 dilution.

Proteolytic assay. Preparation of *E. coli* cell lysates and measurement of their proteolytic and elastolytic activities were as previously described (31). Protein concentration was determined by the method of Lowry et al. (20).

In vitro expression of cloned genes. The cloned genes were expressed in vitro by using the *E. coli* cell-free coupled transcription-translation system (42) as described by the manufacturer for use with [³⁵S]methionine (Amersham Corp., Arlington Heights, Ill.). The resulting gene products

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FIG. 1. Restriction endonuclease maps of plasmids containing the *P. aeruginosa* *lasB* gene and the protease activities of cell-free lysates from *E. coli* TB1 transformed with the corresponding plasmids. Symbols: —, PAO1 DNA insert; —, pUC18; —, direction of transcription of the *lac* promoter; □, complete *lasB* gene; □, truncated *lasB* gene. The proteolytic and elastolytic activities were obtained by extrapolation from a standard curve made by using commercial *P. aeruginosa* elastase. Numbers were converted to micrograms of elastase per milligram of total protein. The limits of detection were 0.1 mg of elastase per mg of total protein with hide powder azure and 5.0 mg of elastase per mg of total protein with elastin Congo red.

were analyzed by SDS-PAGE, followed by autoradiography or immunoblotting (12) with elastase-specific antiserum.

DNA sequence analysis. DNA was sequenced by a modification of the Sanger dideoxy-chain termination method (3, 30, 34, 41) by using the Sequenase Kit (United States Biochemicals Corp., Cleveland, Ohio) as described by the manufacturer. Subclones for sequencing were generated on the basis of the restriction map shown. Additional overlapping clones were generated by the method of Dale et al. (5) as described in the Cyclone Kit (International Biotechnology Inc., New Haven, Conn.). Certain regions were sequenced by using oligonucleotide primers specific for the *lasB* gene by the method of Sanchez-Pescador and Urdea (29). The oligonucleotide primers were made on an Applied Biosystems 380 B nucleotide synthesizer by using β -cyanoethyl-phosphoramidite chemistry (1). Isotope [35 S]dCTP (1,000 Ci/mmol) was from Amersham. The 3.0-kilobase (kb) DNA fragment which contains the *lasB* gene and flanking regions was sequenced in both directions from 333 base pairs upstream of the translational start site to 262 base pairs downstream of the translational stop site. The region of DNA located more than 262 base pairs downstream of the stop codon was sequenced in one direction. Sequence data were analyzed by using International Biotechnology Inc. computer software and University of Wisconsin Genetics Computer Group software (6). The codon usage algorithm of Gribskov et al. (10) was used with a *P. aeruginosa* codon usage table which was compiled from published sequence data (S. E. H. West and B. H. Iglewski, submitted for publication).

RESULTS

Subcloning of the *lasB* gene and expression in *E. coli*. Plasmid pRB1808 includes an 8.0-kb *P. aeruginosa* PAO1 chromosomal DNA insert which contains the structural gene (*lasB*) that encodes *P. aeruginosa* elastase (31). This 8.0-kb fragment was characterized by restriction enzyme analysis

(Fig. 1) and subcloned into the *EcoRI* site of pUC18 in both orientations. No proteolytic or elastolytic activity was detected in the cellular lysates of *E. coli* containing only the pUC vectors. General proteolytic and elastolytic activities were higher in cellular lysates of *E. coli* cells transformed with pRB1808 than in those transformed with pRB1807 but were clearly detectable in both cells. The *SmaI* site of the 8.0-kb insert is proximal to the *lac* promoter in pRB1808 and is distal to the *lac* promoter in pRB1807. The ability of the *lasB* gene to be expressed in both orientations indicates that its promoter can function in *E. coli*. The higher elastase activity of *E. coli* cells transformed with pRB1808 suggests that in this orientation *lasB* is transcribed off the more efficient *lac* promoter and that the direction of transcription of the *lasB* gene is from the *EcoRI* site toward the *KpnI* site.

Deletion analysis of pRB1808 indicated that the 3.0-kb *EcoRI-KpnI* fragment in pUC18 (pRB1803) is sufficient to produce proteolytic and elastolytic activities in *E. coli* lysates (Fig. 1). Elastase antigen was detected in *E. coli* TB1(pRB1803) lysates by immunoblot analysis with elastase-specific antiserum (Fig. 2B). This elastase produced in *E. coli* had the same molecular weight (33,000) as mature elastase purified from *P. aeruginosa* culture supernatants.

Elastase activity was not detected in *E. coli* transformants containing a 1.75-kb *EcoRI-SmaI* fragment in pUC18 (pRB1801). However, these transformants did produce a truncated elastase peptide with a molecular weight of 31,000 which reacted with elastase-specific antiserum (data not shown).

In vitro transcription and translation of the *lasB* gene. The *lasB* gene contained on pRB1803 was expressed in vitro by using a commercially available *E. coli* cell-free transcription-translation system, and the products were analyzed by SDS-PAGE (Fig. 2A) and immunoblots (data not shown). The autoradiograph shows three distinct bands in the lane containing the proteins synthesized from pRB1803. The fastest migrating protein corresponds to a molecular weight

FIG. 2. Characterization of plasmid-encoded proteins. (A) Autoradiogram of SDS-PAGE of [35 S]methionine-labeled proteins synthesized in the *E. coli* cell-free transcription-translation system. Lanes a and b contain the proteins synthesized in vitro directed by pUC18 and pRB1803, respectively. The position of nonradioactive extracellular *P. aeruginosa* elastase is designated by the arrow. (B) Immunoblot analysis of *E. coli* whole-cell extracts and extracellular *P. aeruginosa* elastase reacted with elastase-specific antiserum. Lanes correspond to *E. coli* cells transformed with pUC18 (lane a) and pRB1803 (contains the *lasB* gene) (lane b). Lane c corresponds to extracellular *P. aeruginosa* purified elastase. The molecular weights of the proteins were determined by their comigration with the Bio-Rad molecular weight standards (10^3) designated to the left of each panel.

of 31,000, does not cross-react with elastase-specific antiserum, and comigrates with β -lactamase synthesized from pUC18. The two larger proteins produced by pRB1803 have molecular weights of approximately 54,000 and 50,000 and cross-react with elastase-specific antiserum (data not shown). The 54-kilodalton (kDa) protein is approximately 20 kDa larger than the purified extracellular mature elastase produced by *P. aeruginosa* or the elastase produced in vivo by *E. coli* containing the cloned *lasB* gene.

Nucleotide sequence of the *lasB* gene. A detailed restriction map and the strategy used for nucleotide sequence analysis of the 3.0-kb *EcoRI-KpnI* fragment which contains the *lasB* gene and flanking regions are shown in Fig. 3. The nucleotide sequence of the *lasB* gene region (Fig. 4) contains an open reading frame of 1,491 base pairs, starting at nucleotide 333 and ending at nucleotide 1827. This open reading frame codes for a 53-kDa protein containing 498 amino acids. This differs substantially from the known molecular weight of extracellular active *P. aeruginosa* elastase, which is 33,000. Using Edman degradation, we previously determined the sequence of the first 40 amino acids of the amino terminus of the purified *P. aeruginosa* extracellular elastase (31). Upon searching the deduced amino acid sequence within the large open reading frame, we located the amino terminus of the mature extracellular elastase at amino acid 199 (+1). There was perfect agreement between the amino acid sequence determined by Edman degradation and that deduced from the DNA sequence for all of the first 40 amino acids (Fig. 4).

The molecular weight of this predicted elastase precursor (preproelastase; 53,600) agrees with the *lasB* gene product produced in vitro (Fig. 2A). Reading back from the first amino acid of the mature enzyme (alanine; amino acid 199),

FIG. 3. Restriction map and sequencing strategy of the 3.0-kb DNA fragment containing the *lasB* gene. Horizontal arrows indicate the direction of sequencing and the extent of information obtained from each clone. Symbols: —, subclones containing a specific restriction fragment; —>, subclones generated from sequential exonuclease digestion; O—>, sequence information generated by the primer extension method with *lasB*-specific oligonucleotides.

two methionine codons are encountered at base pairs 333 and 376. The methionine encountered at base pair 333 is assumed to be the translation initiation codon based on the presence of the ribosome-binding site (AGGAGA) 16 nucleotides upstream from this ATG site (33) and the absence of a recognizable ribosome-binding site appropriately located upstream of the ATG at base pair 376.

DISCUSSION

The *P. aeruginosa lasB* gene was localized on a 3.0-kb *EcoRI-KpnI* DNA fragment, expressed in *E. coli* cells and in a procaryotic cell-free transcription-translation system, and characterized by nucleotide sequence analysis. The level of expression of the *lasB* gene when subcloned into pUC18 in both orientations indicates that whereas the *lasB* promoter is expressed in *E. coli* it is not as active in this host as is the *lac* promoter. *P. aeruginosa* elastase produced in *E. coli* is not found in the culture supernatants but in the whole-cell lysates, and it is elastolytic (31), whereas in *P. aeruginosa* the elastase is not elastolytic until released into the supernatant (8, 16). This lack of elastolytic activity in *P. aeruginosa* may be due to a specific intracellular inhibitor (8), which is apparently not present in *E. coli*. The location and processing of elastase in *E. coli* are currently under investigation.

The *E. coli* cell-free transcription-translation system containing the *lasB* gene produced two proteins which cross-react with elastase-specific antiserum. There are several possible explanations for the production of the 54,000- and 50,000-molecular-weight proteins. The predominant 50-kDa protein may be a degradation product of the 54-kDa protein. Alternatively, the *E. coli* cell-free transcription-translation system may recognize a later translational start site or an earlier transcriptional terminator encoded by *lasB*. No such transcriptional termination sequences were detected within the *lasB* gene, and no ribosome-binding sites were identified which would give rise to a 50-kDa protein. Another possibility may be the contamination of the cell-free transcription-translation system with membrane components containing an *E. coli* signal peptidase. Conceivably, this peptidase

FIG. 4. Nucleotide sequence of the *lasB* gene and adjacent DNA. The deduced amino acid sequence, starting with the translation initiation site, is presented. The putative ribosome-binding site is designated by an overline and by S.D. (Shine-Dalgarno site). PRE, PRO, and MATURE designate different regions of the predicted protein. The first 40 amino acids (previously determined by Edman degradation [30]) of the mature protein are underlined. Symbols: -----, possible cleavage sites of the signal peptide; *, predicted zinc ligands; Δ , predicted catalytic site.

The nucleotide sequence of the 3.0-kb fragment containing the *lasB* gene revealed an open reading frame of 1,491 nucleotides which can code for a 497-amino-acid peptide with a molecular weight of 53,600. Evidence supporting the use of this open reading frame includes (i) the similarity in size of the predicted *lasB* gene product (53.6 kDa) and one of the *lasB* gene products expressed in the bacterial cell-free transcription-translation system (molecular weight; approximately 54,000) (Fig. 2), (ii) absolute agreement of the predicted amino acid sequence within the open reading frame with the previously determined sequence of the first 40 amino acids of the amino terminus region of the mature *P. aeruginosa* elastase (Fig. 4), (iii) the similarity of the amino acid compositions of the predicted mature elastase and the

The molecular weight of the predicted protein encoded by the *lasB* gene (53,600) is similar to the molecular weight of the *lasB* gene product expressed in vitro (54,000). The difference in molecular weights between the predicted protein and the extracellular elastase produced by *P. aeruginosa* indicates that elastase is synthesized as a large precursor. Others have observed elastase precursors in *P. aeruginosa* extracts which range in size from 33 to 47 kDa (8, 9, 16). Our data indicate that the elastase precursor is a prepropeptide which is 20 kDa larger than the mature elastase. The N-terminal region of the predicted preproelastase contains a putative signal sequence presumably necessary to cross the cytoplasmic membrane. This putative signal sequence is similar to the signal sequences of other secreted proteins of procaryotic origin. The general format of a signal peptide includes a charged residue within the first five amino acids, a core of at least nine hydrophobic amino acids, and a carboxy-terminal region containing polar amino acids (15, 36). Ninety percent of known procaryotic signal

TABLE 1. Amino acid composition of *P. aeruginosa* elastase

Amino acid	No. of residues	
	Deduced from DNA sequence	Amino acid analysis ^a
Ala	27	31.9
Cys	4	4.6
Asp	22	48.7 ^b
Asn	20	
Glu	9	19.9 ^c
Gln	7	
Phe	16	18.9
Gly	33	38.7
His	7	7.4
Ile	9	9.6
Lys	11	11.9
Leu	14	14.9
Met	9	8.6
Pro	10	11.8
Arg	15	16.3
Ser	25	25.5
Thr	18	18.6
Val	19	19.7
Trp	4	4.9
Tyr	22	24.0

^a Values are those reported by Morihara and Homma (24).^b Value represents the sum of Asp plus Asn residues.^c Value represents the sum of Glu plus Gln residues.

sequences end in either glycine or alanine. A helix-breaking molecule (Pro or Gly) or a large polar residue (Glu) occurs four to eight amino acids before the cleavage site (36). The signal sequence of preproelastase contains two positively charged lysine molecules within the first 5 amino acids, followed by a long hydrophobic core of 8 to 36 amino acids. The length of the signal sequence is ambiguous because of the presence of four possible cleavage sites. Preproelastase contains four Ala-Ala residues at amino acids 20 and 21, 23 and 24, 36 and 37, and 48 and 49 which are located four to six residues downstream of proline or glycine. The presence of more than one cleavage site suggests that this signal sequence is cleaved from the precursor in several steps. Alternatively, any one of these sites may function as the actual cleavage site. We are currently investigating whether this region of the preproelastase functions as a signal sequence.

The predicted signal sequence is followed by a propeptide containing approximately 150 amino acids. The exact function of this propeptide is not known. Alkaline and neutral proteases of *Bacillus amyloliquefaciens* and *B. subtilis* also have large propeptides between their signal sequences and mature proteins (35, 38, 40) which are thought to be removed during secretion. The propeptide may block the proteolytic activity of elastase to prevent damage to cellular proteins, as suggested for the propeptide of *Serratia* sp. protease (27). Several investigators have found inactive precursors of elastase in the periplasm or membrane fraction of *P. aeruginosa* (8, 17). Furthermore, an inactive extracellular proelastase which is the same size as the mature active elastase has been demonstrated (9, 28, 31a). This precursor was activated by the *lasA* gene product which is membrane associated in *E. coli* (31a). This final activation, which broadens the substrate specificity of elastase to include elastin, did not involve proteolytic cleavage. It seems likely that elastase is processed in three distinct steps: removal of a signal sequence, removal of a propeptide, and a final activation step. The exact location and sequence of process-

FIG. 5. Comparison of the regions containing the zinc ligands and active sites of *P. aeruginosa* elastase, *B. thermoproteolyticus* thermolysin, *Serratia* sp. protease, and *B. subtilis* neutral protease. Symbols: *, zinc ligands; +, active site; □, regions of elastase which are homologous to that of at least one other metalloprotease; —, gaps introduced into the amino acid sequence.

ing events of the proelastase in *P. aeruginosa* is currently being investigated.

Elastase is a neutral metalloprotease containing one zinc atom which is essential for its activity (23, 24). The predicted amino acid sequence of elastase was compared with those of other well-characterized procaryotic metalloproteases (Fig. 5). Elastase shows considerable similarities with the primary structure of thermolysin. The overall amino acid homology between mature elastase and thermolysin is 49%. The amino and carboxy termini of the two molecules are quite different; however, the central portions of the two molecules share 67% amino acid homology. *B. thermoproteolyticus* thermolysin was characterized by X-ray crystallography and shown to contain three zinc ligands at His-142, His-146, and Glu-166, an active site at His-231, and two additional sites required for catalysis at Glu-143 and Asp-226 (13, 14, 22, 37). The regions from amino acids 136 to 144, 163 to 168, and 221 to 227 of elastase are very similar to the zinc-binding or active-site regions of thermolysin (amino acids 138 to 146, 165 to 170, and 226 to 231) (Fig. 5). On the basis of these similarities, the predicted zinc ligands of *P. aeruginosa* elastase are His-140, His-144, and Glu-164 and the predicted active site is His-223. Glu-141 and Asp-221 may also be essential for the full catalytic activity of elastase, as suggested for Glu-143 and Asp-226 of thermolysin. X-ray analysis of thermolysin indicates that the three zinc ligands occur in helical regions of the protein (13, 22). Chou-and-Fasman analysis (4) of the protein secondary structure of the mature elastase indicates that its three predicted zinc ligands also occur in helical regions. Confirmation of this awaits analysis of the crystalline structure of *P. aeruginosa* elastase.

Three zinc ligands and one active site were also predicted for *B. subtilis* neutral protease (40) and *Serratia* sp. protease (27) on the basis of their comparison with thermolysin. The predicted zinc-binding regions of elastase are also similar to the zinc-binding regions of these two other metalloproteases. However, only slight similarity is found between the amino acid sequences surrounding the predicted enzyme active site of elastase (His-223) and those of the *Serratia* sp. and *B. subtilis* metalloproteases.

The availability of the *lasB* clone and its nucleotide sequence will allow us to address questions relating to the processing, secretion, and excretion of elastase, the function of the propeptide, the relationship between the structure of elastase and its function; and the regulation of the *lasB* gene in *P. aeruginosa*.

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