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Substitution of Active-Site His-223 in *Pseudomonas aeruginosa* Elastase and Expression of the Mutated *lasB* Alleles in *Escherichia coli* Show Evidence for Autoproteolytic Processing of Proelastase

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The neutral metalloprotease elastase is one of the major proteins secreted into the culture medium by many *Pseudomonas aeruginosa* strains. Encoded by the *lasB* gene, the 33-kDa elastase is initially synthesized as a 53-kDa preproenzyme which is processed to the mature form via a 51-kDa proelastase intermediate. To facilitate studies on proteolytic processing of elastase precursors and on secretion, we developed systems for overexpression of *lasB* in *Escherichia coli* under the control of the inducible T7 and *tac* promoters. Although the 51-kDa proelastase form was detectable in *E. coli* under inducible conditions, most of the elastase produced under these conditions was found in an enzymatically active 33-kDa form. The amino-terminal sequence of the first 15 amino acid residues of this 33-kDa elastase species was identical to that of the mature *P. aeruginosa* enzyme, suggesting that processing was autocatalytic. To test this possibility, the codon in *lasB* encoding His-223, a presumed active-site residue, was changed to encode Asp-223 (*lasB1*) and Tyr-223 (*lasB2*). The effects of these mutations on enzyme activity and processing were examined. No proteolytic or elastolytic activities were detected in extracts of *E. coli* cells containing the *lasB* mutant alleles. Overexpression of the mutated *lasB* genes in *E. coli* resulted in the accumulation of the corresponding 51-kDa proelastase species. These were processed in vitro to the respective 33-kDa forms by incubation with exogenous purified elastase, without an increase in proteolytic activity. Molecular modeling studies suggest that the mutations have little or no effect on the conformation of the mutant elastases. In addition, wild-type elastase and the mutant proelastases were localized to the periplasm of *E. coli*. The present results confirm that His-223 is essential for elastase activity and provide evidence for autoproteolytic processing of proelastase.

Pseudomonas aeruginosa is an opportunistic pathogen known to cause severe and lethal infections of compromised hosts such as patients with cystic fibrosis, burns, cancer, and ocular trauma. Much of its virulence is attributed to the ability of *P. aeruginosa* to secrete toxic and degradative enzymes into the environment. Among these, elastase is believed to be a major contributor to the pathogenesis of this organism. Elastase degrades a number of biologically important proteins including elastin (19), some collagens (10), immunoglobulins G (3) and A (9), serum α_1-proteinase inhibitor (20), and complement components (27). It can also activate the host kinin cascade to cause inflammation (11). The elastolytic activity of elastase is enhanced by the product of the *lasA* gene (7, 24, 26) which has recently been demonstrated in and purified from culture supernatants of *P. aeruginosa* (24).

Elastase is a neutral metalloprotease containing 1 zinc atom per mol that is essential for its activity (19). In the periplasm of *P. aeruginosa*, elastase is found in an inactive form that is proteolytically activated (12, 13). Although secreted as a 33-kDa protein, larger precursors of the enzyme have been demonstrated within the cells (7, 14). These were characterized by Kessler and Safrin (13, 14) who proposed the following model for elastase biosynthesis and secretion: elastase is made by the cells as a preproenzyme of about 60-kDa molecular size containing a signal sequence of approximately 4-kDa molecular size. The signal peptide is removed during translocation through the inner membrane to form a proelastase (~56 kDa) which is rapidly processed to generate a periplasmic elastase (~36 kDa) and a propeptide (~20 kDa). The proteolytic removal of the propeptide was found to be inhibited by EDTA (14) and other metal chelators, including 1,10-phenanthroline and the Zn-specific chelator tetraethylenepentamine (14a). The periplasmic elastase and propeptide accumulate temporarily in the periplasm and remain noncovalently associated until further processing and/or dissociation of the complex occurs, and then the mature enzyme is secreted through the outer membrane.

The subsequent cloning and sequencing of the elastase structural gene (*lasB*) from *P. aeruginosa* PAO1 (1) and IFO 3455 (5) established accurate sizes for some elastase precursors. The primary gene product is a preproelastase of 498 amino acids (53.6 kDa), and the propeptide is located on the amino-terminal end of the molecule, between the signal peptide and the N-terminal end of mature elastase (33 kDa). The amino-terminal sequence of the propeptide and the periplasmic elastase were recently determined (14a), demonstrating that the signal sequence is 23 amino acids (3-kDa) long and that the propeptide consists of 174 amino acid residues (18 kDa).

Elastase shows considerable amino acid sequence homology with other microbial neutral metalloproteases, especially thermolysin (1). Elastase has recently been crystallized, and its three-dimensional structure was determined by Thayer et al. (30). They found that all of the active-site residues, required for interaction with substrates and catal-

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ysis, are identical in both elastase and thermolysin. The two residues implicated in interactions with substrate carbonyl oxygen atoms, Tyr-155 and His-223, are conserved, as is an active-site glutamic acid, Glu-141, that is considered essential for catalysis. The sequence alignment and structural comparisons of elastase and thermolysin indicate that the zinc ligands in elastase are His-140, His-144, and Glu-164.

To gain a better understanding of the mechanisms governing the proteolytic processing of proelastase and secretion of the mature form, we developed systems whereby the *lasB* gene could be overexpressed in *Escherichia coli*. These systems were used to examine the effect of modifying a substrate-binding residue, His-223, on the enzyme activity and proelastase processing.

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. aeruginosa* FRD2 was a spontaneous nonmucoid revertant of FRD1, an isolate from a cystic fibrosis patient (22). *E. coli* HB101 (*proA2 leuB6 thi-1 lacY1 hsdRM recA13 supE44 rpsL20*) and TB1 [*ara*Δ(*lac-proAB*) *rspL* ϕ80 *lacZ*Δ*M15 hsdR*] (Bethesda Research Laboratories) were used in the manipulation of recombinant plasmids. *E. coli* BMH71-18mutS [*thi supE lac-proAB mutS*::Tn*10* (F′ *proA*$^+$*B*$^+$ *lacI*^{q}*Z*Δ*M15*)] (Promega) was used to propagate DNA in pSelect-1 (Promega) which was subjected to site-directed mutagenesis. *E. coli* JM109 [*endA1 recA1 gyrA96 thi hsdR17*(rK$^-$ mK$^+$) *relA1 supE44* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}Δ*M15*)] (Promega) was used for induction of genes in plasmid pKK223-3 (Pharmacia) under *tac* promoter control. *E. coli* BL21(DE3) [*ompT hsdS gul* (λDE3 *lacI lacUV5 lacZ*′ T7-*rnaP*)] (29), which contains the T7 RNA polymerase in the chromosome under the inducible control of the *lacUV5* promoter, was used for induction of genes under T7 promoter control in T7-1 or pT7-2 (U.S. Biochemical).

Media. *E. coli* and *P. aeruginosa* strains were cultured in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl [pH 7.5]). Antibiotics were used at the following concentrations (per milliliter): ampicillin (100 μg) or tetracycline (15 μg for *E. coli* or 100 μg for *P. aeruginosa*). Casein-agar plates contained 0.8% nutrient broth (Difco), 1.5% skim milk (Difco), and 1.5% agar. Elastin-agar plates contained 0.5% elastin (Sigma) instead of skim milk.

DNA manipulations. Most routine genetic manipulations and plasmid extractions were performed as described by Maniatis et al. (18). Triparental matings were used to mobilize recombinant plasmids from *E. coli* to *P. aeruginosa* as described previously (6). DNA sequences were determined by the chain termination technique with Sequenase (U.S. Biochemical) at 42°C by using 5′-[α-^{32}P]dCTP (>6,000 Ci/mmol, 10 mCi/ml; Amersham) and 7-deaza-dGTP. Oligonucleotides used for sequencing primers and oligonucleotide-directed site-specific mutagenesis were synthesized on an Applied Biosystems 380B DNA synthesizer.

Cloning and site-directed mutagenesis of *lasB* from *P. aeruginosa* FRD1. After nitrosoguanidine mutagenesis (6) of *P. aeruginosa* PAO1, a mutant called PDO31 was detected as protease deficient on the basis of the small zones of clearing observed around colonies grown on casein-agar and elastin-agar plates. A gene bank of strain FRD1 (6) containing *Eco*RI genomic fragments in pLAFR1 (4) was transferred to PDO31 by triparental mating (6). One selected transconjugant demonstrated partial complementation of the elastolytic defect as determined by an increased zone of clearing on elastin-agar plates. The recombinant plasmid, pKDF1, in this transconjugant contained an 8-kb *Eco*RI fragment of *P. aeruginosa* FRD1 DNA with the same restriction map as that reported to contain the PAO1 *lasB* gene (1). pKDF1 was subsequently shown to contain the *lasB* gene by Southern blot analysis (data not shown) by using synthetic oligonucleotide probes made according to the published PAO1 *lasB* sequence (1, 5). Also, DNA sequence analysis of approximately 60% of the FRD1 *lasB* gene revealed identity with the published sequence of the PAO1 *lasB* gene (1) (data not shown).

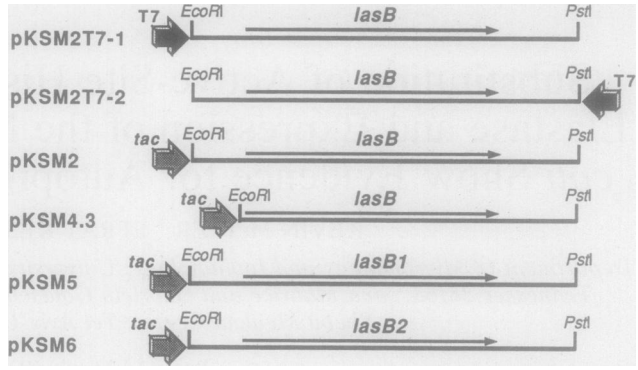


FIG. 1. Maps of *P. aeruginosa* DNA in plasmids used for *lasB* expression in *E. coli*. pT7-1 was the vector for pKSM2T7-1, and pT7-2 was the vector for pKSMT7-2. pKK223-3 was the vector for pKSM2, pKSM4.3, pKSM5, and pKSM6. The *Eco*RI site in pKSM4.3 was introduced by oligonucleotide-directed mutagenesis (see Materials and Methods). The extent of the *lasB* coding sequence is indicated by arrows along the DNA length. T7 and *tac* promoters are indicated by arrowheads. The *lasB*, *lasB1*, and *lasB2* alleles contain His-223, Asp-223, and Tyr-223, respectively.

A 2.5-kb *Eco*RI-*Pst*I DNA fragment from pKDF1 containing the *lasB* gene was cloned into pT7-1, forming pKSM2T7-1, with *lasB* in the transcribing orientation downstream of the T7 promoter (Fig. 1). pKSM2T7-2 was the same as pKSM2T7-1 except that *lasB* was in the nontranscribing orientation (Fig. 1). pKSM2 contained the 2.5-kb *Eco*RI-*Pst*I fragment in pKK223-3 with *lasB* downstream of the *tac* promoter (Fig. 1). pKSM4 contained the 2.5-kb *Eco*RI-*Pst*I fragment in pSelect-1 and was subjected to oligonucleotide-specific mutagenesis with the Altered Sites (Promega) in vitro mutagenesis system. The mutagenic oligomer 5′-AAAAAACCAGGAGAATTCAACAAGATG AAG-3′ (altered sites underlined) was used to create a new *Eco*RI site 8 bp upstream of the initiation codon (shown in boldface) of the *lasB* coding sequence. The 2.15-kb *Eco*RI-*Pst*I DNA fragment generated, which contained the *lasB* gene without its own promoter or ribosomal binding site, was then cloned into pKK223-3 to form pKSM4.3, placing *lasB* under the control of the *tac* promoter (Fig. 1). The two mutagenic oligomers which were used to introduce specific changes in active-site residue His-223 of elastase (altered sites underlined) were 5′-GCATCGACGTGGACCACTCC AGCGG-3′ (oligo *lasB1*) and 5′-GCATCGACGTGTACC ACTCCAGCGG-3′ (oligo *lasB2*). The mutagenic oligomers and a *bla* gene repair oligomer (Promega) were annealed to pKSM4, and colonies with potentially mutated plasmids were obtained by selection for ampicillin resistance according to the manufacturer's instructions. The active-site mutations were first verified by the disappearance of a *Sno*I restriction site, and all mutations were ascertained by DNA sequence analysis. The 2.5-kb *Eco*RI-*Pst*I fragments in the

pKSM4 derivatives containing *lasB1* and *lasB2* alleles were ligated into the expression vector pKK223-3, forming plasmids pKSM5 and pKSM6, respectively (Fig. 1).

***lasB* expression in *E. coli*.** *E. coli* JM109 containing pKK223-3 derivatives, or BL21(DE3) containing pT7-1 or pT7-2 derivatives, were grown in L broth at 37°C to an A_{580} of 0.6. To induce expression of proteins under the control of the *tac* or *lacUV5* promoters, isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM) was added, and the cultures were incubated an additional 4.5 h. Aliquots (0.1 ml) were centrifuged for 2 min at 10,000 × *g*, suspended in sodium dodecyl sulfate (SDS) sample buffer (60 mM Tris-HCl, 2% SDS, 10% glycerol, 0.1 mg of bromophenol blue per ml, 5% 2-mercaptoethanol [pH 6.8]), and loaded onto either SDS-12.5% or SDS-10% polyacrylamide gels for electrophoresis (SDS-PAGE) (17). Proteins were stained with Coomassie brilliant blue R250 (Bio-Rad).

Cell fractionation and localization. *E. coli* cells containing both wild-type and mutant *lasB* alleles were induced with IPTG as described above and fractionated by using a modification of the method of Koshland and Botstein (16). Cultures (15 ml) were collected by centrifugation (5,000 × *g* at 4°C for 10 min), and pellets were washed twice with M9 salts and then resuspended in 0.5 ml of sucrose buffer (10 mM Tris-HCl, 20% sucrose, 5 mM EDTA [pH 7.5]). After 10 min of incubation on ice, samples were centrifuged (5,000 × *g*, 4°C, 10 min), and cells were resuspended in 0.5 ml of cold H_2O and incubated for 10 min on ice. Cells were removed by centrifugation (5,000 × *g*, 4°C, 10 min), and the supernatant was collected as the periplasmic-enriched fraction. The pellets were resuspended in 0.5 ml of TC buffer (5 mM Tris-HCl, 0.5 mM $CaCl_2$ [pH 7.5]) and sonicated for 45 s by use of a Branson 450 sonifier. Sonicates were centrifuged once (5,000 × *g* for 10 min at 4°C) to remove whole cells and again (100,000 × *g* for 45 min at 4°C) to obtain the cytoplasmic-enriched fraction. The pelleted membrane-enriched fraction was resuspended in 0.5 ml of TC buffer. Samples (20 μl) of periplasmic-, cytoplasmic-, and membrane-enriched fractions (each in 0.5 ml) were applied to SDS-12.5% polyacrylamide gels and immunoblotted as described below. To determine the purity of the fractions, β-lactamase (encoded by the ampicillin resistance gene on plasmid vectors) was assayed by examining its ability to degrade cephaloridine (21), and its activity was found only in the periplasmic fractions.

Assays of proteolytic and elastolytic activities. *E. coli* cells containing *lasB* alleles were induced with IPTG as described above, collected by centrifugation, and suspended in TC buffer. Cells were disrupted by sonication (five 20-s bursts at 5°C), and unbroken cells and membranes were removed by centrifugation (100,000 × *g*, 30 min). Proteolytic activity was determined by an azocasein assay as previously described (15). Briefly, 10 μl of sample was added to 1 ml of 0.3% azocasein (Sigma) in TC buffer and incubated for 30 min at 37°C. Undigested substrate was precipitated with 3.3% trichloroacetic acid and removed by centrifugation (10,000 × *g*, 10 min), and the A_{400} of the supernatant was measured. Proteolytic activity was expressed as the increase in A_{400} per minute per milligram of total protein. Elastolytic activity was determined as previously described (23). The reaction was performed in 3 ml of 0.1 M Tris-maleate–1 mM $CaCl_2$ (pH 7.0), containing 10 mg of elastin Congo red (Sigma). After the addition of 50 μl of sample and incubation (2 h, 37°C) with agitation, 2 ml of 0.7 M phosphate buffer (pH 6.0) was added to stop the reaction. Undigested elastin was removed by centrifugation (10,000 × *g*, 10 min), and the A_{495} of the supernatant was measured. Units of elastolytic activity were expressed as the increase in A_{495} per minute per gram of protein. Assays with different enzyme dilutions were performed to establish the linear range of the reaction. Protein determinations were performed by the Bradford method (2), with bovine serum albumin as the standard.

Partial purification and N-terminal analysis of elastase produced in *E. coli*. A 1-liter culture of JM109(pKSM2) (i.e., P*tac-lasB*$^+$) was induced with IPTG as described above. Cells were collected by centrifugation, resuspended in 40 ml of TC buffer, and disrupted in a French pressure cell (12,000 lb/in^2). Unbroken cells were removed by centrifugation (5,000 × *g* for 30 min at 4°C). Proteins in the supernatant were precipitated with 50% saturated ammonium sulfate at 4°C overnight, collected by centrifugation (10,000 × *g* for 30 min at 4°C), and dissolved in 12 ml of TC buffer. After dialysis against the same buffer, a 2.5-ml (40 mg of protein) aliquot was applied to a DEAE (Protein-PAK 40HR; Waters) anion-exchange column equilibrated and eluted with 0.02 M Tris-HCl–0.5 mM $CaCl_2$ (pH 7.5) on a Waters 650E Advanced Protein Purification System. Purified elastase emerged from the column in the run-through fractions, whereas the bulk of the *E. coli* proteins adsorbed to the column and were eluted in the subsequent NaCl gradient (0 to 2 M). Samples of column fractions were examined by SDS-PAGE and immunoblotting. Elastase-containing fractions were electrotransferred to Immobilon-P membranes (Millipore) for N-terminal analysis by Edman degradation by use of an Applied Biosystems 470A Protein Sequencer.

Immunoblotting. Proteins in SDS-polyacrylamide gels were electrotransferred to nitrocellulose in a Trans-Blot apparatus (Bio-Rad) overnight at 14 V and 4°C. The nitrocellulose filters were blocked for 1 h with agitation in 3% gelatin in TBS (20 mM Tris-HCl, 500 mM NaCl [pH 7.5]). Primary antibody was an immuno-affinity-purified anti-elastase immunoglobulin G raised in rabbits against denatured elastase (14) which was used at 0.5 μg/ml in 1% gelatin-TTBS (0.05% Tween-20–TBS) and incubated with the filters for 2 h with agitation. Elastase-related proteins were visualized after incubation (2 h) with goat anti-rabbit horseradish peroxidase conjugate (Sigma; 1:2,000 dilution) and subsequent incubation in horseradish peroxidase color developer (60 mg of 4-chloro-1-napthol, 20 ml of methanol, 60 μl of H_2O_2, and 100 ml of TBS).

Molecular modeling of mutated elastases. The three-dimensional structure of *P. aeruginosa* elastase was recently solved at a 0.15-nm resolution (30), and the crystalline coordinates were kindly provided by D. B. McKay. These were used to compare the structures of the mutant elastases, encoded by the *lasB1* and *lasB2* alleles, with that of wild-type elastase. The molecular modeling of these proteins was performed on an Evans and Southerland graphics workstation at the Molecular Resources Center, University of Tennessee, Memphis, by using the SYBYL Molecular Modeling Software (Tripos Associates, Inc., St. Louis, Mo.) for quantitative comparisons of closely related molecules.

RESULTS

Overexpression of *lasB* in *E. coli*. When *lasB* was expressed in *E. coli* under the control of its native promoter in pKSM2T7-2 (Fig. 1), a 33-kDa elastase species was barely detectable in immunoblots (Fig. 2, lanes 2 and 3). Neither preproelastase (53.6 kDa) nor proelastase (51 kDa) was discernible under these conditions. To enhance production of elastase precursors, the *lasB* gene was overexpressed in

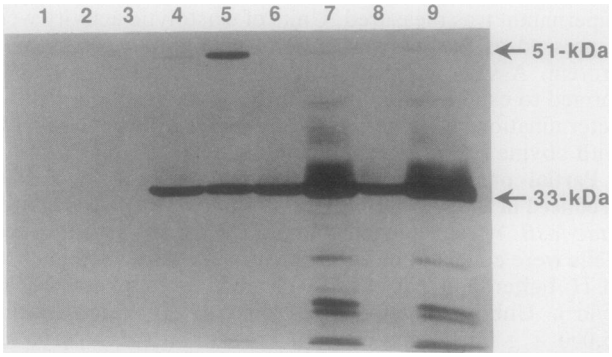


FIG. 2. Overexpression of wild-type *lasB* under control of either *tac* or T7 promoters in *E. coli*. Immunoblots of proteins from noninduced and IPTG-induced JM109 or BL21(DE3) cells harboring the following plasmids are shown: pKK223-3 (lane 1); pKSM2T7-2, uninduced (lane 2) and induced (lane 3); pKSM2T7-1, uninduced (lane 4) and induced (lane 5); pKSM2, uninduced (lane 6) and induced (lane 7); pKSM4.3, uninduced (lane 8) and induced (lane 9). Elastase-related proteins were visualized with rabbit antibodies against denatured *P. aeruginosa* elastase and a horseradish peroxidase-conjugated secondary antibody. Migration positions of the 33-kDa mature elastase and the 51-kDa proelastase are indicated with arrows on the right.

E. coli by using inducible promoters. Expression of *lasB* under the induced T7 promoter from pKSM2T7-1 (Fig. 1) revealed production and some accumulation of the 51-kDa proelastase species; however, much of the proenzyme was processed to the 33-kDa form (Fig. 2, lane 5). Comparable amounts of elastase, predominantly in the processed 33-kDa form, were also produced by the uninduced pKSM2T7-1-containing cells, indicating leakiness in the *lacUV5* promoter controlling expression of T7 RNA polymerase.

When the *lasB* gene was placed under the control of the strong, IPTG-inducible *tac* (*trp-lac*) promoter, uninduced pKSM2-containing cells (Fig. 2, lane 6) produced considerable amounts of the 33-kDa elastase species, comparable to those observed with the uninduced T7 promoter. This indicates that the *tac* promoter was active even without the addition of IPTG. Induction of P*tac-lasB* in pKSM2 increased the amount of total elastase antigen at least twofold; however, it was found mostly in the processed 33-kDa elastase species. Although detectable, the 51-kDa proelastase did not accumulate under these conditions (Fig. 2, lane 7), which may be due to differences in the strain backgrounds. In an effort to further improve control of *lasB* expression, the 0.35-kb segment of *P. aeruginosa* DNA located upstream of the *lasB* initiation codon (ATG) was removed to form pKSM4.3 (Fig. 1) and thus place the *tac* promoter 38 bp from *lasB*. After IPTG induction, this P*tac*-promoterless *lasB* construction yielded high amounts of elastase; however, most of it was again in the 33-kDa form (Fig. 2, lane 9). Other sizes of elastase-related molecules were also detected in both systems, especially upon induction with IPTG, and these were probably degradation products. In all of the systems, overexpression of elastase in *E. coli* had no apparent toxic or detrimental effects on the growth or appearance of the bacteria.

The 33-kDa elastase produced in *E. coli*(pKSM2) cells was partially purified, and an Edman degradation analysis was performed to determine its N-terminal amino acid sequence. The sequence obtained was Ala-Glu-Ala-Gly-Gly-Pro-Gly-Gly-Asn-Gln-Lys-Ile-Gly-Lys-Tyr, which was identical with that of purified *P. aeruginosa* extracellular elastase (33) as well as that predicted from our DNA sequence analysis of the cloned FRD1 *lasB* gene (data not shown). Thus, proelastase overproduced in *E. coli* was rapidly and correctly processed to the mature 33-kDa species.

Site-directed mutagenesis of active-site His-223 in *lasB*. We hypothesized that the rapid proteolytic processing of the 51-kDa proelastase in *E. coli* to the 33-kDa form could be the result of self processing. To examine this, we altered the *lasB* gene by site-specific mutagenesis to encode an inactive elastase. On the basis of the homology between *P. aeruginosa* elastase and *Bacillus* thermolysin, His-223 of elastase is predicted to be an integral part of the active site of this protease involved in substrate binding (1, 5). Recent crystallographic studies support this hypothesis (30). By using oligonucleotide-directed mutagenesis of *lasB*, the codon (CAC) coding for the positively charged His-223 of elastase was changed to encode a negatively charged Asp-223 (GAC) and formed the *lasB1* allele (Fig. 3). The His-223 codon was also changed to encode the bulky Tyr-223 (TAC) and formed the *lasB2* allele. The DNA encoding His-223 was contained within a *Sno*I restriction site (GTGCAC), and the loss of this site after site-directed mutagenesis was used as a diagnostic test for the mutations. After mutagenesis, approximately 80% of the plasmids obtained had lost the *Sno*I restriction site. Two representatives of the *lasB1* and *lasB2* alleles were sequenced in the region encoding residue 223, and all contained the sequence directed by the respective mutagenic oligonucleotide (data not shown). The *lasB1* and *lasB2* alleles were placed under the control of the *tac* promoter to form pKSM5 and pKSM6, respectively (Fig. 1).

Effect of the active-site mutations in *lasB* on the enzyme activity. To determine the consequence of each *lasB* mutation on catalysis, proteolytic activities of the elastase molecules expressed from the *lasB*, *lasB1*, and *lasB2* alleles were determined. Cultures of JM109 containing pKSM2 (i.e., P*tac-lasB*$^+$), pKSM4.3 (i.e., P*tac*-promoterless-*lasB*$^+$), pKSM5 (i.e., P*tac-lasB1*), and pKSM6 (i.e., P*tac-lasB2*) were grown under inducing conditions, cells were disrupted by sonication, and enzymatic activities in the sonicates were determined (Table 1). The hydrolysis of two substrates was examined: azocasein, for quantitation of general proteolytic activity, and elastin Congo red, for quantitation of elastolytic activity. Lysates of cells containing the wild-type *lasB*$^+$ allele demonstrated high levels of both proteolytic and elastolytic activities. These lysates had approximately twice as much proteolytic and elastolytic activity per unit volume as a typical culture supernatant of FRD2 grown under conditions favorable for elastase production (data not shown). Lysates of cells containing pKSM4.3 (where 0.35 kb containing the *P. aeruginosa* promoter was deleted) contained about twofold more proteolytic and elastolytic activities than those obtained from pKSM2 (Table 1). This is consistent with results observed in the immunoblot analysis (Fig. 2) which suggested that somewhat more elastase was produced in *E. coli* from pKSM4.3 than pKSM2 after induction from the *tac* promoter. The levels of proteolytic and elastolytic activities in *E. coli* cells expressing either the *lasB1* or *lasB2* alleles were approximately 2 orders of magnitude lower than those observed in cells expressing the wild-type (*lasB*$^+$) allele (Table 1) and were practically identical with those obtained with control lysates of JM109 cells that contained the vector alone. These results verified the prediction that His-223 is important for the activity of *P. aeruginosa* elastase and provided us with enzymatically

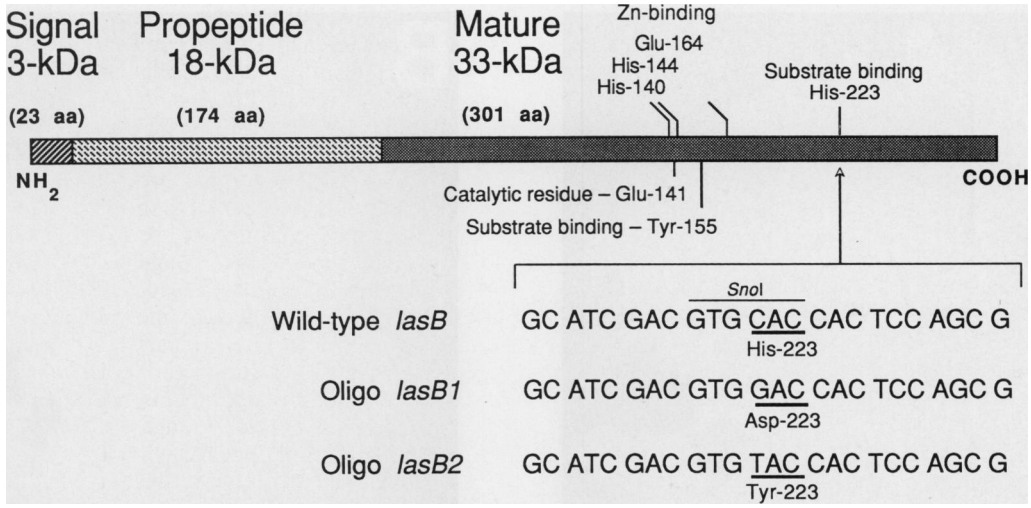


FIG. 3. Schematic representation of preproelastase (53.6-kDa) encoded by the *lasB* gene and locations of the signal sequence (23 amino acids), propeptide (174 amino acids), and the mature sequence (301 amino acids). Putative sites involved in the catalytic activity of the 33-kDa mature elastase are shown. Oligonucleotides used for site-directed mutagenesis are compared with the wild-type *lasB* sequence. The *lasB1* and *lasB2* oligonucleotides were used to replace the His-223 codon with residues Asp-223 and Tyr-223, respectively. His-223 is a residue implicated in substrate binding and activity of the enzyme. The *Sno*I restriction site in the CAC codon for His-223 provided a diagnostic assay for mutations in this codon.

inactive elastase molecules for further studies on proelastase processing.

Molecular modeling of elastases with substitutions at His-223. A possible explanation for the lack of elastase activity in cells expressing *lasB1* and *lasB2* was that the substitution of active-site His-223 with Asp or Tyr changed the conformation of the molecules. To examine this possibility, a molecular model of elastase was constructed by using the coordinates recently published for the crystalline structure of *P. aeruginosa* elastase (30). When the respective mutant residues were introduced into the elastase molecule, the three-dimensional structures obtained appeared essentially unchanged (Fig. 4). In addition, when an energy minimization of a 0.4-nm region surrounding the mutations was performed on the proteins encoded by the *lasB1*, *lasB2*, and *lasB*$^+$ genes, neither mutation resulted in a significant change or disruption of the predicted conformation of the mature elastase molecule (data not shown).

TABLE 1. Proteolytic and elastolytic activities in extracts of *E. coli* cells expressing *lasB*$^+$, *lasB1*, and *lasB2* alleles under the *tac* promoter

Plasmid	Relevant genotype	General proteolytic activity[a]		Elastolytic activity[b]	
		−IPTG	+IPTG	−IPTG	+IPTG
pKSM2	*tac-lasB*$^+$	1.40	8.10	0.67	3.80
pKSM4.3	*tac*-promoterless *lasB*$^+$	2.00	18.2	1.10	9.60
pKSM5	*tac-lasB1*	0.01	0.13	0.05	0.03
pKSM6	*tac-lasB2*	0.01	0.14	0.01	0.10
pKK223-3	Vector	0.08	0.14	0.01	0.08

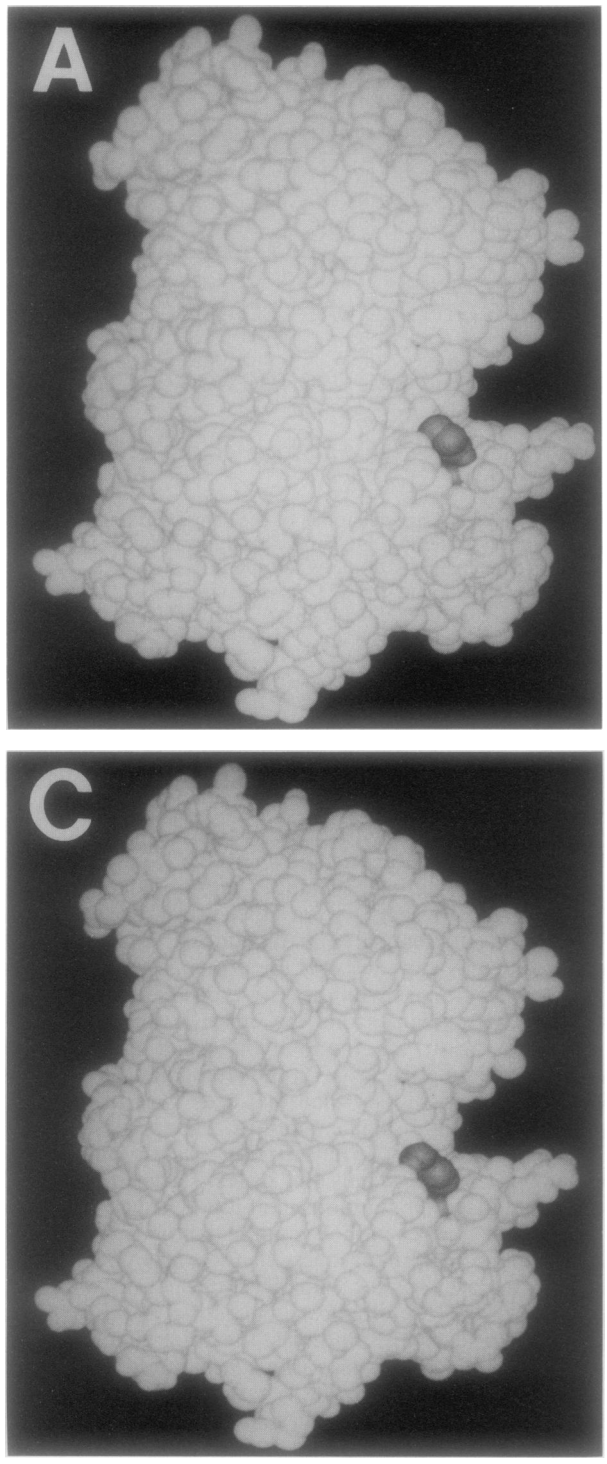
[a] Activity is shown on azocasein as the substrate with values expressed in units (increase in A_{400} per minute) per milligram of protein. Values represent one of several experiments which produced comparable results.

[b] Activity is shown on elastin-Congo red as the substrate with values expressed in units (increase in A_{495} per minute) per gram of protein. Values represent one of several experiments which produced comparable results.

Substitutions of His-223 inhibit processing of proelastase in *E. coli*. The effects of the active-site mutations on proelastase processing were examined by immunoblotting. The results showed that most of the *lasB1*- (Fig. 5A, lane 2) and *lasB2*-encoded (Fig. 5A, lane 4) proteins remained in the 51-kDa precursor form. As shown above (Fig. 2), the wild-type *lasB* products in the induced and uninduced cultures (Fig. 5A, lanes 5 and 6) were efficiently processed to the 33-kDa form. Interestingly, the uninduced mutant alleles did not exhibit leaky expression of the proteins (Fig. 5A, lanes 1 and 3) as was the case with the wild-type allele (Fig. 5A, lane 5). Also, as evident from the relative intensity of the elastase-positive bands, the total amount of elastase-related material accumulating in IPTG-induced cells containing the mutant alleles was lower than that seen with the wild-type *lasB* gene under the same conditions. Thus, unless substitutions of His-223 affect transcription or translation, these mutant proelastase proteins may be less stable in *E. coli*. The finding that these substitutions inhibit proelastase processing suggests that this reaction was autocatalytic.

Localization of *lasB* products in *E. coli*. JM109(pKSM2) colonies on casein plates with IPTG did not show zones of clearing, as did *P. aeruginosa*, indicating that *E. coli* cells are unable to secrete elastase past the outer membrane (data not shown). To localize the various *lasB* products within the cells, JM109 expressing wild-type or mutant alleles were fractionated to enrich for material from the periplasm, membrane, and cytoplasmic compartments, and each fraction was analyzed by immunoblotting. Essentially all of the wild-type *lasB* product expressed from pKSM2 was found in the periplasmic fraction and migrated as a 33-kDa protein (Fig. 6, lane 4). Most of the proelastases expressed by the mutant alleles *lasB1* and *lasB2* were also secreted into the periplasm (lanes 7 and 10), although small amounts of the 51-kDa species were detected in the membrane fraction (lanes 9 and 12).

In vitro processing of mutant proelastases. To exclude the possibility that the mutations rendered the proelastases resistant to proteolytic processing rather than blocked auto-



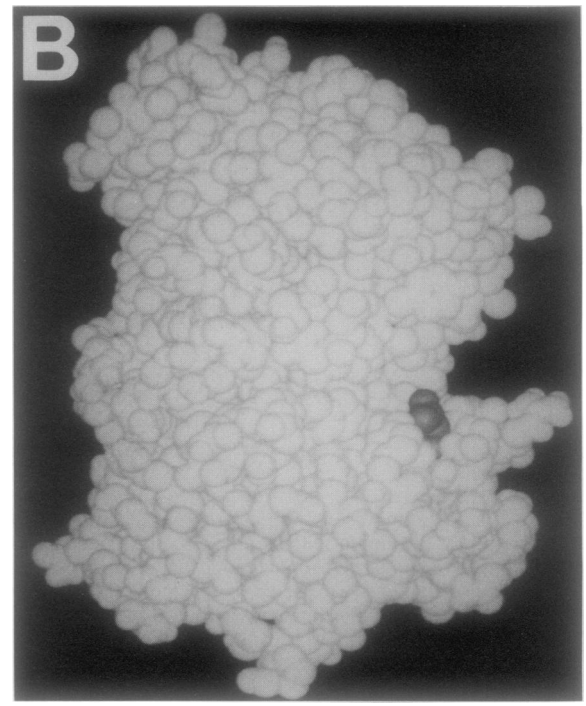


FIG. 4. Molecular models of crystalline structures predicted for *P. aeruginosa* mature elastases encoded by wild-type *lasB*, *lasB1*, and *lasB2* alleles. Crystal coordinates from an X-ray diffraction analysis of elastase (30) were kindly provided by D. B. McKay and used to visualize the wild-type molecule on an Evans and Sutherland graphics computer. The molecular modeling of altered elastases was performed by using the SYBYL Molecular Modeling Software for quantitative comparisons of closely related molecules. An energy minimization algorithm was used to detect predicted changes in conformation resulting from substitutions at His-223. Space-filling molecular models, with residue 223 shaded, are shown for the following: A, wild-type *lasB*-encoded elastase (His-223); B, *lasB1*-encoded elastase (Asp-223); C, *lasB2*-encoded elastase (Tyr-223).

proteolysis, *E. coli* lysates containing the *lasB1* and *lasB2* products were incubated with purified mature elastase and then examined by immunoblot analysis. Incubation of the *lasB1*- and *lasB2*-containing preparations alone left most of the elastase-related proteins in the 51-kDa form (Fig. 5B, lanes 2 and 4). In contrast, incubation (45 min at 37°C) with purified elastase (0.1 μg) resulted in the disappearance of the 51-kDa species with the concomitant increase in the intensity of the 33-kDa form (lanes 3 and 5). Thus, mutant proelastases encoded by *lasB1* and *lasB2* were still processable in an intermolecular reaction by active elastase. Conversion to the 33-kDa elastase did not give rise to new proteolytic activity, further substantiating the conclusion that His-223 is essential for activity (data not shown).

DISCUSSION

Although elastase is secreted by *P. aeruginosa* as a 33-kDa protein, larger precursors of the enzyme are formed and undergo proteolytic processing within the cell before secretion. To gain a better understanding of the mechanisms underlying the proteolytic processing of proelastase and secretion of the mature form, we cloned the elastase structural gene, *lasB*, from *P. aeruginosa* and developed systems for *lasB* expression in *E. coli*. Under the control of its own promoter, *lasB* was weakly expressed in *E. coli* (1) (this study). However, *lasB* was well expressed under the control of inducible T7 and *tac* promoters. Placing the *tac* promoter just 38 bp from the initiation codon of *lasB* also improved overall expression. Although high-level expression of *lasB* was attained in *E. coli*, the 53.6-kDa preproelastase species was never detected in these studies. This was presumably due to the rapid removal of the signal peptide by *E. coli* signal peptidase. The 51-kDa proelastase precursor was

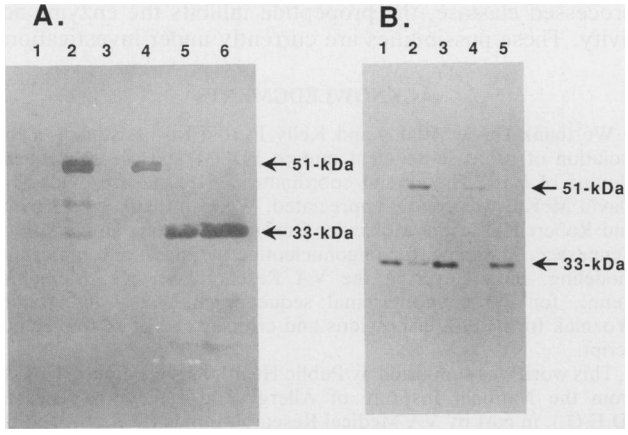


FIG. 5. (A) Overexpression of mutant *lasB* alleles and accumulation of the mutant proelastases in *E. coli*. An immunoblot is shown of proteins from noninduced and IPTG-induced JM109 cells harboring the following: pKSM5 encoding *lasB1*, uninduced (lane 1) and induced (lane 2); pKSM6 encoding *lasB2*, uninduced (lane 3) and induced (lane 4); and pKSM2 encoding wild-type *lasB*, uninduced (lane 5) and induced (lane 6). Elastase-related proteins were visualized with rabbit antibodies against denatured *P. aeruginosa* elastase and a horseradish peroxidase-conjugated secondary antibody. Positions corresponding to the 33-kDa mature elastase and the 51-kDa proelastase are indicated. (B) In vitro processing of mutant proelastases by mature elastase. An immunoblot is shown of the following: 0.1 μg of purified mature elastase from *P. aeruginosa* (lane 1); extracts of IPTG-induced JM109(pKSM5 *lasB1*) cells alone (lane 2) or with 0.1 μg of elastase (lane 3); and extracts of IPTG-induced JM109(pKSM6 *lasB2*) cells alone (lane 4) or with 0.1 μg of elastase (lane 5). All samples were incubated for 45 min at 37°C before SDS-PAGE and subsequent electroblotting.

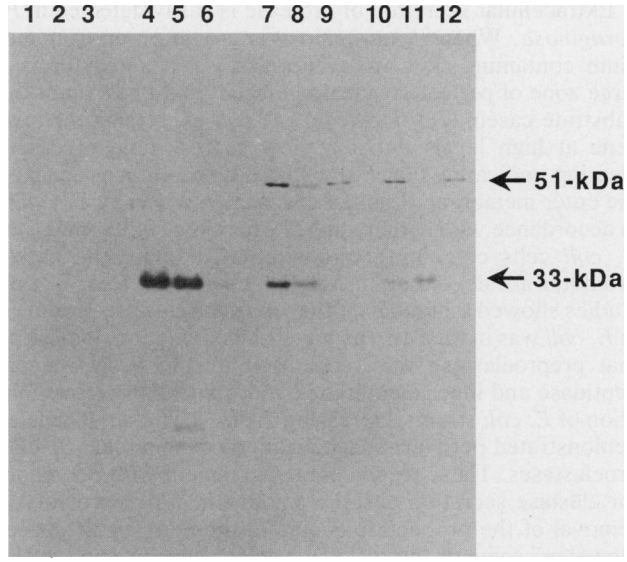


FIG. 6. Localization of products expressed from the *lasB*, *lasB1*, and *lasB2* alleles in *E. coli*. SDS-PAGE and immunoblots with anti-elastase of proteins from periplasmic- (P), cytoplasmic- (C), and membrane- (M) enriched fractions of IPTG-induced JM109 cells are shown harboring the following: pKK223-3 (P [lane 1], C [lane 2], M [lane 3]); pKSM1 *lasB*$^+$ (P [lane 4], C [lane 5], M [lane 6]); pKSM5 *lasB1* (P [lane 7], C [lane 8], M [lane 9]); and pKSM6 *lasB2* (P [lane 10], C [lane 11], M [lane 12]). The *lasB1* and *lasB2* mutant fractions were loaded in fourfold excess to compensate for the reduced accumulation of their products compared to those of the wild type. Positions corresponding to the 33-kDa mature elastase and the 51-kDa proelastase are indicated.

detected; however, most of the elastase-related material observed in *E. coli* was in the processed 33-kDa form. Amino-terminal sequence analyses of the 33-kDa elastase produced in *E. coli* revealed that processing of the 51-kDa proelastase occurred at the physiological cleavage site used in the *P. aeruginosa* secretion pathway. The finding that rapid and accurate processing of proelastase occurred in the absence of the secretion machinery of *P. aeruginosa* raised the possibility that processing could be autocatalytic.

On the basis of its similarity to thermolysin, active-site residues of elastase are likely to include His-223 (1, 5), a prediction which was substantiated by analysis of the elastase crystalline structure (30). To study processing of proelastase molecules devoid of enzymatic activity, oligonucleotide-directed mutagenesis of the *lasB* gene was used to replace the positively charged His-223 codon with those encoding the negatively charged Asp-223 (*lasB1*) or the bulky Tyr-223 (*lasB2*). Expression under the *tac* promoter in *E. coli* revealed that the wild-type allele expressed high levels of proteolytic and elastolytic activity, whereas no proteolytic or elastolytic activity was detectable upon expression of the mutated alleles, *lasB1* and *lasB2*. This verified the prediction that His-223 is essential for enzymatic activity. When the effects of the active-site mutations on proelastase processing were examined, most of the *lasB1*- and *lasB2*-encoded proteins remained in the 51-kDa precursor form. This was in contrast to the product of the enzymatically active wild-type *lasB* allele which was efficiently processed to the mature 33-kDa form. These results suggest that removal of the propeptide from proelastase is an autoproteolytic reaction. Such a mechanism is consistent with the observations that metal chelators, which act as elastase inhibitors, also inhibit proelastase processing (14, 14a).

The results of the computer modeling studies also support the autoproteolytic processing hypothesis, suggesting that the substitutions made at residue 223 caused no overt conformational alterations in the proteins. These results were anticipated because the R group of amino acid 223 is in the aqueous milieu of the active-site cleft of elastase and should have had little influence on the surrounding amino acid residues of the protein (Fig. 4). Further evidence for autoprocessing came from our demonstration that the mutant proelastases were readily processed *in trans*. Mild proteolytic treatment of mutant proelastases with *P. aeruginosa* elastase was sufficient to convert them to mature-sized proteins. It was interesting, however, that accumulating mutant proelastases were processed, albeit inefficiently, to the 33-kDa form. This was especially noticeable upon storage of the cell extracts and may be due to endogenous *E. coli* proteases.

We observed that the total amount of elastase-related material that accumulated in *E. coli* after induction of the *lasB* gene was reduced (about fivefold) when codon 223 was altered. It seems unlikely that a substitution at His-223 would affect *lasB* transcription or translation. The codon changes made in *lasB1* and *lasB2* are commonly used in abundant proteins from *E. coli* (8) and should not have affected translation. Thus, failure to remove the propeptide, which would prevent the subsequent folding to the mature molecule, may cause proelastase to be more susceptible to degradation by *E. coli* intracellular proteases. This possibility is under investigation.

Extracellular secretion of protease is easily detected in *P. aeruginosa*. When *P. aeruginosa* is grown on an agar medium containing skim milk, the elastase secreted forms a large zone of pericolony clearing because of digestion of the substrate casein (28). However, *E. coli* expressing the *lasB* gene at high levels did not show such a zone of casein clearing, indicating that *E. coli* did not secrete elastase past the outer membrane. Lack of elastase secretion by *E. coli* is in accordance with earlier studies where the culture media of *E. coli* cells containing *lasB* expressed under the native promoter are devoid of elastolytic activity (1). Localization studies showed that much of the wild-type elastase produced in *E. coli* was in the periplasmic-enriched fraction, indicating that preproelastase was recognized by the *E. coli* signal peptidase and inner membrane export machinery. Fractionation of *E. coli* strains expressing the *lasB1* and *lasB2* alleles demonstrated periplasmic localization of the mutant 51-kDa proelastases. These results are in agreement with the model for elastase secretion described above in which proteolytic removal of the propeptide occurs in the periplasmic space. However, some of the 51-kDa species were detected in the membrane fraction, suggesting that translocation through the inner membrane is more efficient if proteolytic processing of proelastase occurs. Studies on the localization of these elastase-related proteins in the native host *P. aeruginosa* are currently in progress. These should shed light on the functions the propeptide may fulfill in translocation through both the inner and outer membranes.

The formation and subsequent removal of a propeptide, which is distinct from the signal peptide, is not unique to *P. aeruginosa* elastase production. The serine endoprotease subtilisin secreted by *Bacillus subtilis* also has a large propeptide between the signal sequence and mature protein, and it is removed during secretion by an autoproteolytic process (32). Mutations in the subtilisin gene that give rise to unprocessed subtilisin molecules include alterations in catalytically critical residues. When processing of the precursor is blocked in a *B. subtilis* host in which the subtilisin gene is deleted from the chromosome, secretion is also defective (25). In contrast, when the mutant subtilisins are expressed in *B. subtilis* hosts that contain an intact chromosomal subtilisin gene, the mutant precursors are processed to a mature form and released to the medium. Such processing *in trans* of the precursor has also been demonstrated in vitro by the addition of active subtilisin (25). The data presented in our study suggest that processing of elastase in *P. aeruginosa* may occur by an autocatalytic process similar to that described for subtilisin.

The function of the elastase propeptide has not been elucidated. Propeptides of other secreted bacterial proteases (e.g., *Bacillus* species subtilisin, *Staphylococcus staphylolyticus* lysostaphin, and *Lysobacter enzymogenes* α-lytic protease) have been proposed to function in restricting the proteolytic activity of the proenzyme within the cell to the cleavage of its own propeptide (31). Also, the propeptide may keep the proenzyme in a specific conformation until subsequent intramolecular processing and secretion releases the mature enzyme to fold into its active form. We observed that overexpression of the wild-type *lasB* allele did not affect the growth rates or colony morphology of *E. coli* hosts despite the fact that processing was efficient and that high levels of proteolytic and elastolytic activities were found in extracts of these cells. This suggested that the proteolytic activity of processed elastase within the *E. coli* cells was inhibited. It is possible that, by forming a complex with the processed elastase, the propeptide inhibits the enzyme activity. These possibilities are currently under investigation.

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