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Demonstration of Cell Envelope-Bound Exotoxin A in *Pseudomonas aeruginosa*

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The quantity of membrane-bound and extracellular exotoxin A in four strains of *Pseudomonas aeruginosa* was investigated. In strain PA-103, which is the prototype strain used for toxin production, all of the toxin was released into the growth medium and little toxin remained with the cell envelope. In a virulent strain from a clinical source, strain 119, exotoxin A was found in equal amounts in the growth medium and in the cell envelopes. An avirulent mutant of this strain, strain 119 (AP−), which was resistant to 800 μg of polymyxin B per ml, had all the exotoxin A in a membrane-bound state and was unable to release exotoxin A into the growth medium. Thus, exotoxin A can be found in the membrane-bound form, in the extracellular form, or in both. The quantities of membrane-bound toxin and extracellular toxin vary with the strain of *P. aeruginosa*. The polymyxin B-resistant mutant which is blocked in toxin release will be useful in studies of exotoxin A secretion.

Pseudomonas aeruginosa secretes several extracellular substances into the growth medium (11). Some of these bacterial products are responsible for the virulence of the organism (11, 14, 15). Exotoxin A, a secreted protein which has nicotinamide adenine dinucleotide (NAD^+) glycohydrolase and adenine diphosphate (ADP)-ribosyl transferase activities, has been shown to inhibit protein synthesis in mammalian cells by the ADP-ribosylation of mammalian elongation factor 2 (EF-2) (7, 8). The toxin is not found in the cytoplasm of the bacterial cell, but is secreted into the growth medium in an inactive form as a proenzyme (18). Activation of this toxin occurs after secretion, possibly by a protease(s) which is also secreted by the organism, or activation in vitro can be accomplished by treatment with urea and dithiothreitol (DTT) (18).

Although the secreted exotoxin A has been well characterized, the process of secretion of exotoxin A has not been studied. The exotoxin, which is synthesized in the cytoplasm or on the inner side of the cytoplasmic membrane, must pass through the cell envelope and then be released into the growth medium. Therefore, the exotoxin must exist in a membrane-bound state during and shortly after synthesis. In this study, cell envelope preparations from four strains of *P. aeruginosa* were examined to determine whether exotoxin A can be found associated with the cell envelope. The amount of exotoxin A activity in the cell envelope fraction was different in the strains examined. One of the strains, a mutant that was resistant to polymyxin B, did not release exotoxin A into the growth medium. In this strain, toxin secretion was possibly blocked at the release step.

MATERIALS AND METHODS

Organisms. *P. aeruginosa* PA-103, a prototype for toxin production, was obtained from P. V. Liu, University of Louisville, Louisville, Ky. This strain is protease deficient (11). *P. aeruginosa* 119 was initially isolated as a mucoid strain from the sputum of a cystic fibrosis patient. This organism became nonmucoid upon subculture, was sensitive to polymyxin B (<12.5 μg/ml), and was virulent for chicken embryos when injected intra-allantoically. Strain 119 (AP−) was a nitrosoguanidine-induced mutant of strain 119 that was resistant to polymyxin B (>800 μg/ml). It did not autoplaque, did not produce fluorescein, was protease deficient, and was avirulent for chicken embryos. Strain 119 (AP+) was a revertant of 119 (AP−). It was polymyxin B sensitive, protease positive, and virulent for chicken embryos, and in all tests it was identical with the wild type, strain 119. Since strain 119 (AP−) was able to revert to its wild-type phenotype, it probably has a single-point mutation. These strains were isolated by B. Wilcke (Ph.D. thesis, Temple University School of Medicine, Philadelphia, Pa., 1975; B. W. Wilcke and K. R. Cundy, Bacteriol. Proc., p. 10, 1975).

Growth conditions. The organisms were stored frozen at −70°C. They were grown in 2 liters of dialyzed Trypticase soy broth supplemented with 1% glycerol and 0.5 M sodium glutamate as described by Liu et al. (11, 12). The cultures were incubated aerobically at 32°C for 18 h (approximately 2×10^9 CFU/ml).

Cell envelope preparation. The organisms were collected by centrifugation and washed once with 50

mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.6). Washed cells were lysed by sonication in 50 mM Tris-hydrochloride buffer (pH 7.6). Deoxyribonuclease (4 μg/ml) and ribonuclease (4 μg/ml) were added to the lysate, and whole cells were removed by slow-speed centrifugation. Cell envelopes were pelleted by ultracentrifugation (60,000 × *g* for 1 h). The cell envelopes were washed twice in 50 mM Tris-hydrochloride buffer (pH 7.6). The protein content in the cell envelope preparations was estimated by the method of Lowry et al. (13), using bovine serum albumin as the standard, and the preparations were diluted to contain 2 mg of protein per ml.

Exotoxin A preparation. Exotoxin A was prepared from the culture supernatants of the 18-h cultures by zinc acetate precipitation and ammonium sulfate fractionation as described by Liu et al (12).

Activation of toxin. Cell envelope and toxin preparations were dialyzed for 48 h at 4°C against: (i) 20 mM Tris-hydrochloride buffer (pH 7.6) containing 4 M urea and 5 mM DTT; or (ii) 50 mM Tris-hydrochloride buffer (pH 7.6) containing 200 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 3 mM sodium azide as described by Moss et al. for the activation of cholera toxin (16).

NAD^+ glycohydrolase assay. NAD^+ glycohydrolase activity was measured as described by Moss et al. (16). The ADP-ribose and nicotinamide formed as end products were separated by using cellulose thin-layer plates or by using Dowex AG1-X2 ion-exchange columns (16). Exotoxin A, prepared from culture supernatants and cell envelope preparations, was activated by dialysis and was mixed with 2 mM NAD^+, [*carbonyl*-^{14}C]NAD^+ (50,000 cpm), or 2 mM [*adenine*-^{14}C]NAD^+ (70,000 cpm), both contained in 40 mM dithiothreitol in 200 mM sodium acetate buffer (pH 6.8) in a total volume of 0.1 ml. The mixtures were incubated for 2 h at 37°C, and 5-μl samples were spotted on cellulose thin-layer plates and developed in isobutyric acid-ammonium hydroxide-water (66:1:33). The ADP-ribose and NAD^+ spots were detected by autoradiography. The nicotinamide released by the breakdown of [*carbonyl*-^{14}C]NAD^+ was quantitated by layering 0.1 ml of the reaction mixture on Dowex AG1-X2 columns (0.5 by 4 cm) and eluting with 20 mM Tris-hydrochloride (pH 7.5).

ADP-ribosylation assay. Wheat germ EF-2 was obtained from A. Marcus, The Institute for Cancer Research, Fox Chase, Philadelphia, Pa. The preparation had 3.96 mg of protein per ml. The assay was carried out as described by Chung and Collier (2) using 0.15 μM EF-2 and 0.25 μM [*adenine-U*-^{14}C]NAD^+ (Amersham/Searle Co; 280 mCi/mmol) in a total volume of 0.1 ml. The reaction was stopped by adding an equal volume of 10% trichloroacetic acid. The precipitate was collected and washed on glass fiber filters and then counted.

RESULTS

Activation of exotoxin A. NAD^+ glycohydrolase and ADP-ribosyl transferase activities in exotoxin prepared from the culture supernatant of strain PA-103 were activated by dialysis against 20 mM Tris-hydrochloride buffer (pH 7.6), 4 M urea, and 5 mM DTT, or by dialysis against 50 mM Tris-hydrochloride (pH 7.6), 200 mM NaCl, 1 mM EDTA, and 3 mM sodium azide buffer. The two buffers activated the toxin to the same extent (Table 1). Therefore, we used the buffer containing 50 mM Tris-hydrochloride (pH 7.6), 200 mM NaCl, 1 mM EDTA, and 3 mM sodium azide for activating the exotoxin in all preparations. Dialysis against Tris-hydrochloride buffer alone did not activate the toxin, showing that the activation was not a result of fragmentation of the toxin. In addition to secreting exotoxin A, *P. aeruginosa* secretes exoenzyme S, which is inactivated by urea. Since the exotoxin activity in our experiment was not inactivated by dialysis against urea, it was not exoenzyme S (9).

NAD^+ glycohydrolase activity of culture supernatants. The NAD^+ glycohydrolase activity of exotoxin A prepared from culture supernatants of the four strains was examined using cellulose thin-layer plates to separate ADP-ribose and nicotinamide. Exotoxin preparations obtained from cultures of strains PA-103 and 119 had NAD^+ glycohydrolase activity, whereas exotoxin preparations from strain 119 (AP−) contained little or no NAD^+ glycohydrolase. Strain 119 (AP+), which is the revertant of strain 119 (AP−), also had NAD^+ glycohydrolase activity and was similar to the wild type, strain 119 (Fig. 1).

The quantity of NAD^+ hydrolyzed by these preparations was measured using Dowex AG1-X2 chromatography (Table 2). The experiment was repeated using preparations that were dialyzed against 4 M urea and 5 mM EDTA (Table 2). The quantity of nicotinamide released from NAD^+ by preparations dialyzed against Tris-hydrochloride–NaCl–EDTA–sodium azide buffer or Tris-hydrochloride–urea–DTT buffer was comparable. Strains PA-103, 119, and 119 (AP+) had significant amounts of NAD^+ glycohydrolase activity in the extracellular medium, whereas strain 119 (AP−) had very little activity

TABLE 1. *Comparison of two different buffers for the activation of exotoxin A using the ADP-ribosyl transferase assay*

Dialysis buffer	cpm[a]
50 mM Tris-hydrochloride (pH 7.6) + 200 mM NaCl + 1 mM EDTA + 3 mM sodium azide (buffer A)	2,460
20 mM Tris-hydrochloride (pH 7.6) + 4 M urea + 5 mM DTT (buffer B)	2,632
50 mM Tris-hydrochloride (buffer C)	128

[a] Radioactivity collected on filters by trichloroacetic acid precipitation of ADP-ribosyl transferase assay reaction mixture.

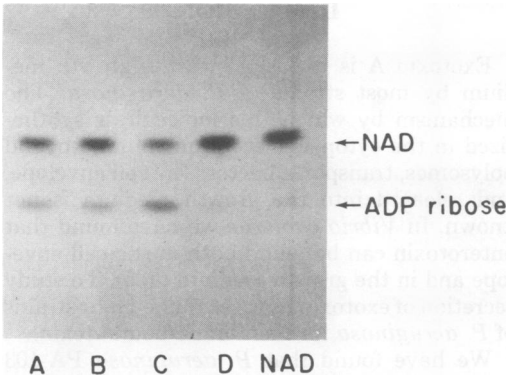

FIG. 1. *NAD⁺ glycohydrolase activity of exotoxin A prepared from culture supernatants of different strains of P. aeruginosa. Autoradiographs of thin-layer cellulose plates used for the separation of [^{14}C]ADP-ribose and nicotinamide from [adenine-^{14}C]NAD⁺. (A) Strain PA-103; (B) strain 119; (C) strain 119 (AP+); (D) strain 119 (AP−); NAD⁺, control.*

TABLE 2. *Quantitation of [^{14}C]nicotinamide released from [carbonyl-^{14}C]NAD⁺ by exotoxin prepared from culture supernatants*

Strain	Percent ^{14}C recovered as nicotinamide		
	Buffer A[a]	Buffer B[a]	Buffer C[a]
PA-103	37	40	<1
119	42	38	<1
119 (AP+)	40	32	<2
119 (AP−)	<1	2	<1

[a] Buffers A, B, and C are described in Table 1.

in the growth medium. Thus, strain 119 (AP−) is unable either to synthesize toxin or to release the toxin into the growth medium.

NAD⁺ glycohydrolase activity in cell envelope preparations. To determine whether the four strains had membrane-bound toxin, cell envelopes were dialyzed against 50 mM Tris-hydrochloride (pH 7.6), 200 mM NaCl, 1 mM EDTA, and 3 mM sodium azide buffer for 48 h and assayed for NAD⁺ glycohydrolase activity. Figure 2 shows that strain PA-103 contains little NAD⁺ glycohydrolase activity bound to its cell envelope. The cell envelopes of strains 119 and 119 (AP+) hydrolyze approximately half of the NAD⁺ in the reaction mixture. When cell envelopes of strain 119 (AP−) were used, all of the NAD⁺ had been hydrolyzed to ADP-ribose and nicotinamide. The quantity of nicotinamide released from NAD⁺ hydrolyzed by these preparations was quantitated using ion-exchange columns (Table 3). Since other investigators have used urea and DTT to activate exotoxin A that is secreted into the growth medium (2, 18), we dialyzed the cell envelope preparations against 20 mM Tris-hydrochloride (pH 7.6), 4 M urea, and 5 mM DTT buffer and quantitated the NAD⁺ glycohydrolase activity of these preparations. The results (Table 3) did not differ from those obtained when the preparations were dialyzed against Tris-hydrochloride-NaCl-EDTA-sodium azide buffer. When cell envelope preparations were not dialyzed against either of the two buffers, no NAD⁺ glycohydrolase activity was demonstrated. Therefore, the membrane-bound toxin is also present as a proenzyme.

ADP-ribosyl transferase activity in culture supernatants and cell envelopes. NAD⁺ glycohydrolase activity associated with cell envelope preparation and exotoxin preparations must have ADP-ribosyl transferase activity in addition to NAD⁺ glycohydrolase activity to be referred to as exotoxin A. Therefore, we examined the ADP-ribosyl transferase activities of the preparations that had been activated

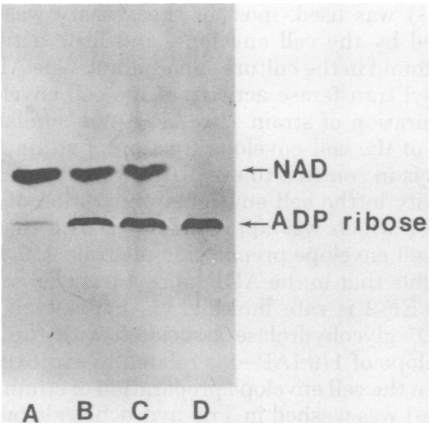

FIG. 2. *NAD⁺ glycohydrolase activity associated with cell envelope preparations of different strains of P. aeruginosa. Autoradiographs of thin-layer cellulose plates used for the separation of [^{14}C]ADP-ribose and nicotinamide from [adenine-^{14}C]NAD⁺. (A) Strain PA-103; (B) strain 119; (C) strain 119 (AP+); (D) strain 119 (AP−).*

TABLE 3. *Quantitation of [^{14}C]nicotinamide released from [carbonyl-^{14}C]NAD⁺ by cell envelopes*

Strain	Percent ^{14}C recovered as nicotinamide		
	Buffer A[a]	Buffer B[a]	Buffer C[a]
PA-103	10	12	<2
119	46	38	<2
119 (AP+)	43	50	<2
119 (AP−)	<98	<98	<2

[a] Buffers A, B, and C are described in Table 1.

by dialysis against Tris-hydrochloride-NaCl-EDTA-azide buffer or Tris-hydrochloride-urea-DTT buffer. The quantity of [^{14}C]ADP-ribose that was transferred to wheat germ EF-2 by exotoxin prepared from culture supernatants and cell envelope preparations is shown in Table 4. In general, the results were similar to those obtained from NAD^+ glycohydrolase assays. Dialysis against either of the two buffers was required to activate the ADP-ribosyl transferase activity, because dialysis against Tris-hydrochloride buffer alone did not activate the toxin. The exotoxin prepared from culture supernatants of strains PA-103, 119, and 119 (AP+) had ADP-ribosyl transferase activity. Strain 119 (AP−) did not secrete any detectable exotoxin A with ADP-ribosyl transferase activity into the growth medium. Strain PA-103 had little or no activity associated with its cell envelope. The cell envelope preparations of strains 119 and 119 (AP+) had the same amount of activity as exotoxin A prepared from the culture supernatant of these cultures. However, when strain 119 (AP−) was used, most of the activity was retained by the cell envelope, and little activity was found in the culture supernatant. The ADP-ribosyl transferase activity of the cell envelope preparation of strain 119 (AP−) was similar to that of the cell envelope fraction of strain 119. This is in contrast to the NAD^+ glycohydrolase activity in the cell envelope preparation of 119 (AP−) which was approximately twice that in the cell envelope preparation of strain 119. It is possible that in the ADP-ribosyl transferase assays EF-2 is rate limiting, or that not all the NAD^+ glycohydrolase associated with the cell envelope of 119 (AP−) is related to exotoxin A. When the cell envelope preparation of strain 119 (AP−) was washed in Tris-hydrochloride buffer (pH 7.5), less than 5% of the activity was released into the buffer. Thus, the exotoxin A was tightly bound to the cell envelope.

DISCUSSION

Exotoxin A is released into the growth medium by most strains of *P. aeruginosa*. The mechanism by which this molecule is synthesized in the cytoplasm or on membrane-bound polysomes, transported across the cell envelope, and released into the growth medium is not known. In *Vibrio cholerae* we have found that enterotoxin can be found both in the cell envelope and in the growth medium (4, 5). To study secretion of exotoxin A, we examined four strains of *P. aeruginosa* for membrane-bound toxin.

We have found that *P. aeruginosa* PA-103 releases all or most of the exotoxin A synthesized into the growth medium. It retains very little exotoxin A in the membrane-bound state. It could be that this is the result of increased activity of the release mechanism, possibly a membrane-bound protease, or that the association between the toxin and membrane is so loose as to allow rapid release of exotoxin A during growth or centrifugation of the organisms during exotoxin A preparation.

P. aeruginosa 119 is virulent for chicken embryos (Wilcke, thesis; Wilcke and Cundy, Bacteriol. Proc., p. 10, 1975) and mice (unpublished data). This strain also releases large amounts of toxin into the growth medium. However, during the secretion of exotoxin A approximately the same amount of exotoxin A remained in the membrane-bound form as was found in the growth medium. Apparently, the release process in strain 119 does not keep pace with the synthetic process, as occurs in strain PA-103.

In contrast to strains PA-103 and 119, the nitrosoguanidine-induced mutant, strain 119 (AP−), is unable to release its exotoxin A after its synthesis. We have found that all the toxin activity is associated with the cell envelope of this strain. Although we have not detected any toxin in the growth medium, it is possible that

TABLE 4. *ADP-ribosyl transferase activity of cell envelope preparations and exotoxin A prepared from culture supernatants*

Strain	Activity (cpm) in:					
	Cell envelope[a]		Culture supernatant		Total	
	Buffer A[b]	Buffer B[b]	Buffer A	Buffer B	Buffer A	Buffer B
PA-103	370	280	2,533	2,306	2,903	2,585
119	2,493	2,836	2,206	2,604	4,699	5,440
119 (AP−)	2,836	3,111	229	132	3,065	3,243
119 (AP+)	2,683	2,526	1,826	2,106	4,509	4,632
Control[c]	115	139	180	122	295	261

[a] Radioactivity collected on filters of trichloroacetic acid precipitates of ADP-ribosyl transferase assay mixture. In all cases preparations dialyzed against buffer C transferred fewer than 150 cpm to EF-2.

[b] Buffers A and B are described in Table 1.

[c] *Escherichia coli* K-12 was used as control.

the toxin could be secreted in a form that has no NAD^+ glycohydrolase or ADP-ribosyl transferase activities, even after the dialysis that is required for the activation of exotoxin. In this case, it may be possible to identify the inactive toxin by using immunological methods. In addition to not being able to release exotoxin A, strain 119 (AP−) is also protease deficient and polymyxin B resistant. Thus, its loss of virulence could be related to any one or all of these properties. The revertant organism, strain 119 (AP+), is identical to the wild type, strain 119. The revertant strain has regained its ability to release both exotoxin A and protease(s), and it has regained its virulence for chicken embryos.

The membrane-bound exotoxin A is not released by suspending the cell envelopes in Tris-hydrochloride buffer during cell envelope preparation. This indicates that the exotoxin A is tightly bound to the cell envelope and possibly requires a special process, such as proteolytic cleavage, to release it into the growth medium. Other investigators have found that extracellular proteases augment the virulence of exotoxin A-producing *P. aeruginosa* (14, 17). Further studies are necessary to determine the process of exotoxin A release from the membrane-bound state.

Strain 119 (AP−) was selected because of its resistance to polymyxin B. Polymyxin B has been shown to act at the cell envelope (1, 3), and polymyxin B-resistant strains have been found to have cell envelope components that have been altered (6). It is possible that changes in membrane components could affect the fluidity of the membrane and the transport and release of membrane-bound precursors such as membrane-bound exotoxin A.

This study also compares the activating buffer, Tris-hydrochloride-NaCl-EDTA-sodium azide, which is used for activating cholera toxin (16), with Tris-hydrochloride-urea-DTT buffer, which is used for activating *P. aeruginosa* toxin. The two buffers were similar in their ability to activate both the membrane-bound and the extracellular toxin. We prefer the former buffer when assaying for NAD^+ glycohydrolase activity using thin-layer cellulose plates, because the urea had to be removed by dialysis before the assay to prevent the smearing of spots. We have also found the Tris-hydrochloride-NaCl-EDTA-sodium azide buffer to be more useful in our attempts to isolate the membrane-bound toxin by affinity chromatography or by immunoprecipitation.

The release of exotoxin A from the cell envelope of *P. aeruginosa* may be an important pathogenic property of the organism. In a clinical situation, a part or all of the exotoxin A synthesized by *P. aeruginosa* may remain cell envelope bound. Disturbance of the physiological balance in the organism, such as by antibiotic treatment, may cause the release of preformed envelope-bound toxin. If the mechanism of secretion of toxin is similar to the secretion of other extracellular proteins in bacteria (10), the membrane-bound toxin may not be identical to the extracellular toxin. Isolation and characterization of this membrane-bound toxin will allow comparison of its properties with the extracellular exotoxin A.

Thus, the exotoxin A formed in the cytoplasm or on the inner side of the cytoplasmic membrane is transported across the *P. aeruginosa* membrane as a membrane-bound exotoxin A. The rapidity of release of this membrane-bound exotoxin A varies according to the activity of the release mechanism in that particular organism. Since the release mechanism could be an important factor in determining the virulence of toxin-producing bacteria, further work is necessary to characterize membrane-bound proteases that may be involved in the release of exotoxin A from the cell envelope of *P. aeruginosa*.

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