Toxin A-Deficient Mutants of *Pseudomonas aeruginosa* PA103: Isolation and Characterization

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An immunological assay utilizing double-diffusion principles was developed for identification of *Pseudomonas aeruginosa* mutants deficient in toxin A. Mutations were chemically induced, and mutants were isolated from *P. aeruginosa* strain PA103. Quantitative assays, both enzymatic and immunological, indicated that five mutants produced toxin A in vitro at levels of 0.3% or less of parental strain levels. Characterization indicated that the mutants fell into four classes and suggested that multiple genes are involved in the regulation of toxin A yields. Classes 1 to 3 produced less than 1% of parental levels of extracellular toxin A. Class 1 mutants are apparently specific for toxin A. Class 2 mutants are pleotropic and produced toxin A, protease, and other extracellular proteins at reduced yields. Class 3 mutants are pleotropic and in addition have relatively high levels of cell-bound toxin A. Class 4 mutants produce toxin A at levels greater than 1% of parental yields. Of 16 toxin A-deficient mutants examined, only 1 was a class 1 mutant. This mutant (PA103-29) was shown to be identical to the parental strain in all respects tested except for its marked deficiency in toxin A. The suitability of this class 1 mutant for use in virulence studies is discussed.

Pseudomonas aeruginosa produces a variety of extracellular products which may play a significant role in the pathogenesis of infections in experimental animal models and in humans (42). Toxin A is the most toxic substance known to be produced by *P. aeruginosa* (42, 46). This toxin has been found to inhibit intracellular protein synthesis by the same mechanism as diphtheria toxin (31, 32). Toxin A is produced in vitro by most clinical isolates of *P. aeruginosa* (6) and appears to be produced in humans during *P. aeruginosa* infections (49). With some strains of *P. aeruginosa*, specific anti-toxin A has a protective effect in experimental burn infections of mice (47). These observations suggest that toxin A is a virulence factor.

The pathogenic role of an individual characteristic of an organism can be analyzed by using mutants deficient in that characteristic (25). A mutant which lacks only one potential virulence factor can be compared to the parental strain in animal infection models which mimic the disease in humans. A decrease in virulence with the mutant indicates that the virulence factor has a role in determining the course of the disease. This study was undertaken to isolate and thoroughly characterize toxin A-deficient mutants of *P. aeruginosa* strain PA103 to identify a non-pleiotropic mutant for virulence tests and to examine the phenotypes associated with pleiotropic-hypotoxigenic mutants.

MATERIALS AND METHODS

Organisms. *P. aeruginosa* strain PA103, isolated by Liu (40), was selected for this study because it is highly toxigenic and well characterized. This strain is virulent in some experimental animal infections (35, 46) despite its relatively low proteolytic activity (40, 58). *Staphylococcus aureus* strain Seattle was used in the staphylolytic enzyme assay. All cultures were stored in sterile, 10% (wt/vol) skim milk (Difco) at -70°C.

Toxin A culture medium. Trypticase soy dialysate (BBL Microbiology Systems) (41) treated with Chelex-100 (TSB-DC) was used for the production of toxin A. To 30 g of Trypticase soy broth (BBL Microbiology Systems) in 90 ml of water, 10 g of sodium Chelex-100 (minus 400 mesh, Bio-Rad) was added as a deferrating agent. This mixture was stirred for 6 h at room temperature then dialyzed against 1 liter of deionized water at 5°C for 15 h. The dialysate was supplemented with 0.05 M monosodium glutamate (Sigma) and 1% glycerol. This TSB-DC had an iron concentration of approximately 0.08 µg/ml (44).

Preparation and purification of antitoxin. *P. aeruginosa* strain PA103 was used as a source of toxin A, which was produced and purified as previously described (33). The purified toxin had a mouse median lethal dose of 0.15 µg/22-g mouse when injected intraperitoneally and migrated as a single band in sodium dodecyl sulfate (SDS)-polyacrylamide gels. Specific antitoxin was obtained by immunization of a sheep as previously described (33). Antitoxin A immunoglobulin was obtained from sheep immune serum as previously described (55). The immunoglobulin fraction was further purified by using a solid-phase immunoab-

sorbant (Sephacrose 4B). Purified toxin A was covalently coupled to Sepharose 4B by a modification (33) of the method of March et al. (43). The immunoabsorbant-purified antitoxin A immunoglobulin contained 0.5 mg of protein per ml.

Isolation of mutants. Log-phase cultures of *P. aeruginosa* PA103 grown in nutrient broth with 0.5% yeast extract (Difco) (NYB) were mutagenized with 100 μ g of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) per ml (Sigma) at 37°C for 60 min (approximately 50% survival) by the method of Finkelstein et al. (22). The cells were washed twice in NYB, resuspended in NYB, and incubated with shaking at 37°C overnight. Mutagenesis with ethyl methane sulfonate (EMS) (Sigma) was performed on log-phase cultures in NYB with exposure to 0.5% (vol/vol) EMS at 37°C for from 1.5 to 5 h. The cultures were then diluted 1/30 in NYB and incubated at 37°C with shaking overnight.

To identify toxin A-deficient mutants, an agar-well assay was developed. NYB was inoculated with the mutagenized overnight cultures and incubated at 37°C with shaking until reaching a cell density of 3×10^8 cells per ml. These cultures were then diluted in saline, and 0.1 ml was spread onto each petri plate containing 10 ml of freshly poured TSB-DC with 1.5% Noble agar (Difco) yielding approximately 50 colonies per plate. The plates were dried and incubated for 24 to 36 h in a humid 32°C incubator. Small wells were made in the media approximately 5 mm from each colony with a sterile Pasteur pipette attached to a vacuum source. Each well was filled with approximately 2 μ l of filter-sterilized sheep antitoxin, and the plates were incubated at 32°C for 18 h. Colonies which produced sufficient amounts of toxin A had a distinct band of precipitation between the colony and an adjacent antiserum well (Fig. 1). The intensity of the precipitin bands was accentuated by substituting a partially purified immune gamma globulin (55) for the whole antisera. Each colony which did not produce a detectable precipitin band was streaked for an isolated colony and retested for toxin A production by the agar well assay with a 1-cm streak of overnight growth on a TSB-DC agar plate instead of an isolated colony. Those isolates which were negative in the agar-well test were subcultured and stored in sterile skim milk at -70°C.

Cultivation for toxin A production. Overnight cultures in TSB-DC (0.1 ml) were used to inoculate 10 ml of TSB-DC in 250-ml Erlenmeyer flasks. The flasks were incubated at 32°C with maximum aeration in a reciprocating shaker (Lab-Line Instruments) for 18 or 22 h. Cell growth was monitored at optical density of 540 nm (OD_{540}) with a Beckman spectrophotometer (model 35). Culture supernatants were obtained by centrifugation at $10,000 \times g$ for 15 min and sterilized via membrane filtration (0.45 μ m, Millipore). These supernatants were either stored at -70°C or dialyzed against 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride (Sigma), pH 8.0, at 5°C and concentrated 10-fold in Minicon-B15 cells (Amicon Corp.) with minimal (<5%) loss of toxin A.

Toxin A yields were also measured in a defined medium for toxin A production (T8S). Medium T8S was medium T8A (18) modified (DeBell, personal

communication) to include succinate (0.021 M), a sevenfold increase in the trace salts (0.6 ml TS2), and the omission of glucose. Nitrilotriacetate and $FeCl_3$ were also omitted. This medium had an iron concentration of 0.03 μ g/ml. Standard sterile solutions of $FeSO_4 \cdot 7H_2O$ were added at various concentrations. One milliliter of log-phase T8S cultures with an OD_{540} of 0.5 was used to inoculate 9.0 ml of medium T8S in 125-ml Erlenmeyer flasks (nitric acid cleaned). The flasks were incubated at 32°C with maximum aeration for 18 h, and the supernatants were obtained by centrifugation.

Extraction of cell-associated toxin A. Cells cultured 22 h in TSB-DC as described above were washed three times in phosphate-buffered saline pH 7.4, resuspended to one-half the original culture volume in phosphate-buffered saline, and disrupted by sonication with 15 20-s bursts (50% maximal output) using a Biosonik IV (Bronwill) sonicator. After each burst, the sample was swirled in an ice water bath for 1 min. Fixed and stained samples examined by light microscopy showed that this procedure resulted in the destruction of greater than 95% of the cells. The cellular debris was removed by centrifugation at $12,000 \times g$ for 30 min. The intracellular extract was stored at -70°C.

ADPR transferase activity. Aminoacyl transferase-containing enzymes were prepared from crude extracts of wheat germ as previously described (15). Extracellular toxin A samples were activated by incubation with an equal volume of 8 M urea-2% dithiothreitol (Sigma) for 15 min at 25°C as previously described (57). The adenosine 5'-diphosphate-ribose

FIG. 1. Agar well assay for the detection of toxin A production by *P. aeruginosa*. Wells were made in the agar 5 mm from each colony and filled with sheep antitoxin A gamma globulin. After incubation at 32°C, a band of precipitation formed between toxin A-producing colonies and the antitoxin-containing wells.

(ADPR) transferase activity of toxin A was measured by the previously described modification (6) of the procedure of Collier and Kandell (16). The reaction was allowed to proceed at 25°C for 60 min. The amount of toxin A present in a given sample was calculated from a standard curve obtained daily with activated purified toxin A (33).

Antiserum neutralization of toxin A. Enzyme neutralization by antibody was determined by assaying the ADPR transferase activity of toxin A after incubation of urea-dithiothreitol-treated samples with equal volumes of antitoxin A or normal sheep serum for 15 min at 37°C as previously described (31).

RPHA assay. The reversed passive hemagglutination (RPHA) assay for toxin A was developed by modifications of the procedure of Holmes et al. (26). A stock solution of bis-diazotized benzidine was prepared and stored exactly as described by Butler (13). Formalinized sheep erythrocytes were prepared and stored at 4°C for up to 2 months (24). The sensitizing mixture varied from batch to batch of bis-diazotized benzidine and had to be determined empirically. A representative sensitizing mixture consisted of: 1.8 ml of buffer A (82 mM NaCl, 43 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 10.7 mM KH_2PO_4 , pH 7.4), 0.24 ml of 10% formalinized sheep erythrocytes, 0.1 ml of purified anti-A immunoglobulin, and 0.24 ml of a 1:10 dilution of stock bis-diazotized benzidine solution in buffer A. The cells were sensitized by incubation of the above mixture for 10 min at 37°C with gentle agitation. The sensitized erythrocytes were washed as described by Holmes et al. (26) and suspended to 0.2% in diluent (buffer A containing 1% crystalline bovine serum albumin and 0.01% merthiolate). The sensitized erythrocytes were used on the same day that they were prepared.

RPHA assays were performed in plastic microtiter trays with V-bottom wells (Linbro Chemical Co.) as described by Holmes et al. (26). Serial twofold dilutions of purified toxin A served as a positive control for passive hemagglutination and were used to determine the endpoint. This assay detected as little as 2 ng of toxin A per ml and did not vary more than one serial dilution between experiments.

Biochemical characterization. The ability to assimilate various organic compounds as sole carbon sources was determined by individually adding to a final concentration of 0.03 M the following filter-sterilized carbon sources (found to be utilized by the parental strain PA103-0) to minimal media (9): D-glucose, acetate, propionate, succinate, DL-lactate, citrate, pyruvate, glycine, L-alanine, L-arginine, DL-aspartate, L-glutamate, L-lysine, and acetamide. Each medium was inoculated from an overnight culture on an NYB agar slant and incubated up to 8 days at 37°C before growth was considered negative.

The ability to oxidize various carbohydrates was determined in OF basal media (Difco). The following filter-sterilized carbohydrate solutions (oxidized by the parental strain PA103-0) were added to individual tubes of melted base medium to 1% (wt/vol) final concentration: D-glucose, D-fructose, D-mannose, D-galactose, L-arabinose, D-xylose, glycerol, and manitol. The reactions were read daily during a 5-day incubation at 37°C.

Other tests were performed as previously described

and included cytochrome oxidase test (20), gluconate oxidation (23), arginine dihydrolase (54), indole test (59), lysine decarboxylase (59), citrate utilization (59), urease activity (59), nitrate reduction (59), and anaerobic fermentation of glucose (59).

Also tested was the ability to grow on cetrимide agar (10), deoxycholate citrate agar (Difco), endo agar (Difco), MacConkey agar (Difco), and salmonella-shigella agar (Difco). These media were lightly inoculated, and growth was ascertained after 24 h at 37°C. Growth at 42°C was determined after 24 h of incubation of inoculated tubes of brain heart infusion broth (Difco).

Assays for extracellular products. The ability to produce various extracellular products was determined on the following plate tests. Unless otherwise stated, the zone of activity around a 15-mm streak of growth was recorded after incubation at 37°C for 48 h. Protease production was determined on agar plates containing 1.5% (wt/vol) skimmed milk (52). Elastase production was detected on nutrient agar (Difco) plates at pH 7.5 containing 0.3% (wt/vol) elastin (Sigma), lipase production was detected on tributyrin agar (29), and esterase production was detected as dark blue colonies on brain heart infusion agar plates at pH 7.6 containing 0.1% indoxyl acetate (Sigma) (28). Hyaluronidase production was determined with nutrient agar plates at pH 6.8 containing umbilical cord hyaluronic acid (Sigma) by the procedure described by Smith (51). Deoxyribonuclease and ribonuclease activities were determined on nutrient agar to which filter-sterilized deoxyribonuclease or ribonuclease solutions were added to a final concentration of 3 mg/ml (34). Hemolysin production by isolated colonies was observed on media containing 0.4% (wt/vol) heart infusion broth (Difco), 1.5% (vol/vol) agar (Difco), and 6% (vol/vol) human or sheep blood. Production of pyocyanin and fluorescein was determined on agar media slants as described by King et al. (36). Gelatinase was determined on a medium of 23 g of nutrient agar, 0.5 g of KH_2PO_4 , 1.5 g of K_2HPO_4 , 4.0 g of gelatin, 0.05 g of glucose, and 1 liter of water (pH 7.0), 20 ml/plate. Cultures were streaked onto plates, incubated at 37°C for 24 h, and then flooded with a solution of 15% (wt/vol) HgCl_2 in 20% (vol/vol) HCl. Clear zones formed around gelatin-liquefying colonies (17).

Cultivation and assay conditions for lecithinase production were those of Stinson and Hayden (53), except that phosphate concentrations were determined by the method of Ames (1). Staphylolytic activity was determined by the method of Burke and Pattée (12) with an overnight NYB culture of *S. aureus* strain Seattle as substrate. Production of alkaline phosphatase was determined by using the medium and culture conditions described by Cheng et al. (14) and assayed by the method of Torriani (56). Production of leukocidin activity was assayed with the culture conditions and cell autolysis method described by Scharmann (50). Leukocidin activity was determined by a modification of the procedure previously described (50) whereby 0.2 ml of autolysis sample was incubated with 0.2 ml of isolated human granulocytes at 5×10^6 /ml in phosphate-buffered saline. The granulocytes were isolated from heparinized, peripheral venous blood via Hypaque-Ficoll separation and 3% dextran sediment-

tation followed by erythrocyte lysis with hypotonic saline (7). Viability of the granulocytes was determined by their exclusion of trypan blue (GIBCO Laboratories, Grand Island, N.Y.).

Serotyping and pyocin typing. Serotypes were determined by slide agglutination with seven immunotype sera kindly provided by M. Fisher (Parke, Davis and Co.). Typing for the production of pyocins was done by the method of Farmer and Herman (21) with 18 indicator strains kindly supplied by B. Minshew (University of Washington, Seattle, Wash.).

Motility and growth curves. Motility was observed microscopically by the hanging-drop method. Growth of the parent and mutant strains was compared *in vitro*. A 50-ml amount of TSB-DC per 1-liter Erlenmeyer flask was inoculated with 0.1 ml of an overnight culture and incubated with maximum aeration in a 32°C shaking water bath. Samples were withdrawn hourly, and the OD₅₄₀ was measured. Growth curves were constructed, and the generation time was calculated by the method of Miller (44).

Antibiotic sensitivity. Sensitivity to ampicillin, cephalothin, carbenicillin, tetracycline, chloramphenicol, kanamycin, amikacin, sulfamethizole, nitrofurantoin, tobramycin, and trimethoprim-sulfamethoxazole was determined by the Kirby and Bauer (3) quantitative disk diffusion method.

PAGE. The SDS-polyacrylamide gel electrophoresis system (SDS-PAGE) was that described by Laemmli (38). Gels contained 10% acrylamide with a stacking gel of 4.5% acrylamide. All samples of culture supernatant preparations were diluted in an equal volume of sample buffer containing 0.0312 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 6.8), 10% 2-mercaptoethanol, 20% glycerol, and 4% SDS. For slab gel electrophoresis, a 10-μl sample containing 7 to 20 μg of protein was heated in sample buffer to 100°C for 5 min before loading. The following proteins (with corresponding molecular weights) were used as standards: phosphorylase B, 93,000; 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. Electrophoresis was performed at room temperature at a constant power of 1 W. Gels were stained at room temperature for 12 to 18 h in a solution of 25% isopropanol, 10% acetic acid, and 0.05% Coomassie brilliant blue. Gels were then decolorized with 10% acetic acid.

Cylindrical gels (5.5 by 0.6 cm) were prepared, electrophoresed, and stained as described above. Samples (50 μl) were applied to these gels without prior heating (57). A duplicate gel of each sample was electrophoresed and immediately sliced (approximately 1.1 mm per fraction). Each fraction was crushed and eluted at 5°C for 24 h with 150 μl of elution buffer [0.125 M tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.0) and 0.1 M dithiothreitol]. The ADPR transferase activity of 2.5 μl of the eluate from each gel fraction was assayed as described above. Previous reports (15, 57) have shown that toxin A and enzymatically active fragments derived from toxin A retain ADPR transferase activity after electrophoresis on SDS-PAGE, providing they are not heated at 100°C before electrophoresis.

Protein determination. Protein was determined by the method of Bradford (8) modified by using a commercial reagent, Bio-Rad Protein Assay Dye Reagent Concentrate, purchased from Bio-Rad Laboratories (Richmond, Calif.). Ovalbumin was used as the standard.

RESULTS

Isolation of toxin A-deficient mutants. An agar-well assay was developed to identify toxin A mutants (Fig. 1). Two mutagenic agents, EMS and NTG, were tested for their ability to induce toxin A mutants. The use of EMS for the induction of toxin A-deficient mutants was found to be ineffective. After incubation with 0.5% EMS, all of approximately 6,000 colonies tested were positive in the agar well assay. After NTG mutagenesis, approximately 15,000 scorable colonies were examined, and colonies were found which were negative in the agar well assay. These were recloned, subcultured, and cultivated for toxin A production in TSB-DC broth. The ADPR transferase activities of the mutant culture supernatants were initially compared to the reactions of 10-fold dilutions of the parental strain supernatant. A total of 16 toxin A-deficient mutants were isolated at a frequency of approximately 10⁻³. The toxin A enzymatic activities of these 16 mutants ranged from <1 to 40% of that produced by the parental strain, designated PA103-0.

Quantitation of toxin A production by PA103 mutants. Only 5 of the 16 mutants, PA103-8, -15, -16, -19, and -29 produced toxin A at 0.5% or less than the parent. These five mutants were independently derived except for mutants PA103-15 and -16. The amount of extracellular toxin A produced by each of these five mutants and the parent was determined by assaying aliquots of 10-fold-concentrated supernatants in the ADPR transferase assay and in the RPHA assay (Table 1). The parental strain produced 25 to 30 μg of toxin A per ml of culture supernatant, and the five mutants produced less than 0.1 μg of toxin A per ml in both assays. Thus, these five mutants produced less than 0.33% of the extracellular toxin A produced by the parent. When the samples were preincubated with specific toxin A antiserum, their ADPR transferase activity was totally inactivated. Preincubation with normal sheep serum had no effect on the ADPR transferase activity of the samples (data not shown).

The amount of cell-associated toxin A in cell lysates of the parent and mutants was quantitated in the ADPR transferase assay and the RPHA assay. The lysates from the parental cells (PA103-0) contained about 0.64 μg of toxin A per ml. Extracts from mutants PA103-8, -15, -16,

and -29 did not contain detectable levels of cell-associated toxin A. However, the cell extract from PA103-19, although not containing toxin A which was detectable in the ADPR transferase assay, gave a positive RPHA reaction. Based on the RPHA assay, mutant PA103-19 had 25% (0.16 $\mu\text{g}/\text{ml}$) as much cell-associated toxin A as the parent.

Characterization of toxin A-deficient mutants. The mutants PA103-8, -15, -16, -19, and -29 were identical to the parental strain PA103-0 (see Materials and Methods) with respect to utilization of organic compounds as their sole carbon source, carbohydrate oxidation, growth on selective media, and growth at 42°C. Positive reactions were observed with all strains in Simmons' citrate, malonate, arginine dihydrolase, oxidase, nitrate-to-gas, and gluconate tests. Lysine and ornithine decarboxylase, indole, and urea reactions were negative. The parent and five mutants were motile, serotype 2, and had the identical pyocin production patterns (611, 231), antibiotic resistance patterns, and generation times (31 min) on TSB-DC at 32°C.

The formation of extracellular products was estimated by the agar and broth methods (Table

2). Production of gelatinase, esterase, lipase, alkaline phosphatase, sheep erythrocyte hemolysin, and fluorescein was not altered in the mutants. However, protease activity varied from that of the parental strain in all but one of the mutants. Three mutants (PA103-15, -16, and -19) showed marked protease deficiencies, and PA103-8 produced a clearing zone that was about 0.5 mm smaller than the parent (PA103-0). Mutant PA103-29 produced an identical protease reaction to the parental strain.

Effect of temperature and iron on yields of toxin A by PA103 mutants. None of the five mutants appears to have a temperature-dependent conditional mutation affecting toxin A production. When the parent and toxin mutants were cultivated for toxin A production in TSB-DC at 24, 28, 37, and 42°C, no increase in ADPR transferase activity could be detected by the mutants at temperatures below or above the optimal (32°C) temperature (data not shown).

The presence of excess iron in the culture medium has been shown to inhibit yields of toxin A (4). At the optimal concentration of iron (0.08 $\mu\text{g}/\text{ml}$) for toxin A yields by the parent (PA103), the mutant strains produced less than 0.1% of

TABLE 1. Quantitation of toxin A in the extracellular supernatants of parental strain PA103-0 and toxin A mutants with ADPR transferase and RPHA assays

Strain	ADPR transferase activity			RPHA		
	cpm ^a	$\mu\text{g}/\text{ml}$ ^b	% Relative activity	Endpoint ^c	$\mu\text{g}/\text{ml}$ ^b	% Relative activity
PA103-0	144,000 ^d	30	100	51,200	25.6	100
PA103-8	4,120	0.10	0.33	64	0.032	0.13
PA103-15	1,394	0.04	0.13	64	0.032	0.13
PA103-16	2,011	0.06	0.20	64	0.032	0.13
PA103-19	1,878	0.06	0.20	8	0.004	0.02
PA103-29	2,136	0.06	0.20	32	0.016	0.06

^a Values obtained with 10 $\times$ -concentrated supernatants. The background has been subtracted.

^b Values corrected to correspond to 1 $\times$ supernatants.

^c Values represent reciprocal of highest dilution giving total agglutination with 10 $\times$ -concentrated culture supernatants.

^d Extrapolated from a 10⁻³ dilution of 10 $\times$ -concentrated supernatant.

TABLE 2. Extracellular products of *P. aeruginosa* strain PA103-0 and toxin A mutants^a

Strain	Protease (zone, mm)	Gelatinase (zone, mm)	Lipase (zone, mm)	Alkaline phosphatase (% relative activity)	Esterase (plate)	Sheep he- molysin (plate)	Fluorescein (slant)
PA103-0	1.5	3.0	2.0	100	+	+	+
PA103-8	1.0	3.0	2.0	100	+	+	+
PA103-15	0.1	3.0	2.0	100	+	+	+
PA103-16	0.1	3.0	2.0	100	+	+	+
PA103-19	0	3.0	2.0	100	+	+	+
PA103-29	1.5	3.0	2.0	100	+	+	+

^a None of the strains produced detectable levels of elastase, human erythrocyte hemolysin, pyocyanin, hyaluronidase, deoxyribonuclease, ribonuclease, staphylolytic enzyme, lecithinase, exoenzyme S, or leukocidin activities. The assay types are noted in parentheses.

parental levels of toxin A (Table 3). When iron was added to the culture media at two suboptimal concentrations (0.03 and 0.05 $\mu\text{g}/\text{ml}$), toxin A yields declined in the mutant cultures as well as in the parental cultures. Thus, toxin A production by the mutants was no more sensitive to iron than the parental strain. It was also possible that some of the mutants required more iron for toxin A production. However, a concentration of iron that was 0.05 $\mu\text{g}/\text{ml}$ higher than the optimum for toxin A yields from the parent (0.13 $\mu\text{g}/\text{ml}$) did not increase toxin A synthesis by the mutants (Table 3).

The effect of iron on yields of total extracellular protein. Excess iron in the culture medium also has been shown to reduce the yield of total extracellular protein produced by *P. aeruginosa* (5). Iron regulation in the toxin A mutant strains was compared with that in the parental strain in TSB-DC medium at 32°C (Table 4). The yields of extracellular protein from mutant PA103-29 low-iron cultures were consistently about 20 to 30 $\mu\text{g}/\text{ml}$ less than parental yields. This can be accounted for by a decrease of approximately 30 μg of toxin A per ml by PA103-29. The cultures of mutants PA103-8, -15, and -16 had yields of extracellular protein that were reduced by about one-half to two-thirds (200 to 310 $\mu\text{g}/\text{ml}$) compared with yields of the parent. Mutant PA103-19 produced less than one-half (150 $\mu\text{g}/\text{ml}$) the parental supernatant level. The markedly reduced yields of extracellular protein in cultures of PA103-8, -15, -16, and -19 could not be accounted for simply by their decreased toxin A yields and suggest that yields of other extracellular proteins are also reduced. When cultured in medium containing an increased iron concentration (5.0 $\mu\text{g}/\text{ml}$), the amount of extracellular protein per milliliter per OD₅₄₀ unit was dramatically decreased for all five mutant strains as well as for the parent (Table 4). Thus, as the iron concentration of the TSB-DC growth medium increased, the yield of other extracellular proteins besides toxin A decreased in the cultures of the five mutants and the parent.

PAGE of extracellular products from PA103 mutants. The TSB-DC supernatants from 18-h cultures of PA103-0 contained 32 visible bands upon SDS-PAGE (Fig. 2). Protein bands of interest or reference are labeled (calculated molecular weights are given below). In all five of the mutant strain supernatants, two proteins were lost, protein 7 (molecular weight, 73,000) and protein 13 (molecular weight, 42,500). The ADPR transferase activity profiles of cylindrical SDS-PAGE of parental strain supernatants showed ADPR transferase activity

TABLE 3. Effect of iron concentration on yields of extracellular toxin A by *P. aeruginosa* mutant strains^a

Strain	Relative ADPR transferase activity (cpm) ^b at Fe concn:			
	0.03 ^c	0.05	0.08	0.13
PA103-0 ^d	6,550	9,750	17,000	8,580
PA103-8	84	79	130	109
PA103-15	38	61	78	69
PA103-16	23	52	124	128
PA103-19	0	0		0
PA103-29	34	68	168	142

^a All cultures (T8S medium) at each iron concentration were at approximately the same cell density when supernatants were obtained by centrifugation at 18 h postinoculation.

^b cpm of sample treated with urea-dithiothreitol (57).

^c Indicates final concentration of iron (micrograms per milliliter) in T8S defined medium.

^d Counts per minute of PA103-0 were extrapolated from 10⁻¹ dilution of supernatant.

TABLE 4. Effect of iron on yields of extracellular protein in TSB-DC cultures of *P. aeruginosa* mutant strains^a

Strain	Low iron ^b		High iron ^b	
	$\mu\text{g}/\text{ml}$ ^c	$\mu\text{g}/\text{OD}_{540}$ ^d	$\mu\text{g}/\text{ml}$ ^c	$\mu\text{g}/\text{OD}_{540}$ ^d
PA103-0	400	47.6	140	6.3
PA103-8	200	22.2	140	6.2
PA103-15	250	27.8	110	4.6
PA103-16	310	34.4	130	5.4
PA103-19	150	16.7	110	4.6
PA103-29	380	42.2	210	8.7

^a All cultures of each iron concentration group were at approximately the same cell density when supernatants were removed by centrifugation at 18 h postinoculation.

^b Low-iron (0.08 $\mu\text{g}/\text{ml}$) medium; high-iron (5.0 $\mu\text{g}/\text{ml}$) medium.

^c Indicates micrograms of extracellular protein per milliliter in dialyzed culture supernatant.

^d Indicates micrograms of extracellular protein per milliliter per OD₅₄₀ unit.

associated with both protein 7 and protein 13 (data not shown). The enzymatic activity associated with both of these proteins was totally inactivated by preincubation with toxin A antiserum but not normal sheep serum. Protein 7 was identified as toxin A on the basis of its enzymatic activity, its neutralization with specific antitoxin, and its comigration on SDS-PAGE with purified toxin A. Based on its enzymatic activity and neutralization by antitoxin, protein 13 produced by the parental strain but not by the mutants was identified as a fragment of toxin A.

FIG. 2. SDS-PAGE of extracellular proteins produced by *P. aeruginosa*. 0: PA103-0 toxin A-producing parental strain; 29, 19, 16, 15, and 8: PA103 toxin A-deficient mutants. Cells were grown in TSB-DC for 18 h to a cell density of approximately OD_{540} 9.0. Supernatants were concentrated fivefold, and 10 μ l of each was applied to the gel. Toxin A (Tox) and protein standards (PS) were used as molecular weight markers.

Based on the SDS-PAGE profiles of the culture supernatants, the mutants had three distinct patterns. First, the pattern of mutant PA103-29 supernatant proteins was the same as the parental extracellular protein pattern except for the loss of the toxin A band (protein 7) and the toxin A fragment band (protein 13). Mutant PA103-29 was the only mutant examined whose extracellular protein profile was apparently identical to that of the parental strain except for the loss of toxin A material. A second altered SDS-PAGE pattern was seen with the supernatants of mutants PA103-8, -15, and -16. Compared with the parent, the supernatants of these mutant strains lacked proteins 1 (molecular weight, 100,500), 7 (toxin A), and 13 (toxin A fragment) and had reduced concentrations of proteins 3 (molecular weight, 93,000), 11 (molecular weight, 50,000), and 25 (molecular weight, 23,500). A third altered SDS-PAGE pattern was obtained with the supernatant of mutant 19. Supernatants of mutant 19 lacked proteins 1, 7, 11, and 13; had reduced concentrations of proteins 3, 11, 23, and 25; had an increased concentration of protein 29 (molecular weight, 17,500); and contained two additional bands, one immediately above and one immediately below protein 5 (molecular weight, 96,500) (Fig. 2).

DISCUSSION

The agar well assay, which was developed to identify toxin A-deficient mutants, was found to have several advantages over modified Elek plates (4). The agar well assay was more rapid, allowing the examination of a larger number of colonies, and was more economical, requiring much less antiserum and fewer petri plates than the Elek technique.

Toxin A-deficient mutants of *P. aeruginosa* strain PA103 were isolated, and their phenotypic properties were compared with the parental strain. Although none of the mutants completely lacked extracellular toxin A, five mutants were isolated whose extracellular yields of toxin A were <0.33% of the parent (Table 1). An extensive characterization indicated that the mutants were derived from the parental strain and were not contaminants. Furthermore, these tests acted as a probe of the genome to show that no secondary mutation affecting these characteristics had occurred.

Additional studies were performed examining properties known to be important to toxin A production in an effort to determine the nature of these mutations. Toxin A is not formed constitutively, and the regulation of its production by *P. aeruginosa* has only been partially elucidated. Excess iron in the culture medium has been shown to inhibit yields of toxin A (4) and other extracellular products (5). The molecular mechanism(s) underlying iron regulation of toxin A yields remains unknown. None of the five PA103 toxin A mutants characterized could be classified as having mutations involved with regulation of toxin A by iron (Tables 3 and 4). Toxin A production is also regulated by the growth temperature (41); however, none of the mutant strains characterized appeared to have conditional temperature-sensitive mutations.

Wretling et al. (61) reported that protease-deficient mutants of *P. aeruginosa* had pleiotropic changes in the activities of other extracellular enzymes. We have found that four of the five chemically induced, severely toxin A-deficient mutants showed reduced yields or lower activities of other extracellular proteins or both. Our data and that of Wretling et al. (61) suggest that a common regulatory mechanism(s) probably exists for the synthesis, processing, or release of toxin A, protease, and other extracellular proteins.

Among the five mutants characterized, four produced toxin A intracellularly at relative concentrations that were less than 1% of the parental strain (PA103-8, -15, -16, and -29). This would indicate that these four are not permease mu-

tants, but either have defects in their ability to synthesize toxin A or possibly an increased rate of intracellular degradation of toxin A. The markedly pleiotropic mutant PA103-19 had a relatively high level of cell-associated, enzymatically inactive toxin A. This may indicate a defect in the intracellular processing or secretion of toxin A by this mutant.

Mutant PA103-29 was found to be identical to the parental strain except for its markedly reduced toxin A yields. This mutant is the most likely among the five mutants to have a specific toxin A (*tox*) gene mutation. SDS-PAGE patterns showed that every protein band in the parent culture supernatant was present in PA103-29 supernatants except for two bands associated with the *tox* structural gene product: toxin A itself (73,000 daltons) and an enzymatically active fragment found at 43,500 daltons (Fig. 2). An enzymatically active fragment of approximately this molecular weight with ADPR transferase activity has been previously observed (S. Lory and R. J. Collier, Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, Abstr. no. B6, p. 16). Firm support for a structural gene mutation would be the presence of toxin A cross-reacting material in culture supernatant of mutant PA103-29. However, it is the nature of our agar well screening technique to detect mutants which produce no extracellular toxin A antigens. Thus, our initial mutant selection process prohibits the identification of a cross-reacting material-producing mutant. However, the mutation in PA103-29 could be very early in the structural gene sequence such that no detectable cross-reacting material product was formed. Alternatively, the specificity of this mutation for toxin A could be due to mutation in a *tox*-specific regulatory gene. Since the locus and the fine structure of *tox* genes are unknown, we cannot distinguish between these two possibilities.

In an effort to categorize the various types of mutations which result in toxin A deficiencies, we have established a broad classification system. Class 1 toxin A mutants, such as PA103-29, have mutations which are apparently specific for toxin A. Other cell functions appear to be normal. The genotype of this mutant is referred to as *tox-1*. Class 2 mutants are also markedly deficient in toxin A production, but in addition are deficient in the production of protease and other extracellular proteins. Mutants PA103-8, -15, and -16 fall into this group. Class 3 mutants have pleiotropic deficiencies in the production of multiple extracellular proteins including toxin A, but have relatively high levels of cell-associated toxin A antigen. Mutant PA103-19 was placed in this category. Such mutants may have

defects in the transport of extracellular products through the cell membrane. Class 4 mutants are those which produce toxin A at levels greater than 1% of parental yields. The wide range of toxin A yields observed in this group suggests that there are several subgroups in this class. The demonstration of four toxin A mutant classes indicates that there are at least four genetic loci involved in the regulation and production of toxin A.

Investigators have studied the purified toxic products of *P. aeruginosa* and their effects when injected into animals (30, 37). Others have compared different strains of *P. aeruginosa* possessing various combinations of pathogenic properties in animal infections in an effort to correlate a suspected virulence trait of the organism with pathogenicity (47). Such a correlation is difficult because independently isolated *P. aeruginosa* strains are so heterogeneous with respect to the production of potential virulence products (60). Genetic techniques have been used to understand the role of colonization factors, invasiveness, toxins, and other virulence factors of other bacterial pathogens (2, 11, 19, 27, 39, 48). A decrease in virulence with a mutant deficient in only a single characteristic, when compared with the parental strain in a suitable animal model, indicates that this characteristic has a role in the disease (25).

The long-term goal of our studies is to determine the effect of a specific *tox* mutation on the virulence of *P. aeruginosa*. The current study is the initial step in using this approach to study virulence in *P. aeruginosa*, the isolation of a specific *tox* mutant. Mutant PA103-29 appears to be identical to the parental strain, except for a specific mutation affecting yields of toxin A (*tox*). The wide range of tests performed not only indicated that no other alterations are associated with this mutation, but also laid a groundwork of characterization for strain PA103 in virulence tests. Thus, PA103-29 is well suited for comparisons with its toxigenic parental strain in experimental animal infection models. Comparisons between these strains should make it possible to determine the relative contribution of toxin A as a virulence factor of *P. aeruginosa*.

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