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Production of Exoenzyme S by Clinical Isolates of *Pseudomonas aeruginosa*

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Exoenzyme S differs from toxin A and diphtheria toxin in that it does not adenosine diphosphate (ADP)-ribosylate elongation factor-2, but rather catalyzes the transfer of the ADP-ribose moiety of nicotinamide adenine dinucleotide to a number of different proteins in extracts of eucaryotic cells. Polyoma-transformed BHK-21 cells were isolated which were resistant to diphtheria toxin and toxin A. Extracts from these cells are ADP-ribosylated by exoenzyme S but not toxin A or diphtheria toxin, providing an assay which distinguishes between S and A activities. A total of 124 clinical isolates of *P. aeruginosa* were analyzed for production of toxin A and exoenzyme S. Exoenzyme S production was detected in 38% of the strains, whereas 80% of the strains produced toxin A.

Pseudomonas aeruginosa is an opportunistic pathogen which can cause serious and lethal infections in compromised patients (19, 20). *P. aeruginosa* produces a variety of extracellular products that may contribute to its pathogenicity (17). Toxin A has been shown to be the most toxic extracellular product of *P. aeruginosa* on a weight basis (19). The mechanism of action of toxin A is identical to that of diphtheria toxin in that it inhibits eucaryotic protein synthesis by catalyzing the transfer of the adenosine 5'-diphosphate ribosyl (ADPR) moiety of nicotinamide adenine dinucleotide (NAD) to elongation factor-2 (EF-2; 6, 10-12). The resultant ADPR EF-2 is inactive in protein synthesis (10, 11).

Some strains of *P. aeruginosa* produce a second extracellular protein (exoenzyme S) that has been shown to have ADPR transferase activity (14). Exoenzyme S differs from toxin A in that it does not ADP-ribosylate EF-2, but rather catalyzes the transfer of the ADPR moiety of NAD to a number of substrate proteins in crude extracts of eucaryotic cells (14). Exoenzyme S also differs from toxin A in its heat stability and its inactivation, rather than potentiation, by pretreatment with urea and dithiothreitol (DTT) (14). In vitro production of S is increased by the addition of 10 mm nitriloacetic acid (NTA) to the culture medium, whereas toxin A yields either remain unchanged or are slightly reduced in the presence of NTA. Furthermore, exoenzyme S is not precipitated or neutralized by antitoxin A (14).

Although exoenzyme S has been shown to be produced in vivo in a burned mouse model (2), its role in human *P. aeruginosa* infections has not yet been determined. Preliminary studies on the production of S by clinical isolates of *P. aeruginosa* involved growing each strain in Trypticase soy broth dialysate (TSBD), with and without NTA, and then determining the ADPR transferase activity of both supernatants in a wheat germ extract with and without prior incubation with 4 M urea + 1% DTT. Confirmation of enzyme phenotype was obtained by neutralization utilizing antisera prepared against pure toxin A or partially purified S (29). Although this method was reasonably accurate, it was extremely slow for screening large numbers of isolates. In some strains that produced low amounts of both A and S, this procedure sometimes yielded ambiguous results depending on the relative amounts of A and S produced. Therefore, a specific assay was needed to detect S.

This study was undertaken to determine the percentage of clinical isolates of *P. aeruginosa* that produce exoenzyme S and to evaluate the possibility of a correlation between exoenzyme S or toxin A production and patient mortality. This report describes the development of an assay specific for S-utilizing polyoma-trans-

formed baby hamster kidney cells (PyBHKR) resistant to both diphtheria toxin and *Pseudomonas* toxin A. This assay was used to determine the ADPR transferase enzyme phenotype of 124 clinical isolates of *P. aeruginosa*.

MATERIALS AND METHODS

Cell and culture conditions. Stocks of baby hamster kidney (BHK-21) and polyoma virus-transformed baby hamster kidney (PyBHK-21) cells were provided by Jules V. Hallum (University of Oregon Health Science Center, Portland, Oreg.). All cells were grown in Eagle minimal essential medium with Hanks salts, supplemented with 10% fetal calf serum and 50 μg of gentamicin per ml. Cells were incubated at 37°C in a 5% CO_2 atmosphere. Cells were transferred by removing the growth medium and washing the cells twice with 0.25% trypsin in 0.5 mM ethylenediaminetetraacetic acid–140 mM NaCl–2.7 mM KCl–8 mM Na_2HPO_4–1 mM glucose–1.5 mM KH_2HPO_4 and then incubating them at 37°C until all cells were lifted.

Toxins. Diphtheria toxin and *P. aeruginosa* toxin A were purified as previously described (13, 16). Partially pure S was kindly provided by Michael R. Thompson. Exoenzyme S was purified from culture supernatants as previously described, except that all buffers contained 10 mM DTT (29). The addition of DTT was found to stabilize the enzymatic activity of S.

Radioactive reagents. [^{14}C]NAD labeled in the adenine moiety (25 μCi/ml, specific activity 302 mCi/mmol) and a ^{3}H-labeled mixture of amino acids (1.0 mCi/ml) were obtained from the Amersham Corp. (Arlington Heights, Ill.).

Clinical isolates of *P. aeruginosa*. Clinical isolates of *P. aeruginosa* were obtained from the Walter Reed Army Institute of Research, U.S. Army Institute of Surgical Research, and the University of Virginia School of Medicine. These isolates were determined to be different strains on the basis of their Fisher-Devlin-Gnabasik serotype (8), colonial morphology, pigment production, and protease production. Seventy-one of the clinical isolates were from (nonburn) bacteremia patients, and 53 of the strains were from burn patients.

Bacteriological medium and growth conditions. The culture medium consisted of TSBD, 1% glycerol, and 0.05 M monosodium glutamate (18) deferrated with Chelex-100 (Bio-Rad Laboratories, Richmond, Calif.). Where indicated, the medium was supplemented with 10 mM NTA (2). A 10-ml volume of medium was added to 250-ml Erlenmeyer flasks and inoculated with 0.1 ml of a 15-h shaking culture of the appropriate strain. The flasks were incubated at 32°C for 20 h in a reciprocating shaker (150 linear excursions per min) (Lab-line Instruments, Melrose, Ill.). Culture supernatants were obtained by centrifugation at 10,000 × *g* for 15 min.

Isolation of diphtheria toxin-resistant cell lines. PyBHK-21 cells were seeded in 25-cm^2 culture flasks at a concentration of 10^6 cells per flask and allowed to adhere overnight. The medium was replaced with 10 ml of fresh medium containing diphtheria toxin. Toxin was removed after 4 h; the cells were washed and incubated at 37°C. Dead cells were washed off every other day, and the medium was replaced. After 5 to 7 days of growth, flasks containing 1 to 20 surviving colonies were kept. The surviving cells were grown in toxin-free medium and then reexposed to higher concentrations of diphtheria toxin. The diphtheria toxin concentrations used were: first passage, 1.2×10^{-1} μg of toxin per ml; second passage, 1.2 μg of toxin per ml; third passage, 12 μg of toxin per ml; and fourth passage, 120 μg of toxin per ml. The final passaged cells, PyBHKR, were grown from a single cell by cloning in 96-well plates.

Protein synthesis inhibition assay. Cells were seeded in Linbro six-well plates (30-mm wells), at a concentration of 5×10^5 cells per well, and allowed to attach overnight. The medium was then removed, and the cells were washed with medium containing one-tenth the normal concentration of amino acids. This was followed by incubation at 37°C in 5% CO_2 for 3 h in a mixture of 0.8 ml of the 1/10 amino acid medium and 0.1 ml of toxin diluted in Tris-Glu buffer [25 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.2)–140 mM NaCl–5 mM KCl–0.7 mM Na_2HPO_4–6 mM glucose (pH adjusted to 7.4 with 1 N HCl)]. Control plates received no toxin. At the end of this time period, 0.1 ml of ^{3}H-amino acid mixture diluted to 4 μCi/ml in 1/10 amino acid medium was added to each plate, and incubation was continued for 2 h. The medium was then decanted, and 1.0 ml of 0.25% trypsin in water was added per plate. After lifting, the cells were lysed by freezing at −20°C overnight and thawing the next day. Proteins in the lysate were precipitated by the addition of trichloroacetic acid to a final concentration of 10%, heating at 90°C for 15 min, and cooling in an ice bath. The precipitated proteins were collected on 0.45-μm membrane filters (Millipore Corp., Bedford, Mass.), and the incorporated radioactivity was measured in a Beckman LS-200B scintillation counter. The triplicate samples were averaged and expressed in graphs as the percentage of control protein synthesis.

Tumor induction and preparation of cell extracts. Three-week-old male golden hamsters were inoculated subcutaneously and intrascapulary with 10^6 diphtheria toxin-resistant PyBHK cells (PyBHKR) or toxin-sensitive (parental) PyBHK cells in growth medium. Five weeks postinoculation, large palpable tumors were found on all of the animals backs. Animals were killed, and tumors were removed aseptically and weighed. Care was taken to remove only the well-encapsulated tumor mass. The tumors were minced and then homogenized in 4 volumes of 0.25 M sucrose at 4°C using a rotary homogenizer. To each milliliter of homogenate, 0.16 ml of diluent (4 M NaCl and 20 mM DTT) and 0.25 g of prewashed activated charcoal was added, and the mixture was shaken at 5°C for 15 min to remove endogenous NAD (9). The extract was centrifuged at 27,000 rpm (type 30 rotor) for 75 min, the supernatant was removed, and the protein concentration was adjusted to 7.2 mg/ml with 200 mM sodium acetate buffer (pH 6.0) (buffer II).

Cell-free ADP-ribosylation of EF-2. Cellular EF-2 was measured by the method of Gill and Dinius (9). A 50-μl volume of cell extract was added to 300 μl of histamine buffer (0.11 M histamine–90 mM Tris-hy-

drochloride [pH 8.0]-70 mM DTT-0.017 mM ethylenediaminetetraacetic acid). Diphtheria toxin fragment A or *Pseudomonas* toxin A was added at a concentration of 6.0 μg/ml. The reaction mixture was equilibrated at 37°C, after which 0.125 μCi of [^{14}C]-NAD in 5 μl was added to each tube. The reaction mixtures were incubated at 37°C for 10 min and then terminated by the addition of 5% trichloroacetic acid. The precipitates were collected by filtration on 0.45-μm filters and counted.

ADPR transferase activity. Toxin A production was identified by a significant increase in ADPR transferase activity in TSBD (no NTA) culture supernatants, after preincubation with 4 M urea-1% DTT (31). Partially purified EF-2 prepared from extracts of wheat germ was used as a substrate (5). The reaction mixture consisted of 10 μl of culture supernatant, 25 μl of wheat germ extract, 25 μl of buffer I (125 mM Tris-hydrochloride [pH 7.0]-100 mM DTT), and 5 μl of ^{14}C-labeled NAD.

Exoenzyme S production was detected in supernatants from cultures grown in TSBD with 10 mM NTA, utilizing the PyBHKR extracts as a substrate. The reaction mixture consisted of 10 μl of culture supernatant, 10 μl of buffer II (200 mM sodium acetate [pH 6.0]), 10 μl of PyBHKR extract, and 5 μl of ^{14}C-labeled NAD.

The reaction mixtures for both toxin A and exoenzyme S assays were incubated at 25°C for 30 min. The reaction was terminated with the addition of 10% trichloroacetic acid. The precipitates were collected and counted as previously described (31).

Protein determination. Protein was determined by the method of Bradford (4) modified by using a commercial reagent, Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad). Lysozyme was used as the standard.

RESULTS

Isolation of diphtheria toxin- and *Pseudomonas* toxin A-resistant cell lines. PyBHK cells are quite sensitive to diphtheria toxin. Diphtheria toxin at a concentration of 3×10^{-3} μg/ml is sufficient to cause 50% inhibition of protein synthesis in a standard 5-h assay system. PyBHK cells were exposed to medium containing diphtheria toxin in varying concentrations for 4 h. The surviving cells were grown in toxin-free medium and then reexposed to greater concentrations of toxin.

Cells (PyBHK) surviving prior exposure to 1.2×10^{-1} μg of toxin per ml (first passage) showed little increase in their subsequent resistance to inhibition of protein synthesis by diphtheria toxin (Fig. 1). However, exposure of these cells to 1.2 μg of toxin per ml yielded survivors with a greatly increased resistance to diphtheria toxin. Subsequent exposure of these diphtheria toxin-resistant PyBHK cells to 12 and 120 μg of toxin per ml further increased this resistance. Many survivors resulted from the final toxin exposure of the PyBHK-resistant cells. These

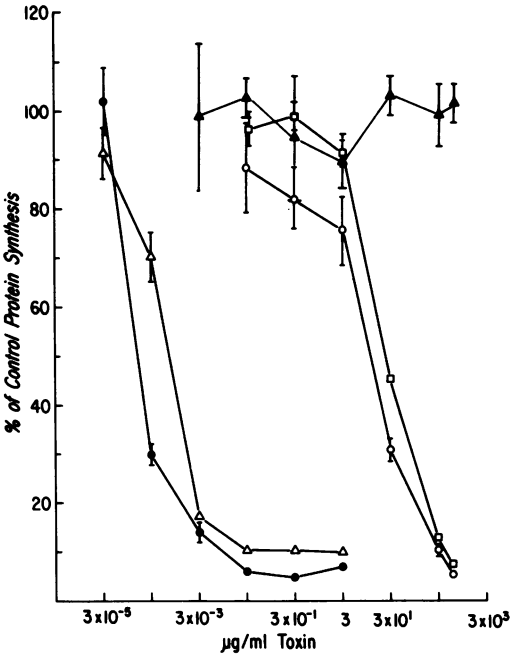

FIG. 1. *Increasing resistance of PyBHK cells to diphtheria toxin. Inhibition of protein synthesis by diphtheria toxin in: ●, PyBHK parent cells; △, first-passage cells surviving prior exposure to 1.2×10^{-1} μg of toxin per ml; ○, second-passage cells surviving exposure to 1.2 μg of toxin per ml; □, third-passage cells surviving exposure to 12 μg of toxin per ml; ▲, a single clone from fourth-passage cells surviving prior exposure to 120 μg of toxin per ml (PyBHKR cells).*

cells, when plated for isolation of single cell clones, varied in their resistance to intoxication by diphtheria toxin. The clone with the highest resistance to toxin was termed PyBHKR (Fig. 1) and used in further experiments. Exposure of these PyBHKR cells to diphtheria toxin concentrations of up to 750 μg/ml for up to 24 h had no discernible effect on the cells.

P. aeruginosa toxin A has been shown to catalyze the same intracellular ADP-ribosylation of EF-2 diphtheria toxin (10, 12). PyBHK cells are sensitive to toxin A, with a concentration of 2×10^{-2} μg/ml sufficient to inhibit protein synthesis by 75% (Fig. 2). PyBHKR cells, on the other hand, were completely unaffected by 20 μg of toxin A per ml.

The cellular attachment to or uptake of toxin A appears to be different than that of diphtheria toxin, with various cell types displaying different sensitivities to the two toxins (21, 22, 30). Since these two toxins utilize different receptors, our results (Fig. 1 and 2) suggest that the toxin resistance of the PyBHKR cells may be mediated at a point common to both toxins, the ADP-ribosylation of EF-2.

A cell-free assay was used to compare the relative abilities of the EF-2 from the sensitive and resistant cells (PyBHK and PyBHKR) to be ADP-ribosylated by diphtheria toxin fragment A and toxin A of *P. aeruginosa*. The results (Table 1) indicate that PyBHK EF-2 is readily ADP-ribosylated by both fragment A and toxin A, whereas PyBHKR EF-2 is not labeled by either toxin. The possibility that the resistant cell extracts contained a factor that inhibited the transfer of ADPR to EF-2 was tested. Resistant cell extracts were mixed with an EF-2 preparation from normal PyBHK cells. The addition of resistant cell extracts did not interfere with the transfer of labeled ADPR to the PyBHK EF-2 (data not shown). These results indicate that the mutation is cytoplasmic in nature and may be due to an altered EF-2 which is incapable of accepting ADPR in the toxin-catalyzed reaction (Table 1).

Characterization of PyBHKR cells. The PyBHK and PyBHKR cells had similar morphology. They both appeared as squamous or somewhat rounded, short fibroblasts with a tendency to overlap and form piles. The cloning efficiencies at various serum levels (2 to 10%) of the PyBHK and PyBHKR cells were indistinguishable. Both PyBHK and PyBHKR cells had similar rates of cell division, with an average doubling time of 13.2 and 12.5 h, respectively, in medium containing 10% fetal calf serum. Karyotypic analysis showed that both the PyBHK and PyBHKR cells contained 84 chromosomes in a similar distribution, and both cell lines induced tumors in golden hamsters. Cells recovered from tumors remained totally resistant to diphtheria toxin, indicating that the mutation conferring toxin resistance was stable during in vivo culture of the cells. The resistance of PyBHKR cells to diphtheria toxin has remained stable during 2 years of passage in cell culture.

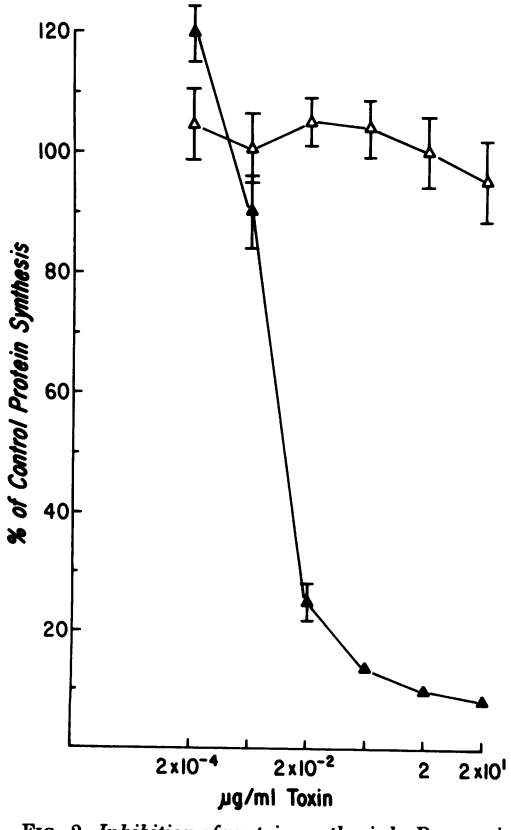

FIG. 2. *Inhibition of protein synthesis by P. aeruginosa toxin A in PyBHK cells (▲) and PyBHKR cells (△).*

TABLE 1. *ADP-ribosylation of EF-2 in PyBHK and PyBHKR cell extracts by diphtheria toxin fragment A and Pseudomonas toxin A*[a]

Cell extract	Toxin	
	Diphtheria fragment A	*Pseudomonas* toxin A
PyBHK	15,714[b]	6,205
PyBHKR	278	214

[a] These experiments were repeated three times with comparable results.

[b] Numbers are expressed in counts per minute per milligram of added EF-2 extract. Cell extract protein concentrations in the reaction mixture were PyBHK (590 μg/ml) and PyBHKR (300 μg/ml).

Comparison of ADPR transferase activities of toxin A and exoenzyme S in PyBHK or PyBHKR extracts. Tumors were induced in hamsters with PyBHKR or PyBHK cells, and extracts were prepared from the tumors as described above. In these extracts, enzyme activities of toxin A, partially purified S, and culture supernatants from various strains of *P. aeruginosa* were compared. Controls contained wheat germ extracts in place of the tumor cell extracts. The results of these experiments (Table 2) showed that EF-2 in the PyBHKR cell extracts is not ADP-ribosylated by either pure toxin A or culture supernatants from A^+S^- strains of *P. aeruginosa*. Identical results were obtained with purified fragment A of diphtheria toxin (data not shown). On the other hand, proteins in the PyBHKR extracts are ADP-ribosylated by partially purified S and by culture supernatants of S^+ strains of *P. aeruginosa*. Thus these extracts provided a simple assay which distinguished S from A.

Production of S by clinical isolates of *P. aeruginosa*. A total of 124 clinical isolates of *P. aeruginosa* were analyzed for production of the

ADPR transferases A and S. Production of S was assayed using the PyBHKR assay described above. Toxin A production was identified by at least a twofold increase in ADPR transferase activity in TSBD (no NTA) culture supernatants after preincubation with 4 M urea-1% DTT (13). The results (Table 3) showed that 48 (38%) of all strains tested produced S. There was no significant difference in the percentage of S producers between the bacteremia and burn patients. The majority of the strains (34/48) that produced S also produced A. The percentage of toxin A-producing strains (80%) agreed with that previously reported (3, 25, 27).

The relationship between S or A production and patient mortality is shown in Table 4. There were no differences between the bacteremic and the burn isolates in the mortality rates for each phenotype. Although the numbers were small, there was an increased mortality rate associated with patients infected with strains of *P. aeruginosa* which produce both S and A. Using a one-tailed T-test for proportions, when the mortality rate associated with the A^+S^+ strains was compared to the mortality rates of the other three phenotypes, a value of 1.66 was obtained. A value of 1.65 or greater is considered significant ($P < 0.05$) (15).

TABLE 2. *Comparison of P. aeruginosa A and S enzyme activities in extracts of wheat germ, ByBHK, and PyBHKR cells*[a]

Toxin or strain	ADPR transferase phenotype	ADPR transferred[b] (pmol/mg of protein)		
		PyBHK[c]	PyBHKR	Wheat germ
Toxin				
A (0.05 μg)		19.4	0	42.4
S (0.06 μg)		757	660	309.3
Strain[d]				
PA 103	A^+S^-	18.8	0	38.1
PS 388	A^-S^+	539	432	181.2
PA 16	A^+S^+	49.8	64	19.9
WR 5	A^-S^-	0	0	0

[a] Enzyme activity was measured by incubating 10 μl of enzyme or culture supernatant with 10 μl of buffer, 10 μl of cell extract, and 5 μl of ^{14}C-labeled NAD for 30 min at 25°C.

[b] These experiments were repeated three times with comparable results.

[c] Source of cell extract.

[d] Supernatants from 20-h cultures.

DISCUSSION

Although there is increasing evidence that toxin A is a major virulence factor of *P. aeruginosa*, little is known about the role of exoenzyme S in *P. aeruginosa* infections. In vivo production of S has been demonstrated in a burned mouse model (2). However, the prevalence of exoenzyme S production by strains of *P. aeruginosa* was unknown before this report.

To determine the incidence of S production by clinical isolates, it was necessary to develop specific methods to detect S and to distinguish it from toxin A. Moehring and Moehring isolated mutants of Chinese hamster ovary cells resistant to diphtheria toxin that were termed presumptive translational mutants (23). The mutation conferring resistance was shown to be in EF-2 (24). More recently, similar mutants were isolated after a single exposure of normal or mutagenized cells to diphtheria toxin (24). Utilizing

TABLE 3. *Production of A and S by clinical isolates of P. aeruginosa*

Toxin phenotype	No. positive[a]		
	Bacteremia patients	Burn patients	Total
A^+S^-	32 (45)	33 (62)	65 (53)
A^+S^+	21 (29)	13 (25)	34 (27)
A^-S^+	7 (10)	7 (13)	14 (11)
A^-S^-	11 (16)	0 (0)	11 (9)
A^+	53 (74)	46 (87)	99 (80)
S^+	28 (39)	20 (38)	48 (38)

[a] Numbers in parentheses are percentages.

TABLE 4. *Association of the production of enzyme A or S with patient mortality*

Enzyme phenotype	Patient mortality					
	Bacteremia		Burn		Total	
	S[a]	D[b]	S	D	S	D
A^+S^-	19 (59)[c]	13 (41)	20 (60)	13 (40)	39 (60)	26 (40)
A^+S^+	9 (43)	12 (57)	5 (38)	8 (62)	14 (41)	20 (59)
A^-S^+	4 (57)	3 (43)	3 (43)	4 (57)	7 (50)	7 (50)
A^-S^-	6 (55)	5 (45)	0 (0)	0 (0)	6 (55)	5 (45)

[a] S, Number of patients surviving.

[b] D, Number of patients dying.

[c] Numbers in parentheses are percentages.

the original methods of Moehring and Moehring (23), PyBHKR cells were isolated which also appear to have an altered EF-2. These cells were resistant to protein synthesis inhibition by both diphtheria toxin and *Pseudomonas* toxin A. The toxin-resistant cells were isolated from polyoma virus-transformed cells that were capable of inducing tumors in hamsters, thus providing a convenient source of cells to prepare extracts. Extracts from these cells are ADP-ribosylated by exoenzyme S, but not toxin A or diphtheria toxin, thus providing an assay highly specific for S. When coupled with the ADPR transferase assay for toxin A, the S-specific assay makes it possible to determine the ADPR transferase enzyme phenotype of strains of *P. aeruginosa*.

Clinical isolates of *P. aeruginosa* were obtained from two groups of patients, those with burn wound infections and those with bacteremia (nonburn). These types of infections were chosen because of their severity. The mortality rate associated with *Pseudomonas* bacteremia is reportedly between 43 and 68% (1, 7, 28), and *Pseudomonas* is also associated with fatal infections of the burn wound (26). Of the 124 strains tested, 38% produced exoenzyme S. Production of S by clinical isolates of *P. aeruginosa* is therefore not a rare event, as was recently suggested (32). There was no difference in the incidence of S production between the two patient populations. It is interesting to note that, out of 53 burn isolates, there were no A^-S^- strains, suggesting that the ability of a strain to produce A or S may provide a selective advantage for the organism in this type of infection. The mortality rate associated with *P. aeruginosa* strains producing both A and S is greater than the mortality rate of patients infected with strains producing either one of these enzymes alone. Although more strains should be analyzed, these data suggest that a patient infected with a strain which produces both S and A has less of a chance of survival than a patient infected with a strain which produces only one or neither of these enzymes.

ACKNOWLEDGMENTS

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