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Isolation and Characterization of Transposon-Induced Mutants of *Pseudomonas aeruginosa* Deficient in Production of Exoenzyme S

THALIA I. NICAS AND BARBARA H. IGLEWSKI*

Department of Microbiology and Immunology, Oregon Health Sciences University, Portland, Oregon 97201

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Exoenzyme S is an extracellular product of *Pseudomonas aeruginosa*. This enzyme catalyzes the transfer of ADP-ribose from NAD to a number of as yet unidentified eucaryotic proteins, but it is distinct from toxin A. To evaluate the role of exoenzyme S in the pathogenicity of *P. aeruginosa*, we isolated transposon-induced mutants of strain 388, a clinical isolate that produces exoenzyme S but no toxin A. The transposon Tn*1* was introduced by using a temperature-sensitive derivative of plasmid RP1. A Tn*1*-induced mutant was found which had no detectable exoenzyme S activity or antigen in culture supernatants or in cell lysates. Except for its lack of exoenzyme S and resistance to carbenicillin, this mutant was indistinguishable from the parent strain. When tested in an experimental mouse burn infection model, this Tn*1*-induced mutant was reduced in virulence by at least 2,000-fold, suggesting a role for exoenzyme S in the virulence of this strain.

Pseudomonas aeruginosa produces a number of extracellular products that may contribute to its pathogenicity (20, 30). Toxin A, the most toxic substance known to be secreted by *P. aeruginosa*, exerts its lethal effect by inhibiting protein synthesis (12). The enzymatic activity of toxin A is the transfer of ADP-ribose from NAD onto eucaryotic elongation factor 2 (12). Exoenzyme S is a second *P. aeruginosa* extracellular product that has been shown to have ADP-ribosyltransferase activity (14). The enzymatic activity of exoenzyme S differs from that of toxin A in that it does not ADP-ribosylate eucaryotic elongation factor 2, but rather catalyzes the transfer of ADP-ribose from NAD to a number of proteins in crude extracts of eucaryotic cells (14). The two enzymes are also distinct in several physical and biochemical properties, and are not antigenically cross-reactive (27).

Production of exoenzyme S is fairly common among clinical isolates of *P. aeruginosa*, occurring in at least 38% of strains from burn infections and bacteremias (24). Although it has been demonstrated that exoenzyme S is produced in vivo (1), the role of this enzyme in the pathogenesis of *P. aeruginosa* infections has not been established.

To assess the role of exoenzyme S in the pathogenesis of *P. aeruginosa*, we isolated mutants specific for exoenzyme S by using Tn*1* insertion in *P. aeruginosa* 388, a virulent clinical isolate that produces exoenzyme S but not toxin A. A mutant deficient in exoenzyme S production which was thus obtained was markedly reduced in its virulence in a mouse burn infection model.

MATERIALS AND METHODS

Bacterial strains, phage, and plasmids. *P. aeruginosa* 388 is an exoenzyme S-producing hospital isolate previously characterized in our laboratory (1, 14). Plasmid pME19 in *P. aeruginosa* PAO 5 was obtained from R. E. W. Hancock (University of British Columbia, Vancouver, British Columbia, Canada). This plasmid is a temperature-sensitive derivative of plasmid RP1, constructed and described by J. Watson (7). Bacteriophage PRD1 was a gift from R. Olsen (University of Michigan Medical School, Ann Arbor). Bacteriophage G101 was obtained from R. E. W. Hancock.

Introduction of plasmid and transposon mutagenesis. Plasmid pME319 was introduced into strain 388 by conjugation (25), selecting for tetracycline resistance on Vogel-Bonner minimal medium (29) supplemented with 200 μg of tetracycline per ml (Sigma Chemical Co., St. Louis, Mo.). Acquisition of the plasmid increased the MIC of carbenicillin (Geopen; carbenicillin disodium, Roerig, New York, N.Y.) for isolated colonies from 200 to 4,000 μg/ml on carbenicillin-supplemented nutrient yeast agar (NYA), and resulted in sensitivity to the plasmid-specific bacteriophage PRD1. We were unable to score for plasmid-encoded kanamycin and neomycin resistance and resistance to bacteriophage G101 as strain 388 was highly resistant to these antibiotics and G101 did not form plaques on this strain. To isolate insertional mutants from 388(pME319), the strain was grown in tetracycline-supplemented Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) overnight at 32°C, diluted in Trypticase soy broth, plated on NYA containing 2,000 μg of carbenicillin per ml, and grown at either 32°C (for viable counts to determine frequencies) or 43°C (for mutagenesis). Colonies from the 43°C selection were then picked to carbenicillin-supplemented NYA, grown at 43°C, and stored at 4°C. Colonies resistant to carbenicillin were scored for tetracycline resistance by replica plating on NYA supplemented with 200 μg of tetracycline per ml and for auxotrophy by plating on Vogel-Bonner medium. Resistance to PRD1 was scored by streaking bacteria across a streak of PRD1 on NYA plates. In vitro stability of mutants was assessed after serially passing cultures grown at 37°C on King B medium (15).

DNA characterization. Cleared lysates for analysis of plasmid DNA were prepared as described by Crosa and Falkow (reference 4, p. 269) and examined by agarose gel electrophoresis (4).

Preparation of culture supernatants and lysates for exoenzyme S assays. Two media were used to grow strains for exoenzyme S assays: (i) TSBD (13), deferrated dialysate from Trypticase soy broth supplemented with 0.1 M monosodium glutamate, 1% glycerol, and 0.01% nitriloacetic acid (Sigma), and (ii) a defined minimal medium consisting of 25 mM KH_2O_4, 95 mM NH_4Cl, 50 mM monosodium glutamate, 110 mM disodium succinate, 10 mM trisodium nitriloacetate, 2.5% glycerol, 5 mM $MgSO_4$, and 18 μM $FeSO_4$ (J. D. Lile and B. H. Iglewski, submitted for publication). For quantitation of exoenzyme S, cultures were grown at 32°C with

* Corresponding author.

vigorous aeration to allow optimum exoenzyme S production. Bacteria were separated from supernatants by centrifugation at 10,000 × *g* for 10 min. Pellets were suspended in one-tenth the original culture volume in 0.02 M Tris-hydrochloride (pH 7.5) with 1 mg of DNase I (Sigma) per ml and lysed by passage through a French pressure cell at 10,000 lb/in^2. Both supernatants and lysates were held on ice and assayed for exoenzyme S activity within 30 min of preparation.

Exoenzyme S assays. ADP-ribosyltransferase activity of exoenzyme S was assayed by a modification of the assay previously described (14). A 0.005-ml volume of culture supernatant or cell lysates was incubated with 0.025 ml of wheat germ extract (13), 0.025 ml of 0.20 M sodium acetate buffer (pH 6.0), and 0.005 ml of 0.03 mM [*adenosine*-^{14}C(U)]NAD (540 Ci/mol, New England Nuclear Corp., Boston, Mass.) for 5 min at 21°C. The reaction was stopped by addition of trichloroacetic acid (TCA), and TCA-precipitable material was washed, collected, and counted as described previously (13). Preliminary experiments established that bacterial cell lysates did not interfere with measurement of the activity of added exoenzyme S. For screening large numbers of colonies for exoenzyme S production, the assay was modified as follows: cultures were grown in 0.10 ml of TSBD in 96-well microtiter plates for 72 h at 32°C. Cells were pelleted by centrifugation, and 0.02-ml culture supernatants were transferred to a second microtiter well containing 0.10 ml of 0.20 M sodium acetate buffer, 0.01 ml of wheat germ extract, and 0.004 ml of 0.03 mM [*adenosine*-^{14}C(U)]NAD. After 30 min of incubation at 21°C, the entire reaction mix was transferred to 0.20 ml of 10% TCA in a well of a Minifold 96-well filtration manifold (Schleicher & Schuell, Inc., Keene, N.H.). The mixture was then filtered through nitrocellulose paper, washed twice with 5% TCA, and counted as described previously (13). Typical cultures of the parent strain 388 gave 1,500 to 3,000 cpm in this assay.

Purification of exoenzyme S and preparation of antiserum. Details of exoenzyme S purification will be described elsewhere (Lile and Iglewski, submitted for publication). Briefly, culture supernatants were concentrated by filtration on nitrocellulose, and proteins in the 54,000- to 49,000-dalton range were separated by preparative gel electrophoresis, using two different detergent-containing gel systems. Two proteins were purified by electrophoretic extraction from sliced gels, a 49,000-dalton protein and a 53,000-dalton protein. ADP-ribosyltransferase activity was associated with the 49,000-dalton protein. Rabbit antisera were prepared from both the 49,000- and 53,000-dalton proteins by using previously described protocols (5; Lile and Iglewski, submitted for publication). Antibody to the 49,000-dalton protein cross-reacted with the 53,000-dalton protein and vice versa, and antisera against both proteins neutralized the ADP-ribosyltransferase activity of the 49,000-dalton protein.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunological detection of proteins. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out as described by Laemmli (19), using 7.5% acrylamide in the running gel. Culture supernatants were concentrated 20-fold by ammonium sulfate precipitation, and 0.020 ml per lane was applied to the gel. Cell lysates were prepared as described above, and 100 μg of protein per lane was applied to the gel. Protein concentrations were determined by the method of Schacterle and Pollack (23). Electrophoretic blotting to nitrocellulose paper and immunological detection of proteins was carried out by the method of Towbin et al. (28), with the following modifications. Electrophoretic transfer was carried out overnight at 4°C by using a constant current of 25 mA. After blocking with 3% bovine serum albumin in 0.9% NaCl with 10 mM Tris-hydrochloride (pH 7.4) for 1 h, the blots were briefly rinsed in water and incubated for 2 h with rabbit antiserum to exoenzyme S (49,000-dalton form) diluted 1,000-fold in 50 mM Tris-hydrochloride (pH 7.4) with 150 mM NaCl, 5 mM EDTA, and 0.05% Triton X-100 (wash buffer). The nitrocellulose was then again briefly rinsed in water, incubated for 2 h in a 1,000-fold dilution of horseradish peroxidase-conjugated goat antirabbit immunoglobulin (Cappel Laboratories, Malvern, Pa.) in wash buffer, and rinsed for 1 h in wash buffer. The blot was then stained with horseradish peroxidase substrate (Bio-Rad Laboratories, Richmond, Calif.), following the manufacturer's recommendations. All incubations and washes were carried out at room temperature. This procedure was capable of detecting as little as 5 ng of exoenzyme S protein in crude preparations.

Assays for extracellular products. All assays have been described previously. Elastase digestion was assayed on elastin nutrient agar (21), casein digestion was assayed on modified brain heart infusion (BHI) agar containing 10% powdered skim milk (11), esterase was assayed on BHI agar containing 0.1% indoxyl acetate (10), and hemolysis was assayed on BHI supplemented with sheep blood. Production of *P. aeruginosa* elastase and alkaline protease was confirmed by radial immunoprecipitation of antibody to purified products placed in wells adjacent to colonies grown on elastin nutrient agar or skim milk-supplemented BHI (11, 21, 22). Pigment production was assessed on plates prepared according to King et al. (15). Total protease production was determined by assay of supernatants of TSBD-grown cultures by measurement of dye released from hide powder blue (11). Phospholipase C and alkaline phosphatase activity were measured as described previously (3, 18), using supernatants of cells grown in low-phosphate medium (8).

Experimental burn infection model. A previously described burned mouse infection model (1, 26) was used. Female Swiss-Webster mice (Simonson, Hayward, Calif.) weighing 17 to 19 g were shaved, anesthetized with methoxyfluorane (Metophane; Abbott Laboratories, North Chicago, Ill.), and subjected to a 15-s alcohol flame burn involving 15% of the total body surface. Immediately after burning, mice were injected subcutaneously at the burn site with 0.5 ml of bacterial suspension. Bacteria used for infection were grown to early logarithmic phase (absorbance at 540 nm [A_{540}], 0.35 to 0.50), washed once in phosphate-buffered 0.9% saline, suspended in phosphate-buffered saline, and appropriately diluted. The number of CFU per milliliter of bacteria injected was determined for each experiment by plate counts on

TABLE 1. Exoenzyme S ADP-ribosyltransferase activity of *P. aeruginosa* 388 and its Tn*1*-derived mutant 388 *exs*1::Tn*1*

Strain	Exoenzyme S activity (cpm)[a] in:			
	TSBD-grown cultures		Defined medium	
	Supernatant	Lysate[b]	Supernatant	Lysate
388	14,540	2	11,610	1,058
388 *exs*1::Tn*1*	5	0	4	0

[a] Cpm transferred from NAD to TCA-precipitable material in a 5-min ADP-ribosyltransferase assay. Background counts (20 to 55 cpm) have been subtracted. Late logarithmic phase cultures (A_{540}, 0.6 to 0.7) were used.

[b] Lysates were prepared from cells suspended at 10% of their original volume. Figures are corrected to the original volume of culture.

King B agar (15). Mean lethal doses (LD_{50}) were calculated by the Spearman-Karber method (6).

Other methods. Serotypes were determined by previously described methods (22). Growth rates in TSBD were determined in logarithmic-phase cultures (A_{540}, 0.20 to 2.0) by monitoring the A_{540}s of vigorously aerated cultures. For determinations of in vivo stability of mutants, heart blood was collected by cardiac puncture within 1 h after death. Samples were plated on King B agar or Vogel-Bonner medium and after growth at 32°C were replica plated to NYA supplemented with 2,000 μg of carbenicillin per ml or transferred to TSBD for growth for determination of exoenzyme S levels.

RESULTS

Isolation of Tn*1*-induced mutations in *P. aeruginosa* 388. When strain 388 harboring plasmid pME319 (which is temperature sensitive for replication [7]) was grown at 43°C, carbenicillin-resistant colonies appeared at a frequency of ca. 10^{-4} to 10^{-5}. This frequency is approximately 10-fold higher than was previously observed for *P. aeruginosa* PAO by Haas et al. (7). In their study with PAO (7), most carbenicillin-resistant clones were unstable at 43°C, whereas virtually all of our isolates appeared to be stable. The majority (ca. 98%) of carbenicillin-resistant colonies also retained the two other plasmid markers scored, tetracycline resistance and sensitivity to the plasmid-specific phage PRD1. To ensure that the retention of plasmid or transposon markers at high temperature could be resulting in insertional mutation, colonies resistant to carbenicillin at 43°C were screened for mutation to auxotrophy. Among colonies expressing resistance to carbenicillin at 43°C, ca. 0.5% were auxotrophic, suggesting that insertional mutation was occurring. One auxotrophic mutant that was carbenicillin resistant but tetracycline sensitive and PRD1 resistant was further characterized as having a requirement for leucine. This strain was designated 388 *leu*::Tn*1*.

Mutations in exoenzyme S were recognized by using an ADP-ribosyltransferase assay adapted for screening colonies grown in microtiter trays. Presumptive exoenzyme S-deficient mutants were recloned and grown under optimal conditions for exoenzyme S production. Of approximately 1,600 carbenicillin-resistant colonies screened, two mutants deficient in exoenzyme S were found. When DNA from these two strains and from 388 *leu*::Tn*1* was compared with that of strain 388(pME319) by agarose gel electrophoresis of cleared lysates, the mutant strains were found to have lost the DNA band corresponding to pME319 (data not shown). One of the exoenzyme S-deficient mutants, designated 388 *exs*1::Tn*1*, was carbenicillin resistant but lacked the other two plasmid markers, as did 388 *leu*::Tn*1*, suggesting integration of the Tn*1* transposon in these two strains. The second exoenzyme S-deficient mutant, designated 388 *exs*2::pME319, was carbenicillin and tetracycline resistant and PRD1 sensitive, suggesting integration of the entire plasmid. This strain was not further characterized.

In vitro stability of transposon-induced mutants. When strains 388 *exs*1::Tn*1* and 388 *leu*::Tn*1* were serially passed 20 times on TSBD agar without antibiotic at 37°C, no loss of either carbenicillin resistance or the mutant phenotype (i.e., exoenzyme S deficiency or leucine requirement) was observed. The reversion frequency of 388 *leu*::Tn*1* grown in TSBD liquid cultures without carbenicillin and then plated on minimal medium was $<5 \times 10^{-10}$.

In vitro characterization of exoenzyme S-deficient mutants. Exoenzyme S activity of culture supernatants of strain 388 *exs*1::Tn*1* was less than 0.01% of that of the parent strain 388 in each of two media designed to optimize production of exoenzyme S (Table 1). Although 10 to 15% of exoenzyme S activity was found in a cell-associated form in the parent strain grown in defined medium, no cell-associated exoenzyme S activity was found in 388 *exs*1::Tn*1*. Protein and antigen corresponding to exoenzyme S was also absent in 388 *exs*1::Tn*1*. The protein bands corresponding to both the enzymatically active 49,000-dalton form and the 53,000-dalton form of exoenzyme S were missing from culture supernatants of the mutant strain 388 *exs*1::Tn*1* (Fig. 1). Several smaller bands previously identified as breakdown products of exoenzyme S on the basis of their weak cross-reactivity to antibody to exoenzyme S and ADP-ribosyltransferase activity (Lile and Iglewski, submitted for publication) were also absent in the mutant strain; other secreted proteins appeared to be unaffected. Electrophoretic blotting to nitrocellulose and staining, using antiserum to purified 49,000-dalton exoenzyme S, indicated the absence of antigen corresponding to the exoenzyme S proteins in concentrated culture supernatants (Fig. 1, lanes E and F). To establish whether the lack of detectable exoenzyme S activity (Table 1) was due to the accumulation of an enzymatically inactive form of the enzyme rather than failure to produce it, lysates

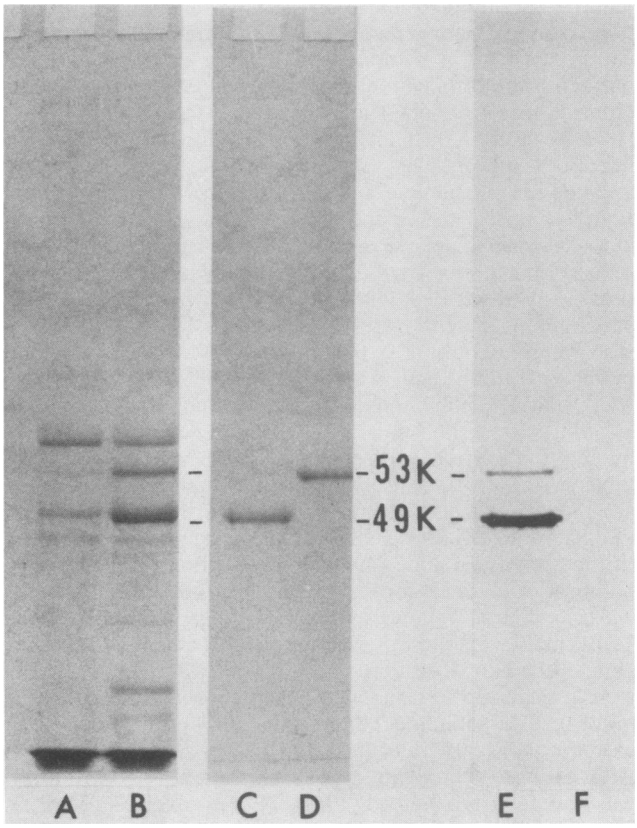

FIG. 1. SDS-PAGE of extracellular products of *P. aeruginosa* 388 and 388 *exs*1::Tn*1*. Lanes A to D were Coomassie blue stained; lanes E and F were blotted to nitrocellulose and stained by using antibody to purified 49,000-molecular-weight (49K) exoenzyme S. Lanes A, B, E, and F: concentrated culture supernatants of strain 388 *exs*1::Tn*1* (A and F) and strain 388 (B and E). Lanes C and D: purified exoenzyme S, active 49K form (C) and inactive 53K form (D). The protein in lane A that appears to be close in size to the 49K band of exoenzyme S is an unrelated protein that does not comigrate with exoenzyme S in other gel systems.

TABLE 2. Plate assays of extracellular products of *P. aeruginosa* 388 and its Tn*1*-derived mutants

Strain	Elastase (zone, mm)	Caseinase (zone, mm)	Sheep hemolysin (zone, mm)	Esterase	Pyocyanin	Fluorescein
388 (parent)	15	15	13	+	+	+
388 *exs*1::Tn*1*	15	15	14	+	+	+
388 *leu*::Tn*1*	15	15	15	+	+	+

of 388 *exs*1::Tn*1* cells grown in either medium were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotting, and staining with anti-exoenzyme S. No exoenzyme S antigen was found in cell lysates, suggesting that the mutation was not causing intracellular accumulation of an enzymatically inactive form of exoenzyme S (data not shown).

To confirm that the transposon-induced mutation in 388 *exs*1::Tn*1* was specific for exoenzyme S and did not affect other secreted products, plate assays were carried out for elastin digestion, casein digestion, hemolytic activity, and esterase (Table 2), and culture supernatants were assayed for levels of activity of phospholipase C and alkaline phosphatase and for total protease activity (Table 3). None of these activities was altered in the mutant strain. Secretion of both major *P. aeruginosa* proteases, elastase and alkaline protease, was confirmed with radial immunoprecipitation, using antibody to these products (11, 19, 22). The mutant was also found to be unaltered in production of pigments and in serotype (Fisher type 1), and its growth rate in TSBD medium was unchanged (generation time, 60 ± 5 min). Strain 388 *leu*::Tn*1* was also unchanged in all properties tested, but in addition, it produced wild-type levels of exoenzyme S activity (Tables 2 and 3).

Virulence of Tn*1*-induced mutants in a burned mouse infection model. *P. aeruginosa* 388 is highly virulent in the burned mouse infection model (1; Table 1). The exoenzyme S-deficient mutant *P. aeruginosa* 388 *exs*1::Tn*1* was much reduced in virulence, with an LD_{50} approximately 2,000- to 12,000-fold greater than that of the parent strain 388 (Table 4). In contrast, the Tn*1*-induced leucine auxotroph, 388 *leu*::Tn*1*, was unchanged in its virulence in this model.

Stability of Tn*1* mutants to passage through mice. Of approximately 2×10^3 colonies replica plated to antibiotic-containing agar after recovery from blood of mice infected with 388 *exs*1::Tn*1* or 388 *leu*::Tn*1*, all were resistant to 2,000 μg of carbenicillin per ml, whereas none of ca. 10^3 colonies recovered from 388-infected mice were carbenicillin resistant. Of 100 colonies of 388 *exs*1::Tn*1* recovered from mice and tested for exoenzyme S production, none had regained exoenzyme S production. Approximately 10^5 388 *leu*::Tn*1* CFU recovered from mice were plated on minimal medium. No revertants to prototrophy were found.

DISCUSSION

The data presented show that exoenzyme S makes a significant contribution to the pathogenicity of *P. aeruginosa* 388 in the mouse burn infection model. *P. aeruginosa* produces a wide variety of extracellular products that may contribute to its pathogenicity. Strain 388 was shown to produce at least two proteases and phospholipase C as well as exoenzyme S, but this strain does not produce toxin A (14). The Tn*1*-induced mutant, 388 *exs*::Tn*1*, appeared to be identical to the parent strain 388, except that this mutant did not produce exoenzyme S and was greatly reduced in virulence. In contrast, the presence of the Tn*1* transposon at another site in strain 388 *leu*::Tn*1* had no discernible effect on virulence. These results, which correlate exoenzyme S production to the virulence of strain 388, extend previous studies that demonstrated that exoenzyme S is produced in vivo in the burned mouse model (1).

The mutation in strain 388 resulted in loss of both the 49,000-dalton protein previously shown to have ADP-ribosyltransferase activity (Lile and Iglewski, submitted for publication) and the antigenically related, enzymatically inactive, 53,000-dalton protein. As transposon mutation generally affects only a single gene or genes in the same operon, this study supports the hypothesis that the two proteins are products of the same gene. This is of interest in light of the recent finding in our laboratory that many strains that lack detectable exoenzyme S ADP-ribosyltransferase activity in vitro do produce the 53,000-dalton protein (J. Lile and B. H. Iglewski, unpublished data). These data suggest that our previous studies indicating the presence of exoenzyme S in 38% of clinical isolates (24) may have underestimated the frequency of exoenzyme S production.

The data presented are compatible with the hypothesis that mutation to exoenzyme S deficiency was the result of insertional mutagenesis. However, as we were unable to isolate revertants, and we are at present unable to introduce the transposon to alternative exoenzyme S-producing backgrounds, rigorous confirmation of insertional inactivation of a gene required for exoenzyme S production has not been possible.

Comparison of isogenic strains altered in a single product is among the most powerful methods for assessing the role of potential virulence factors. However, the use of chemical mutagens to generate such strains in *P. aeruginosa* has proven very difficult and inefficient (5, 21, 22), and even when careful evaluation of mutant phenotype is undertaken, the possibility of undetected second mutations is still present. Transposon mutagenesis has the potential advantages of

TABLE 3. Secretion of extracellular enzymes by *P. aeruginosa* 388 and Tn*1*-derived mutants[a]

Strain	Protease (PU/ml)[b]	Phospholipase activity (U/ml)[c]	Alkaline phosphatase activity (U/ml)[d]	Exoenzyme S activity (cpm)[e]
388 (parent)	1.50	0.38	2.14	5,600
388 *exs*1::Tn*1*	1.53	0.40	2.25	5
388 *leu*::Tn*1*	1.52	0.39	2.18	5,415

[a] All values are means of duplicate assays of supernatants of three to five separate cultures.

[b] PU, Protease units (increase in A_{595} of 1.0 per h at 37°C) in hide powder blue digestion assay of supernatants of cultures grown in TSBD.

[c] U, Increase in A_{410} of 1.0 per min at 25°C in nitrophenylphosphorylcholine chromogenic assay of supernatants of cultures grown in low-phosphate medium.

[d] U, Increase in A_{420} of 1.0 per min at 25°C in nitrophenylphosphate chromogenic assay of supernatants of cultures grown in low-phosphate medium.

[e] Counts per minute transferred from NAD to TCA-precipitable material in a 5-min ADP-ribosyltransferase assay of supernatants of stationary-phase cultures (A_{540}, 2.0) grown in TSBD.

TABLE 4. Virulence of *P. aeruginosa* 388 and Tn*1*-derived mutants in a burned mouse infection model

Strain	LD_{50}[a] (CFU per animal)	
	Expt 1	Expt 2
388 (parent)	21	39
388 *exs*1::Tn*1*	250,000	69,000
388 *leu*::Tn*1*	11	59

[a] LD_{50}s were calculated by the Spearman-Karber method (6).

very low probability of double mutation (16) and relatively high efficiency of mutagenesis (16). As indicated in this and other studies (9), Tn*1* insertions have the additional advantage of being very stable both in vitro and in vivo.

Previous studies with Tn*1* in *P. aeruginosa* (18) and in other species of bacteria (2, 9) have suggested a relatively high degree of regional specificity of insertion for Tn*1*, a factor which would limit its suitability as a potential mutagen. Krishnapillai et al. (17) mapped 12 Tn*1* insertion sites in *P. aeruginosa* PAO and found only 6 unique sites, all distal to 55 min on the PAO chromosome. Their study excluded insertions leading to auxotrophic mutation and used a transduction system to generate insertion, factors which may have influenced the apparent site specificity. Despite this previously reported regional specificity of Tn*1* insertion, we were able to find auxotrophic mutants and mutants in exoenzyme S production at relatively high frequencies in *P. aeruginosa* 388. Furthermore, we have used Tn*1* insertion to isolate auxotrophs and mutants deficient in proteases in a second *P. aeruginosa* strain, PAO (unpublished data). These observations suggest that Tn*1* mutagenesis will prove to be a useful tool in the study of the genetics of *P. aeruginosa* pathogenesis.

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