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Biological Effects of *Pseudomonas aeruginosa* Type III-Secreted Proteins on CHO Cells

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A strain of *Pseudomonas aeruginosa* that fails to express known type III-secreted effector proteins was constructed as an expression host. Individual effectors were expressed in *trans*, and their biological effects on CHO cells were assessed in an acute cellular infection model. Intoxication with ExoS, ExoT, or ExoY resulted in alterations in cell morphology. As shown in previous genetic studies, ExoU expression was linked to acute cytotoxicity.

The exoenzyme S regulon, which encodes the type III secretion system of *Pseudomonas aeruginosa*, consists of coordinately regulated secretion, translocation, regulatory, and effector genes (6). To date, four type III-secreted effector proteins have been identified; ExoS, ExoT, ExoU, and ExoY (3, 10, 21–23). ExoS and ExoT are members of the family of bacterial ADP-ribosyltransferases (10, 21). Despite having 75% amino acid identity, ExoT possesses only 0.2% of the enzymatic activity of ExoS (21). ExoU functions as an acute cytotoxin in vitro and is associated with lung injury in vivo (3). The mechanism of ExoU-mediated toxicity remains unknown. ExoY is a recently discovered adenylate cyclase, which is activated by a eukaryotic protein that is distinct from calmodulin (23). Because *P. aeruginosa* produces multiple effector proteins in a strain-specific manner, it is difficult to determine the role of the individual products in pathogenesis. The goal of this study was to construct a strain of *P. aeruginosa* which fails to express the known effector proteins for use as an expression host. By constructing a host without effectors but possessing a functional secretion and delivery apparatus, the cellular effects of individual virulence determinants could be assessed.

Construction of a *P. aeruginosa* type III-secreted effector mutant. *P. aeruginosa* PA103 was chosen as the parental strain (Table 1). PA103 produces significant amounts of ExoU and ExoT (3) but fails to express ExoY (23) and does not possess *exoS* (5). In addition, strain PA103 is easy to genetically manipulate and displays virulence in both tissue culture and acute lung infection models of *P. aeruginosa* pathogenesis (1, 3, 5, 11). Although strain PA103 produces large amounts of exotoxin A in vitro, this toxin appears to play no role in the tissue culture and acute infection models developed to measure the contribution of the type III-secreted products (1, 11). In previous studies, individual mutations in *exoU* (PA103Δ*exoU*) and *exoT* (PA103*exoT*::Tc) were constructed (3, 4). In this study, we constructed the double mutant PA103Δ*exoUexoT*::Tc and compared its properties with those of the parental (PA103) and individual mutant (PA103Δ*exoU* and PA103*exoT*::Tc) strains in a Chinese hamster ovary (CHO) cell model of infection.

To construct the double *exoU-exoT* mutation, pMOB*exoT*::Tc (3) was conjugated into strain PA103Δ*exoU* (4). Tetracycline-resistant merodiploids were selected and passaged on Vogel-Bonner minimal medium (20) with 100 μg of tetracycline per ml. Plasmid sequences and the wild-type *exoT* allele were resolved from the chromosome by selecting for strains resistant to 5% sucrose and tetracycline (7, 18). Isolates exhibiting the correct phenotype were grown under inducing conditions for the exoenzyme S regulon, and their extracellular protein profiles were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. Chromosomal DNA was isolated (7) from strains defective for the extracellular production of ExoU and ExoT and subjected to Southern blot analysis (14). We selected a single isolate, PA103Δ*exoUexoT*::Tc, which failed to hybridize to the *exoU* probe, exhibited tetracycline resistance, and failed to express extracellular ExoU and ExoT.

Single (PA103*exoT*::Tc and PA103Δ*exoU*) and double (PA103Δ*exoUexoT*::Tc) mutant strains were analyzed for their extracellular protein profiles by SDS-PAGE and Western blot analysis. In the parental PA103 strain, all of the tested extracellular proteins of the regulon (ExoU, ExoT, PcrV, and PopD) were induced by the inclusion of the chelator nitrilotriacetic acid (NTA) in the growth medium (Fig. 1A and B). Induction and secretion of type III proteins also occurred in each mutant strain. These results indicated that type III-mediated regulation and secretion were unaffected by the introduction of mutant alleles. Introduction of either single or double mutant alleles resulted in the absence of only the respective protein products (Fig. 1A and B).

Parental and mutant *P. aeruginosa* strains were transferred from Vogel-Bonner minimal medium to serum-free tissue culture medium and used to infect CHO cells at a multiplicity of infection of ≈5:1. Following either a 3-h (strains PA103 and PA103*exoT*::Tc) or a 4-h (uninfected; strains PA103Δ*exoU* and PA103Δ*exoUexoT*::Tc) infection at 37°C in 5% CO_2, duplicate wells were washed with phosphate-buffered saline and either fixed in 2% paraformaldehyde or stained for 5 min with trypan blue and photographed. An additional quantitative 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay for cell viability was performed for each strain 4 h postinfection (2). CHO cells infected with parental strain PA103 differed in morphology, were permeable to trypan blue, and showed significant differences in cell viability compared to uninfected cells (Fig. 1C and Table 2). CHO cells infected with PA103Δ*exoU* (expressing ExoT) appeared rounded, were not permeable to trypan blue, and retained viability (Fig. 1C and Table 2). Cells infected with a strain expressing only ExoU (PA103*exoT*::Tc) possessed a phenotype similar to that of cells

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Known type III effector protein(s) expressed or relevant characteristic(s)	Reference(s) or source
P. aeruginosa strains		
PA103	ExoT, ExoU	B. H. Iglewski
PA103Δ*exoU*	ExoT	4
PA103*exoT*::Tc	ExoU	3
PA103Δ*exoUexoT*::Tc	None	This study
PA103*toxA*::Ω	ExoT, ExoU	B. H. Iglewski
PA103*exsA*::Ω	None	7
Plasmids		
pMOB*exoT*::Tc	Allelic replacement vector encoding tetracycline-interrupted *exoT*	3, 18
pUCP18	pUC-derived cloning vector able to replicate in *P. aeruginosa*	17
pUCP*exoS*	Encodes the wild-type ADP-ribosyltransferase ExoS (100% activity)	12
pUCP*exoS*E381A	Encodes the noncatalytic mutant ExoSE381A (0.02% activity)	This study and 13
pUCP*exoT*	Encodes the ADP-ribosyltransferase ExoT (0.2% activity)	21
pUCP*exoY*	Encodes the adenylate cyclase ExoY	23
pUCP*exoY*K81M	Encodes the noncatalytic mutant ExoYK81M	23

infected with the parental strain. Finally, when a strain of *P. aeruginosa* which fails to express any of the known type III-secreted effector proteins, PA103Δ*exoUexoT*::Tc, was used, the infected cells were indistinguishable from the uninfected control cells (Fig. 1C and Table 2). We interpreted these results as suggesting that part of the ExoU-mediated cytotoxic response (3) may involve cellular morphology changes and/or membrane damage. On the other hand, ExoT appears to cause cell rounding in the absence of membrane damage or changes in cell viability at this time point. Elimination of ExoT and ExoU appears to result in an avirulent strain in this tissue culture model.

Expression of individual effector proteins in PA103Δ*exoU exoT*::Tc. In the acute in vitro infection model, cocultivation of strain PA103Δ*exoUexoT*::Tc with CHO cells resulted in no observable effect. This result suggested that this might be an ideal host strain from which to assess the biological effects of the individual type III-secreted effector proteins of *P. aeruginosa*. Strain PA103Δ*exoUexoT*::Tc was transformed with a vector control (pUCP18); pUCP*exoS*, a plasmid encoding a noncatalytic derivative of ExoS (pUCP*exoS*E381A); pUCP*exoT*; pUCP*exoY*; or a plasmid expressing a noncatalytic adenylate cyclase (pUCP*exoY*K81M). Expression plasmid pUCP*exoS*E381A was constructed by replacing the *Nsi*I-*Bam*HI fragment of pUCP18*exoS* (12) with that from pET16bΔRI*exoS*E381A (13). The strains were induced for expression of the exoenzyme S regulon, and the extracellular protein profile was analyzed by SDS-PAGE and Western blot analysis (Fig. 2A and B). This analysis indicated that expression of each product was variable. ExoS and the noncatalytic mutant ExoSE381A appeared to be equally expressed and secreted, as has been shown in previous studies (Fig. 2A and B, compare lanes 2 and 3) (22). When *exoT* was provided in *trans*, the protein was made and secreted in relatively large quantities (Fig. 2A and B, lanes 5). Both forms of ExoY were expressed and secreted in much smaller quantities than either ExoS or ExoT.

CHO cells were infected for 4 h and subsequently stained with trypan blue or fixed in paraformaldehyde and observed by phase-contrast microscopy or subjected to the MTT assay. At this point in infection, permeability to trypan blue or changes in cell viability were not observed with strains expressing ExoS, ExoSE381A, ExoT, ExoY, or ExoYK81M (Table 2). Compared to the vector control, however, expression of ExoS, ExoSE381A, ExoT, and ExoY, but not of ExoYK81M, resulted

TABLE 2. Biological effects of the various bacterial strains on CHO cells

Strain	Effector(s)	% Altered morphology[a]	% Trypan blue stained[a]	Decreased viability[b]
None (uninfected)		6 ± 2	0	NA[c]
PA103	ExoT, ExoU	53 ± 2	53 ± 2	+
PA103*exoT*::Tc	ExoU	46 ± 8	46 ± 8	+
PA103*toxA*::Ω[d]	ExoT, ExoU	ND[e]	ND	+
PA103Δ*exoU*	ExoT	98 ± 2	0	−
PA103Δ*exoUexoT*::Tc	None	6 ± 2	0	−
PA103*exsA*::Ω	None	NC[f]	0	−
PA103Δ*exoUexoT*::Tc pUCP	None	5	0	−
PA103Δ*exoUexoT*::Tc pUCP*exoS*	ExoS	90 ± 3	0	−
PA103Δ*exoUexoT*::Tc pUCP*exoS*E381A	ExoSE381A	99 ± 1	0	−
PA103Δ*exoUexoT*::Tc pUCP*exoT*	ExoT	99	0	−
PA103Δ*exoUexoT*::Tc pUCP*exoY*	ExoY	95 ± 6	0	−
PA103Δ*exoUexoT*::Tc pUCP*exoY*K81M	ExoYK81M	7 ± 4	0	−

[a] Percentage of CHO cells displaying an altered morphology or trypan blue staining via phase-contrast microscopy; results are averaged from photomicrographs taken of three random fields of infected monolayers.
[b] Presence or absence of a significant reduction in cell viability compared to uninfected control cells by an MTT viability assay.
[c] NA, not applicable; uninfected control cells were used as the basis of comparison for the MTT assay.
[d] Control for the MTT assay; a strain used to show that exotoxin A does not mediate the acute cytotoxic response in this tissue culture model.
[e] ND, not done.
[f] NC, no change in cellular morphology relative to the uninfected control.

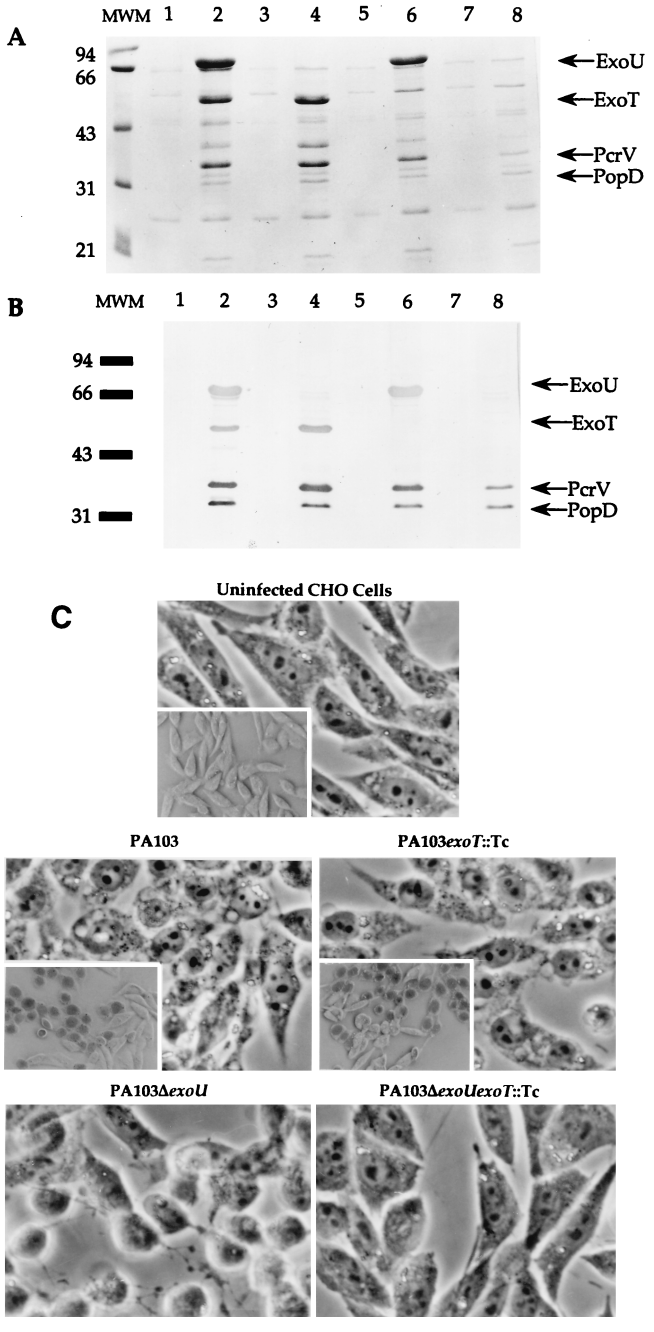

FIG. 1. Extracellular protein profiles, Western blot analysis, and infection of CHO cells with parental (PA103) and mutant (PA103Δ*exoU*, PA103*exoT*::Tc, and PA103Δ*exoUexoT*::Tc) strains of *P. aeruginosa*. (A) Coomassie blue-stained polyacrylamide gel (10%) of concentrated culture supernatants from strains grown in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of 10 mM NTA, a chelator that induces the expression of the exoenzyme S regulon. Supernatant fractions were collected and concentrated 20-fold by the addition of a saturated ammonium sulfate solution to 55% from strains PA103 (lanes 1 and 2), PA103Δ*exoU* (lanes 3 and 4), PA103*exoT*::Tc (lanes 5 and 6), and PA103Δ*exoUexoT*::Tc (lanes 7 and 8). Molecular mass markers (MWM; in kilodaltons) and the relative mobilities of ExoU (72 kDa), ExoT (53 kDa), PcrV (32.2 kDa), and PopD (31 kDa) are indicated. (B) Western blot of a duplicate gel as shown in panel A. A mixture of specific antisera reactive to ExoU, ExoS-ExoT, PcrV, and PopD was used as the primary antibody. Bound antibodies were visualized with a peroxidase-labeled secondary antibody and 4-chloro-1-naphthol and peroxide as substrate. (C) Phase-contrast microscopy (40× objective) of CHO cell morphology following infection with parental or mutant strains of *P. aeruginosa*. The results of the trypan blue staining for uninfected cells, PA103, and PA103*exoT*::Tc (10× objective) are shown in the insets. Only infections with bacterial strains expressing ExoU resulted in trypan blue staining 3 to 4 h after bacterial infection.

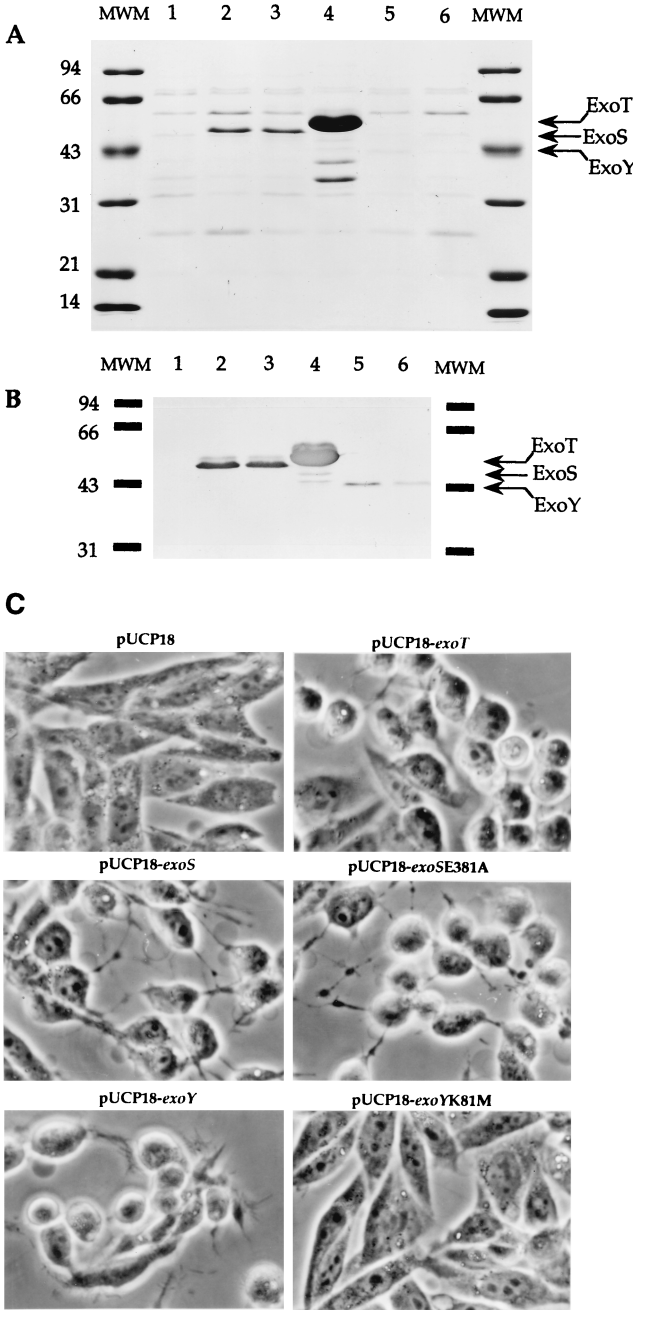

FIG. 2. Extracellular protein profiles and Western blot analysis of PA103Δ*exoUexoT*::Tc expressing various effector proteins in *trans* and their effects on CHO cells. (A) Coomassie blue-stained polyacrylamide gel (11%) of concentrated culture supernatants from strains grown under inducing conditions (growth in the presence of 10 mM NTA). Supernatants are from strains PA103Δ*exoUexoT*::Tc pUCP18 (lane 1), PA103Δ*exoUexoT*::Tc pUCP*exoS* (lane 2), PA103Δ*exoUexoT*::Tc pUCP*exoS*E381A (lane 3), PA103Δ*exoUexoT*::Tc pUCP*exoT* (lane 4), PA103Δ*exoUexoT*::Tc pUCP*exoY* (lane 5), and PA103Δ*exoUexoT*::Tc pUCP*exoY* K81M (lane 6). Molecular mass markers (MWM; in kilodaltons) and the relative mobilities of ExoT (53 kDa), ExoS (49 kDa), and ExoY (42 kDa) are indicated. (B) Western blot of a duplicate gel as shown in panel A. A mixture of specific antisera to ExoS-ExoT and ExoY was used, and the bound antibodies were visualized with a peroxidase-labeled secondary antibody. (C) Cellular morphology of CHO cells infected with PA103Δ*exoUexoT*::Tc expressing various effector proteins in *trans*. The name of the expression plasmid in each strain is given above the appropriate picture.

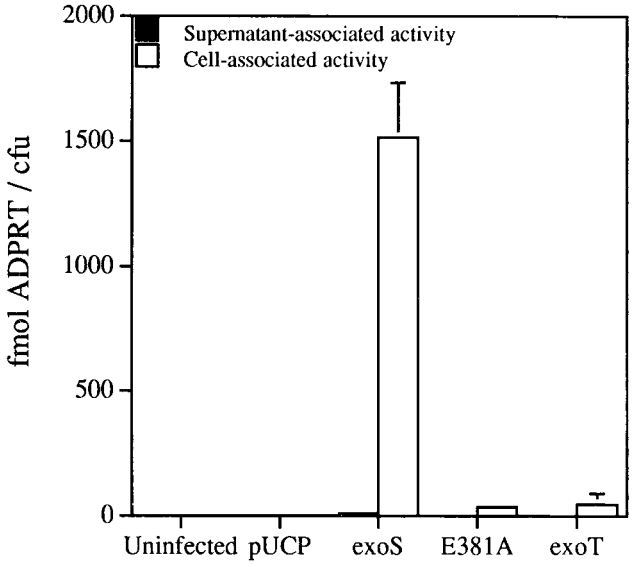

FIG. 3. Cell- and supernatant-associated ADP-ribosyltransferase activity of PA103Δ*exoUexoT*::Tc expressing ExoS, ExoSE381A, or ExoT in *trans*. ADP-ribosyltransferase activity assays were performed on supernatant- and cell-associated samples collected from uninfected CHO cells or CHO cells infected with PA103Δ*exoUexoT*::Tc pUCP18, PA103Δ*exoUexoT*::Tc pUCP*exoS*, PA103Δ*exoUexoT*::Tc pUCP*exoS*E381A, or PA103*exoUexoT*::Tc pUCP*exoT*. Activity is normalized to CFU and expressed as 10^{-4} femtomoles of ADPRT (femtomoles of ADP-ribose transferred to soybean trypsin inhibitor).

in an altered cellular morphology (Fig. 2C and Table 2). Cells appeared rounded and eventually detached from the surface of the well. Our data confirm that the ADP-ribosyltransferase activity of either ExoS or ExoT is not required to cause a rounding of CHO cells, supporting earlier observations of HeLa cell morphology changes when ExoS and ExoSE381A were delivered by the *Yersinia* type III apparatus (8). In addition, we confirm that ExoY is capable of causing a similar morphological effect on CHO cells which is dependent on adenylate cyclase activity (23). As positive controls for cell morphological changes, CHO cells were intoxicated with *Escherichia coli* heat-labile enterotoxin or pertussis toxin. Heat-labile enterotoxin mediated CHO cell elongation while pertussis toxin mediated CHO cell clustering, indicating that the cell line we are using responds to changes in cyclic AMP levels as previously reported (data not shown) (9, 23).

Measurement of effector translocation into CHO cells. In previous studies, we have used ExoS ADP-ribosyltransferase activity to measure type III-mediated translocation from *P. aeruginosa* 388 (expresses ExoS and ExoT) into CHO cells (19). To determine if PA103Δ*exoUexoT*::Tc could be used to study translocation of individual components, we pretreated CHO cells with cytochalasin D to inhibit the uptake of bacteria. Treated cells were infected with an inoculum of PA103Δ*exoUexoT*::Tc pUCP*exoS* in serum-free medium for 4 h at a multiplicity of infection of ≈5:1. The supernatant was removed, the number of viable bacteria was measured from a small aliquot, and the remaining sample was subjected to centrifugation at 14,000 × *g*, 4°C. A portion of the soluble fraction was retained for ADP-ribosyltransferase activity assays (supernatant-associated activity). The CHO cell monolayer was washed and treated for 2 h with 100 μg of ciprofloxacin per ml and 200 μg of gentamicin per ml to kill the extracellular bacteria. Infected CHO cells were lysed with 150 μl of distilled water. The lysate was subjected to centrifugation at 14,000 × *g* (4°C), and a portion of the soluble fraction was retained to perform ADP-ribosyltransferase activity assays (lysate-associated activity) as described previously (19). A duplicate well, not treated with antibiotics, was used to perform viable counts. Supernatant and lysate fractions from uninfected CHO cells or cells infected with strains containing the vector control plasmid (pUCP18), pUCP*exoS*E381A, or pUCP*exoT* were included as negative controls. Under these conditions, ExoS ADP-ribosyltransferase activity was predominantly associated with the CHO cell lysate, rather than the supernatant, indicating that PA103Δ*exoUexoT*::Tc is able to translocate ExoS (Fig. 3). Similar results were obtained when CHO cells were infected with PA103Δ*exoUexoT*::Tc pUCP*exoY* and cyclic AMP accumulation was measured (23). Activities that were slightly above background levels were measured from cells infected with strains expressing either ExoSE381A or ExoT. This amount of activity may represent the residual ADP-ribosyltransferase activity of the mutant proteins.

TABLE 3. Biological effects of *P. aeruginosa* type III-secreted proteins on CHO cells

Protein	Enzymatic activity	Effect on CHO cells
ExoS	ADP-ribosyltransferase	Morphological alterations
ExoT	ADP-ribosyltransferase	Morphological alterations
ExoU	Unknown	Acute cytotoxicity: membrane permeability, loss of viability
		Morphological alterations
ExoY	Adenylate cyclase	Morphological alterations

Concluding remarks. We constructed a strain of *P. aeruginosa*, PA103Δ*exoUexoT*::Tc, which fails to express any of the known *P. aeruginosa* type III-secreted effector proteins. Strain PA103Δ*exoUexoT*::Tc was used as an expression host, and the effects of individual translocated proteins were assessed in a cellular acute infection model. CHO cell viability was measured by using trypan blue staining and an MTT assay. Our results indicate that PA103Δ*exoUexoT*::Tc expresses and secretes type III effectors and that translocation into CHO cells is measurable by using either activity assays or changes in cell morphology or viability. Our analysis confirmed that ExoS, ExoSE381A, and ExoY alter CHO cell morphology but do not result in an acute cytotoxic response. ExoS, however, has been shown to mediate cytotoxic responses after longer infection (15) or transfection (16) periods. We demonstrated that delivery of ExoT also results in morphology changes and confirmed that ExoU is responsible for acute cytotoxicity. The biological effects of each of the type III-secreted effectors are summarized in Table 3.

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