Pseudomonas aeruginosa lasB1 Mutants Produce an Elastase, Substituted at Active-Site His-223, That Is Defective in Activity, Processing, and Secretion

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Pseudomonas aeruginosa secretes elastase in a multistep process which begins with the synthesis of a preproelastase (53.6 kDa) encoded by lasB, followed by processing to proelastase (51 kDa), and concludes with the rapid accumulation of mature elastase (33 kDa) in the extracellular environment. In this study, mutants of P. aeruginosa were constructed by gene replacement which expressed lasB1, an allele altered in vitro at an active-site His-223-encoding codon. The lasB1 allele was exchanged for chromosomal lasB sequences in two strain backgrounds, FRD2 and PAO1, through a selectable-cassette strategy which placed a downstream Tn501 marker next to lasB and provided the selection for homologous recombination with the chromosome. Two lasB1 mutants, FRD720 and PDO220, were characterized, and their culture supernatants contained greatly reduced proteolytic (9-fold) and elastolytic (14- to 20-fold) activities compared with their respective parental lasB+ strains. This was primarily due to the effect of His-223 substitution on substrate binding by elastase and thus its proteolytic activity. However, the concentration of supernatant elastase antigen was also reduced (five- to sevenfold) in the mutant strains compared with the parental strains. An immunoblot analysis of cell extracts showed a large accumulation of 51-kDa proelastase within lasB1 mutant cells which was not seen in wild-type cell extracts. A time course study showed that production of extracellular elastase was inefficient in the lasB1 mutants compared with that of parental strains. This showed that expression of an enzymatically defective elastase inhibits proper processing of proelastase and provides further evidence for autoproteolytic processing of proelastase in P. aeruginosa. Unlike the parental strains, culture supernatants of the lasB1 mutants contained two prominent elastase species that were 33 and 36 kDa in size. Extracellular 51-kDa proelastase was barely detectable, even though it accumulated to high concentrations within the lasB1 mutant cells. These data suggest that production of an enzymatically defective elastase affects proper secretion because autoproteolytic processing of proelastase is necessary for efficient localization to the extracellular milieu. The appearance of reduced amounts of extracellular elastase and their sizes of 33 and 36 kDa suggest that lasB1-encoded elastase was processed by alternate, less-efficient processing mechanisms. Thus, proelastase must be processed by removal of nearly all of the 18-kDa propeptide before elastase is a protein competent for extracellular secretion.

Pseudomonas aeruginosa is an opportunistic pathogen which causes a variety of disease manifestations in compromised hosts. The ability of P. aeruginosa to secrete several toxic and degradative enzymes into the environment is a major contributor to the pathogenesis of the organism. Elastase is one of several extracellular proteases secreted by P. aeruginosa and is considered a major virulence factor. This is supported by its ability to degrade a number of biologically important proteins, including elastin (18), some collagens (8), immunoglobulins G (2) and A (7), serum α2-proteinase inhibitor (19), and complement components (23), and it releases iron bound to transferrin (3).

Elastase is a neutral metalloprotease requiring one zinc ion per molecule that is essential for its activity and a calcium ion for stability (18). Elastase production and processing are facilitated by a growth medium containing both zinc and calcium ions (22). On the basis of the inferred amino acid sequence (1, 5, 24) and crystallographic structure (25), elastase shares a high degree of sequence and functional homology with the zinc metalloprotease thermolysin of Bacillus thermoproteolyticus. These similarities to thermolysin have allowed the prediction of specific residues involved in elastase enzymatic activity and substrate binding, as well as zinc and calcium binding.

Kessler and Safrin (9, 10) proposed a model for elastase secretion, now refined by DNA sequence information, which involves two proteolytic processing steps. Elastase, encoded by lasB, is initially synthesized as a proproelastase with a molecular mass of 53.6 kDa. During translocation through the inner membrane, a 2.6-kDa signal sequence is removed to form a 51-kDa proelastase (12). The proelastase is rapidly processed to a 33-kDa mature form by cleavage of an 18-kDa N-terminal propeptide. The model proposes that the propeptide remains noncovalently associated with a 33-kDa periplasmic elastase until further processing or dissociation of the complex occurs, which is followed by secretion of the mature enzyme through the outer membrane.

We have recently shown that overexpression of lasB in Escherichia coli results in the intracellular accumulation of processed and enzymatically active 33-kDa elastase; however, little 51-kDa proelastase is seen (16). When the codon in lasB encoding His-223, an active-site residue, is changed to encode Asp-223 (lasB1) or Tyr-223 (lasB2), overexpression of these mutant alleles in E. coli results in both loss of enzymatic activity and accumulation of the unprocessed

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51-kDa proelastase (16). These results suggest that the rapid processing of proelastase to mature elastase is autocatalytic.

In the present study, we developed a gene replacement strategy to construct defined mutants of \textit{P. aeruginosa} using a cloned gene modified in vitro by a single base pair change (e.g., lasB1). To study processing and secretion of an enzymatically defective elastase in the native host, we constructed mutants of \textit{P. aeruginosa} with a chromosomally encoded lasB1 allele expressed under its native promoter. These studies showed that modifying the substrate-binding residue, His-223, affected not only enzyme activity but also proelastase processing and extracellular secretion of elastase in \textit{P. aeruginosa}. These results indicate that the pathway of elastase secretion in \textit{P. aeruginosa} includes autoprocessing.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids and media.** The bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were cultured in L broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl [pH 7.5]) or a minimal medium (27). Media were solidified with 1.5% Bacto agar (Difco). Unless otherwise specified, antibiotics were used at the following concentrations (per milliliter): ampicillin, 100 \(\mu\)g for \textit{E. coli}; carbenicillin, 300 \(\mu\)g for \textit{P. aeruginosa}; kanamycin, 30 \(\mu\)g for \textit{E. coli} or 500 \(\mu\)g for \textit{P. aeruginosa}; tetracycline, 15 \(\mu\)g for \textit{E. coli} or 100 \(\mu\)g for \textit{P. aeruginosa}; and mercuric chloride, 18 \(\mu\)g for both \textit{E. coli} and \textit{P. aeruginosa}. Casein-agar plates contained 1.5% skim milk (Difco) and 0.8% nutrient broth (Difco). Elastin-agar plates contained 0.5% elastin (Sigma) and 0.8% nutrient broth (Difco).

**DNA manipulations.** Routine DNA manipulations and plasmid extractions were performed as described elsewhere (14). Triparental matings were used to mobilize recombinant plasmids from \textit{E. coli} to \textit{P. aeruginosa} as previously described (6). DNA sequences were determined by the chain termination technique with Sequenase (U.S. Biochemical) at 42°C by using 5'-[\(\alpha\)-\(32\)P]dCTP (>6,000 Ci/mmol, 10 mCi/ml; Amersham) and 7-deaza-dGTP. Oligonucleotides used for sequencing primers were synthesized on an Applied Biosystems 380B DNA synthesizer in the Molecular Resources Center of the University of Tennessee, Memphis.

SDS-PAGE and immunoblotting. Protein samples were 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 a 12.5% polyacrylamide gel for polyacrylamide gel electrophoresis (PAGE) (13). Proteins in polyacrylamide gels were electrotransferred to nitrocellulose in a Trans-Blot apparatus (Bio-Rad) for 2 h at 160 mA and 4°C. Immunoblotting was performed as previously described (16) with rabbit anti-elastase immunoglobulin \(G\) (a gift of E. Kessler) as the primary antibody and then with a goat anti-rabbit horseradish peroxidase conjugate (Sigma).

**Gene replacement in \textit{P. aeruginosa} FRD2 with selectable cassettes.** An adjacent \textit{tnsO1} (encoding mercury resistance) was used as a selectable marker to recombine the \textit{lasB1} mutant allele into the chromosome of \textit{P. aeruginosa}. Adjacent to \textit{lasB} are a 2.2-kb \textit{PstI} fragment and a 4.3-kb \textit{KpnI} fragment (Fig. 1). Because \textit{Tn501} contains no \textit{PstI} or \textit{KpnI} sites, such restriction fragments containing \textit{Tn501} can be used as selectable cassettes that can be ligated next to a DNA fragment containing \textit{lasB1}. \textit{pKSM15} (Fig. 1) and plasmids containing fragments of \textit{pKSM15} were subjected to \textit{Tn501} mutagenesis as previously described (21). The relative positions of insertions, mapped by restriction analysis, are shown on the map of \textit{pKSM15} (Fig. 1). The DNA fragments with \textit{Tn501} insertions were exchanged for chromosomal sequences in FRD2 by a transduction method and with phage F116L as previously described (21), and the strains constructed were examined for any defects in protease production that might occur as a result of the insertion. The 2.2-kb \textit{PstI} and 4.2-kb \textit{KpnI} fragments containing \textit{Tn501-6} (8.3 kb) were cloned from \textit{pKSM15::Tn501-6} into pBlue-Script KS- to provide a source of these selectable cassettes.

### TABLE 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or phenotype</th>
<th>Source or reference</th>
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</thead>
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<td>D. Haas</td>
</tr>
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</tr>
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</tr>
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</tr>
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<td><strong>Plasmids</strong></td>
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<td>Stratagene</td>
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<td>Inducible tac promoter expression vector; Ap'</td>
<td>Pharmacia</td>
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<td>pLAFR3 (IncP1) with 8-kb \textit{EcoRI} \textit{P. aeruginosa} DNA containing lasB; Te'</td>
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<tr>
<td>p720-BAM</td>
<td>pEMRZ23 with 15.2-kb \textit{BamHI} \textit{FRD720 DNA containing lasB1 and \textit{Tn501-6}; Ap' Km' Hg'}</td>
<td>This study</td>
</tr>
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* Abbreviations for genotypes: Te', tetracycline resistance; Hg', mercury resistance; Ap', ampicillin resistance; Km', kanamycin resistance; Cb', carbenicillin resistance; Tra, transfer by conjugation.
Genes replacement in *P. aeruginosa* PAO1. A *lasB* mutant was constructed in the PAO strain background as follows. FRD720 (*lasB* Tn501-6) genomic DNA was digested with *BamH*I, ligated into the *BamH*I site of the gene replacement vector pEMR2, packaged in vitro into λ particles (Gigapack II λ packaging kit; Stratagene), and transduced into *E. coli* HB101 with selection on L agar containing HgCl₂. Tn501 does not have a *BamH*I site. Plasmids from mercury-resistant colonies were screened by restriction analysis, and a clone (p720-Bam) was identified containing a single *BamH*I fragment which included Tn501-6 (8.3 kb) and 19.6 kb of *P. aeruginosa* DNA. Sequence analysis was used to verify that the *lasB* mutation and the downstream Tn501-6 were present in this clone (data not shown). The p720-Bam construct was used to introduce the *lasB* mutation into the *P. aeruginosa* PAO1 chromosome by the excision marker rescue method as described above. Gene replacement was verified through a Southern blot analysis (data not shown).

Growth curves and sampling for elastase production. Logarithmic-phase (optical density at 600 nm [OD<sub>600</sub>] of 0.6) cultures of *P. aeruginosa* strains were used to inoculate (1:100) L broth (250 ml, 1-liter flask), and then they were incubated at 37°C with maximum aeration. Sample sizes withdrawn each hour were 1 ml for the first 5 h and 10 ml for the next 13 h. OD<sub>600</sub> was used to approximate the cell density. Samples were prepared for immunoblot analysis and enzyme assays as follows: a 0.5-ml aliquot was centrifuged (14,000 × g for 2 min at room temperature), and the pellet was resuspended in SDS sample buffer and incubated at 100°C for 5 min (cell extract fraction). The remainder of the sample was centrifuged (8,000 × g for 10 min at 4°C), and 2 ml of the resulting supernatant was stored at −70°C until used in assays for enzyme activity and elastase antigen.
(described below). To prevent further nonspecific proteolysis, phenylmethylsulfonyl fluoride (1 mM) and EDTA (5 mM) were added to the supernatant remaining, which was then concentrated 25-fold in a Minicon B15 unit (Amicon). SDS sample buffer (25 μl) was added to each, and samples were incubated for 6 min at 95°C (supernatant fraction). Cell extract and supernatant samples from each strain were immunoblotted with antielastase as described above.

**Assays of proteolytic and elastolytic activities.** Standardized cultures of *P. aeruginosa* strains were used for assays of enzyme activities. L broth was inoculated (1:100) with overnight cultures, grown to an OD₆₀₀ of 0.6, and the cultures were used to inoculate (1:100) 10 ml of Luria broth, which was incubated at 37°C with aeration. Maximal elastase accumulated in the extracellular medium by 18 h. Dilutions of samples were assayed for proteolytic and elastolytic activities to establish the linear range of the reactions. Proteolytic activity was determined as previously described (11, 15). Elastolytic activity was determined as previously described (15, 20).

**Quantitation of elastase by ELISA.** Relative concentrations of elastase antigen in culture supernatants were measured by a modified direct-binding enzyme-linked immunosorbent assay (ELISA) as previously described (22). Briefly, samples of culture supernatants (stored at −70°C) were diluted 1:50 in ELISA coating buffer (5 mM sodium carbonate [pH 9.0]), and 100 μl of each diluted supernatant was added to ELISA wells. The ELISA plate was placed in a 100°C water bath for 3 min and then placed at 4°C overnight. Under these conditions, the proteolytic activity of elastase on immunoglobulins was inhibited; however, elastase maintained the potential to be specifically recognized by antibodies as determined by reproducible quantitation of various dilutions of supernatant aliquots (22). Elastase was quantified by adding 100 μl of a 1:250 dilution of rabbit antielastase serum to each well. Addition of a 1:1,000 dilution of peroxidase-conjugated goat anti-rabbit (Cappel Research) and then of the substrate 4-chloro-1-naphthol (Sigma) allowed detection. All assays were performed in triplicate, with the mean ELISA value recorded as OD₆₀₀ per milliliter of culture supernatant.

**RESULTS**

**Construction of a lasB1 mutant of *P. aeruginosa* FRD2 utilizing an adjacent selectable marker.** To obtain a selectable marker next to the lasB gene, DNA downstream of lasB was subjected to Tn501 mutagenesis, and four distinct sites of insertion were mapped (Fig. 1). Since these insertions are in unknown loci that could potentially affect elastase production, each was exchanged for chromosomal sequences by gene replacement in FRD2. None of the transposon insertions shown in Fig. 1 had any apparent effect on expression of lasB or extracellular proteolytic activity (data not shown). Although our preliminary studies (15) suggested that recombinants with chromosomal insertions at Tn501-1 were adversely affected in elastase production, further study showed that this phenotype was related to a spontaneous mutation (lasC) in the strain's background that was not directly associated with the insertions. The nature of this mutation is under investigation.

We previously described the lasB1 allele which contains a single base pair mutation that changed the codon for His-223 (a substrate-binding residue) to encode Asp-223, a mutation which adversely affects proteolytic activity when expressed in *E. coli* (16). By a selectable cassette strategy, DNA containing the lasB1 allele was cloned next to DNA containing Tn501-6 in the gene replacement vector pEMRZ23 (see Materials and Methods). The plasmid formed, pKSM20 (Fig. 1), was used in a gene replacement procedure to exchange the chromosomal wild-type allele in FRD2 for the mutant lasB1 allele with the adjacent Tn501 for selection. Among the colonies tested, about 4% had undergone gene replacement as evidenced by the presence of Tn501 (mercury resistance) and sensitivity to vector-encoded markers (carbenicillin and kanamycin). All eight of the potential lasB1 mutants obtained exhibited a reduction in extracellular proteolytic activity and had barely detectable elastolytic activity on plate assays. One of these mutant strains, designated FRD720, was chosen for further characterization.

To verify that FRD720 contained the lasB1 allele, a BamHI fragment was cloned from FRD720 genomic DNA which conferred mercury resistance in *E. coli*. p720-Bam was a 19-kb BamHI fragment of *P. aeruginosa* DNA from the FRD720 chromosome in pEMR2 which contained Tn501-6 and the presumptive lasB1 allele. The mutation causing the His-223 substitution disrupts a restriction site recognized by SncI and ApaLI within the lasB gene coding sequence (16). Digestion of p720-Bam with SncI (or ApaLI) demonstrated the loss of this restriction site and suggested the presence of the lasB1 mutant allele (data not shown). This was confirmed by sequence analysis of DNA that included codon 223 on p720-Bam, which showed the presence of the lasB1 mutation (data not shown).

**Construction of a lasB1 mutant of *P. aeruginosa* PA01 by using the cloned mutant allele from FRD720.** A lasB1 mutant was also made in the *P. aeruginosa* PA01 background for comparison with FRD720 and to control for any strain-dependent phenomena. Gene replacement with pKSM20 (lasB1 Tn501-6) in FRD2 was not a frequent event, and limited attempts to construct lasB1 mutants in PA01 with pKSM20 were unsuccessful. However, p720-Bam (lasB1 Tn501-6), described above, was a clone similar to pKSM20 and contained another 11 kb of *P. aeruginosa* DNA. Following conjugation of p720-Bam into PA01, mercury-resistant (Tn501-6) colonies showed loss of vector-encoded antibiotic resistance markers at high frequency, approaching 80%. All 80 colonies examined that showed loss of the vector sequences exhibited reduced proteolytic activity and barely detectable elastolytic activity in agar plate assays. One lasB1 mutant of PA01, designated PDO220, was used in the subsequent studies.

**Proteolytic and elastolytic activities in supernatants of lasB1 mutant strains.** To determine the consequence of the lasB1 mutation on extracellular proteolytic activity in *P. aeruginosa*, supernatants from 18-h standardized cultures were obtained from the lasB1 mutants (FRD720 and PDO220) and their respective wild-type strains (FRD2 and PA01). FRD706, which contains a chromosomal Tn501-6 insertion and wild-type lasB allele, was also included in this analysis to control for any effects due to the transposon. The hydrolysis of two substrates was examined: azocasein, for quantitation of general proteolytic activity, and elastin Congo red, for quantitation of elastolytic activity. All three strains (FRD2, FRD706, and PA01) expressing the wild-type lasB allele exhibited high levels of both proteolytic and elastolytic activities (data not shown). In contrast, the levels of proteolytic activity in the culture supernatants of the two lasB1 mutant strains (FRD720 and PDO220) were eight- to ninefold lower than those observed with their respective wild-type strains. Compared with the activity of parent strains, elastolytic activity was reduced in the lasB1 mutants by 14- and 20-fold (FRD720 and PDO220, respectively) (data not shown).
shown). These data supported earlier lasB1 expression studies in E. coli which established that the His-223 residue was important for the enzymatic activity of P. aeruginosa elastase.

**Reduced extracellular elastase in lasB1 mutant cultures.** The reduction in supernatant proteolytic and elastolytic activities observed above with lasB1 mutants was not attributable to reduced growth rates. Measurements of culture turbidity, taken at 1-h intervals over an 18-h period of growth, showed no difference between FRD720 (lasB1 Tn501-6) and FRD706 (lasB+ Tn501-6) (Fig. 2A [open symbols]). Likewise, PDO220 (lasB1 Tn501-6) and PAO1 (lasB+) showed the same growth pattern over time (Fig. 2B [open symbols]) and demonstrated that the Tn501-6 insertion did not affect growth under these conditions. The aliquots removed during the growth analysis were also examined for the concentration of elastase protein by ELISA. The two wild-type strains, FRD706 (Fig. 2A) and PAO1 (Fig. 2B), exhibited a biphasic expression of elastase antigen, with an initial burst between 6 and 9 h and then a plateau in production between 10 and 12 h, which was followed by a rapid rise in elastase concentration through the final time point at 18 h. In contrast, cultures of the lasB1 mutants (FRD720 and PDO220) demonstrated a much slower rise in elastase concentration, and 18-h supernatants contained approximately five- and sevenfold less elastase antigen, respectively, compared with the amounts of their wild-type strains (Fig. 2).

**Effect of lasB1 mutation on proelastase processing and secretion.** Expression of wild-type lasB in E. coli resulted in translocation of some mature (33-kDa), enzymatically active elastase to the periplasm, although this heterologous host (E. coli) was unable to secrete elastase to the extracellular medium (16). The product of lasB1 in E. coli was defective not only in activity but also in processing and translocation to the periplasm (16). Here, we examined the potential for a similar lasB1-mediated defect in P. aeruginosa which affected both processing and translocation. A defect in translocation, in this case, to the extracellular medium would explain the reduced extracellular elastase antigen in cultures of lasB1 mutants described above. To test this, cell extracts and supernatant fractions were taken during the time course study described above and analyzed for elastase antigen by immunoblot analysis. Cell extracts of the lasB+ strains, FRD706 (Fig. 3A) and PAO1 (Fig. 4A), showed mature-size (33-kDa) elastase both within the cell and localized to the supernatants (Fig. 3B and 4B) at all time points. The cell-bound elastase species (33 kDa) from both wild-type strains appeared to form a doublet, which suggests the possibility of two intracellular species, a feature previously noted by Kessler and Safrin (9, 10). In the lasB1 mutants, proelastase (51 kDa) was the dominant species in cell extracts of FRD720 (Fig. 3C) and PDO220 (Fig. 4C), indicating a defect in processing. Both lasB1 mutants accumulated large amounts of 51-kDa proelastase within the cell as early as 6 h. In these P. aeruginosa lasB1 extracts, many sizes of proelastase breakdown products were observed (Fig. 3C and 4C), including a 33-kDa form, which was similar to that seen in our previous E. coli expression studies (16) and which suggests that the lasB1 51-kDa proelastase was susceptible to general proteolytic digestion. Interestingly, the elastase appearing in the lasB1 mutant culture supernatants was of two sizes, a 33-kDa mature-size species and a novel 36-kDa elastase species.

**DISCUSSION**

The secretion of P. aeruginosa elastase is a multistep process which begins with the synthesis of a 53.6-kDa preproelastase and results in the rapid accumulation of
mature 33-kDa elastase in the extracellular environment. In an effort to better understand this pathway, we altered the active-site His-223-encoding codon in the structural gene for elastase to form the lasB1 allele to study its plasmid-borne expression in E. coli (16). In the present study, we constructed P. aeruginosa strains which expressed the lasB1 allele from the chromosome to examine the effect of this enzymatic defect on proelastase processing and secretion in the native organism. The lasB1 allele was exchanged for wild-type lasB sequences in two strain backgrounds, FRD (FRD720) and PAO (PDO220). This was accomplished through a selectable-cassette strategy which placed a downstream Tn501 marker next to lasB1 and provided the selection pressure needed for homologous recombination with the chromosome. In general, the strategy employed here allowed the introduction of a defined, single-base pair alteration into the chromosome of P. aeruginosa and should have general application to other genetic studies of P. aeruginosa.

Supernatants from the two lasB1 mutant strains, FRD720 and PDO220, contained greatly reduced proteolytic (9-fold) and elastolytic (14- to 20-fold) activities compared with those of their respective wild-type parent strains. In part, this could be attributed to the low concentration of supernatant elastase, as detected by ELLA, which was reduced by five- to sevenfold in the mutant strains (FRD720 and PDO220, respectively) compared with parental strains grown under the same conditions. However, reduction in proteolytic and elastolytic activities in the supernatants of lasB1 mutants was primarily due to the His-223 substitution, which affects substrate binding by elastase and thus its proteolytic activity. This is in agreement with our previous studies with lasB1 expression in E. coli, which resulted in almost total loss of both proteolytic and elastolytic activities (16). There are other proteases in the supernatant of P. aeruginosa, including alkaline protease and LasA, which have been reported to exhibit some elastolytic activity (26, 28), and they may account for the residual elastolytic activity. Determination of the specific proteolytic activity of lasB1 elastase, purified from mutant culture supernatants, is in progress and will show whether any residual enzymatic activity remains following a substitution at His-223.

The five- to sevenfold reduction in the level of elastase produced by the lasB1 mutants was not attributable to any detectable growth defect, since the two mutants demonstrated growth patterns comparable to those of the wild-type strains. This was also not due to reduced rates of lasB transcription in the mutants; a lasB-cat operon fusion, constructed in a low-copy-number plasmid, produced chloramphenicol acetyltransferase levels that were almost identical in all strains (17). However, the immunoblot analysis of cell extracts showed a large accumulation of the 51-kDa proelastase form in lasB1 mutant cells which was not seen in wild-type cell extracts. This is further evidence that expression of an enzymatically defective elastase inhibits proper processing of proelastase. We recently have shown that cultures of wild-type P. aeruginosa deprived of zinc and calcium ions, which are required for elastase enzymatic function, also result in an accumulation of proelastase (22). It was of interest that the accumulating proelastase in P. aeruginosa lasB1 cell extracts generally appeared to degrade in a nonspecific fashion, although much of the products was found in a stable 33-kDa form. Even 51-kDa lasB1 proelastase that was overproduced in E. coli, which also underwent nonspecific proteolysis, formed significant

![Immunoblots of samples taken in a time course study showing elastase-related proteins in cell extracts and supernatants of wild-type and lasB1 mutant strains of P. aeruginosa FRD.](http://jb.asm.org/)

**FIG. 3.** Immunoblots of samples taken in a time course study showing elastase-related proteins in cell extracts and supernatants of wild-type and lasB1 mutant strains of *P. aeruginosa* FRD. The number above each lane corresponds to the time point (hours) at which each sample was taken and can be directly compared with Fig. 2. Positions corresponding to the 33-kDa mature elastase and the 51-kDa proelastase are indicated. (A) FRD706 lasB+ cell extract samples; (B) FRD706 lasB+ supernatant samples; (C) FRD720 lasB1 cell extract samples; (D) FRD720 lasB1 supernatant samples.
amounts of 33-kDa mature-size elastase (16). These results suggest that the lasB1 protease may have residual autoprocessing enzymatic activity or that the normal processing site in proelastase may be a preferred site for other proteases within the cell, thus generating the 33-kDa elastase. These possibilities are under investigation.

Extracellular secretion of elastase by the wild-type strains was efficient, with 33-kDa mature elastase appearing in relatively large amounts by 6 h and continuing to accumulate through the 18-h time point. This was consistent with the ELISA results, which showed a rapid increase in elastase supernatant concentration well into the stationary phase (Fig. 2). On the other hand, the mutant strains lagged behind in their ability to secrete elastase across the outer membrane, with significant amounts not appearing until 9 h. In addition, there were clearly two elastase species found in lasB1 mutant supernatant fractions, an approximately 36-kDa form as well as the mature-size 33-kDa form. The 33-kDa form was detectable first, and the larger species began to appear soon afterward. Interestingly, the supernatant fractions of lasB1 mutants contained only barely detectable amounts of 51-kDa proelastase, even though it accumulated to significant amounts within the cell. These data suggest that production of an enzymatically defective elastase perturbs secretion because proelastase must be processed for efficient extracellular secretion to take place. The appearance of the extracellular 33- and 36-kDa species suggests that nearly all of the propeptide must be removed before elastase becomes a protein that is competent for secretion. Thus, the propeptide may contain sequences which, under most circumstance, prevent exoproteins from traversing the membrane. In vitro degradation experiments were performed with trypsin on lasB1 proelastase produced by E. coli, and a similar 36-kDa protein product was observed (data not shown). Thus, there may be a general protease cleavage site upstream of the normal maturation site, at which other proteases in the cell can cleave proelastase. This may provide a less-efficient secondary pathway for processing and secretion of an inactive proelastase. Both the smaller 33- and the 36-kDa species were secreted across the outer membrane, suggesting that secretion through the outer membrane can proceed once processing has occurred by either method.

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