***Pseudomonas aeruginosa* cystic fibrosis clinical isolates produce exotoxin A with altered ADP-ribosyltransferase activity and cytotoxicity**

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The role of *Pseudomonas aeruginosa* exotoxin A (ETA) as a virulence factor in the lung infections of cystic fibrosis (CF) patients is not well understood. Transcript-accumulation studies of bacterial populations in sputum reveal high levels of transcription of *toxA*, which encodes ETA, in some patients with CF. However, in general, tissue damage in the lungs of patients with CF does not seem to be consistent with a high level of expression of active ETA. To address this discrepancy the authors analysed the production and activity of ETA produced by a number of *P. aeruginosa* CF isolates. One CF isolate, strain 4384, transcribed *toxA* at levels similar to the hypertoxigenic strain PA103 but produced an ETA with reduced ADP-ribosyltransferase (ADPRT) activity. Complementation *in trans* of strain 4384 with the wild-type *toxA* and a mixed toxin experiment suggested the absence of inhibitory accessory factors within this strain. The *toxA* gene from strain 4384 was cloned and sequenced, revealing only three mutations in the gene, all within the enzymic domain. The first mutation changed Ser-410 to Asn. The second mutation was located within an α -helix, altering Ala-476 to Glu. The third mutation, Ser-515 to Gly, was found at the protein surface. To date, Ser-410, Ala-476 and Ser-515 have not been reported to play a role in the ADPRT activity of ETA. However, it may be the combination of these mutations that reduces the enzymic activity of ETA produced by strain 4384. Expression of 4384 *toxA* and wild-type *toxA* in an isogenic strain revealed that 4384 ETA had 10-fold less ADPRT activity than wild-type ETA. ETA purified from strain 4384 also demonstrated 10-fold less ADPRT activity as compared to wild-type ETA. Cytotoxicity assays of purified ETA from strain 4384 indicated that the cytotoxicity of 4384 ETA is not reduced; it may be slightly more toxic than wild-type ETA. Analysis of five other CF isolates revealed a similar reduction in ADPRT activity to that seen in strain 4384. Sequence analysis of the enzymic domain of *toxA* from the five CF strains identified a number of mutations that could account for the reduction in ADPRT activity. These results suggest that some CF isolates produce an ETA with reduced enzymic activity and this may partially explain the pathogenesis of chronic lung infections of CF due to *P. aeruginosa*.

Keywords: cystic fibrosis, *Pseudomonas aeruginosa*, exotoxin A, ADPRT, cytotoxicity

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Abbreviations: ADPRT, ADP-ribosyltransferase; CF, cystic fibrosis; EF-2, elongation factor 2; ETA, exotoxin A; TSBDC, trypticase soy broth dialysate chelexed. The GenBank accession numbers for the *toxA* sequences are: strain 4384, AF227419; strain 5154, AF227420; strain 5166, AF227421; strain 5552, AF227422; strain 5585, AF227423; strain 5588, AF227424.

INTRODUCTION

Pseudomonas aeruginosa is the bacterium most frequently associated with pulmonary infection in cystic fibrosis (CF) patients. Interestingly, *P. aeruginosa* infections in CF patients rarely disseminate from the lungs, in contrast to other *P. aeruginosa* infections. However, in lung infections associated with CF, *P. aeruginosa* still produces some of its toxic virulence factors involved in adherence, growth and persistence. The role of individual virulence determinants in the persistence and lack of dissemination of the bacterium in CF patients is not well understood. In particular, the involvement of exotoxin A (ETA), one of the most cytotoxic virulence factors produced by this bacterium, is not known (Iglewski & Sadoff, 1979).

ETA production in the lungs of patients with CF has been demonstrated using a variety of methods. Storey *et al.* (1992) and Raivio *et al.* (1994) demonstrated that *toxA* transcript accumulation was detectable in bacterial populations directly isolated from sputum of CF patients chronically infected with *P. aeruginosa*. Subsequently, Jaffar-Bandjee *et al.* (1995) detected the presence of ETA in sputum samples from CF patients. High levels of circulating antibodies to ETA are also found in CF patients (Jaffar-Bandjee *et al.*, 1995; Klinger *et al.*, 1978; Moss *et al.*, 1986), suggesting repeated exposure to the ETA antigen. In addition, a strong correlation exists between high serum antibody levels to ETA and increased mortality in CF patients (Moss *et al.*, 1986). Preliminary evidence also indicates that patients with more severe pulmonary complications have *P. aeruginosa* populations that transcribe higher levels of the ETA-encoding *toxA* gene (T. L. Raivio & D. G. Storey, unpublished result). These data suggest that *in vivo* production of ETA is common and may be an important virulence determinant in patients with CF.

ETA possesses an ADP-ribosyltransferase (ADPRT) activity. In eukaryotic cells, ETA catalyses the transfer of the ADP-ribose moiety from NAD⁺ to elongation factor 2 (EF-2). As a result, protein synthesis ceases and intoxicated cells die since elongation of polypeptide chains no longer occurs (Iglewski *et al.*, 1977). ETA causes cell intoxication via a three-step mechanism. The first step involves binding of ETA to a specific receptor, the α^2 -macroglobulin receptor/low-density lipoprotein (Kounnas *et al.*, 1992). The second step consists of the internalization of the toxin by target cells through endocytosis. In the third step, the toxin is cleaved by a cellular protease (Ogata *et al.*, 1992), reduced, and translocated to the cytosol, where it ADP-ribosylates EF-2. A better understanding of the mechanism of action of ETA was made possible by the characterization of its three-dimensional structure (Allured *et al.*, 1986). Several studies have been performed to characterize this protein and assign specific functions to its three domains (Allured *et al.*, 1986; Hwang *et al.*, 1987; Jinno *et al.*, 1988, 1989). In addition, the importance of each domain in the cytotoxicity of ETA has been demonstrated (Jinno

et al., 1989; Ogata *et al.*, 1992; Seetharam *et al.*, 1991; Wick & Iglewski, 1988).

Domain I, located at the N-terminal end of the protein, is required for binding of ETA to target cells. Hwang *et al.* (1987) showed that deletion of domain I abolished cell binding and decreased ETA cytotoxicity against Swiss 3T3 cells. Jinno *et al.* (1988) demonstrated the importance of Lys-57 for cell binding by constructing a mutant that had decreased cytotoxicity and failed to compete for binding with native ETA.

Domain II has been implicated in the translocation of ETA into the cytosol. Hwang *et al.* (1987) determined that deleting portions of domain II reduces the toxicity of ETA without abolishing cell binding. Furthermore, mutation of all arginine residues in domain II resulted in a decrease in cytotoxicity, presumably due to reduced translocation efficiency (Jinno *et al.*, 1989). In addition, it was recently demonstrated that a deletion of the smallest helix in domain II, helix F, increased translocation and cytotoxicity of the mutated toxin (Taupiac *et al.*, 1999).

Domain III is responsible for the ADP-ribosylating activity of the protein (Hwang *et al.*, 1987; Siegall *et al.*, 1989). This domain possesses an endoplasmic reticulum retention sequence which is critical for intracellular targeting of the toxin (Chaudhary *et al.*, 1990; Seetharam *et al.*, 1991). Using deletion analysis, Chow *et al.* (1989) and Siegall *et al.* (1989) demonstrated that amino acids 400–608 were sufficient for full enzymic activity of ETA. In addition, several studies of domain III have indicated the importance of amino acids His-426, His-440, Tyr-470, Tyr-481 and Glu-553 for the enzymic activity and cytotoxicity of ETA (Brandhuber *et al.*, 1988; Carroll & Collier, 1987; Han & Galloway, 1995; Wick & Iglewski, 1988; Wick *et al.*, 1990).

The role of ETA in chronic lung infections associated with CF has yet to be defined. In particular, it is curious that some bacterial populations in the lungs of the affected patients transcribe high levels of this toxin (Raivio *et al.*, 1994; Storey *et al.*, 1992) but the infection rarely spreads beyond the lungs. In this study, to address the role of ETA in the pathogenesis of CF lung infections we analysed the ETA produced by a number of *P. aeruginosa* CF isolates. We found that *P. aeruginosa* strains from the lungs of patients with CF produced ETA with altered ADPRT activity and cytotoxicity, and identified several mutations in the *toxA* gene that could account for the reduction in ADPRT activity.

METHODS

Bacterial strains, media and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Strains of *P. aeruginosa* containing plasmids were grown in TSBDC containing 400 µg carbenicillin ml⁻¹. *Escherichia coli* strains containing plasmids were grown in Luria–Bertani

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Genotype/phenotype	Reference
Strains		
<i>P. aeruginosa</i>		
PA103	<i>regA</i> ⁺ <i>regB</i> ⁺ hypertoxigenic laboratory strain	Liu (1966)
PA103 <i>toxA</i> :: Ω	Inactivated <i>toxA</i> by Ω insertion, produces no ETA	Hamood <i>et al.</i> (1989)
4384, 5154, 5166	Clinical isolates from the lungs of a chronically infected CF patient (FH1)	Raivio <i>et al.</i> (1994)
5552, 5585, 5588	Clinical isolates from a patient (FH2)	This study
<i>E. coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU1699</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Pharmacia
JM109	<i>endA1</i> <i>recA1</i> <i>gyrA96</i> <i>thi</i> <i>hsdR17</i> (<i>r</i> _k ⁻ <i>m</i> _k ⁺) <i>relA1</i> <i>supE44</i> Δ (<i>lac</i> - <i>proAB</i> (F' <i>traD36</i> <i>proAB</i> <i>lacI</i> ^q Δ M15)	Yanisch-Perron <i>et al.</i> (1985)
Plasmids		
pCG5	Ap ^r , pKK223-3 containing <i>EcoRI</i> - <i>PstI</i> <i>toxA</i> gene from strain 4384	This study
pCR2.1	PCR cloning vector	Invitrogen
pKK223-3	Ap ^r , expression vector	Pharmacia
pMS151-1	Ap ^r , pUC9 containing the <i>toxA</i> gene from PAK	Hamood <i>et al.</i> (1989)
pUC12	Ap ^r , general cloning vector	Yanisch-Perron <i>et al.</i> (1985)

medium supplemented with 100 μ g ampicillin ml⁻¹. For growth analysis, all *P. aeruginosa* cultures were grown overnight at 32 °C in 10 ml TSBDC (Liu, 1973). Secondary cultures were inoculated the following day to obtain an OD₅₄₀ of 0.02. Aliquots of the secondary cultures were taken at different time points and centrifuged for 10 min at 8000 g. The supernatant was removed and stored at -70 °C for future analysis.

ETA purification. Culture conditions for maximal production of ETA were as previously described (Liu, 1973). ETA from strains 4384 and PA103 was purified using methodology based on the original purification scheme (Liu, 1966, 1973). Ammonium sulfate was added slowly to 5 l culture supernatant to 60% saturation and stirred overnight at 4 °C. Purification involved four major steps: membrane ultrafiltration, hydroxylapatite chromatography, ion-exchange chromatography and gel-filtration chromatography. The presence of ETA was monitored at each purification step using ADPRT assays, SDS-PAGE analysis and Western immunoblotting.

Cloning of *toxA* from strain 4384. Chromosomal DNA from *P. aeruginosa* strain 4384 was digested with *EcoRI* and *PstI* and ligated into the corresponding sites of pKK223-3. The ligation mixture was then transformed into *Escherichia coli* strain JM109. Colony hybridization using the internal 1.53 kb *toxA* fragment from pMS151-1 was performed to identify positive clones. Southern blot analysis was carried out as recommended by Schleicher & Schuell, using the internal 1.53 kb *BamHI* *toxA* probe. The *toxA* fragment was labelled with [α -³²P]dCTP. Hybridization products were visualized by autoradiography.

Sequencing of the *toxA* gene. Various subclones were generated from pCG5 using specific restriction enzymes. All

DNA fragments were subcloned into the pUC12 vector and sequenced using universal primers via the dideoxy chain-termination method (Sanger *et al.*, 1977) by the University of Calgary Core DNA Services.

PCR amplification. Amplification of domain III of ETA was performed using Ulta polymerase (Perkin-Elmer Cetus), which possesses 3' to 5' proof-reading ability, with primers complementary to the known DNA sequence. The amplified fragment was cloned into a pCR2.1 cloning vector (Invitrogen) and transformed into *E. coli* strain JM109. Mutations identified in domain III were confirmed by sequencing the cloned chromosomal DNA (Sanger *et al.*, 1977).

ADP-ribosylation assays. ADPRT activity was assayed as described by Chung & Collier (1977). ADPRT assays were performed in triplicate on each supernatant sample and the results averaged.

SDS-PAGE and immunoblots. Proteins were separated by the method of Laemmli (1970) using a 5% (w/v) stacking gel and a 10% (w/v) resolving acrylamide gel. To estimate the *M_r* of the proteins, a prestained standard of known *M_r* was run on each gel. Immunoblots were performed according to the method of Towbin *et al.* (1979). Primary antibodies to ETA were used at a 1:2000 dilution. Specific reactions were detected by incubation with the secondary antibody, anti-rabbit IgG coupled with horseradish peroxidase. Colour was developed with a solution of Tris/saline containing 3 mg horseradish peroxidase ml⁻¹. Supernatants isolated from each CF strain were concentrated using a Microcon Centrifugal Filter Device. Briefly, the supernatant of each sample was collected at an OD₅₄₀ of 4.0, and 500 μ l of supernatant was applied to the filter possessing an *M_r* 10000 cut-off range and centrifuged at 8000 g for 30 min. The filter containing the concentrated

samples was inverted and recentrifuged for another 15 min. The retentate was then applied to an SDS-PAGE gel.

Mixed toxin experiments. Strains 4384, PA103 and PA103*toxA*:: Ω were grown in low-iron conditions to an OD₅₄₀ of 4, and 10 μ l of supernatant was then collected and mixed with 10 μ l of purified toxin from the Swiss Serum Institute (1/100 dilution). The samples were incubated for up to 6 h at 32 °C. The ADPRT activity was assayed as previously described (Chung & Collier, 1977).

Cytotoxicity assays. The cytotoxicity of ETA was measured using a colorimetric microtitre assay. Briefly, HeLa cells were grown to confluency (1×10^6 per well) and incubated overnight at 37 °C. Serial dilutions of ETA were added to the HeLa cells and incubated for 48 h at 37 °C. Twenty microlitres of tetrazolium dye (MTT; 5 mg ml⁻¹) was added to each well and incubated for 4 h at 37 °C to allow the viable cells to reduce the tetrazolium dye to purple formazan. The dye was solubilized by the addition of 100 μ l 2-propanol/HCl solution. The A₅₇₀ of each well was measured using an EIA reader.

RESULTS

ETA production and corresponding ADPRT activity of selected *P. aeruginosa* isolates from two chronically infected CF patients

We have examined the population transcript accumulation from a number of CF patients over a 3–4 year period and found that patients with more severe lung disease tend to harbour bacterial populations that transcribe higher levels of *toxA* (Storey *et al.*, 1992). We examined *P. aeruginosa* isolates from two of these patients to determine if they produced higher levels of ETA under laboratory conditions. We found that all six strains examined produced low levels of ETA activity based on ADPRT activity when compared to the hypertoxigenic strain PA103 (data not shown). Since ETA is implicated in the deterioration of the lungs of CF patients, it was of interest to further analyse the production of ADPRT-deficient ETA by these strains.

Specific activity calculated from *P. aeruginosa* hypertoxigenic strain PA103 and CF isolate strain 4384

To confirm whether the activity of ETA from our six CF isolates was significantly different from that of PA103 we decided to further characterize the production and enzymic activity of ETA by one CF isolate, strain 4384. Of the six strains, 4384 was chosen because its level of transcription of *toxA* is similar to that of the hypertoxigenic strain PA103 (Storey *et al.*, 1991). To analyse the ETA from 4384 it was necessary to concentrate the toxin using a microconcentrator filter. As a control, we concentrated ETA from strain PA103 using the same method. ADPRT assays revealed that concentrated ETA from strain 4384 still had reduced enzymic activity (approx. 800 c.p.m. per 10 μ l supernatant) compared to ETA from strain PA103 (approx. 32000 c.p.m. per 10 μ l supernatant). Subsequently, Western blot analysis using anti-ETA antibodies was performed on the concentrated supernatant from strains 4384 and PA103 grown in low-

Fig. 1. Western blot analysis of ETA from strain 4384 (lanes 1 and 2) and PA103 (lanes 3 and 4). Western blot analysis was performed on 10 μ l of supernatant from each strain using purified anti-ETA antibodies. The lane separating lanes 2 and 3 contains Benchmark prestained marker.

iron conditions. Fig. 1 shows that significantly less ETA was purified from strain 4384 (0.16 μ g per 10 μ l) as compared to strain PA103 (1.8 μ g per 10 μ l) even when the two strains were grown under comparable conditions. We used the results from these assays to calculate the specific activity of the two ETA preparations. The specific activity of ETA from PA103 was determined to be 58898 c.p.m. (μ g protein)⁻¹, whereas the specific activity of ETA from strain 4384 was only 5104 c.p.m. (μ g protein)⁻¹. Thus ETA produced by strain 4384 had approximately a 12-fold reduction in its enzymic activity when compared to ETA from strain PA103.

Absence of an accessory toxin-modifying factor in strain 4384

It is possible that the reduced ADPRT activity of ETA from strain 4384 is due to processing of the toxin by an accessory factor. To address this possibility, we complemented strains 4384 and PA103*toxA*:: Ω with plasmid pMS151-1 containing wild-type *toxA*. PA103*toxA*:: Ω was used as a negative control, because ETA cannot be expressed due to the insertion of the omega fragment in the *toxA* gene. Strain PA103*toxA*:: Ω produced approximately 70 c.p.m. per 10 μ l as compared to the complemented strain PA103*toxA*:: Ω (pMS151-1), which produced an extracellular ETA level of 1400 c.p.m. per 10 μ l. Uncomplemented strain 4384 produced similar levels of extracellular ETA to PA103*toxA*:: Ω , 110 c.p.m. per 10 μ l, and the complemented strain [4384(pMS151-1)] produced 619 c.p.m. per 10 μ l. The different levels of complementation observed between PA103*toxA*:: Ω and 4384 may be explained by a difference in the expression of *toxA* by the two strains, the presence of an accessory factor, or differences in secretion patterns. ETA produced by complemented strain 4384 was not as enzymically active as ETA produced by the complemented strain PA103*toxA*:: Ω , suggesting that an accessory factor may be influencing ETA activity in strain 4384. To investigate this possibility, supernatants

Fig. 2. ADPRT activity of ETA when mixed with the supernatant from strain 4384 or strain PA103. Supernatants from strain 4384, PA103, PA103tox $\Delta\Omega$ were mixed in equal proportions with ETA and incubated for 6 h at 32 °C. The enzymic activity of each sample was determined using the ADPRT activity assay. A 1/100 dilution of the native ETA was used as a control. The supernatant of strain 4384 alone or mixed with equal amounts of native ETA was assayed for ADPRT activity. Supernatant from hypertoxigenic strain PA103 was mixed with the native ETA or was used by itself as a positive control for this experiment. Supernatant from strain PA103tox $\Delta\Omega$ with or without wild-type ETA was also measured.

from strains 4384, PA103 and PA103tox $\Delta\Omega$ were mixed with purified ETA to look for a reduction in enzymic activity. After a 6 h incubation of the supernatant from strain 4384 with the purified ETA, the ADPRT activity of the purified toxin was retained, eliminating the possibility of a toxin-modifying accessory factor in strain 4384 (Fig. 2). These experiments suggested that the decreased ADPRT activity observed in strain 4384 may be an inherent property of the ETA produced rather than a property of the strain.

Secretion of ETA from strain 4384

A possible explanation of the results could also be that ETA is not efficiently secreted from strain 4384 and this accounts for the lower ADPRT activity from this strain. This was a possibility since the ADPRT activity of strain 4384 was calculated from the bacterial supernatant and so only accounts for secreted toxin. We therefore examined the intracellular ADPRT activity of ETA in strain 4384 and compared this to the activity of strain PA103. We found 10-fold less ETA activity in the intracellular fractions from strain 4384 (approx. 170 c.p.m. per 10 µl) as compared to the intracellular fraction of strain PA103 (approx. 1705 c.p.m. per 10 µl). These results suggested that a defect in secretion did not result in an intracellular build-up of ETA in strain 4384.

Comparison of ADPRT activity from 4384 tox Δ to that from PA103 tox Δ in strain PA103tox $\Delta\Omega$

To obtain a more direct comparison of the 4384 and the PA103 ETAs we expressed each cloned tox Δ in the ETA-deficient strain PA103tox $\Delta\Omega$. The strains were grown

Fig. 3. SDS-PAGE of purified ETA from *P. aeruginosa* strains 4384 and PA103 for comparison of ETA production. The strains were grown in two different batches of low-iron TSBDC. All samples were taken at an OD₅₄₀ of 4. Each sample was run on a 10% (w/v) polyacrylamide gel, blotted to nitrocellulose and stained using anti-ETA antibody. Lanes 1 and 2, 10 µl purified ETA from strain 4384; lanes 3 and 4, 10 µl purified ETA from strain PA103. ETA, 1 µl purified ETA from the Swiss Serum Institute. The ETA band is marked with an arrow.

to an OD₅₄₀ of 4, in iron-depleted TSBDC. Culture supernatants from strain PA103tox $\Delta\Omega$ (pCG5), containing the 4384 tox Δ , had levels of activity (approx. 130 c.p.m. per 10 µl) that were only marginally higher than the negative control strain PA103tox $\Delta\Omega$ (approx. 68 c.p.m. per 10 µl) and strain 4384 itself (approx. 98 c.p.m. per 10 µl). In contrast, culture supernatants from PA103tox $\Delta\Omega$ (pMS151-1), containing a wild-type tox Δ , had ADPRT activity (approx. 890 c.p.m. per 10 µl) similar to that of the culture supernatants from the hypertoxigenic strain PA103 (approx. 700 c.p.m. per 10 µl). These results again suggest that 4384 ETA has a lower ADPRT activity than wild-type ETA even when produced in an isogenic strain.

Comparison of ETA production in strains 4384 and PA103

To compare the enzymic activity of ETA produced by each strain, ETA was purified from both 4384 and PA103. Western blot analysis, using anti-ETA antibodies, showed that ETA purified from strains 4384 and PA103 is a protein of M_r 66000 that co-migrates with purified ETA (Swiss Serum Institute) (Fig. 3). Fig. 3 also indicates that strain 4384 produces significantly less ETA than strain PA103. This may be due to the presence of high levels of proteases in strain 4384 (T. L. Raivio, D. E. Woods & D. G. Storey, unpublished result) which are absent in the protease-deficient strain PA103 (Liu, 1966). The ADPRT activity of the two purified ETA preparations from strain 4384 (approx. 100 and 50 c.p.m. per 10 µl respectively) was lower than that of the two preparations from strain PA103 (approx. 1800 and 2500 c.p.m. per 10 µl). The highest level of ADPRT activity was obtained from the commercially available purified Swiss Serum ETA (approx. 2800 c.p.m. per 10 µl).

Fig. 4. Specific activity of purified ETA from strain 4384. The concentration of purified ETA from this strain was determined by running 5 and 10 µl of ETA purified from strain 4384 and comparing the level to known concentrations of the purified Swiss Serum toxin. Lanes 1 and 2 contain 5 and 10 µl, respectively, of purified ETA from strain 4384. The next lane contains the BenchMark prestained protein marker. Lanes 3–9 represent various concentrations of the Swiss Serum ETA: 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.01563 and 0.0078 µg, respectively.

Fig. 5. Cytotoxic effect on HeLa cells of ETA isolated from strain 4384 and PA103. Increasing concentrations of ETA from strain 4384 (▲) and Swiss Serum ETA (■) were used to intoxicate HeLa cells.

Cytotoxicity of ETA from strain 4384 and PA103

We calculated the specific activity of the purified ETA from strain 4384 using known concentrations of the purified Swiss Serum ETA (Fig. 4). The specific activity of the strain 4384 ETA was 11000 c.p.m. µg⁻¹ whereas that for the strain PA103 ETA was 110000 c.p.m. µg⁻¹. Thus, we again observed a 10-fold difference in activity between the ETA from the two strains. To compare the cytotoxicity of native ETA with that of the purified ETA from strain 4384, we performed a colorimetric microtitre assay. The ability of both ETA preparations to intoxicate HeLa cells is illustrated in Fig. 5. At low concentrations, both ETA from strain 4384 and Swiss Serum ETA killed equivalent numbers of HeLa cells. However, at higher toxin concentrations the percentage of HeLa cell viability was less in the presence of ETA from 4384 than it was in the presence of the Swiss Serum ETA. These results suggested that ETA from strain 4384 was slightly more cytotoxic than Swiss Serum ETA.

tox A	389	LLERNYPTGA	EFLDGGDVSF	STRGTQNW	TVERLLQ	AHRQ	428
4384	389	LLERNYPTGA	EFLDGGDVSF	STRGTQNW	TVERLLQ	AHRQ	428
5166	389	LLERNYPTGA	EFLDGGDVSF	STRGTQNW	TVERLLQ	AHRQ	428
5552	389	LLERNYPTGA	EFLDGGDVSF	STRGTQNW	TVERLLQ	AHRQ	428
5585	389	LLERNYPTGA	EFLDGGDVSF	STRGTQNW	TVERLLQ	AHRQ	428
5588	389	LLERNYPTGA	EFLDGGDVSF	STRGTQNW	TVERLLQ	AHRQ	428
5154	389	LLERNYPTGA	EFLDGGDVSF	STRGTQNW	TVERLLQ	AHRQ	428
tox A	429	LEERGVFV	GYHGTFL	EAAQSI	VFGGVR	ARSQDL	DAIWRG
4384	429	LEERGVFV	GYHGTFL	EAAQSI	VFGGVR	ARSQDL	DAIWRG
5166	429	LEERGVFV	GYHGTFL	EAAQSI	VFGGVR	ARSQDL	DAIWRG
5552	429	LEERGVFV	GYHGTFL	EAAQSI	VFGGVR	ARSQDL	DAIWRG
5585	429	LEERGVFV	GYHGTFL	EAAQSI	VFGGVR	ARSQDL	DAIWRG
5588	429	LEERGVFV	GYHGTFL	EAAQSI	VFGGVR	ARSQDL	DAIWRG
5154	429	LEERGVFV	GYHGTFL	EAAQSI	VFGGVR	ARSQDL	DAIWRG
tox A	469	FYIAGDP	ALAYGYA	QDQEPD	ARGIRN	GALLRV	YVPRSS
4384	469	FYIAGDP	ALAYGYA	QDQEPD	ARGIRN	GALLRV	YVPRSS
5166	469	FYIAGDP	ALAYGYA	QDQEPD	ARGIRN	GALLRV	YVPRSS
5552	469	FYIAGDP	ALAYGYA	QDQEPD	ARGIRN	GALLRV	YVPRSS
5585	469	FYIAGDP	ALAYGYA	QDQEPD	ARGIRN	GALLRV	YVPRSS
5588	469	FYIAGDP	ALAYGYA	QDQEPD	ARGIRN	GALLRV	YVPRSS
5154	469	FYIAGDP	ALAYGYA	QDQEPD	ARGIRN	GALLRV	YVPRSS
tox A	509	PGFYRT	GLTLAA	PEAA	GEVERL	IGHPLP	LRLDAITGPEEE
4384	509	PGFYRT	GLTLAA	PEAA	GEVERL	IGHPLP	LRLDAITGPEEE
5166	509	PGFYRT	GLTLAA	PEAA	GEVERL	IGHPLP	LRLDAITGPEEE
5552	509	PGFYRT	GLTLAA	PEAA	GEVERL	IGHPLP	LRLDAITGPEEE
5585	509	PGFYRT	GLTLAA	PEAA	GEVERL	IGHPLP	LRLDAITGPEEE
5588	509	PGFYRT	GLTLAA	PEAA	GEVERL	IGHPLP	LRLDAITGPEEE
tox A	549	GGRLETIL	GWPLA	ERTV	VPISAI	PTDPRN	VGGDLDPSSIP
4384	549	GGRLETIL	GWPLA	ERTV	VPISAI	PTDPRN	VGGDLDPSSIP
5166	549	GGRLETIL	GWPLA	ERTV	VPISAI	PTDPRN	VGGDLDPSSIP
5552	549	GGRLETIL	GWPLA	ERTV	VPISAI	PTDPRN	VGGDLDPSSIP
5585	549	GGRLETIL	GWPLA	ERTV	VPISAI	PTDPRN	VGGDLDPSSIP
5588	549	GGRLETIL	GWPLA	ERTV	VPISAI	PTDPRN	VGGDLDPSSIP
tox A	589	DKEQA	ISALPD	YASQ	PGKPP	REDLK	613
4384	589	DKEQA	ISALPD	YASQ	PGKPP	REDLK	613
5166	589	DKEQA	ISALPD	YASQ	PGKPP	REDLK	613
5552	589	DKEQA	ISALPD	YASQ	PGKPP	REDLK	613
5585	589	DKEQA	ISALPD	YASQ	PGKPP	REDLK	613
5588	589	DKEQA	ISALPD	YASQ	PGKPP	REDLK	613

Fig. 6. Schematic representation of mutations found in the enzymic domain of *tox A* from the six clinical isolates. The enzymic moieties of *tox A* from strain 4384 and the remaining five CF strains were amplified using two different sets of primers. The amplified fragments were then cloned into a T-tailed vector (pCR2.1) (Invitrogen) and sequenced by The University of Calgary Core DNA services. Changes in the amino acid composition of the CF isolates are boxed.

Cloning and sequencing of *tox A* from strain 4384

It was previously demonstrated that several amino acids in the enzymic domain of ETA are essential for ADPRT activity. We amplified and sequenced the DNA encoding the entire *tox A* of strain 4384 in order to investigate the possibility of mutations in this gene. Sequence analysis revealed three mutations in domain III of 4384 ETA. The first mutation alters Ser-410 to Asn, the second mutation replaces Ala-476 with Glu and the third mutation is a change of Ser-515 to Gly (Fig. 6). No other mutations were found in the 4384 *tox A* gene, suggesting that any or all of the mutations found in domain III may account for the observed reduction in ADPRT activity.

Western blot analysis of the 66 kDa ETA protein from five other CF strains

To confirm that ETA was being produced by other CF strains with low ADPRT activity, Western blot analysis using anti-ETA antibodies was performed on the supernatant of five CF strains grown in low-iron conditions (data not shown). We were able to identify an immunoreactive band of *M_r* 66000 in the supernatants of CF strains 5166, 5552, 5585 and 5588. This indicates that the

ETA protein is expressed and secreted in all four strains. No detectable bands were observed in the supernatant of strain 5154.

Sequencing of the DNA encoding the enzymic moiety of *toxA* from five *P. aeruginosa* CF isolates

We cloned and sequenced the DNA encoding the enzymic moiety of *toxA* from the remaining CF strains in order to investigate the possibility of conserved mutations which could account for the reduced ADPRT activity (Fig. 6). All the CF strains tested had the same alteration of Ser-515 to Gly. The two other mutations identified in 4384, Ser-410 to Asn and Ala-476 to Glu, were also present in strain 5166. Strains 5552 and 5154 both had the mutation of Ala-476 to Glu. Two mutations in domain III of *toxA* from strain 4384 were absent from strains 5585 and 5588. In addition, strain 5154 contained a stop codon that would prematurely truncate the ETA protein in this strain, explaining the absence of an ETA band on the Western blots of strain 5154. Other point mutations were observed throughout the coding region for the enzymic moiety of *toxA*; however, these were unique to each strain and were not conserved among the six CF isolates examined.

DISCUSSION

Previous studies have suggested that the majority of *P. aeruginosa* strains isolated from the lungs of CF patients produce low levels of virulence determinants other than alginate (Woods *et al.*, 1986; Burke *et al.*, 1991; Jaffar-Bandjee *et al.*, 1995). We were particularly interested in the levels of ETA produced by CF isolates because we have observed high levels of transcription of *toxA* in the lungs of some patients with CF. In the current study, we have examined the production and function of ETA from six CF isolates. We were able to detect ETA in the supernatants from five of the six isolates, using anti-ETA antibodies and Western blotting. This suggested that at least some CF strains were able to produce ETA. However, when these isolates were grown in optimal conditions for ETA production, very little ADPRT activity was detected in the culture supernatants. The apparent lack of ADPRT activity of ETA in supernatants from CF strains that appear to express *toxA* might explain why others have reported low levels of ETA from CF isolates.

We selected one strain, 4384, to further characterize the ETA produced. Previously, CF isolate 4384 has been shown to transcribe *toxA* at a level similar to the hypervirulent strain PA103 (Raivio *et al.*, 1994). The low ADPRT activity from this isolate could be due to a high level of protease production, a defect in secretion by strain 4384 or an inhibitory accessory factor, or it may be an inherent property of the toxin produced. Complementation studies, intracellular ADPRT activity and mixed toxin studies (Fig. 2) showed that neither proteases, inhibitory accessory factors nor a secretory defect are likely to be responsible for the reduced toxin

enzymic activity. An analysis of the specific activity of strain 4384 suggested that the reduced ADPRT activity of 4384 ETA may be due to an altered amino acid sequence rendering a functionally deficient protein.

It has previously been shown that amino acids 400–608 in domain III were sufficient for full enzymic activity of ETA (Chow *et al.*, 1989; Siegall *et al.*, 1989). In addition, several residues in domain III were shown to be critical for enzymic activity (Brandhuber *et al.*, 1988; Carroll & Collier, 1987; Han & Galloway, 1995; Wick & Iglewski, 1988; Wick *et al.*, 1990). The observed reduction in the specific activity of strain 4384 ETA may be due to the presence of mutations within the domain III sequence which would render the toxin partially inactive. Sequence analysis revealed three mutations in domain III of *toxA* from strain 4384 (Fig. 6). The position of mutation Ala-476 to Glu is within an α -helix which is believed to be an important part of the catalytic site (Domenighini *et al.*, 1994). The presence of a mutation within the helix may modify the dimensions of the active site, possibly interfering with substrate binding or transfer of the NAD⁺ moiety. A second mutation, which is at the protein surface, is a substitution of a Gly for Ser-515. This amino acid may be essential for the protein integrity or as a protein modification site. The third mutation, of Ser-410 to Asn, was outside the region normally considered to be involved in either substrate binding or the catalytic site. None of these mutations (positions 410, 476, 515) had previously been reported to be involved in ADPRT activity. These mutations alone may have an effect on the activity of the protein, or the mutations could act in concert to reduce the ADPRT activity of the ETA. The presence of these mutations may affect proper folding of the protein and render the catalytic site inaccessible to the substrate.

The ADPRT activity of ETA is directly responsible for the inhibition of protein synthesis in eukaryotic cells (Iglewski & Kabat, 1975; Iglewski *et al.*, 1977; Iglewski & Sadoff, 1979). Our findings suggested that the specific activity of purified ETA from strain 4384 (approx. 11000 c.p.m. μg^{-1}) was about 10-fold lower than that of the toxin from strain PA103 (approx. 110000 c.p.m. μg^{-1}). As such, we expected that strain 4384 ETA would have a reduced cytotoxicity to coincide with its reduced ADPRT activity. However, Fig. 5 shows that despite the low ADPRT activity of this ETA, the cytotoxic activity of the toxin from strain 4384 was slightly higher than the toxin from strain PA103. An increased cytotoxic activity could be explained in a number of ways. Mutations in domains I and II have previously been shown to alter the cytotoxicity of ETA (Siegall *et al.*, 1989; Taupiac *et al.*, 1999). However, sequence analysis of the *toxA* from strain 4384 showed no mutations in either domains I or II. Alterations in the C-terminal end of the protein also seem to affect the cytotoxicity of the protein. Once again, sequence analysis of *toxA* from strain 4384 suggested no alterations in this portion of the gene. This leads us to hypothesize either that ETA may have a second cytotoxic activity in domain III or that the cytotoxicity of the

toxin is not tightly linked to the ADPRT activity. The nature of the increased cytotoxicity remains to be elucidated.

A similar pattern of reduced ADPRT activity was also observed among five other CF isolates. To investigate this deficiency, we amplified and sequenced the ETA enzymic moiety of these five strains to see if the amino acid sequences differed from that of the wild-type. Surprisingly, we found a number of mutations which could potentially be responsible for the reduced ADPRT activity in the CF strains. A stop codon was identified in strain 5154, resulting in the production of a truncated protein. Thus, the lack of ADPRT activity from this strain results from lack of production of ETA. Interestingly, all five of the other strains had the conversion of Ser-515 to Gly. However, this mutation is predicted to be on the outside surface of the ETA molecule and therefore not directly involved in either substrate binding or catalysis. Four strains, including 4384, had the alteration of Ala-476 to Glu in a position predicted to be located in the active site of the enzyme. This mutation alone could potentially account for the alteration in ADPRT activity of the ETA in these strains. Finally, two strains (5166 and 5154) had the third mutation also present in strain 4384. This mutation, Ser-410 to Asn, is also outside the region believed to be directly involved in NAD⁺ binding and catalysis. Other point mutations were found in the five additional strains but none of those appear to be consistent among the strains. Except for the mutation of Ser-515, no clear pattern has emerged that could account for the reduction of ADPRT activity in all the strains. Further research will be necessary to clarify the role of these mutations.

Previous research has revealed that in strain PAO1, an insertion sequence in the region upstream of *tox*A altered the phenotype of the strain (Woods *et al.*, 1991; Sokol *et al.*, 1994). In particular, when the insertion was present the strain became mucoid and the levels of ADPRT activity in the supernatant of the culture dropped by 20%. A possible explanation of our findings is that some or all of our CF isolates may have an insertion in the region upstream of *tox*A. However, both the purified 4384 ETA and the 4384 ETA produced in the transformed strain PA103*tox*A::Ω had reduced ADPRT activity. These results suggested that the ETA itself was modified. Additionally, we have shown that an inhibitory accessory factor is probably not involved in the lower ADPRT activity of ETA from strain 4384. While an insertion sequence may not play a role in the reduced ADPRT activity of strain 4384, it still could account for the reduced activity of the other strains used in this study.

Even though we have examined only a small number of isolates, our results suggest that mutations in *tox*A may be common among CF isolates. Our results also suggest that the proposed role of ETA in the pathogenesis of CF lung infection may have to be altered, especially during the chronic phase of the infection. It is likely that during the initial infections with *P. aeruginosa*, ETA plays a role in establishment and persistence of the infection.

Once the infection becomes chronic, mutations may arise in the colonizing strains. Our research has indicated that these mutations occur in *tox*A, but they may arise in other genes as well (Taylor *et al.*, 1992). As a result of these mutations, at least some of the bacterial population in the lungs may lose or have reduced ADPRT activity. Our results may explain why other researchers have found that CF isolates produce lower levels of ETA. The correlation between ETA production, the immune response to ETA and morbidity and mortality in CF suggests a role for ETA in the chronic phase of the disease (Moss *et al.*, 1986). Our data also suggest that the cytotoxicity of the toxin, rather than the ADPRT activity, may be the more important feature of the toxin in the chronic phase of CF lung disease. On the other hand, we cannot rule out the possibility that the mutations have left the organism less pathogenic, thereby allowing the host, and thus the bacteria, to survive for a longer period of time. The chronic production of an immunogenic protein such as ETA in this situation could lead to immunopathological effects in the lungs of patients with CF.

In summary, this work has shown that strains producing ETA with reduced ADPRT activity commonly occur among *P. aeruginosa* CF isolates. In at least one strain, the ETA produced had a slightly higher level of cytotoxicity despite a 10-fold reduction in ADPRT activity. Other clinical isolates are currently under investigation to determine the prevalence of these mutations and perhaps lead to a better understanding of the factors contributing to mutations among CF isolates.

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