Isolation and Characterization of a *Pseudomonas aeruginosa* PAO Mutant That Produces Altered Elastase

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Pseudomonas aeruginosa PAO mutants defective in elastase were isolated by plate assays of nitrosoguanidine-mutagenized clones. A total of 75 elastase mutants were isolated from 43,000 mutagenized clones. One mutant (PAO-E64) was apparently identical to the parental strain except for its deficiency in elastase activity. This mutant produced an enzyme which was antigenically indistinguishable from parental elastase. Furthermore, equal levels of elastase antigen were produced by this mutant and its parental strain. The mutant elastase, however, had greatly reduced enzymatic activity. Mutant PAO-E64 is presumed to have a mutation in the structural gene for elastase. We have designated the genotype of the mutation in PAO-E64 as *lasA1*.

Pseudomonas aeruginosa is an opportunistic pathogen which is associated with a variety of severe and often fatal infections (15). This organism produces a number of extracellular substances, including proteases, which may play a role in pathogenesis (15). The proteolytic activity of *P. aeruginosa* was first characterized by Morihara (18). Two distinct *P. aeruginosa* proteases have been isolated and purified to homogeneity (17, 22). These enzymes, alkaline protease (17) and elastase (protease II) (19, 21), are classic metalloproteases, although their substrate specificities and pH optima are different (23). *P. aeruginosa* elastase is of particular interest due to its ability to produce corneal ulcers (9, 12), necrotic skin lesions (10, 16), and pulmonary hemorrhages (12) and to inactivate human plasma α_1-proteinase inhibitor (23).

This report describes the identification and characterization of a mutation which effects elastase activity but not the amount of elastase protein produced.

Protease mutants previously isolated from *P. aeruginosa* have been pleiotropic (27). This is the first report to describe the isolation and characterization of a mutant of *P. aeruginosa* with a specific protease mutation.

MATERIALS AND METHODS

Bacterial strain. *P. aeruginosa* strain PAO1, which was isolated by B. W. Holloway (7), was used in this study. Strain PAO1 was chosen for this study as it produces toxin A, elastase, and alkaline protease. In addition, it is virulent in several animal models, and its genome is well characterized (7).

Isolation of mutants. Log-phase cultures of *P. aeruginosa* PAO1 grown in nutrient broth with 0.5% yeast extract (Difco) (NYB) were mutagenized with 50 µg of *N*-methyl-*N'*-nitrosoguanidine (Sigma Chem-

ical Co.) per ml at 37°C for 30 min by the method of Finkelstein et al. (5). The cells were washed twice in NYB, resuspended in NYB, and incubated with shaking at 37°C overnight. NYB was inoculated with the mutagenized overnight cultures and incubated at 37°C with shaking until density was approximately 3×10^8 cells per ml. The cultures were diluted in sterile saline such that 0.1 ml yielded approximately 50 colonies per plate of nutrient agar containing 0.3% elastin (elastin-NA). These elastin-NA plates were incubated at 37°C for 48 h. A zone of clearing was observed surrounding colonies producing elastase. Those colonies which showed no zone of clearing were recloned, tested again on elastin-NA plates, and stored in sterile skim milk at −70°C.

Culture conditions for toxin A production. The culture medium for the production of toxin A contained deferrated Trypticase soy broth dialysate, 1% glycerol, and 0.05 M monosodium glutamate (TSBD), as previously described (1). Samples (1 ml) of log-phase cultures (optical density at 540 nm, 0.5) were used to inoculate 9.0-ml amounts of medium in 125-ml Erlenmeyer flasks (nitric acid cleaned). The flasks were incubated at 32°C with maximum aeration for 20 h. Bacterial growth was measured by changes in turbidity (optical density at 540 nm). The supernatants were obtained by centrifugation, dialyzed against 0.05 M Tris-hydrochloride buffer (pH 8.0) at 5°C for approximately 18 h, and sterilized by membrane filtration (0.45 µm; Millipore Corp.). Portions of these supernatants were concentrated 10-fold in Minicon-B15 cells (Amicon Corp.) before storage at −70°C.

ADP ribose transferase activity. Partially purified EF-2 was prepared from extracts of wheat germ as described by Chung and Collier (3). The ADP ribose transferase activity of activated (urea- and dithiothreitol-treated) supernatants was measured as previously described (25). Toxin A was quantitated by comparing its enzymatic activity to standard curves obtained daily with pure toxin. The amount of toxin A present in a crude supernatant was then calculated from the standard curve (8).

Culture conditions for elastase production. The liquid medium employed for elastase production contained 5% peptone (Difco) and 0.25% Trypticase soy broth (PTSB) (4); 2-ml amounts of log-phase cultures (optical density at 540 nm, 0.5) were used to inoculate 18.0-ml amounts of medium in 250-ml Erlenmeyer flasks. The flasks were incubated with maximum aeration at 37°C for 16 h or at 21°C for 36 h. Supernatants were obtained by centrifugation, sterilized by membrane filtration, and stored at −70°C. Other liquid media tested for elastase production were TSBD, MTYG medium, which was developed by Wretlind et al. (27), nutrient broth (Difco), and NYB.

Elastase activity assays. Elastase activity was quantitated by a modification of a procedure described previously (2). To 15-ml glass tubes were added 10 mg of elastin-Congo red (Sigma) and 2.0 ml of reaction buffer (0.1 M Tris-maleate buffer, pH 7.0, 1 mM $CaCl_2$). After equilibration to the reaction temperature, 1.0 ml of PTSB culture supernatant was added. The tubes were capped and incubated horizontally with rapid shaking at room temperature (21°C) for 3 h or at 37°C for 2 h. The reaction was terminated by adding 2.0 ml of 0.7 M sodium phosphate buffer (pH 6.0), and the tubes were placed in an ice water bath. The substrate was removed by membrane filtration (0.45 μm; Millipore). The culture supernatant was replaced with reaction buffer in the blank. The absorbance of the filtrates was read at 495 nm. The background absorbance at 495 nm was obtained by mixing culture supernatants with the assay buffers in the proportions described above but omitting the substrate. Background absorbance was subtracted from enzyme sample absorbance to obtain final values. Elastase activity was expressed as absorbance at 495 nm per hour per milliliter, and the specific activity was calculated as the elastase activity per nanogram of elastase. Elastase protein was quantitated in a radioimmunoassay (RIA) as described below. Elastase activity was also quantitated by using elastin-NA plates. Elastase production was measured as the zone of clearing extending from the edge of a 1.5-cm streak of growth.

Elastase antigen was detected in an agar well assay adapted for elastase. Cultures (1.5-cm streaks) were incubated on a nutrient agar plate at 37°C for 48 h. Small wells were placed between cultures approximately 5 mm from the end of the streaks of growth and filled with elastase antiserum. After overnight incubation at 10°C, precipitin bands formed between the antiserum wells and the strains that produced elastase.

RIA for elastase. The following procedures were used to prepare radiolabeled preparations of elastase for use in RIAs. Highly purified elastase was the kind gift of K. Morihara, Shionogi and Co. Ltd., Osaka, Japan. A 1-mCi amount of carrier-free $Na^{125}I$ (Amersham Corp., Arlington Heights, Ill.) was added to 37.5 μg of elastase and 10 μg of lactoperoxidase in 1 ml of phosphate-buffered saline (pH 7.2). The enzymatic iodination reaction was initiated by adding 25 μl of 0.03% hydrogen peroxide (29). After a 5-min incubation an additional 25 μl of 0.03% H_2O_2 was added, and the reaction continued for 5 min. The labeled preparation was dialyzed for 18 h at 4°C against 1 liter of

phosphate-buffered saline (pH 7.2). The dialyzed sample was applied at 4°C to a Sephadex G-100 column (1.2 by 30 cm; bed volume, 88 ml) equilibrated with phosphate-buffered saline (pH 7.2) containing 1 mg of bovine serum albumin per ml and eluted with the same buffer. Fractions containing the radiolabeled elastase were pooled; sodium azide was added to a final concentration of 0.02%, and the samples were stored at 4°C. Samples possessed specific activities of at least 1 μCi of elastase protein per μg and were more than 85% immunoprecipitable in the presence of excess elastase antiserum (see below).

IgG Sorb (Enzyme Center, Boston, Mass.), a Formalin-fixed preparation of a protein A-bearing strain of *Staphylococcus aureus*, was used as a particulate immunoadsorbent for immune complexes containing immunoglobulin (11, 14). Lyophilized samples were reconstituted to 10% (vol/vol) in NET buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.4) and stored at 4°C. Immediately before use in RIAs, cells were pelleted by centrifugation at 4,000 × g for 15 min at 4°C. Cells were suspended to 10% (vol/vol) in NET buffer containing 0.5% Nonidet P-40 (Particle Data Laboratories, Elmhurst, Ill.) and were incubated for 10 min at 22°C. The cells were then washed once with NET buffer containing 0.05% Nonidet P-40 and finally suspended to 10% (vol/vol) in assay buffer (NET buffer containing 0.05% Nonidet P-40 and 1 mg of bovine serum albumin per ml).

Specific anti-elastase antiserum was the kind gift of B. Wretlind, Naval Medical Research Institute, Bethesda, Md. This antiserum contained no anti-alkaline protease activity, nor was it able to precipitate alkaline protease.

RIAs were carried out in a final volume of 0.5 ml in polystyrene test tubes (10 by 75 mm). Assay buffer was used as the diluent for all reagents. Reagents were added in the following sequence: (i) assay buffer; (ii) approximately 10,000 cpm of [^{125}I]elastase in 10 μl of buffer; (iii) 5 to 100 μl of nonradioactive antigen (either purified elastase or culture supernatants sterilized by membrane filtration) per assay; and (iv) 0.01 μl of elastase antiserum, which was sufficient to immunoprecipitate 50 to 60% of the total radioactivity in the absence of competing antigen. Assay mixtures were incubated for 15 min at 22°C, and then 25 μl of prepared IgG Sorb was added. The samples were incubated for an additional 10 min at 22°C. Immune complexes were collected by centrifugation at 4,000 × g for 15 min at 4°C and washed twice with 1 ml of assay buffer, and the final pellets were counted in a Beckman Biogamma counter.

Biochemical characterization. The tests, media, and procedures for the determination of biochemical activities, serotype, pyocin type, motility, and generation time were as described elsewhere (D. E. Ohman, J. C. Sadoff, and B. H. Iglewski, submitted for publication).

Extracellular product characterization. Production of the following extracellular products was determined as described elsewhere (Ohman et al., submitted for publication): caseinase, esterase, hyaluronidase, DNase, RNase, hemolysins, pyocyanin, fluorescein, gelatinase, lecithinase, staphylolytic activity, and alkaline phosphatase. Lipase production in TSBD

culture supernatants was determined by the method of Wretlind et al. (27), using p-nitrophenyl caprylate (Sigma) as the substrate. The production of alkaline protease in TSBD culture supernatants was determined by an RIA, as described elsewhere (Cryz and Iglewski, submitted for publication).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. TSBD culture supernatants were dialyzed and concentrated as described above. Extracellular proteins were analyzed by electrophoresis on sodium dodecyl sulfate–10% polyacrylamide slab gels as described elsewhere (Ohman et al., submitted for publication).

Genetic techniques. Mutagenesis with ethyl methane sulfonate and enrichment of auxotrophs were as previously described (7, 26). The marker *argF* (55 min) was identified by growth on both citrulline and ornithine (6). The donor strain PAO505 *met*9011 *ami*200 FP2 was the kind gift of D. Haas, Eidgenossische Technische Hochschule, Zurich, Switzerland. Matings on the plates were performed as previously described (24). Recombinants were purified on selective media, and coinheritance of the elastase-positive phenotype was tested on elastin-NA plates incubated at 37°C.

RESULTS

Mutant isolation. Elastase mutants were identified by their failure to produce a zone of clearing on elastin-NA plates when incubated at 37°C for 48 h. A total of approximately 43,000 clones were examined after 12 independent nitrosoguanidine mutagenesis treatments; 75 mutants were identified by reduced elastase activity.

Toxin A production by elastase mutants. Among the 75 elastase mutants identified, 31 produced toxin A, as determined by an agar well assay in which specific antitoxin was used. The loss of toxin A production by the other 44 mutants may have been due to membrane alterations. The relative amounts of toxin A produced by the 31 toxin A$^+$ mutants were determined in the ADP ribose transferase assay. Only 2 mutants (PAO-E23 and PAO-E64) from the original 75 produced parental levels (3 µg/ml) of toxin A. These two mutants were selected for further study.

Biochemical characterization. Elastase mutants PAO-E23 and PAO-E64 were tested for the ability to utilize 24 organic compounds which were found to serve as sole carbon sources for the parental strain, PAO1 (Table 1). Mutant PAO-E64 utilized all 24 compounds, but mutant PAO-E23 was not able to utilize glutamate. Both elastase mutants were able to grow on five selective media, and their generation times (36 min) were the same as the generation time of the parent. Five biochemical tests (arginine dihydrolase, gluconate, malonate, nitrate to gas, and Simmons citrate) were positive for the parental

TABLE 1. *Biochemical characterization of P. aeruginosa strain PAO1*

Tests	Result with strain PAO1[a]
Carbon assimilation	
Acetate	+
Acetamide	+
Adipate	+
Alanine	+
Arabinose	+
Arginine	+
Aspartate	+
Citrate	+
Fructose	+
Fumarate	+
Galactose	+
Glucose	+
Glutamate	+
Glycine	+
Lactate	+
Lysine	+
Malate	+
Mannitol	+
Mannose	+
Propionate	+
Pyruvate	+
Ribose	+
Succinate	+
Xylose	+
Growth	
Cetrimide agar	+
Deoxycholate citrate agar	+
Endo agar	+
MacConkey agar	+
Salmonella-shigella agar	+
Other tests	
Arginine dihydrolase	+
Gluconate	+
Malonate	+
Motility	+
Nitrate to gas	+
Simmons citrate	+

[a] Mutant PAO-E64 gave the same results as the parent (PAO1) in all tests, whereas mutant PAO-E23 did not utilize glutamate and was nonmotile. Other test results were as follows: generation time on TSBD, 36 min; serotype, 3, 7; pyocin pattern, 111, 424.

strain and the two elastase mutants. The pyocin patterns and serotypes of the two mutants were identical to those of the parent (Table 1). The lack of diffusion of growth through motility agar indicated that the motility of mutant PAO-E23 was altered. These results indicated that mutant PAO-E23 did not have a single mutation which affected only the activity or production of elastase.

Production of extracellular products. Release of extracellular products by strain PAO1 and mutant PAO-E64 was determined by using agar plate and liquid culture assays (Table 2). The production of the following extracellular

TABLE 2. *Extracellular products of P. aeruginosa PAO1 and PAO-E64*

Strain	Extracellular products[a]												
	Zone of activity (mm)					Growth on agar			Activity of:				
	Elas-tase	Casei-nase	Gela-tinase	He-mo-lysis (hu-man)	He-mo-lysis (sheep)	Ester-ase	Pyo-cyanin	Fluo-res-cein	Alka-line phos-pha-tase (U/ml)	Lipase (U/ml)	Leci-thi-nase (U/ml)[b]	Alka-line pro-tease (μg/ml)	Toxin A (μg/ml)
PAO1	4.5	6.0	9.0	2.0	0.5	+	+	+	3.9	16.0	0.18	2.7	3.0
PAO-E64	0	6.0	9.0	2.0	0.5	+	+	+	3.8	16.0	0.18	3.4	3.0

[a] Neither strain produced detectable levels of exoenzyme S, RNase, DNase, staphylolytic enzyme, and hyaluronidase. Zones of activity for protease and gelatinase were measured after 24 h, those for hemolysis were measured after 48 h, and those for elastase were measured after 72 h of incubation at 37°C.

[b] One lecithinase unit of activity was defined as the liberation of 1 μM water-soluble organic phosphorus from phosphatidylcholine (from egg yolk; type IXE; Sigma).

products was not detectably altered as a result of the mutation affecting elastase production in mutant PAO-E64: gelatinase, caseinase, human and sheep hemolysins, esterase, pyocyanin, fluorescein, and toxin A. In addition, strain PAO1 and mutant PAO-E64 produced the same levels of alkaline phosphatase, lipase, lecithinase, and alkaline protease. Thus, the elastase mutant PAO-E64 was identical to the parental strain by all of the above criteria, except for its lack of elastase activity.

Extracellular protein profiles on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The culture supernatants of strain PAO1 and mutant PAO-E64 each contained 25 visible bands on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). These gel profiles were indistinguishable. Despite the lack of elastase activity in mutant PAO-E64, no band corresponding to elastase (molecular weight, 23,000 [13, 28] or 39,000 [22]) was missing or reduced in the gel profile of this mutant (Fig. 1).

Production of elastase in liquid medium by PAO1. PTSB was found to be best for elastase production by strain PAO1 compared with the other media tested. The supernatants of PTSB cultures contained at least fourfold-more elastase activity than the supernatants of TSBD, MTYG (27), nutrient broth, and NYB cultures (data not shown). Therefore, PTSB was used for all studies on elastase production.

Elastase activity, as measured in the elastin-Congo red assay at 37°C, was determined as a function of both time of incubation and bacterial cell density. When cells were grown at 37°C, maximum elastase activity and maximum cell density (optical density at 540 nm, approximately 10) was observed 15 h after inoculation. When PTSB cultures were incubated at 21°C, maximum growth and elastase activity were at

36 h after inoculation (data not shown).

Production of elastase antigen. To measure accurately the production of elastase protein by strain PAO1 and mutant PAO-E64, a highly sensitive liquid-phase RIA specific for *P. aeruginosa* elastase was developed. The standard curve for the elastase RIA was linear when the concentration of elastase was between 5 and 400 ng/ml. Purified alkaline protease exhibited no cross-reactivity with elastase (Fig. 2). The addition of PTSB growth medium produced no nonspecific displacement of labeled antigen from precipitated immune complexes (data not shown). The limit of detection with this assay was approximately 5 ng of elastase per ml.

The RIA for elastase showed that strain PAO1 and mutant PAO-E64 produced the same amount of elastase antigen when they were cultured at 37°C (approximately 150 μg/ml) and 21°C (approximately 200 μg/ml) (Table 3). The higher levels of elastase antigen obtained with the 21°C culture supernatants may have been due to increased production or increased stability of this protein at the lower temperature. In addition, the RIA for elastase could not detect any loss of antigenic determinants on mutant PAO-E64 elastase protein. These observations were confirmed by the agar well assay, which was adapted for elastase detection. A precipitin band of complete identity and uniform intensity formed between both parental and mutant colonies and wells containing elastase antiserum (data not shown).

Effect of assay and growth temperature on elastase activity. The observation that mutant PAO-E64 had parental yields of elastase antigen but did not produce a detectable zone of clearing on elastin-NA plates (at 37°C) suggested that this mutant may produce an altered enzyme. This possibility was examined by com-
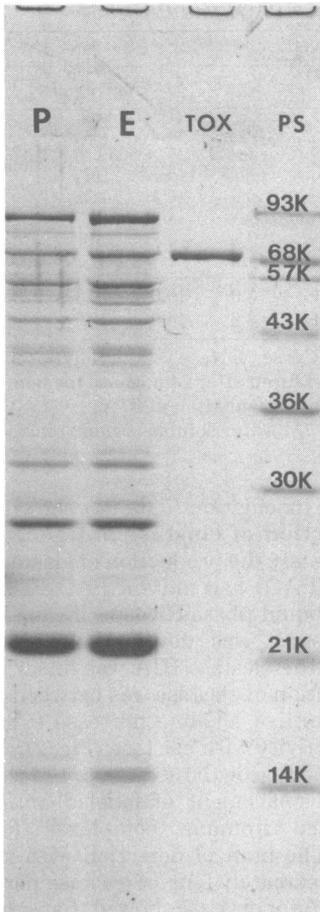
FIG. 1. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of extracellular proteins produced by P. aeruginosa parental strain PAO1 (P) and elastase mutant PAO-E64 (E). Cells were grown in TSBD for 20 h, supernatants were concentrated 10-fold, and 10 μl of each supernatant was applied to the gel. Toxin A (TOX) and the following protein standards (PS) were used as molecular weight markers: phosphorylase B (molecular weight, 93,000 [93K]), bovine serum albumin (68,000), pyruvate kinase (57,000), ovalbumin (43,000), lactate dehydrogenase (36,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (21,000), and lysozyme (14,000).*

paring the specific activities of the mutant PAO-E64 and strain PAO1 elastases (Table 4). The specific activity of the mutant enzyme was much lower (66% less at 37°C) than that of the parental enzyme. Furthermore, when the assay temperature was increased from 21 to 37°C, the specific activity of the parental elastase markedly increased, whereas the specific activity of the mutant elastase increased only slightly. Increasing the growth temperature from 21 to 37°C had no appreciable effect on the specific activity of the mutant or parental enzyme (Table 4). The re-

duced specific activity of the mutant elastase was not due to the synthesis of an inhibitor by the mutant since the activities of mixtures of mutant and parental supernatants gave additive results (data not shown).

The observation that the specific activity of the mutant PAO-E64 elastase did not increase when the temperature of the assay was increased suggested that this mutant may have a temperature-dependent conditional mutation affecting
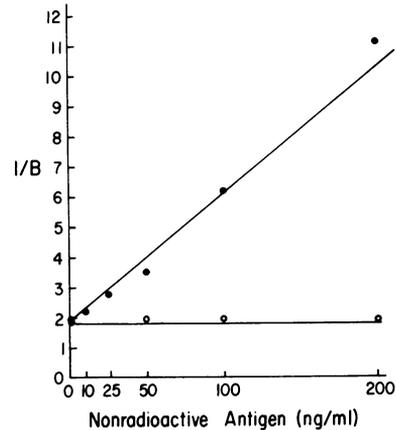
FIG. 2. *RIA for P. aeruginosa elastase. B, Fraction of total labeled antigen immunoprecipitated. Symbols: ●, purified elastase; ○, purified alkaline protease.*

TABLE 3. *Effect of growth temperature on the production of elastase antigen as determined by RIA[a]*

Strain	Elastase concn (μg/ml) at:	
	21°C	37°C
PAO1	218	147
PAO-E64	216	168

[a] Strains were cultured in PTSB at 21°C for 36 h or at 37°C for 15 h.

TABLE 4. *Effect of growth and assay temperatures on elastase specific activity[a]*

Growth temp (°C)[b]	Strain	Sp act at the following assay temp:	
		21°C	37°C
21	PAO1	0.43	1.00
	PAO-E64	0.28	0.32
37	PAO1	0.66	0.98
	PAO-E64	0.28	0.33

[a] Elastase specific activity was defined as the change in absorbance at 495 nm per hour per nanogram of elastase. This experiment was repeated twice with comparable results.

[b] Strains were grown in PTSB at 21°C for 36 h or at 37°C for 15 h.

elastase activity. This possibility was explored by incubating cultures of strain PAO1 and mutant PAO-E64 on elastin-NA plates at varying temperatures (Fig. 3). After 72 h of incubation, strain PAO1 produced zones of clearing at 21°C (3.0 mm), 32°C (4.0 mm), and 37°C (4.5 mm), but not at 42°C. No elastin clearing was observed with mutant PAO-E64 at 37 and 42°C. However, at 32°C, mutant PAO-E64 produced detectable elastase activity. The zone of clearing surrounding mutant PAO-E64 increased to 1.0 mm when a plate was incubated for 72 h at 21°C (Fig. 3). At 21°C in this assay, the mutant produced approximately one-third the elastase activity of the parent. In addition, an elastin-NA plate inoculated with mutant PAO-E64 and incubated at 37°C for 48 h showed no elastin clearing, but subsequent incubation of that plate at 10°C overnight produced a zone of clearing around mutant PAO-E64 (data not shown). This indicates that the elastase produced by PAO-E64 was not irreversibly inactivated at 37°C.

The genotype of mutant PAO-E64 was designated *lasA1*. Preliminary mapping experiments failed to locate the *lasA1* gene.

DISCUSSION

Morihara and Tsuzuki (20) observed that *P. aeruginosa* produces two major extracellular proteases, which are termed alkaline protease and elastase (or protease II). These enzymes have been implicated in the pathogenesis of a variety of *P. aeruginosa* infections (9, 10, 12, 16, 23). Both of these enzymes hydrolyze casein, but only elastase degrades elastin (17, 18). Protease-deficient mutants of *P. aeruginosa* have been

isolated previously on the basis of reduced casein digestion, which is not specific for any one of the three known *P. aeruginosa* proteases (27). As one might expect, all of these mutants have been pleiotropic (27). However, to evaluate the contribution of each of these proteases to virulence, mutants with specific elastase or alkaline protease deficiencies are needed. The use of elastin-NA plates enabled us to isolate a mutant (PAO-E64) with a specific elastase deficiency (Table 1). This mutant produces parental levels of alkaline protease and other extracellular proteins (Table 2 and Fig. 1). This elastase-deficient, apparently nonpleiotropic mutant was isolated at a frequency of 2.3×10^{-5}. We have designated the genotype of the mutant PAO-E64 as *lasA1*. Preliminary genetic mapping studies failed to locate the *lasA1* gene.

Despite the fact that mutant PAO-E64 was isolated as deficient in elastase activity, it produced the same amount of elastase antigen as the parental strain, PAO1. Furthermore, the mutant enzyme was indistinguishable from native elastase in an RIA and in an immunodiffusion assay. However, the specific activity of the mutant elastase was greatly reduced, and it did not increase appreciably with increasing temperature, suggesting that the mutant enzyme may have a temperature-sensitive alteration.

Our data (Tables 3 and 4 and Fig. 1 through 3) suggest that mutant PAO-E64 has a mutation in the structural gene for elastase which results in the formation of an altered enzyme. A single base pair change in the structural gene, resulting in one amino acid alteration, could be sufficient to completely alter the kinetic properties of the mutant elastase. However, we cannot discount the possibility that a preenzyme elastase failed to be converted to an active form due to a defect in a post-translational processing system. Definitive data on the nature of the alteration and thermal stability of the mutant elastase await the purification of the mutant enzyme.

FIG. 3. *Comparison of the zones of elastase activity produced by P. aeruginosa strain PAO1 (P) and elastase mutant PAO-E64 (E) after 3 days of incubation on elastin-NA plates at 21°C (a), 32°C (b), 37°C (c), and 42°C (d).*

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