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Pseudomonas aeruginosa Cystic Fibrosis Clinical Isolates produce Exotoxin A with altered ADP-Ribosyl Transferase Activity and Cytotoxicity

by

Claude Marie Violaine Audette Gallant

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Abstract

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 *toxA* transcription in some patients with CF. 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 exotoxin A. To address this discrepancy we have analyzed the production and activity of exotoxin A produced by a number of CF isolates. One CF isolate, strain 4384, transcribed *toxA* at levels similar to the hypertoxigenic strain PA103 but produced an exotoxin A with reduced ADP-ribosyl transferase (ADPRT) activity. Complementation *in trans* of this CF isolate with the wild type *toxA* restored the ADPRT activity which suggested the absence of inhibitory accessory factors within strain 4384. The *toxA* gene from strain 4384 was sequenced, and three mutations were found in domain III. One mutation altering Ser-410 to Asn was found outside of the active site of ETA. The two remaining mutations were isolated in the active site of ETA. The first mutation is located within an α-helix altering amino acid Ala-476 to Glu. The second mutation is found at the protein surface and is a replacement of Ser-515 with Gly. To date, 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 both mutations that reduces the enzymatic activity of ETA produced by strain 4384. Cytotoxicity assays of ETA from strain 4384 have shown that the cytotoxicity is not reduced, and it may be slightly more toxic than wild type ETA. Analysis of five other CF isolates revealed the same reduction in ADPRT activity. Sequence analysis of the enzymatic domain of *toxA* from the five CF strains identified a number of mutations which

could account for the reduction of ADP-ribosyl transferase (ADPRT) activity. These results suggest that some CF isolates produce an exotoxin A with reduced enzymatic activity and this may partially explain the pathogenesis of chronic lung infections due to *P. aeruginosa*.

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Dedication

For my family; my wonderful parents, Hugues and Nicole and my two amazing sisters Nathalie and Marie-Josée. Thank you for your love and for always believing in me when I needed it the most.

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List of Abbreviations

Å	angstrom
Ala	alanine
aa	amino acid
ADP	adenosine diphosphate
ADPRT	ADP-ribosyl transferase
Amp	ampicillin
Ap^r	ampicillin resistance cassette
Arg	arginine
bp	base pairs
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
CPM	counts per minute
C-terminal	carboxy terminal
Cys	cysteine
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
dGTP	deoxyguanosine triphosphate
dTTP	deoxythymidine triphosphate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EF-2	elongation factor 2
ER	endoplasmic reticulum
ETA	exotoxin A
ExoS	exoenzyme S
g	grams
Glu	glutamic acid
Gly	glycine
His	histidine
IgA	immunoglobulin A
IgG	immunoglobulin G
IL-1	interleukine 1
IPTG	isopropyl-β-D-galactopyranoside
kb	kilobase pairs
kDa	kilodalton
Km	kanamycin
L	liter
LB	Luria Bertani Broth
LD_{50}	lethal dose to kill 50%
Leu	leucine
LPS	lipopolysaccharide
LRP	Low Density Lipoprotein Receptor Protein

Lys	lysine
M	molar
mg	milligram
ml	millilitre
mM	millimolar
mRNA	messenger ribonucleic acid
MW	molecular weight
NAD	nicotinamide adenine dinucleotide
ng	nanogram
N-terminal	amino terminal
O.D	optical density
ORF	open reading frame
P1	promoter 1 of *regAB* operon
P2	promoter 2 of *regAB* operon
PAGE	polyacrylamide gel electrophoresis
PAI	*Pseudomonas* autoinducer
PEG	polyethylene glycol
PCR	polymerase chain reaction
Phe	phenylalanine
PLC	phospholipase C
PLC-H	hemolytic phospholipase C
PLC-N	non-hemolytic phospholipase C
RBS	ribosome binding site
RNA	ribonucleic acid
RNAP	RNA polymerase
rpm	rotations per minutes
SDS	sodium dodecyl sulphate
sec	seconds
Ser	serine
SSC	standard sodium citrate
SSPE	standard sodium phosphate EDTA
SF	Pseudomonas stabilizing fragment
t	time
T1	transcript 1 encoding the *regAB* operon
T2	transcript 2 encoding regA
TAE	Tris-acetate-EDTA
TBS	Tris buffered saline
TCA	trichloroacetic acid
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethyl-1,2-diaminoethane
TES	Tris-EDTA-sodium chloride
TFB	transformation buffer
Thr	threonine
TNF	tumor necrosis factor
Tris	(hydroxymethyl) aminomethane
Trp	tryptophan

TSBDC	trypticase soy broth dialysate, chelated
Tyr	tyrosine
UV	ultra-violet
X-gal	5-choloro-4-bromo-3-β-galactoside
°C	degrees Celsius
α	alpha
β	beta
Ω	omega fragment
Δ	deletion
μg	micrograms
μl	microliter
μM	micromolar

Chapter 1

Introduction

1.1 Cystic Fibrosis

1.1.1 Genetic Defect

Cystic Fibrosis (CF) is a lethal genetic recessive disease with an estimated frequency of 1 in 2500 people of European descent (Welsh and Smith, 1995). Patients with CF have mutations on a gene located on the long arm of chromosome 7, which encodes a cAMP-regulated chloride ion channel termed the cystic fibrosis transmembrane conductance regulator (CFTR) (Rommens *et al.*, 1989).

To date, more than 700 mutations have been identified in the CFTR gene, which give rise to varying degrees of the CF disease phenotype (Pilewski and Frizzell, 1999). These mutations include missense mutations, small insertions and deletions causing frameshifts, in-frame deletions, mutations affecting splicing, and non-sense mutations (Davis *et al.*, 1996). The severity of this inherited disease varies, and it affects organs such as the pancreas, liver, intestines and lung. These abnormalities are caused by defective water and electrolyte transport across the apical membrane of epithelial cells.

Morbidity and mortality of CF patients is due to the progressive deterioration of pulmonary function (Hoiby, 1977; Pilewski and Frizzell, 1999). In the airways of CF patients, abnormalities in the CFTR protein give rise to hyperabsorption of sodium chloride which causes a reduction in water and salt

content in the periciliary environment. This results in the hyperviscous mucous secretions, which compromises the mucociliary clearance (Pilewski and Frizzell, 1999). In addition, abnormal CFTR protein also affects the clearance by submucosal gland of mucins and various defense substances onto the airway surface (Hoiby, 1977; Pilewski and Frizzell, 1999). The impairment of the mucociliary clearance and the unusually thick mucus in the airway of CF patients causes a susceptibility to infection by opportunistic pathogens (Baltimore *et al.*, 1989; Gilligan, 1991).

1.1.2 Airway infections

Even though there is variation in the severity of disease among CF patients, deterioration of the lung function is one of the most serious sequelae of the disease. Recurrent and chronic pulmonary infections due to bacterial colonization are the leading cause of fatality in CF patients. Even though considerable progress has been made in the CF field, the link between the genetic disease and the associated lung infection that ultimately leads to chronic lung infection and respiratory failure still remains to be elucidated (Bals *et al.*, 1999).

Several factors play a critical role in the development of lung infections in CF patients. First, the defective CFTR protein causes hyperabsorption of salt by the epithelium due to enhanced Na^+ conductance, and electrolyte secretion is principally inhibited due to the lack of CFTR Cl^- conductance (Kunzelmann and Schreiber, 1999). This eventually leads to hyperviscous mucus, which impairs

the mucocilliary clearance mechanism therefore predisposing CF patients to lung infection and colonization. As well, Imundo *et al.* (1995) suggest that in CF patients there is an abnormality in the respiratory epithelium causing enhanced levels of tetrasaccharide and asiloganglioside-1 receptors that would ultimately increase binding of opportunistic pathogens to the airway epithelial cells. In addition, Pier *et al.* (1996) propose that the CFTR protein is a binding receptor for *P. aeruginosa,* and that in its absence, the pathogen can not be internalized and cleared by the epithelial cells. Inflammation associated with a host immune response to clear the bacteria is detrimental to the host since it releases proteases, oxidants, defensins and DNA from lysed neutrophils, epithelial cells and bacteria in the airway, further contributing to tissue damage (Bals *et al.*, 1999).

Many microorganisms are capable of infecting the lungs of CF patients. *Staphylococcus aureus*, *Haemophilus influenzae*, *Klebsiella pneumoniae* and *Escherichia coli* are mostly isolated in young children and can easily be treated with antimicrobial therapies (Hoiby, 1977; Bals *et al.*, 1999). However, almost all CF patients eventually become infected with *Pseudomonas aeruginosa*. Infection with *P. aeruginosa* is chronic due to the inability to clear the pathogen even with aggressive antimicrobial chemotherapies. In addition, *Burkholderia cepacia* is an emerging pathogen which correlates with rapid clinical deterioration of some CF patients (Bals *et al.*, 1999).

To date, most treatment protocols have concentrated on controlling lung infection and the associated airway inflammation.

1.1.3 Treatment for Cystic Fibrosis Lung Disease

To date, the development of new treatments against the lung infections associated with this disease have improved the life of CF patients. Aggressive antibiotic chemotherapies are commonly used in order to reduce the bacterial load in the airway. Aerosolized antibiotics are widely used since they are delivered in high doses directly into the airway, thereby, decreasing systemic toxicity (Rubin, 1999). However, the problem with antibiotic treatment is the ease with which the bacteria can mutate and develop resistance (Steen, 1997).

The majority of the tissue damage observed in CF patients may be due to host inflammatory response and not the offending pathogen. One way to circumvent this detrimental effect and control the inflammation is to administer anti-inflammatory medication (Rubin, 1999). Another treatment frequently employed is the use of inhaled DNAse which helps to clear the airway secretions which are viscous as a result of cellular and bacterial debris (Rubin, 1999). Recently, attempts have been made to restore the CF phenotype using gene therapy delivering vectors to transfer normal CFTR into the airway epithelia. However, targeting the appropriate cells, evading an inflammatory response and obtaining a stable transfer still remains to be perfected before gene therapy can be safely and widely implemented.

The most common mutation found in caucasians, ΔF508, is a three base pair deletion of a phenylalanine codon at position 508 in the CFTR protein. The CFTR protein accumulates in the cytosol of epithelial cells carrying this mutation.

This suggests an intracellular trafficking defect in CF patient carrying the ΔF508 mutation. At this point, increasing progress has been made in finding ways to glycosylate the ΔF508 protein and transport it into the outer membrane of cells (Rubin, 1999).

Although many microorganisms are capable of colonizing the lungs of CF patients, *P. aeruginosa* has clearly become the predominant pathogen.

1.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a gram-negative rod, which is ubiquitous in nature but has a preference for moist environments with an optimal temperature range between 37°C and 42°C. This bacterium is a facultative aerobe, except if certain substrates such as nitrates or arginine are made available in the media (Vasil, 1986).

P. aeruginosa is a prevalent opportunistic human pathogen among immunocompromised patients (Van Delden and Iglewski, 1998). Approximately 90% of CF patients become infected with *P. aeruginosa,* however, loss of pulmonary function and the development of chronic infection is inconsistent between patients (Zar *et al.,* 1995). 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. A large number of cell-associated factors and exoproducts have been

demonstrated to be involved in the pathogenesis of *P. aeruginosa* and will be further discussed.

1.3 Cell-Associated Virulence Factors

P. aeruginosa possess a number of cell-associated virulence factors such as pili, non-pilus adhesins, a polar flagellum, lipopolysaccharide, and alginate which promote the initial colonization by the bacterium at the site of infection.

1.3.1 Pili and non-pilus adhesins

Attachment of bacteria to host cells is a crucial step in the initial colonization of the respiratory airway of CF patients. *P. aeruginosa* adherence has been found to be associated with the production of two adhesins, pili and non-pilus adhesins. It has been proposed that type IV pili of *P. aeruginosa* play an important role in pathogenesis by promoting adherence to human cells since alteration of the pilin subunit decreases adherence of the bacteria to human pneumocyte cells and results in a drastic decline in virulence in the mouse infection model (Farinha *et al.*, 1997). In addition, type IV pili are required by the bacteria to form a surface-associated movement called twitching motility. This movement is believed to be due to the extension and retraction of type IV pili, which bring the bacterium in close proximity with the epithelial cells (Bradley, 1980; O'Toole and Kolter, 1998).

RpoN is an alternative sigma subunit of RNA polymerase, which is essential for pilin expression and is required for the expression of non-pilus

adhesins of *P. aeruginosa* (Simpson *et al.*, 1992). This was demonstrated by transposons mutagenesis, which allowed the isolation of two distinct classes of adhesins, the first class can bind to epithelial cells and mucins, whereas the second class can only recognized receptors on mucins (Simpson *et al.*, 1992).

1.3. 2 Flagellum

P. aeruginosa are characterized by the presence of a single polar flagellum. The role of flagellar motility as a potential virulence determinant was demonstrated by several studies. A ten-fold decrease in virulence was observed with a Fla$^-$ mutant in a mouse thermal-injury model as compared to the wild type strain (Drake and Montie, 1988). As well, active immunization with flagellar antigen protects vaccinated burned mice (Montie *et al.*, 1982). Finally, absence of the flagellum in an isogenic strain is linked to loss of virulence (Montie *et al.*, 1982)

1.3.3 Lipopolysaccharides

P. aeruginosa, like other gram negative bacteria, produce LPS which is composed of a lipid A, a polysaccharide core and a variable repeating O-antigen unit. Lipid A of *P. aeruginosa* comprises the endotoxin portion of LPS, which is less inflammatory than that of other gram negative bacteria (Goldberg and Pier, 1996). In addition, Goldberg *et al.* (1995) demonstrated that by mutating one of the LPS synthesis genes, the resulting LPS$^-$ mutant strain was less virulent than the wild type. Expression of LPS by *P. aeruginosa* promotes entry of bacteria

into eukaryotic cells and protects the organism from host defences (Goldberg and Pier, 1996). It was also observed that during the chronic phase of the lung infection there is a loss of sugar units in the O-side chain (rough LPS) which helps *P. aeruginosa* to evade host defense mechanisms (Goldberg and Pier, 1996).

1.3. 4 Alginate

Alginate is a polymer made of the β1-4 linked uronic acid β-D mannuronate and its C-5 epimer α–L guluronate (Gacesa, 1998).

To date, at least 24 genes have been implicated in the biosynthesis of alginate and are located at various loci on the chromosome of *P. aeruginosa* (Gacesa, 1998). One critical gene in the biosynthesis pathway, *algD*, encodes a GDP-mannose dehydrogenase which catalyses the conversion of GDP-mannose to a precursor of alginate the GDP-mannuronic acid (Attree *et al.,* 1997). It was demonstrated that *algD* could be activated by several environment signals such as high osmolarity, limiting levels of nitrogen and phosphate, and ethanol-induced perturbation (Berry *et al.*, 1989; Devault *et al.*, 1990; Delic-Attree *et al.,* 1997). Each of these stress factors could mimic conditions observed in the lungs of CF patients.

The presence of *P. aeruginosa* non-mucoid strains in the lungs of CF patients characterizes the initial colonization step of the disease. However, as the disease progresses to a more chronic infection the cells turn rapidly to a mucoid phenotype. This rapid conversion is due to the expression of alginate.

Production of alginate by *P. aeruginosa* during chronic lung infection contributes to bacterial resistance to phagocytosis, the action of neutrophils and opsonization (Kim *et al.*, 1998)

1.4 Exoproducts

After initial colonization by *P. aeruginosa,* large arrays of extracellular virulence factors are expressed by the bacterium at low levels during a chronic infection and at increasing levels during an acute exacerbation (Van Delden and Iglewski, 1998). Extracellular virulence factors include proteases such as alkaline protease, LasA and LasB elastase, hemolysins, two siderophores; pyochelin and pyoverdin, and two ADP-ribosyl transferases, exoenzyme S and exotoxin A.

1.4.1 Proteases

Proteases such as alkaline protease, LasB elastase and LasA are important in P. *aeruginosa* virulence. The exact role of alkaline protease during acute and chronic infections is not fully understood, though its expression is critical during corneal infections.

LasB elastase is a zinc metalloprotease that affects the host immune response by degrading a variety of compounds such as IgA and IgG immunoglobins (Doring *et al.*, 1981), complement components (Schultz and Miller, 1974) and human plasma α-proteinase inhibitor (Morihara *et al.*, 1965).

LasB is also associated with tissue destruction since it can degrade elastin and collagen (Morihara and Tsuzuki, 1975).

A 22-kilodalton protein, LasA, was shown to increase the elastolytic activity of LasB. LasA is a serine protease that is able to nick the elastin structure rendering it more susceptible to the proteolytic activity of LasB (Peters and Galloway, 1990; Galloway, 1991). It was established that both lasA and lasB are transcribed in the lungs of CF patients (Storey *et al.*, 1992; Storey *et al.*, 1998). Both lasA and lasB are regulated at the transcriptional level by LasR, which is a global transcriptional activator that induces specific genes in a cell-density-dependent manner termed quorum sensing.

1.4.2 Phospholipase C

Maximal expression of phospholipase C (PLC) is achieved during late log and early stationary phase of growth in low phosphate conditions (Ostroff and Vasil, 1987). Two distinct PLC's; hemolytic (PLC-H) and non-hemolytic (PLC-N) are produced by *P. aeruginosa* (Ostroff and Vasil, 1987). Phosphatidylcholine is one of the major constituents of lung surfactant and is the preferred substrate for both PLC-H and PLC-N (Berka and Vasil, 1982). Degradation of lung surfactant would provide essential nutrients to allow colonization of *P. aeruginosa* in the lung tissue (Ostroff and Vasil, 1987). PLC is an important virulence determinant since mutations in PLC-H result in a tremendous increase in the LD_{50} as compared to the parent strain PA01 (Ostroff *et al.*, 1989). The role of PLC is multifactorial, causing tissue damage and necrosis, and can also cause

aggregation of blood platelets (Sage *et al.*, 1997). However, the impact of phospholipase C in disease severity and pathogenesis still remains to be elucidated.

1.4. 3 Siderophores

In the human host the availability of free iron is a limiting requirement for bacterial growth (Schalk *et al.*, 1999). In low iron conditions, *Pseudomonas aeruginosa* synthesizes two known high-affinity iron-chelating siderophores; pyochelin (Cox, 1980) and pyoverdin (Cox and Adams, 1985). Pyochelin is a thiazol derivative that exhibits a relatively low affinity for iron (Cox *et al.,* 1981; Schalk *et al.,* 1999). Pyoverdin is a hydroxymate-cathecholate characterized by a conserved hydroxyquinoline chromophore bound to an amino acid tail of variable length (Stintzi *et al.*, 1999). In contrast to pyochelin, pyoverdin has a high affinity for iron (III) and is characterized by a yellow-green fluorescent pigment (Schalk *et al.*, 1999).

1.4.4 Exoenzyme S

Exoenzyme S (ExoS) is an ADP-ribosyl transferase which targets several eukaryotic proteins. Exoenzyme S production by *P. aeruginosa* plays a critical role during infection. Using transposon mutagenesis, Nicas and Iglewski (1984) observed a 2000-fold decrease in virulence in the burned mouse model with the exoenzyme S mutant. In addition, Nicas *et al.* (1985) also demonstrated that the

exoenzyme S deficient strain was still able to colonize the site of infection, however, it was unable to disseminate to the blood and other tissues.

In vitro studies have demonstrated that ExoS exhibits substrate specificity for G-proteins including, vimentin, Ras, Rasb, IgG3, and apolipoprotein A1 (Coburn *et al.*, 1989 a,b; Coburn and Gill, 1991; Bette-Bobillo *et al.*, 1998; Knight and Barbieri., 1997). Exoenzyme S is produced as two immunologicaly related forms, a 49 kDa form (ExoS) and a 53 kDa form (ExoT). The two proteins are encoded by separated genes on the chromosome of *P. aeruginosa* and are 76% homologous (Yahr *et al.*, 1996). ExoT is deficient in its catalytic activity since it expressed 0.2% of the ADP-ribosyl transferase activity of ExoS (Yahr *et al.*, 1996). However, the ADPRT deficient ExoT is still cytotoxic to cells and mediates resistance to phagocytosis (Fleizig *et al.*, 1997). These studies suggest that the toxin has two independent functional domains or mechanisms for inactivating target cells. Recently, Goranson and Frank (1996) and Yahr *et al.* (1996) demonstrated that exoenzyme S was delivered into eukaryotic cells via a type III secretion pathway. The type III secretion system requires three functional sets of genes: a secretion and chaperone protein, translocators of effector proteins and effector proteins (Sawa *et al.*, 1999). In addition, the type III secretion system is induced by direct contact of the bacterium with the target cell (Rosqvist *et al.*, 1994). ExoS and ExoT were the first identified effector proteins produced by *P. aeruginosa*. Both proteins are cytotoxic to target cells causing disruption of the actin microfilament structure (Frithz-Lindsten *et al.*, 1997). More recently, Finck-Barbançon *et al.* (1997) and Yahr *et al.* (1998) identified two new effector

proteins that are also secreted by the type III secretion pathway. The first one, ExoU, is not catalytically active but is required for an acute cytotoxic response and is also associated with fatal infection in an animal model (Finck-Barbançon *et al.*, 1997). The second effector protein, ExoY, is an adenylate cyclase whose production correlates with a drastic increase in intracellular levels of cAMP (Yahr *et al.*, 1998).

1.4.5 Exotoxin A

1.4.5.1 Structural Gene of Exotoxin A

toxA encodes a 2.760 kilobase mRNA that is translated as a monocistronic message (Gray *et al.*, 1984). The ATG initiation start codon is located 75 base pairs upstream of the mature exotoxin A (ETA) coding sequence and encodes a 25-amino acid hydrophobic signal peptide that is removed during secretion (Gray *et al.*, 1984). Southern blot analysis of various *P. aeruginosa* strains indicates that sequences downstream of *toxA* are well conserved, whereas much dissimilarity is observed upstream of the ETA structural gene (Vasil *et al.*, 1986). In addition, the structural gene for *toxA* was mapped around 85 minutes on the PA01 chromosome (Hanna *et al.*, 1983). Vasil *et al.* (1986) also confirmed, using three non-overlapping ETA specific probes, that there is no duplication of the *toxA* gene in any strain studied, therefore establishing that there is only one copy of the *toxA* gene per genome. As well, Vasil *et al.* (1986)

showed that among the *Pseudomonads,* the production of ETA was restricted to *P. aeruginosa.*

1.4.5.2 Environmental factors affecting production of ETA

Analysis of the production of ETA by *P. aeruginosa* indicates that the toxic protein is not constitutively expressed (Liu, 1966; Liu, 1973). Various growth parameters such as aeration, carbon source, temperature and nutritional factors were shown to influence the yield of ETA (Liu, 1966). Optimal ETA production occurs at 32°C during the late log phase of growth with glycerol as a carbon source. Bjorn *et al.* (1978) demonstrated that production of ETA is influenced by the level of iron in the media. Maximal production of ETA is achieved when the level of Fe^{3+} in the culture media reaches 1 μM, whereas at concentration of 5 μM or above the expression of ETA is repressed. Recently Somerville *et al.* (1999) were able to demonstrate that glutamate increases expression of ETA. This increase is due to the enhanced number of toxin-producing cells and not by increased level of *toxA* transcription.

ETA is an A-B type toxin; the A fragment contains the enzymatic portion of the toxin, whereas the B fragment is responsible for the binding of the toxin to target cells. *Diphtheria* toxin shares the closest homology with *P. aeruginosa* exotoxin A (Gray *et al.,* 1984). Both toxins are similar in size and are secreted as a proenzyme form that must undergo activation in order to be enzymatically toxic. In addition, both toxins are regulated by the concentration of iron in the medium, and they both modify the dipthamide residue of EF-2, thereby inhibiting protein

synthesis (Gray *et al.*, 1984). However, both proteins are distinct since they show no cross-reactivity, bind to different cell receptors and their amino acid composition shows no or very little homology. Gill (1988) demonstrated that the homology between the toxins is mostly located near the NAD hydrophobic pocket.

1.4.5.3 Regulation of Exotoxin A

Gene duplication of *toxA* is not responsible for the different levels of ETA production seen between different strains. This observation suggests the presence of a complex regulatory mechanism controlling the expression of ETA in *P. aeruginosa*. Using a PA103 chromosomal gene bank, *reg*A was cloned and shown to complement a hypotoxigenic mutant strain PA103-29 (Hedstrom *et al.*, 1986). A ten-fold increase in ETA production was observed when multiple copies of *reg*A were added *in trans* to the cells suggesting that *reg*A is an activator (Hedstrom *et al.*, 1986). *reg*A encodes a 29-kDa protein that is located in the inner membrane (Zimniak *et al.*, 1989).

Analysis of the transcription of *reg*A and *tox*A revealed that in low iron conditions, synthesis of both mRNAs follows a biphasic pattern (Frank and Iglewski, 1988). In addition, using an internal *Sal*I probe, Frank and Iglewski (1988) observed the size distribution of the *reg*A mRNA during growth. Early in the growth cycle, they were able to detect a large transcript (T1) whereas a smaller transcript (T2) appears during late exponential and early stationary phase. Wick *et al.* (1990) were able to identify a second gene, *reg*B, located

downstream of *regA* that confers increased levels of ETA production. RegB is predicted to be a 75-amino acid protein with a molecular mass of 7527 Da (Wick *et al.*, 1990). Storey *et al.* (1990) showed that *regA* and *regB* form an operon that is under the control of two promoters. Early in the growth cycle under high or low iron conditions, transcription from the P1 promoter results in transcription of both *regA* and *regB*, which can be detected as the larger T1 transcript. ETA produced as a result of activity from this promoter remains cell-associated. During the late exponential phase or early stationary phase the smaller T2 transcript, under the control of the P2 promoter, is activated solely in iron-limiting conditions and encodes only *regA*. This enhances transcription of *toxA* gene that can be secreted outside of the cells (Wick *et al.*, 1990). The exact mechanisms by which *regA* and *regB* act as positive regulators of *toxA* are still unknown. Walker *et al.* (1995) demonstrated that RegA expression in *E. coli* containing a *toxA* gene was able to direct ETA expression. In addition, they showed that RegA was able to initiate *toxA* transcription with RNAP holoenzyme isolated from either *P. aeruginosa* or *E. coli*. They also suggested that the failure to show binding between purified RegA and the *toxA* upstream region is due to its requirement for RNAP holoenzyme (Walker *et al.*, 1994; Walker *et al.*, 1995). Therefore, the *regAB* operon plays the most direct and critical role in the production of exotoxin A. However, several other positive and negative regulatory genes are also implicated in exotoxin A production.

P. aeruginosa lasR gene encodes a transcriptional activator protein, the LasR protein (Gambello *et al.*, 1993). LasR is a global virulence regulator since it

is involved in the regulation of several genes including *toxA*. The LasR protein displays 27% homology to the LuxR protein of *Vibrio fischeri* (Gambello and Iglewski, 1991). It was previously demonstrated that LuxR functions in a cell-density-dependent manner called quorum sensing (Shadel *et al.*, 1990). After reaching a threshold concentration, small homoserine-lactone molecules trigger a LuxR-type transcriptional activator that activates specific target genes. In *P. aeruginosa* two quorum sensing systems have been identified the *las* and *rhl* system. The *las* system contains the AI synthase encoded by *lasI* which produces a N-(3-oxododecanoyl) homoserine lactone (PAI-1) (Pearson *et al.*, 1994). The transcriptional activator LasR protein encoded by *lasR* is activated in high cell density and regulates several target genes including *lasA*, *lasB*, *apr*, *toxA lasI* and *rhlR* (Gambello *et al.*, 1991; Passador *et al.*, 1993; Pesci *et al.*, 1997; Toder *et al.*, 1991). The *rhl* system contains a different autoinducer synthase, RhlI, that produces a N-butyryl homoserine lactone (PAI-2) autoinducer molecule (Pearson *et al.*, 1995). When the autoinducer reaches a certain threshold, it binds to the transcriptional activator, RhlR, which induces transcription of *lasB*, *rpoS, rhlA, rhlI* genes (Brint and Ohman, 1995; Latifi *et al.*, 1996; Pearson *et al.*, 1997).

West *et al.* (1994) identified a gene, *vfr* (virulence factor regulator), that is able to increase the expression of exotoxin A through activation from the *regAB* P1 promoter. The *vfr* gene encodes a 28 kDa protein which is 92% homologous to the cyclic AMP receptor protein (CRP/CAP) in *Escherichia coli* (West *et al.*, 1994).

In addition, production of exotoxin A has been shown to be dependent upon the expression of an alternate sigma factor PvdS (Ochsner *et al.*, 1996). PvdS is negatively regulated by Fur, and the promoter region of *pvdS* harbors the characteristic palindromic Fur box sequence (Cunliffe *et al.*, 1995; Miyazaki *et al.*, 1995).

Production of exotoxin A in *P. aeruginosa* was also enhanced up to five times when *ptxR* was provided *in trans* (Hamood *et al.*, 1996). This gene encodes a 34-kDa activator protein that belongs to the LysR family of transcriptional regulators. More recently, Swanson *et al.* (1999) identified *ptxS* as a negative regulator of exotoxin A synthesis. *ptxS* is located upstream of *ptxR* and is divergently transcribed. PtxS is a 37-kDa protein that belongs to the GalR family of repressors and is able to negatively regulate its own expression. However, the mechanisms of regulation of both *ptx*R and *ptx*S on *toxA* remain elusive.

1.4.5.4 Structure of Exotoxin A

One important contribution to the study of ETA was the 3 Å resolution of the proenzyme form of exotoxin A achieved by Allured *et al.* (1986). The crystallized ETA revealed the presence of three distinct domains.

Domain I is located at the N-terminal end of the protein and is divided into two subdomains, Domain Ia (1-252) and Domain Ib (365-404). These two domains are separated in the DNA sequence but lie closely together in the three-

dimensional structure (Pastan and FitzGerald., 1989). This domain is composed of 17 β–strands with the primary 13 strands forming the structural core of a β-barrel (Allured *et al.*, 1986).

Domain II encompasses residues 253-364 and is characterized by the presence of six consecutive α-helices. The crystallographic structure of ETA also discloses the presence of four disulphide bridges linking Cys-11 with Cys-15, Cys-197 with Cys-214, Cys-265 with Cys-287 and finally Cys-372 with Cys-379 (Allured *et al.*, 1986). The first two helices of domain II, A and B, were also shown to be linked together by the presence of a disulfide bridge.

Domain III is located at the C-terminal end of the protein and is comprised of residues 405-613. The most fascinating trait of this domain is the existence of an extended cleft. The exact location of this cleft was made possible by the refined 2.5 Å crystal structure of the active form of ETA in the presence of NAD (Li *et al.*, 1995). This active cleft starts from the helix at residue 523-531 and finishes at the helix at residue 445-453, with the presence of a loop at the surface comprising residues 445-453 and critical residues 481, 440 and 553 at the bottom of the cleft (Li *et al.*, 1995).

1.4.5.5 Functional Analysis of ETA Domains

1.4.5.5.1 Domain I

Several studies implicate domain I as having a role in recognizing and binding to a eukaryotic cellular receptor. Initially, to gain a better understanding of the structure and function of this domain, Hwang *et al.* (1987) constructed a

series of plasmids that express different portions of ETA under the control of a T7 promoter. Plasmid pJH8 expresses ETA that contains a deletion of domain Ia which was purified to 95% homogeneity and injected into mice. A 200-fold decrease in toxicity was observed with mice challenged with the Δ1a strain when compared to native ETA. Competition analysis with native ETA demonstrated that strains expressing domain Ia, half of domain II, and domain III could successfully prevent inhibition of protein synthesis, whereas a strain lacking domain Ia could not block this effect (Hwang *et al.*, 1987). Taken together these data strongly indicate that domain Ia is responsible for binding to a specific eukaryotic receptor. In 1992, Kounnas *et al.* were able to identify the α_2-macroglobulin receptor/low density lipoprotein as the specific receptor for ETA.

In addition, Pirker *et al.* (1985) observed a decrease in cytotoxicity when *P. aeruginosa* ETA was treated with reagents that react with lysine residues. There is a total of 15 lysine residues in ETA, 12 are located in domain I, whereas the remaining 3 are found in domain III. Since ETA still contains its enzymatic property, this implies that the reduced toxicity is due to a change in the structure of domain I resulting in its inability to bind to sensitive cells.

Subsequently, Jinno *et al.* (1988) used site-directed mutagenesis to convert all of the 12 lysines found in domain I to glutamates. Substitution of lysine 57 to glutamate reduced the cytotoxicity of ETA towards 3T3-cells about 50-100 fold and 5 fold when injected into mice. The location of lysine 57 at the surface of the protein may be critical for the interaction of domain I with the cellular receptor. In addition, deletion of amino acids 4-224 of domain I results in

the same reduced level of toxicity as *P aeruginosa* ETA Glu-57, whereas a larger deletion of amino acids 4-252 causes a further reduction in toxicity. This suggests that amino acids 225-252 play a critical role in toxicity of ETA (Jinno *et al.*, 1988).

In addition, construction of a chimeric protein with domain II, Ib and III attached to a growth factor such as TGFα provides further evidence that domain Ia is responsible for cell binding to target cells (Siegall *et al.*, 1989). Their study was able to show that the chimeric toxin was able to bind to and kill TGF receptor-bearing cells. As well, Chia *et al.* (1986) were able to neutralize the cytotoxicity action of ETA using monoclonal antibodies against domain Ia. This implies that the reduction in cytotoxicity is caused by inhibiting the binding of ETA to target cells.

The function of domain Ib still needs to be clarified as Siegall *et al.* (1989) observed no decrease in cytotoxicity when residues 365-380 were deleted.

1.4.5.5.2 Domain II

Upon binding to the α_2-macroglobulin receptor, the toxin is internalized via coated pits into an endosome (Kounnas *et al.*, 1992). Acidification of the endosome is believed to be the signal required for translocation of ETA across the endosome membrane into the cytosol. It was demonstrated that the binding of ETA to the membrane was increased in an acidic environment, with optimal binding achieved at pH of 4-5, which is consistent with the pH found inside the endosome (Rasper and Merrill, 1994). The permeabilization of the lipid bilayer

by ETA involves four steps: 1) acidification of the endosome exposing a buried hydrophobic fragment; 2) a lipid-dependent binding of the toxin to the membrane surface; 3) insertion of the hydrophobic fragment into the lipid bilayer and 4) formation of a "pore" in the membrane lipid bilayer by the toxin (Nordera *et al.*, 1997). Inside the endosome, ETA is cleaved by the membrane associated cellular protease furin (Ogata *et al.*, 1990; Fryling *et al.*, 1992). The cellular protease cleaves between Arg-279 and Gly-280 releasing a 28 kDa fragment containing the N-terminal binding domain of ETA and a 37 kDa fragment containing the C-terminal portion of ETA (translocation and ADP-ribosylating activity domains). After the toxin is cleaved by the protease, separation of the 37 kDa fragment from the 28 kDa fragment is completed following reduction of a disulfide bond between Cys-265 and Cys-287 (Ogata *et al.*, 1990). Domain II of ETA mediates this translocation of ETA from the endosome into the cytosol of the target cell.

The first half of domain II is extremely hydrophobic which suggests that this region could potentially interact with lipids present in the membrane of endocytic vesicles (Hwang *et al.*, 1987). Initially, Hwang *et al.* (1987) demonstrated that an ETA lacking the hydrophobic fragment of domain II is still able to bind to the specific receptors and ADP-ribosylate EF-2. However, this construct is devoid of all cytotoxicity suggesting that the hydrophobic region is essential for the translocation of ETA (Hwang *et al.*, 1987).

In addition, Zdanovsly *et al.* (1993) also noticed a decrease in cytotoxicity when three amino acids, Trp-281, Leu-284 and Tyr-289, found at the N-terminal

end of the 37 kDa fragment were substituted with alternate amino acids. To examine the role of the amino acids positioned at the surface of domain II, Kasturi *et al.* (1992) mutated all of the 27 surface amino acids in favour of an alanine residue. Their investigation showed a decreased cytotoxicity for each of the 27 amino acid substitutions suggesting a role for these surface residues in translocation activity.

Madshus and Collier (1989) also noticed a reduction in cytotoxicity when they eliminated the disulfide bridge linking Cys-265 with Cys-287. This implies that removal of this disulfide bridge in domain II somehow puts constraints on the structure of ETA affecting efficient translocation. More recently, Taupiac *et al.* (1999) deleted all six α-helices positioned in domain II of ETA to investigate their role in translocation. All deletion mutants still retained full binding and enzymatic activity, signifying that each deletion only affects translocation. Removal of helix B, C and D which make up the core of domain II results in a non-toxic and non-translocating protein, while deletion of helix A and E forms a protein which still retains some cytotoxicity. The authors concluded that helix B, C and D are crucial for translocation, whereas helix A and E are expendable. Surprisingly, removal of helix F results in a 60% increase in translocation efficiency and a 3-6 fold increase in cytotoxicity which suggests that the presence of this helix is inhibitory to translocation. This novel finding is exciting since they demonstrated that cytotoxicity of the toxin could be improved by increasing translocation of the protein across the membrane.

1.4.5.5.3 Domain III

After successful translocation across the membrane of the endosome into the cytosol, the C-terminal fragment of ETA catalyzes the irreversible inactivation of eukaryotic elongation factor 2 (EF-2). The main enzymatic reaction catalyzed by ETA is the transfer of ADP-ribose from NAD^+ to the dipthamine residue of EF-2 (Iglewski *et al.*, 1977).

$$\text{NAD}^+ + \text{EF-2} \xrightarrow{\text{ETA}} \text{ADP-ribose-EF-2} + \text{Nicotinamide} + \text{H}^+$$

EF-2 is responsible for the catalysis of the final elongation step of protein synthesis (Iglewski, 1994). Thus, covalent modification of EF-2 effectively shuts down polypeptide chain elongation, thereby killing the cells and releasing nutrients to the environment. As well, in the absence of EF-2 as a substrate, ETA can hydrolyse NAD^+ to ADP-ribose and nicotinamide (Chung and Collier, 1977; Leppla *et al.*, 1978)

$$\text{NAD}^+ + \text{H}_2\text{O} \xrightarrow{\text{ETA}} \text{ADP-ribose} + \text{Nicotinamide} + \text{H}^+$$

Activation of ETA can also be mimicked *in vitro* by treatment of ETA with a reducing agent such as dithiothreitol and a denaturing agent such as urea (Leppla *et al.*, 1978; Vasil *et al.*, 1977).

Previous studies have established that domain III of ETA contains the enzymatic moiety of the toxin. Initially, Hwang *et al.* (1987) showed that domain III and 20 adjacent amino acids from domain Ib were required for full enzymatic activity. However, Chow *et al.* (1989) and Siegall *et al.* (1989) demonstrated that a smaller fragment, containing amino acids 400-608, was sufficient for full enzymatic activity. Further analysis of domain III has identified specific residues that play a critical role in the catalytic function of ETA. Carroll and Collier (1987), using photoaffinity labelling, demonstrated that Glu-553 was crucial for the enzymatic activity of ETA. Their model suggests that the side chain of Glu-553 is close to the nicotinamide-ribose bond that is cleaved when ADP-ribose is transferred to EF-2. The hydrophobic cavity is defined by the presence of aromatic side chain of His-440, Trp-466, Tyr-470, Tyr-481 and Trp-558 that may participate in the enzymatic reaction. Brandhuber *et al.* (1988) showed that iodination of Tyr-481 drastically reduced the enzymatic activity of ETA. Thus, Brandhuber *et al.* (1988) proposed a model where the nicotinamide ring of NAD is stacked on the indole ring of Trp-466 and Tyr-470 phenolic hydroxyl group would somehow be involved in NAD binding. However, using site-directed mutagenesis, Lukas and Collier (1988) demonstrated that substitution of Tyr-470 with Phe resulted in no decrease in NAD-glycohydrolase and ADP- ribosyl transferase activity.

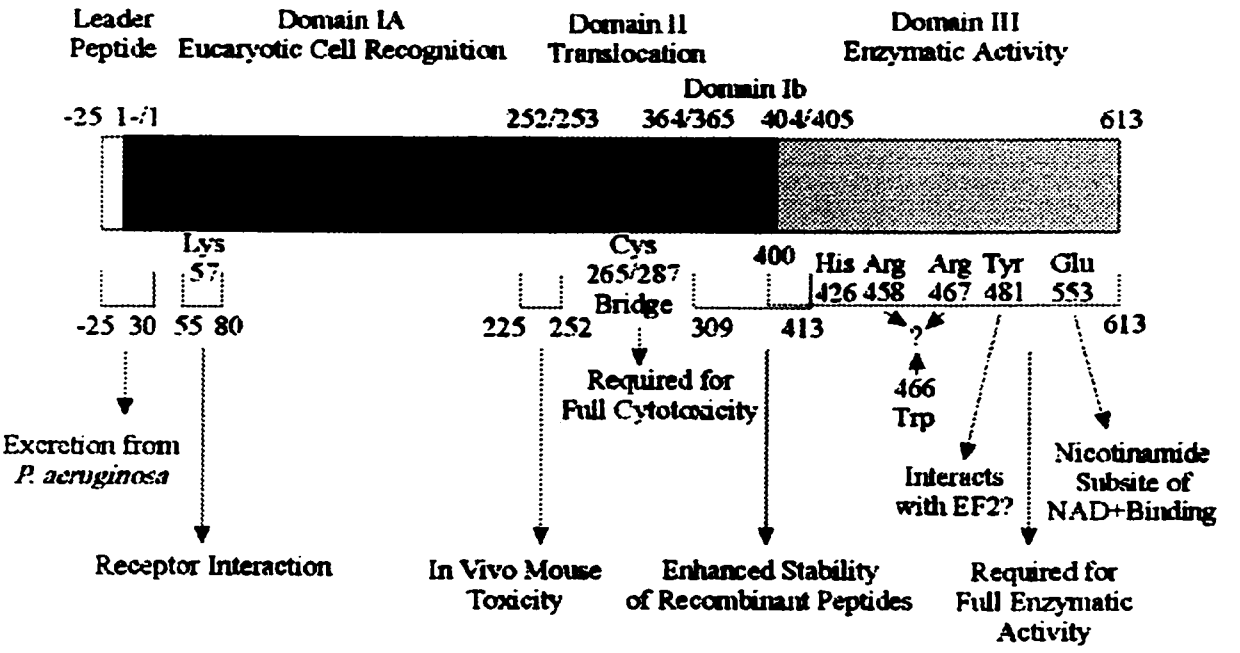
In addition, substitution of Tyr-481 with Phe caused a 10-fold reduction in ADP-ribosyl transferase activity but no reduction in NAD-glycohydrolase activity (Lukas and Collier, 1988). This suggests that the phenolic hydroxyl group of Tyr 481 must interact with the substrate EF-2. Wick and Iglewski (1988) also observed that substitution of His-426 for Tyr-426 is responsible for the enzymatically inactive phenotype seen with the cross-reactive ETA protein CRM66. This residue is located in the inner face of a α-helix opposite the active cleft. Disruption of this α-helix most likely renders the active site inaccessible to the substrate.

More recently, Domenighini and Rappuoli (1994) presented a model in which conserved consensus sequences make up the NAD-binding site of ADP-ribosyl transferase. In their model, the NAD-binding site of ETA is created by a α-helix bent over two β-strands. In *P. aeruginosa* ETA, the common structure of the NAD-binding site includes: 1) a conserved glutamic acid residue which sits on the bottom β-strand and is essential for catalysis; 2) formation of a β-strand characterized by a hydrophobic region surrounded by two conserved residues, Tyr-470 and Tyr-480, which may anchor the nicotinamide ring of NAD. The stability of the NAD molecule inside the hydrophobic cavity permits specific interaction with several residues allowing the electophillic carbon atom of ribose to freely interact with the diphtamine residue of EF-2.

Domain III is also characterized by the presence of the sequence Arg-Glu-Asp-Leu-Lys (REDLK) at its C-terminal end (Chaudhary *et al.*, 1990). The enzymatic activity of ETA is not affected by a deletion of this specific sequence,

but its cytotoxicity is drastically reduced (Chaudhary *et al.*, 1990). The presence of the amino acids 609-613 (REDLK) at the C-terminal end of ETA suggests that this sequence is required for directing the toxin to the endoplasmic reticulum. This was further supported by an observation that a severe increase in cytotoxicity of exotoxin A occurred when the REDLK signal was replaced with the endoplasmic retention signal KDEL, or $(KDEL)_3$ (Saraswathy *et al.*, 1991). Kreitman and Pastan (1995) determined that the presence of a glutamate residue in the signal retention sequence increased the cytotoxicity of ETA by possibly improving binding to a sorting receptor involved in transporting the toxin from the transreticular golgi apparatus to the ER. In addition, Jackson *et al.* (1999), using immunofluorescence, observed a decrease in immunofluorescence when the retrograde transport of the KDELK receptor from the golgi complex to the ER was prevented by antibodies.

Figure 1. Diagrammatic representation of residues required for full ETA toxicity (adapted from Wick *et al.*, 1990).


Leader Peptide
Domain IA Eucaryotic Cell Recognition
Domain II Translocation
Domain III Enzymatic Activity
Domain Ib
-25 1-/1
252/253
364/365
404/405
613
Lys 57
Cys 265/287 Bridge
400
His Arg Arg Tyr Glu
426 458 467 481 553
-25 30 55 80
225 252
309 413
?
613
Required for Full Cytotoxicity
466 Trp
Excretion from P. aeruginosa
Interacts with EF2?
Nicotinamide Subsite of NAD+Binding
Receptor Interaction
In Vivo Mouse Toxicity
Enhanced Stability of Recombinant Peptides
Required for Full Enzymatic Activity

1.4.5.6 Clinical manifestations of Exotoxin A

Exotoxin A is one of the most cytotoxic virulence factor produced by *P. aeruginosa* with an LD_{50} of 0.2 µg upon intraperitoneal injection into mice (Iglewski and Sadoff, 1979). In addition, Iglewski *et al.* (1977) noticed that injection of ETA in mice caused a decrease in EF-2 in several organs such as the heart, kidneys, spleen, lungs, and liver. The sensitivity of these organs to the toxin was due to the presence or over abundance of the specific receptor for *P. aeruginosa* ETA (Forristal *et al.*, 1991). Several studies have shown that the largest reduction in EF-2 was located in the liver, likely targeted by ETA due to elevated levels of cellular low–density lipoprotein (LDL) receptor on the surface of hepatocytes and Kupffer cells (Iglewski *et al.*, 1977; Pavloski *et al.*, 1974). The differing levels of EF-2 inhibition detected in each organ can be explained by: 1) distribution of the toxin via the circulatory system; 2) difference in the processing of the toxin by cells or 3) by the number of *P. aeruginosa* ETA receptors present in the tissues (Forristal *et al.*, 1991). Recently, Laithwaite *et al.* (1999) observed that the presence of LPS decreased the sensitivity of macrophages to *P. aeruginosa* exotoxin A. The investigators explained that the reduced expression of LDL-receptor protein on the surface of the macrophage is responsible for the decrease in ETA cytotoxicity. Finally, they hypothesize that the regulated expression of LRP at the surface of susceptible cells could be a mechanism controlled by the host that could directly influence which cells are affected by *P. aeruginosa* ETA during an infection (Laithwaite *et al.*, 1999).

In addition, Kohzuki *et al.* (1993) demonstrated in a mouse model that strains expressing ETA were more toxic than strains defective in ETA production. As well, their research indicates that antibodies against ETA act to decrease the toxic effect of this protein. The cytotoxicity of ETA is multifactorial since it affects several mammalian cell types (Middlebrook and Dorland, 1977). It was demonstrated that ETA inhibits human granulocyte and macrophage progenitor cell proliferation (Pollack and Anderson, 1978; Stuart and Pollack, 1982). Many reports have shown the immunological activity of ETA can be observed by alteration in the production of tumor necrosis factor (TNF-α) by leukocytes and the production of IL-1 by macrophages (Staugas *et al.*, 1992)

The biological implication of exotoxin A for the pathogenesis of *P. aeruginosa* 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 *toxA* (Raivio et al., 1994). These data suggest that *in vivo* production of ETA is common and may be an important virulence determinant in patients with CF.

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 these patients transcribe high levels of *toxA* (Storey *et al.*, 1992; Raivio *et al.*, 1994), but the infection rarely spreads beyond the lungs. To address the role of ETA in the pathogenesis of CF lung infections, we have analyzed the ETA produced by a number of CF isolates. One strain, 4384, which transcribes *toxA* at high levels, produces a toxin with a 12-fold reduction in enzymatic activity compared to wild type ETA. Inhibitory factors that might alter the activity of ETA are not produced by strain 4384. Sequence analysis of the *toxA* gene from strain 4384 suggests that the loss of activity may be due to the presence of mutations in domain III. These mutations have not previously been identified as causing alterations in ADP-ribosyl transferase activity. Interestingly, analysis of the cytotoxicity of ETA produced by strain 4384 indicated that it is equally as cytotoxic to HeLa cells as the native ETA. Five CF isolates were identified which displayed a similar reduction in ADPRT activity. We have amplified and sequenced regions encoding the enzymatic domains of *toxA* in five strains and found several mutations which could account for the observed reduction in ADPRT activity. This is the first demonstration that *P. aeruginosa* strains producing ETA with altered ADPRT activity and cytotoxicity are found in the lungs of patients with CF.

1.5 Project objectives.

The objectives of these studies were two-fold. The first aim was to characterize the altered ADPRT activity of ETA isolated from *P. aeruginosa* clinical strain, 4384. Initially, we wished to determine if there are any mutations in the *toxA* gene from strain 4384 that could be responsible for the reduced ADPRT

activity. In addition, we wanted to identify any accessory factor which could act to modify the activity of ETA in strain 4384 by complementation analysis. Finally, we compared the expression of ETA from strain 4384 and strain PA103. My second objective was to look at the prevalence of mutations in domain III of a series of CF isolates with diminished ETA activity. This addressed the possibility that CF strains with altered ADPRT activity are common among CF isolates.

Chapter 2

Materials and Methods

2.1 Strains and Plasmids.

The bacterial strains and plasmids employed in this study are listed in Table 1. Strain JM109 was used consistently for plasmid manipulations. All strains were stored and maintained at -70°C in 15% glycerol (BDH).

Table 1. Bacterial Strains and plasmids used in this study.

Strain/Plasmid	Genotype/phenotype	Reference
Strains		
Pseudomonas aeruginosa		
-PA103	*regA*$^+$*regB*$^+$hypertoxigenic laboratory strain	Liu, 1966
-PA103*toxA*::Ω	Inactivated *toxA* by Ω insertion, produces no ETA	Hamood *et al.*, 1989
-4384,5154,5166	Clinical isolates from the lung of a chronically infected CF patient (FH1)	Raivio *et al.*, 1994
-5552,5585,5588	Clinical isolates from patient (FH2)	Raivio *et al.*, 1994
-2770,2850,2906, 3540, 3546	Environmental isolates	Woods, unpublished
Escherichia coli		
DH5α	*sup*E44Δ*lacU*1699*f80lacZ*Δm15 *hsd*R17*rec*A1 *end*A1 *gyr*A96 *thi-1 rel*A1	Pharmacia
JM109	*end*A1 *rec*A1 *gyr*A96 *thi hsd*R17 (R_k^- m_k^+) *rel*A1 *sup*E44 Δ*lac-pro*AB(*F'* *tra*D36 *pro*AB *lacI*^{q} ΔM15)	Yanisch-Perron, 1985

M15[pREP4]	Nal^S Str^S rif^S, lac^- ara^- gal^- mtl^- F^- $recA^+$ uvr^+	Qiagen
Plasmids		
pCG5	Ap^r, pKK223-3 containing *Eco*R1-*Pst*1 *toxA* gene from strain 4384	This study
pCG (*tox*-SF)	Ap^r, pKK223-3 containing *Eco*R1-*Pst*1 *toxA* gene from strain 4384 and the 1.8kb stabilizing fragment at the *Pst*I site	This study
PCR 2.1™	PCR cloning vector	InVitrogen
PCR2.1™ (4384*toxA*$^{1986-2341}$)	Ap^r, PCR2.1™ vector with the 358 base pair amplified fragment from the first half of the enzymatic domain of *tox*A from CF strain 4384.	This study
PCR2.1™ (5154*toxA*$^{1986-2341}$)	Ap^r, PCR2.1™ vector with the 358 base pair amplified fragment from the first half of the enzymatic domain of *tox*A from CF strain 5154.	This study
PCR2.1™ (5166*toxA*$^{1986-2341}$)	Ap^r, PCR2.1™ vector with the 358 base pair amplified fragment from the first half of the enzymatic domain of *tox*A from CF strain 5166.	This study
PCR2.1™ (5552*toxA*$^{1986-2341}$)	Ap^r, PCR2.1™ vector with the 358 base pair amplified fragment from the first half of the enzymatic domain of *tox*A from CF strain 5552.	This study
PCR2.1™ (5585*toxA*$^{1986-2341}$)	Ap^r, PCR2.1™ vector with the 358 base pair amplified fragment from the first half of the enzymatic domain of *tox*A from CF strain 5585.	This study
PCR2.1™ (5588*toxA*$^{1986-2341}$)	Ap^r, PCR2.1™ vector with the 358 base pair amplified fragment from the first half of the enzymatic domain of *tox*A from CF strain 5588.	This study
PCR2.1™ (4384*toxA*$^{2336-2662}$)	Ap^r, PCR2.1™ vector with the 329 base pair amplified fragment from the second half of the enzymatic domain of *tox*A from 4384.	This study

PCR2.1™ (5154*toxA*$^{2336-2662}$)	Ap^r, PCR2.1™ vector with the 329 base pair amplified fragment from the second half of the enzymatic domain of *tox*A from CF strain 5154.	This study
PCR2.1™ (5166*toxA*$^{2336-2662}$)	Ap^r, PCR2.1™ vector with the 329 base pair amplified fragment from the second half of the enzymatic domain of *tox*A from CF strain 5166.	This study
PCR2.1™ (5552*oxA*$^{2336-2662}$)	Ap^r, PCR2.1™ vector with the 329 base pair amplified fragment from the second half of the enzymatic domain of *tox*A from CF strain 5552.	This study
PCR2.1™ (5585*toxA*$^{2336-2662}$)	Ap^r, PCR2.1™ vector with the 329 base pair amplified fragment from the second half of the enzymatic domain of *tox*A from CF strain 5585.	This study
PCR2.1™ (5588*toxA*$^{2336-2662}$)	Ap^r, PCR2.1™ vector with the 329 base pair amplified fragment from the second half of the enzymatic domain of *tox*A from CF strain 5588.	This study
pKK223-3	Ap^r, Expression vector	Pharmacia
pMS151-1	Ap^r, pUC9 containing the *tox*A gene from PAK and the 1.8 stabilizing fragment	Hamood, 1989
pUC12	Ap^r, General cloning vector	Yanisch-Perron, 1985
pUC12(EcoRI-PstI 4384toxA)	Ap^r, pUC base vector with the entire 4384 *tox*A	This study
pUC12 (PstI-BamHI)	Ap^r, pUC12 vector with the 760 base pair PstI-BamHI fragment from 4384*toxA*	This study
pUC12 (BamHI)	Ap^r, pUC12 vector with the internal 1.530 kb fragment from 4384*toxA*	This study
pUC12 (SalI)	Ap^r, pUC12 vector with the 834 base pair fragment from 4384*toxA*	This study
pUC12(BamHI-EcoRI)	Ap^r, pUC12 vector with the 460 base pair fragment from 4384*toxA*	This study

pUCSF	Ap^r, pUC19 with the 1.8 kb stabilizing fragment inserted into the *Pst*I site	Hamood *et al.*, 1989
pUCSF(4384*toxA*)	Ap^r, pUCSF with the *EcoR*I-*Pvu*II *toxA* from clinical strain 4384	This study
pUCSF (WT*toxA*)	Ap^r, pUCSF with the *EcoR*I-*Pvu*II *toxA* from pMS151-1	This study
pALTER-1	Tet^r, mutagenesis vector	Promega
pALTER (WT*toxA*)	Tet^r, *EcoR*I-*PST*I *toxA* gene from pMS151 into the *EcoR*I-*Pst*I site of pALTER-1 vector	This study
pALTER(4384*toxA*)	Tet^r, *EcoR*I-*PST*I *toxA* gene from pCG5 into the *EcoR*I-*Pst*I site of pALTER-1 vector	This study
pQE-32	Ap^r, expression vector with a 6xHis affinity tag at the N-terminal end	Qiagen
pQE-32 (4384*toxA*-C-terminal end)	Ap^r, *Bam*HI-*Eco*RI 4384*toxA* fragment inserted into the *Bam*HI-*Pst*I site of pQE-32	This study
PQE-32 (WT*toxA*-C-terminal end)	Ap^r, *Bam*HI-*Eco*RI WT*toxA* fragment inserted into the *Bam*HI-*Pst*I site of pQE-32	This study
pQE-32 (4384*toxA*)	Ap^r, *BamH*I-*EcoR*I *toxA* gene minus the RBS isolated from pUCSF (4384*toxA*) and inserted into the *BamH*I-*Pst*I site of pQE-32	This study
pQE-32 (WT*toxA*)	Ap^r, *BamH*I-*EcoR*I *toxA* gene minus the RBS isolated from pUCSF (WT*toxA*) and inserted into the *BamH*I-*Pst*I site of pQE-32	This study

2.2 Media

2.2.1 Liquid Media

All strains were grown in LB Broth (10 g of NaCl (BDH), 5 g of yeast extract (Difco Laboratories) and 10 g of Bacto tryptone (Difco Laboratories) per liter). Unless otherwise specified, all strains of *E.coli* carrying a plasmid were grown in

LB Broth supplemented with 100 µg/ml of ampicillin (Sigma Chemical Co.), whereas *P. aeruginosa* containing plasmids were grown in the presence of 400 µg/ml of carbenicillin (Sigma Chemical Co.). A low-iron media, TSBDC was also routinely used for maximal ETA expression. Briefly, 30 g of TSB (BDH) was mixed with 5 g of Chelex 100 resin (Bio-Rad Laboratories) in 90 ml of water for 5 hours at room temperature. This mixture was then dialysed against 1 liter of water overnight at 4°C. The following morning the dialysis tubing MWCO: 6-8,000 Da (Spectra Chemical Co.) and its contents were discarded, and the remaining media was autoclaved and stored at 4°C until needed. Prior to use, 20 ml of 2.5 M of monosodium glutamate (Sigma Chemical Co.) and 20 ml of 50% glycerol was added to the TSBDC media.

2.2.2 Solid media

E. coli transformed cells were screened on LB Agar plates (15 g of agar (Gibco/BRL) was added per liter of LB Broth media) supplemented with ampicillin (100 µg/ml). *P. aeruginosa* transformed cells were grown on VBMM agar plates (3 g of trisodium citrate (OmniPur), 2 g of citric acid (Bio-Rad Laboratories), 10 g of dipotassium hydrogen orthophosphate (BDH), 3.5 g of sodium ammonium phosphate (Fisher Scientific), 0.2 g of magnesium sulphate (Fisher Scientific) and 15 g of agar per liter of media) containing 400 µg/ml of carbenicillin. All *P. aeruginosa* strains were streaked on *Pseudomonas* isolation agar (Difco Scientific) for single colonies prior to storing in 15% glycerol.

2.2.3 Antibiotics concentrations

Ampicillin was used at concentrations of 100 µg/ml of media for all the genetic manipulations of plasmids involving *E. coli* host strains. For the purification of ETA in the QE-vector system (Qiagen), all positive transformants in the *E.coli* M15 host cells were screened on media supplemented with 100 µg/ml of ampicillin and 25 µg/ml of kanamycin (Sigma Chemical co.). Carbenicillin was used at a concentration of 400 µg/ml for transformants in *P. aeruginosa* host strains.

2.3 Growth curve methodology

All growth curves were carried out in low iron conditions to achieve maximal ETA expression (Liu, 1966). All flasks used in the experiments were acid washed (20% HCL, Fisher Scientific) for 4 hours at room temperature, rinsed three times in ddH_2O and autoclaved. For growth analysis, all *P. aeruginosa* cultures were grown overnight at 32°C in 10 ml of TSBDC. Secondary cultures were inoculated the following day to obtain a starting $O.D_{540}$ of 0.02. Aliquots of the secondary cultures were taken at different time points and centrifuged for 10 minutes at 13000 RPM. The supernatant was removed and frozen at -20°C. All growth curves were repeated at least three times.

2.4 Assays

2.4.1 Exotoxin A assays

All strains were previously grown in low iron conditions until O.D_{540} of 4.0 where 1.5 ml of the cells were pelleted at 13 000 RPM for 10 minutes. The supernatant was removed and stored at –70°C. The ADP-ribosyl-transferase activity was assayed as previously described by Chung and Collier (1977). Briefly, ETA activity was measured by mixing 10 µl of bacterial supernatant with the same volume of urea-DTT solution (0.5 ml of 8M urea (BDH), 0.01 g of DTT (Sigma Chemical Co.). ETA was activated for 15 minutes at room temperature. After the incubation, 25 µl of T-II-C (125mM Tris pH 7 (ICN), 100 mM DTT), 25 µl of wheatgerm extract (containing the EF-2), and 5 µl of ^{14}C-NAD (NEN Dupont) was added to each tube and incubated for 30 minutes at room temperature. The proteins were TCA precipitated, filter dried and then assessed using a liquid scintillation counter. ADPRT assays were performed in triplicate on each supernatant sample and the results averaged.

2.4.2 Cytotoxicity Assays

The cytotoxicity of exotoxin A was measured using a colorimetric microtiter plate assay. Briefly, HeLa cells were grown to confluency (1×10^6/well) and incubated overnight at 37°C. Serial dilutions of ETA were added to the HeLa cells and incubated for 48 hours at 37°C. Twenty µl of a tetrazolium dye (MTT [5mg/ml]) was added to each well and incubated for 4 hours 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 µl of isopropanol-HCL solution. The $O.D_{570}$ of each well was measured using an EIA reader.

2.5 DNA Methodology

2.5.1 Plasmid isolation

2.5.1.1 Small Scale Isolation of Plasmid DNA (Alkaline lysis method)

A single bacterial colony was used to inoculate 3 ml of LB broth containing a suitable concentration of antibiotics. The following morning, 1.5 ml of the overnight culture was centrifuged at 13 000 rpm for 3 minutes. The pelleted cells were then resuspended in 100 µl of Solution A (50mM glucose (Sigma Chemical co.), 10mM EDTA (BDH), 25mM Tris-HCL (pH 8) (Gibco/BRL) and * 4mg/ ml of lysozyme (Sigma Chemical Co.). The cells were then lysed by the addition of 200 µl of ice cold Solution B (0.2M NaOH (BDH), 1% SDS) for 5 minutes on ice. 150 µl of Solution C (3M potassium 5M acetate pH 4.8 (BDH) was then added to the mixture and store on ice for an additional 5 minutes. The cellular debris was centrifuged for 5 minutes at 13 000 rpm, and the remaining supernatant was then transferred to a fresh Eppendorf tube. Following a chloroform:isoamyl alcohol (24:1) extraction**, the plasmid was precipitated on ice for 15 minutes by the addition of 900 µl of 95% ethanol. The mixture was centrifuged for an additional ten minutes. The plasmid was then rinsed with 900 µl of 70% ethanol and then recentrifuged for 5 minutes. The plasmid was resuspended in 40 µl of water containing 2 µl of RNAse A (10 mg/ml) (Gibco/BRL).

* Only added if the host strain was *Pseudomonas aeruginosa*. The cells were then incubated for 5 minutes at room temperature.

** 3 phenol: chloroform: isoamyl alcohol (25:24:1) extractions were performed if the plasmid was isolated from *P.aeruginosa*

2.5.1.2 Large Scale Isolation of Plasmid DNA:

The bacteria were grown overnight in 250 ml of LB broth containing the appropriate amount of antibiotic. The cells were centrifuged for 15 minutes at 10 000 RPM and resuspended in 6 mls of Glucose buffer (50 mM glucose, 25 mM Tris-HCL, 10mM EDTA). One ml of a lysozyme solution (20mg/ml) was added to the resuspended cells and allowed to incubate for 10 minutes at room temperature. Next, 14 mls of a lysis solution (0.2 M NaOH, 1% SDS) was added to the mixture and placed on ice for 5 minutes. Lastly, 7 mls of sodium acetate (3M sodium, 5M acetate pH 4.8 (OmniPur) was added to each tube. The cellular debris was centrifuged at 10 000 rpm for 10 minutes. The supernatant was then subjected to a phenol:chloroform:isoamyl alcohol (25:24:1) extraction. The aqueous solution was removed, and the nucleic acids were precipitated with 0.6 volume of isopropyl alcohol (BDH) for 10 minutes at room temperature. The DNA was then obtained by centrifugation at 10 000 RPM for 15 minutes. The resulting pellet was air dried and resuspended in 1 ml of water. One gram of cesium chloride (Gibco/BRL) was added per ml of the nucleic acid-water mixture. Eighty µl of ethidium bromide (10mg/ml) was added for every ml of CsCl-nucleic acid-water mixture. The DNA solution was then transferred to a 1.2 ml tube. The

centrifugation tubes were then balanced and heat-sealed. The tubes were then loaded in the TLN-120 rotor and centrifuged at 120 000 rpm for 1 hour at 20°C in a Tabletop Ultracentrifuge. After the centrifugation, the plasmid DNA was visualized using UV light and removed as follows: the top of the tube was pierced with a needle to allow the air to enter, and the tip of the needle was inserted bellow the lowest band, and gently the plasmid was withdrawn. The ethidium bromide was then removed from the sample by adding TES-saturated isobutanol. The sample was then dialysed overnight in 1 liter of dialysis buffer (1.27 g Tris-HCl, 0.236 g of Tris, 1.68 g of EDTA per liter).

2.5.1.3 Isolation of plasmids for sequencing

The culture was grown overnight in 20 ml of LB supplemented with the appropriate antibiotic concentration. The following morning, 1.5 ml of the cells were centrifuged at 13 000 rpm for 10 minutes in a microcentrifuge. The supernatant was removed by aspiration, and the pellet was resuspended in 200 µl of glucose buffer (50mM glucose, 25 mM Tris-HCL (pH 8), 10 mM EDTA (pH 8)). Next, the cells were lysed on ice for 5 minutes by the addition of 300 µl of the lytic solution (0.2M NaOH, 1% SDS). Finally, the solution was neutralized by adding 300 µl of 3M potassium acetate (pH 4.8) for five minutes on ice. The cellular debris was then separated by centrifugation at 13 000 RPM for 10 minutes. The supernatant was then treated with 4 µl of Rnase A (10mg/ml) for a minimum of 2 hours at 37°C. After the incubation, the supernatant was extracted twice with 400 µl of chloroform:isoamyl alcohol. The DNA was then precipitated

with an equal volume of 100% isopropanol. Subsequently, each tube was centrifuged at 13 000 RPM for 10 minutes at room temperature to pellet nucleic acid. 500 µl of a solution of 70% ethanol was then used to wash the DNA pellet. The air dried pellet was then resuspended in 32 µl of H_20 and precipitated on ice for 30 minutes by the addition of 8 µl of NaCl and 40 µl of 13% PEG (Sigma Chemical co.). The plasmid DNA was recovered by centrifugation for 30 minutes at 4°C. The DNA was rinsed with 500 µl of 70% ethanol and finally resuspended in 20 µl of H_20. Before sending the plasmid DNA to the University of Calgary Core DNA Service, 2 µl of the sequencing mini-prep was electrophoresed on a 0.8% agarose gel to confirm size and purity.

2.5.2 Isolation of chromosomal DNA

Each strain was grown overnight in 5 ml of LB broth. The following morning the cells were centrifuged at 5000 rpm for 10 minutes. 1.5 ml of a proteinase K (Gibco/BRL) solution (50mM NaCl, 2% SDS and 400 µg of proteinase K per 1.5 ml) were used to resuspend each pellet and incubated at 42°C until the solution became clear. Next, 250 µl of 5M NaCl and 200 µl of CTAB/NaCl (10%CTAB in 0.7M NaCl) were added to each tube and incubated at 65°C for 30 minute. This incubation was followed by four phenol:chloroform:isoamyl extractions. Subsequently, the chromosomal DNA was precipitated with 100% ethanol and 5% of 5M NaCl. The DNA was then centrifuged at 13 000 rpm for 10 minutes. The pellet was washed with 1 ml of 70% ethanol and air dried until the ethanol

had evaporated. The pellet was then resuspended in 40 µl of H_2O and 2 µl of RNAse A (10mg/ml).

2.5.3 Transformation.

2.5.3.1 *Pseudomonas aeruginosa*

The *P. aeruginosa* strain was streaked on VBMM agar and incubated overnight at 37°C. The next morning a single colony was used to inoculate 25 ml of LB Broth and incubated at 37°C until the culture reached an $O.D_{540}$ of 0.125. The cells were centrifuged at 8 000 rpm for 10 minutes and then resuspended in 12.5 ml of a sterile 0.15 M $MgCl_2$ (BDH) solution and incubated on ice for 5 minutes. The two previous steps were repeated, and the resuspended cells were put on ice for an additional 20 minutes. The cells were centrifuged one last time as above, and the pellet was resuspended in 2.5 ml of 0.15 M $MgCl_2$. Two hundred µl of competent *P. aeruginosa* cells were used per transformation with 1 µg of plasmid DNA. The transformation reaction was incubated on ice for one hour. The mixture was then heat shocked at 37°C for 3 minutes and put on ice for 5 minutes. Five hundred µl of LB broth was added to each tube and incubated at 37°C for 2 hours. The transformed cells were then plated (200µl) on VBMM agar supplemented with 400 µg/ml of carbenicillin.

2.5.3.2 *Escherichia coli*

The competent JM109 cells were thawed on ice for 20 minutes prior to use. The ligation mixture was then mixed with 200 µl of competent cells and incubated on

ice for 45 minutes. The cells were then heat shocked at 42°C for 2 minutes and put on ice for an additional 2 minutes. Three ml of LB broth was added to the ligation mixture and incubated at 37°C for 90 minutes. The cells were then centrifuged for 5 minutes at 5 000 rpm and resuspended in 800 µl of LB broth. Two hundred µl of the transformed *E. coli* cells were plated on the appropriate media and incubated at 37°C overnight.

2.5.4 Electroporation

2.5.4.1 *Pseudomonas aeruginosa*

The *P. aeruginosa* strain was grown overnight in 10 ml of LB broth. The next morning, 1 ml of the overnight culture was transferred to 50 ml of fresh LB broth and incubated at 37°C until the $O.D_{540}$ reached between 0.3-0.5. The cells were then harvested by centifugation at 5 000 rpm for 10 minutes. The pelleted cells were resuspended in 50 ml of 300 mM sucrose solution. The cells were recentrifuged and resuspended in 25 ml of the previous solution. Finally, after another round of centrifugation, the cells were resuspended in 250 µl of 300 mM sucrose solution and chilled on ice for 30 minutes. The electrocompetent cells (40 µl) were then mixed with 5 µl of the plasmid DNA and transferred to a chilled 0.2 cm gap cuvette. Maximun electroporation efficiency was achieved at 1.6-kV/0.2 cm. After the electroporation, 3 ml of LB broth was added to each tube. Each electroporation reaction was then incubated at 37°C for 90 minutes. The transformed cells were then centrifuged at 5 000 rpm for 10 minutes and

resuspended in 1 ml of LB broth. Two hundred µl of cells were then spread on VBMM agar plates containing 400 µg/ml of carbenicillin.

2.5.4.2 *Pseudomonas aeruginosa* Cystic fibrosis isolates

Preparation of CF isolate electrocompetent cells was as described above. However, it was noticed that maximum electroporation effiency for CF strains was obtained at 2.5 kV/0.2cm. Following delivery of the pulse, 3 ml of LB broth was added to each tube, and incubate at 37°C for 90 minutes. After incubation the cells were centrifuged at 5 000 rpm for 10 minutes. The transformed cells were then resuspended in 200 µl of LB broth and spread on one VBMM agar plate supplemented with 400 µg/ml of carbenicillin.

2.5.5 Preparation of Competent Cells

2.5.5.1 *Escherichia coli* strain JM109

E.coli JM109 was streaked on LB agar plates and incubated overnight at 37°C. The following morning a single colony was used to inoculate twenty-five ml of LB broth and incubated overnight at 37°C. Five ml of the overnight culture was used to subculture 500 ml of fresh LB media, and incubated at 37°C until the cells reached an $O.D._{600}$ between 0.45-0.55. After reaching the proper cell density, the cells were chilled on ice for two hours. The cells were centrifuged for ten minutes at 5 000 rpm. The pelleted cells were gently resuspended in 20 ml of ice-cold trituration buffer, diluted to 500 ml with the same buffer, and set on ice

for an additional 45 minutes. The cells were then collected by centrifugation at 5 000 rpm for 10 minutes and resuspended in a final volume of 50 ml of the trituration solution. A final concentration of 15% (v/v) was achieved by adding drop wise an 80% glycerol solution to the resuspended cells. The competent cells were then freeze dried in 200µl aliquots and stored at –80°C

2.5.5.2 K-12 derived *Escherichia coli* strains M15 [pREP4]

The *E. coli* cells were streaked on an LB agar plate supplemented with 25µg/ml of kanamycin and incubated overnight at 37°C. A single colony was then used to inoculate 10 ml of LB/Km (25µg/ml) media and incubated overnight at 37°C. The following morning, 1 ml of the culture was used to inoculate 100 ml of LB/Km (25µg/ml) media. The cells were incubated at 37°C until they reached an $O.D_{600}$ of 0.5. The cells were then collected by centrifugation at 5 000 rpm for 10 minutes. The pelleted cells were then resuspended in cold TFB1 buffer (100mM RbCl (Sigma Chemical co.), 50 mM $MbCl_2$, 30 mM KAc, 10 mM $CaCl_2$ (Fisher Scientific), 15% glycerol, pH 5.8) and recentrifuged for another 10 minutes. The cells were gently resuspended in 4 ml of TFB2 (10mM MOPS (Sigma Chemical co.), 10 mM RbCl, 75 mM $CaCl_2$, 15% glycerol, pH. 8). The competent cells were then prepared in aliquots of 500 µl, snap frozen on dry ice, and stored at –70°C.

2. 5. 6 Agarose gel electrophoresis

The DNA fragments were fractionated on a 0.8% agarose gel (Gibco/BRL) in TAE buffer (4.84 g of Tris, 1.14 ml of glacial acetic acid (BDH) and 0.675 g of EDTA per liter). Each sample was mixed with 1/10 volume of loading DNA dye which consist of two tracking dyes (0.25% bromophenol blue, 0.25% xylene cyanol and 30% Glycerol). The DNA gel was electrophoresed at 85 volts for approximately 1 hour on a Mini or Maxi Horizontal Agarose Submarine Unit (Hoefer Scientific Instruments). As a standard, a molecular weight marker (1 kb Plus Ladder) (Gibco/BRL) was electrophoresed beside the sample under investigation to confirm sample size. After the separation of the DNA was completed, the gel was stained in 1μg/ml of ethidium bromide for 10 minutes at room temperature. The DNA was visualised using a gel photodocumentation system.

2.5.7. Manipulation of DNA

2. 5. 7. 1. Restriction endonuclease digestion.

All restriction enzymes were purchased from Gibco/BRL. Briefly, all DNA digestions included approximately 1 μg of DNA, 1 μl of enzyme (1-20 units) and 1/10 volume of 10x reaction buffer. The digestion reaction was carried out at 37°C for approximately 2 hours for plasmid DNA. For chromosomal digests, the reaction was incubated overnight at 37°C.

2. 5. 7. 2 Partial digestion.

*Bam*HI was selected to isolate the *toxA* fragment less the RBS from pUCSF (4384*toxA*) and pMS151-1. Briefly, the enzyme was diluted 1/20 in react 3 buffer. The DNA samples were incubated at 37°C for 10 minutes prior to digestion. Each tube comprises 10.5 µl of diluted plasmid DNA and 0.5 µl of react 3 buffer. After the incubation time, 1 µl of the diluted enzyme (1/20) was added to each tube every 30 seconds for a period of 10 minutes. To terminate the reaction, 5 µl of 50 mM of EDTA and 2 µl of loading DNA dye were added to each sample and it was electrophoresed on a 0.8% agarose gel.

2.5.7.3 Ligation reaction.

Each ligation reaction included 4 µl of 5x ligation buffer (Gibco/BRL), 1 µl of T4 DNA ligase (Gibco/BRL), 2 µl of vector (1µg/µl) and X µl of DNA insert and H_2O to a final volume of 20 µl. When possible, a 1:2 ratio (vector:insert) was obtained which was found to be optimal. The ligation reaction was incubated overnight at 16°C. A control ligation reaction was always included which consisted of the vector without the insert. In some instances, we also assessed the formation of ligated products by electrophoresing 4 µl of the ligation reaction on a 0.8% agarose gel.

2.5.7.4 Dephosphorylation reactions

The dephosphorylation reaction was performed in a final volume of 100 µl, which included 10 µl of CIAP 10x buffer (Gibco/BRL), 10 µl of 1/1000 diluted CIAP and

X µl of the digested vector DNA. For protruding 5'- termini, the dephosphorylation reaction was incubated at 37°C with 5 µl of the enzyme for 30 minutes. An additional 5 µl was then added to the reaction and incubated an additional 30 minutes. For recessed 5' termini or blunt ends, the dephosphorylation reaction was first incubated for 15 minutes at 37°C then at 56°C for an additional 15 minutes. At this point, 5 µl of CIAP enzyme (1/1000) was added, and the dephosphorylation reaction conditions were repeated. The reaction was stopped by the addition of 2 ul of 0.5M EDTA and incubated at 75°C for 20 minutes. The dephosphorylation reaction was extracted once with an equal volume of phenol:chloroform:isoamyl solution.

2. 5. 8. Isolation and purification of DNA fragments

The DNA band of interest was excised from the 0.8% agarose gel previously stained with ethidium bromide. Nine hundred µl of NaI solution (Bio 101 Inc) was added to each tube and incubated at 55°C until the gel fragment was fully dissolved. Next, 5 µl of GLASSMILK per 5 µg of DNA was added to each sample. The DNA was allowed to bind to the silica matrix for approximately 10 minutes at room temperature. The silica matrix which contained the bound DNA was then pelleted by centrifugation at 13 000 rpm for 1 minute. The pellet was then washed three times with ice cold NEW WASH solution. The DNA was then eluted from the GLASSMILK with water (used same volume as with the GLASSMILK) at 55°C for 4 minutes. The DNA was recovered by centifugation at 13 000 rpm for 30 seconds. To increase the DNA yield, the elution step was

repeated a second time. Finally, 5 µl of the gene clean product was electrophoresed on a 0.8% agarose gel to determine the quantity and quality of the isolated DNA.

2.5.9. Southern Blotting

2.5.9.1 Membrane preparation

The DNA fragments were separated on a 0.8% agarose gel and visualised using a gel documentation system. The gel was then immersed in 0.25M HCl to allow fragmentation of DNA. The DNA gel was then rinsed two times in water and soaked twice for 15 minutes in 400 ml of a 1M NaCl/0.5M NaOH solution. Finally, the gel was neutralized in 400 ml of 0.5M Tris pH 7.4/ 1.5M NaCl solution twice for 15 minutes. At this point, a piece of Nytran membrane (Schleicher and Schuell) was cut exactly the same size as the gel, rinsed in water, and then soaked in 10X SSC. Meanwhile, a tray was filled with 20X SSC (175.3g of NaCl and 88.2g of tri-sodium citrate per liter of H_2O, pH 7), and a glass plate was put across the tray. A long piece of Whatman paper (Whatman 3 mm Chromatography paper, Whatman International, Ltd.) was placed across the plastic bridge to allow both ends to be immersed in buffer. The gel was then placed face down on the Whatman paper and covered with the Nytran membrane. A glass pipet was then used to remove air trapped between the gel and the membrane. A plastic wrap was put around the gel, and 4 pieces of Whatman paper were set on top of the membrane. At last, half a stack of paper towels was positioned on top of the membrane and held in place with 500g

weights. The blot was secured with plastic wrap and tape and left overnight at room temperature. The following morning, the Nytran membrane was washed for 5 minutes at 60°C in 5X SSC. The DNA was fixed either by baking at 80°C for 2 hours in a vacuum oven (Johns Scientifics) or by cross-linking the DNA to the membrane by exposure to UV light for 5 minutes.

2.5.9.2 Pre-Hybridization and Hybridization

The pre-hybridization solution (6 ml of 20X SSC, 4 ml of 50X Denhart' s, 2 ml of 10%SDS and 7.9 ml of water) and 100µl of denatured salmon sperm DNA was added along with the Nytran membrane to a plastic bag and incubated at 42°C for 2 hours. After the period of incubation, the pre-hybridization solution was discarded and replaced with the hybridization solution (6 ml of 20X SSC, 2 ml of 10% SDS, 10 ml of 50% deionized Formamide, 2 ml of water, 100µl of denatured salmon sperm (Sigma Chemical co.) and the labelled DNA probe (previously boiled for 5 minutes). The plastic bag was carefully sealed and incubated overnight at 42°C. The following morning, the excess probe was removed by washing the Nytran as outline below. The membrane was first washed twice in 300 ml of a solution consisting of 6X SSC/0.1 %SDS. This process was performed twice for 15 minutes at room temperature. Next, the blot was transferred to a second solution (2X SSC/0.5%SDS) and incubated twice at 37°C for 15 minutes each. Finally, the last wash was performed at 65°C for 30 minutes in 0.1X SSC/0.5%SDS. After washing, the membrane was placed on a piece of Whatman paper to remove most of the excess moisture. The Nytran membrane

was then wrapped in plastic and placed inside an X-ray cassette and exposed to XAR-5 Kodak Scientific Imaging Film (Kodak) at –70°C

2.5.9.3 ^{32}P Random Labelling and Purification of the DNA probe

The internal *toxA* fragment was isolated from pMS151-1 (Hamood, 1989). Briefly, the plasmid was digested with *Bam*HI for several hours at 37°C. The digested DNA was then electrophoresed on a 0.8% agarose gel and the 1.53 kb band was electrophoresed using a Gene Clean II Kit (Bio 101 Inc.). Five µl of the purified fragment was electrophoresed on another agarose gel to estimate the amount of DNA prior to labelling. Next, the DNA probe was mixed with water to a final volume of 34 µl. The probe was denatured for 5 minutes in boiling water and set on ice for an additional 2 minutes. After chilling, 10 µl of reaction mix (Pharmacia), 5 µl of ^{32}P-dCTP (NEN Dupont) and 1 µl of Klenow (Pharmacia) was added to the DNA probe. The probe was then left at room temperature for 1 hour. After the incubation, the probe was purified from excess isotope using a NENSORB 20 cartridge (NEN-Dupont). First the column was equilibrated by adding 2 ml of methanol (BDH), followed by 2 ml of react A buffer (0.1 M Tris-HCl, 10mM triethylamine and 1 mM EDTA) and allowed to pass slowly through the column. At this point the probe was mixed with 200 µl of reactive A and passed through the column. The column was then washed with 3 ml of reactive A and the DNA probe recovered by the addition of 1 ml of 50% ethanol. The first ten drops were collected, and 1 µl of the probe was counted in a scintillation counter (Beckman LS-6500) to determined the quality of the probe.

2.5.10 Cloning of *toxA* from clinical strain 4384

Chromosomal DNA from *P. aeruginosa* strain 4384 was digested with *Eco*RI and *Pst*I and ligated into the corresponding sites of pKK223-3. The ligation mixture was then transformed into *E. coli* strain JM109. Colony hybridization using the internal 1.53kb *toxA* fragment as a probe was performed to identify positive clones. Southern Blot analysis was carried out as described using the internal 1.53 kb *Bam*HI *toxA* sequence as a ^{32}P labelled probe. Hybridization was carried out in 4X SSPE (35.03 g of NaCl, 5.52 g of NaH_2P0_4 . H_20, 1.48 g of EDTA pH 7.4) containing 100 μg/ml of sheared denatured salmon sperm DNA at 65°C for 15 hours. The next morning, the surplus of probe was washed firstly in a solution consisting of 2X SSC/1% SDS two times for ten minutes each at room temperature. Next, the membrane was washed in 1X SSC/0.1%SDS for 15 minutes at 65°C. The final washes were also done at 65°C in 0.1X SSC/0.11%SDS for 10 minutes. Hybridization products were visualized by autoradiography.

2.5.11 Sequencing of the *toxA* gene

Various subclones were generated from pCG5 using specific restriction enzymes purchased from Gibco-BRL. All DNA fragments were subcloned into the pUC12 (Pharmacia) vector and sequenced using universal primers via the dideoxy chain termination method by the University of Calgary Core DNA services, Calgary, Canada.

2.5.12 Site-Directed Mutagenesis

The *EcoR*I-*Pst*I digested *toxA* fragments from pMS151 and pCG5 were cloned separetely into the *EcoR*I-*Pst*I site of the pALTER-1 vector (Promega). The resulting plasmids, pALTER (4384*toxA*) and pALTER (WT*toxA*), were isolated by the alkaline lysis plasmid isolation method and the constructions were confirmed using restriction endonucleases. Two mutagenic oligos were used for creating mutations in pALTER (WT*toxA*). The first mutagenic oligo, $^{5'}$(GAA CCC GAC GCG CGC GGC CGG)$^{3'}$, was used to change Ala-476 to Glu-476 and the second mutagenic oligo, $^{5'}$(TAC CGC ACC GGC CTG ACC CTG GCC)$^{3'}$, was used to substitute the Ser-515 for Gly-515. Two repair oligos were used for restoring the mutations observed in the *toxA* gene from pCG5 to the wild type genotype in pALTER (4384*toxA*). The first repair oligo, $^{5'}$(GAA CCC GAC GCA CGC GGC CGG)$^{3'}$, was used to restore the Glu-476 to an Ala-476 and a second oligo, $^{5'}$(TAC CGC ACC GGC CTG ACC CTG GCC)$^{3'}$, changed Gly-515 back to an Ser-515. The dsDNA from both pALTER (4384*toxA*) and pALTER (WT*toxA*) were alkaline denatured. The mutagenic and repair oligos were then annealed to the ssDNA. As a control, an ampicillin repair oligo and a tetracycline knockout oligo were added to each reaction. The mutant strand was synthesized using T4 DNA Polymerase and T4 Ligase. The *E. coli* ES1301 *mutS* strain was used for transforming the mutagenesis reactions. The transformants were screened on LB Amp-100 plates and on LB Tet-10 plates. Positive transformants were identified by growth on Amp-100 and sensitivity to tetracycline.

2.6. PCR Methodology.

2.6.1. PCR Amplification

Amplification of domain III of *toxA* was performed using Ultma polymerase (Perkin-Elmer Cetus), which is a thermostable polymerase which possesses 3' to 5' proofreading ability, with primers complementary to the known DNA sequence. The PCR reaction was divided in two main steps which included a lower and an upper mix. The lower mix consisted of 1.25 µl of 10X Ultma buffer, 4 µl of 25 mM Mg Cl_2, 4µl of mixed dNTP's (10mM of each dNTP), 2 µl of each primer (20µm) and 0.25 µl of water. The first amplified product was generated with a forward KF10 primer $^{5'}$(TGC TGG AGC GCA ACT ATC CCA)$^{3'}$, which comprised nucleic acids 1986-2006 of *toxA*, and a reverse KR12 primer $^{5'}$(GCT CGA GCG CGG CAC ATA GAC)$^{3'}$ that consisted of nucleic acids 2321-2341 of *toxA*. These primers allowed the amplification of a 358 bp fragment, which is located in the upstream half of the enzymatic domain of *toxA*. The remaining half of the enzymatic domain was amplified as a 328 bp product using an upstream KF6 primer $^{5'}$(TCG AGC CTG CCG GGC TTC TAC)$^{3'}$ which encompass nucleic acids 2336-2356 of *toxA*, and a reverse CL1 primer $^{5'}$(TTA CTT CAG GTC CTC GCG CGG CGG TTT)$^{3'}$ which cover nucleic acids 2636-2662 of *toxA*. An AmpliWax PCR Gem 100 pellet (Perkin Elmer) was added to each PCR tube and passed through a cycle which allowed the melting of the wax at 80°C for 5 minutes and a slow cool down to 25°C to allow the wax to solidify. The upper mix (21 µl of water, 5µl of 10X Ultma buffer, 10 µl of chromosomal DNA (diluted 1/1000) and 0.5 µl of Ultma enzyme was added on top of the hardened wax. Each PCR tube was pre-

incubated at 98°C for 8 minutes prior to amplification. The PCR tubes were then submitted to 30 PCR amplification cycles which consisted of a denaturation step at 98°C for 1 minute, an annealing period of 1 minute at 60°C and a DNA elongation temperature of 72°C for 1 minute. A negative control with water in place of template was also included during each amplification to make sure there was no DNA contamination. After the amplification cycle was complete, 5 µl was electrophoresed on an agarose gel to ensure amplification and for visualization of product.

2.6.2. Cloning of PCR products

After confirmation of the amplified fragment on an agarose gel, the PCR product was cloned into a pCR2.1™ cloning vector (Invitrogen). For optimal ligation, this was performed the same day as the amplification to make sure that the $^{3'}$A-overhangs on the PCR product were not lost. The ligation reaction contained 2 µl of pCR2.1™ vector (25 ng/ul), 1 µl of 10X ligation buffer, 1 µl of T4 DNA ligase and 6 µl of PCR product diluted in water to a ratio of 1:2 (vector: insert). This ligation reaction was incubated overnight at 16°C. The following morning the ligation mix was transformed into *E. coli* competent strain JM109. The transformants were spread onto $LBAmp_{100}$ plates supplemented with 10µl of 1M IPTG (Gibco/BRL) and 40 µl of 20mg/ml of X-gal (Gibco/BRL) dissolved in dimethylformamide (BDH). Mutations identified in domain III were confirmed by sequencing the cloned chromosomal DNA.

2.6.3 Arbitrary-Primed PCR

The AP-PCR reaction based on the procedure by Caetano-Anolles (1993) was divided in two main reactions. The original reaction consisted of 5 µl of 10X Taq PCR reaction buffer, 4 µl of 25 mM $MgCl_2$, 4µl of dNTPs (25mM of each dNTP), 2 µl of ARB1 primer (GGCCACGCGTCGACTAGTACNNNNNNNNNGATAT) (1 µm), 2 ul of genomic DNA (1/100 dilution) and 0.5 µl of Taq polymerase in a total volume of 50 µl. The PCR conditions used were: 95°C for 5 minutes followed by 5 cycles of 94°C for 30 seconds, 30°C for 30 seconds and 72°C for 1.5 minutes. Thirdy cycles of 94°C for 30 seconds, 45°C for 30 seconds and 72°C for 2 minutes were performed, ending with a final step at 72°C for 5 minutes, and holding at 4°C. The second reaction mix consisted of 1µl of the initial reaction to eliminate non-specific bands, 5 µl of 10X Taq PCR reaction buffer, 4 µl of 25 mM $MgCl_2$, 4µl of mixed dNTPs (25mM of each dNTP), 1 µl of ARB2 primer (GGCCACGCGTCGACTAGTAC) (1 µm), and 0.5 µl of Taq polymerase in a total volume of 50 µl. The PCR cycle for the second reaction was as follow: Twenty-nine cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1.5 minutes. This was followed by a final extension at 72°C for 5 minutes and holding the reaction at 4°C. Ten µl of the reaction was run on a polyacrylamide to visualize the PCR product pattern for each strain tested.

2.7. Protein Methodology

2.7.1. SDS-polyacrylamide gel electrophoresis analysis

Proteins were separated by the method of Laemmli (1970) using a 5% stacking gel and a 10% resolving acrylamide gel. The resolving gel was prepared with 30.3 ml of H_2O, 24.3 ml of 30% acrylamide (Gibco/BRL), 18.75 ml of Tris-HCl (pH 8.8), 750 µl of 10% SDS, 750 µl of 10% ammonium persulfate (Bio-Rad) and 100 µl of TEMED (Bio-Rad). The stacking gel was composed of 6 ml of 30% acrylamide solution, 300 µl of 10% SDS, 7.5 ml of 0.5M Tris (pH 6.8), 15.6 ml of H_20 and was polymerized with 30 µl of TEMED and 300 µl of 10% ammonium persulfate. For determination of ETA expression between the CF isolates, 20 µl of the supernatant of each strain previously grown in low iron conditions to an $O.D_{540}$ of 4 were used. To determine the specific activity of strain 4384 and PA103, the supernant from both strains were concentrated. Supernatants isolated from strains 4384 and PA103 were concentrated using Microcon Centrifugal Filter Devices (Amicon). Briefly, the supernatant of each sample was collected at $O.D_{540}$ of 4.0. Five hundred µl of supernatant was applied to the filters that possess a M_r 10,000 cut-off range and centrifuged at 13000 RPM for 30 minutes. The filter containing the concentrated samples was inverted and recentrifuged for another 15 minutes. The retentate was then applied to a SDS-PAGE gel.

In order to approximate the molecular weight of the proteins, a prestained standard of known molecular weight (Bio-Rad/BRL) was electrophoresed on each gel. As a positive control, 1 µl of the Swiss Serum purified exotoxin A

protein was also included in each gel. Duplicate gels were electrophoresed at 125 volts in a Gibco BRL mini-V8-10 Vertical System (Gibco/BRL). The SDS electrophoresis buffer was prepared with 6.05 g Tris, 28.8 g Glycine and 2 g of SDS per 2 liters. After electrophoresis, one gel was stained for one hour at room temperature in 0.5% Coomassie Brilliant Blue (Sigma Chemical co.). The gel was then destained for at least 5 hours in 30% methanol, 10% acetic acid. The duplicate gel was then used for Western Blot analysis.

2.7.2. Western Blotting

Immunoblots were performed according to the method of Towbin *et al.* (1979). Briefly, the proteins were transferred to a nitrocellulose membrane in ice chilled transfer buffer (1.875 g of Tris, 1.525 g of Tris-HCl, 14.425 g of glycine and 50 ml of methanol per liter) at 100 volts using a Gibco BRL mini-V8-10 Vertical System (Gibco/BRL). The membrane was then immersed in 50 ml of a 1% skim milk solution in Tris Saline Buffer (1.32 g of Tris-HCl, 9 g of NaCl and 0.194 g of Tris per liter) for at least 1 hour to block non-specific binding. After blocking the membrane, antibodies to ETA (graciously provided by Dr. J. Olson) were used at a 1:2000 dilution in 1% skim milk and incubated at 37°C for 1 hour. The membrane was then washed three times with 100 ml of tris saline buffer. Specific reactions were detected by incubation at 37°C for 1 hour with the secondary antibody, anti-rabbit IgG coupled with horseradish peroxidase (Sigma Chemical co.). Color was developed with a solution of tris saline containing 3mg/ml of horse radish peroxidase (Sigma Chemical co.) in methanol.

2.7.3. Mixed toxin experiments

Strains 4384, PA103 and PA103*toxA*::Ω were grown in low iron conditions to an A_{540} of 4. ten µl of supernatant were then collected and mixed with ten µl of the purified Swiss Serum toxin (1/100 dilution). The samples were then incubated for up to six hours at 32°C. The ADP-ribosyl transferase activity was assayed as previously described by Chung and Collier (1977).

2.7.4. Expression of ETA in pQE-32 vector

2.7.4.1. Construction of the expression vectors

The *BamH*I-*EcoR*I *toxA* gene from pUCSF (4384*tox*A) and pUCSF (WT*tox*A) was cloned in the *Bam*HI-*Pst*I site of the pQE-32 vector in two separate steps. First, both plasmids were digested with *Bam*HI and *EcoR*I to isolate the C-terminal end of the *toxA* gene. The 454 base pair fragment was isolated using a GENE Clean Kit and ligated into the *Bam*HI-*Pst*I site of the pQE-32 vector using an *Eco*RI-*Pst*I linker (AATTTGCA). This construct was then digested with *Bam*HI and CIAP treated to reduce vector self-religation. At the same time, both pUCSF (4384*tox*A) and pUCSF (WT*tox*A) were digested with *Bam*HI. The 1.53 kb internal *toxA* gene was isolated from both plasmids and ligated into the *Bam*HI site of the dephosphorylated vector. Both pQE-32 (4384*toxA*) and pQE-32 (WT*toxA*) were digested with several restriction enzymes to verify that the *toxA* fragment was cloned in the correct orientation.

2.7.4.2. Rapid Screening of Small-scale expression of pQE-32 (4384 *toxA*) and pQE-32 (WT*toxA*) in M15

Both strains were streaked on LB-Amp100 and Km-25 plates and incubated overnight at 37°C. The following day a single colony was used to inoculate 10 ml of LB supplemented with Amp100 and Km-25. The next morning, 1.25 ml of the overnight-saturated culture was used to inoculate 8.75 ml of fresh LB media with Amp100 and Km-25. The culture was incubated with shaking for one hour before induction with IPTG to a final concentration of 2mM. Before induction, 1 ml of the culture was transferred to a microcentrifuge tube. The tube was centrifuged for 30 seconds at 13 000 rpm. The pellet was saved at –20°C and referred to as sample t=0 (before induction). After induction, 1 ml samples were collected every hour for 4 hours. Each sample was centrifuged for 30 seconds, and the pellet was collected and saved at –20°C. When all the samples were collected, each was resuspended in 200 µl of Buffer B (8M urea, 0.1 M Na-phosphate, 0.01 M Tris/HCl pH 8). The cells were lysed by gentle vortexing until the solution became translucent. The cellular debris was then pelleted for 10 minutes at 13 000 rpm. Next, 50 µl of a 50% slurry of Ni-NTA resin (Qiagen) were added to each sample and mixed gently for 30 minutes at room temperature. The resin was then centrifuging by spinning for 10 seconds at 13 000 rpm. The resin containing the bound ETA was washed three times with one ml of Buffer C (Buffer B but pH adjusted to 6.3 with HCl). Twenty µl of Buffer C/100mM EDTA was then added to each tube. The EDTA is able to chelate the Ni^{2+} ions from the

NTA resins thus, eluting the bound proteins. Each tube was centrifuged for 10 seconds at 13 000 rpm, and 20 µl of the supernatant containing the ETA protein was transferred to a fresh microcentrifuge tube. Each sample was then analyzed by SDS-PAGE analysis and Western immunoblotting using anti-ETA antibodies.

Chapter 3

Characterization of the altered ADPRT activity of ETA isolated from *P. aeruginosa* clinical strain 4384

3.1 ETA production and corresponding ADP-ribosyl transferase activity of selected *P. aeruginosa* isolates from two chronically infected CF patients

The population transcript accumulation was examined from a number of CF patients over a 3 to 4 year period and it was found that patients with more severe lung disease tend to harbor bacterial populations that transcribe higher levels of *toxA* (Storey *et al.*, 1994, Raivio *et al.*, 1994). Accordingly, *P. aeruginosa* isolates from two of these patients were examined to determine if they produced higher levels of ETA under laboratory conditions. All six strains examined produced low levels of ETA activity based on an ADP-ribosyl transferase assay when compared to the hypertoxigenic strain PA103 (Table 2). Interestingly, despite higher levels of transcription for ETA, in all of the strains which were isolated from the sputa of the two patients, the ETA produced exhibited a drastically reduced ADPRT activity. In addition, Ujack and Rabin (data not shown) measured the amount of ETA produced by the six CF isolates using an ELISA assay (unpublished data). Their data indicated that 4 out of 6 CF isolates produced similar levels of ETA protein when compared to the hypertoxigenic laboratory strain, PA103. CF strains 4384 and 5154 were isolated from patient FH1 and secreted more than 200 μg/ml of ETA.

Table 2: ADPRT activity of *P. aeruginosa* CF isolates

Clinical isolates and laboratory strains of *P. aeruginosa*	ADP-ribosyl transferase activity (CPM·10⁻¹)
CF isolate, strain 4384 (FH1)	**90**
CF isolate, strain 5154 (FH1)	**61**
CF isolate, strain 5166 (FH1)	**86**
CF isolate, strain 5552 (FH2)	**78**
CF isolate, strain 5585 (FH2)	**28**
CF isolate, strain 5588 (FH2)	**65**
Laboratory strain, PA103	**1649**
Laboratory strain, PA103toxA::Ω	**44**

Strains 5552 and 5585 were isolated from patient FH2 and produced more then 400 μg/ml of ETA calculated by ELISA. Taken together, these results indicate that 4 out of 6 CF isolates produced 2 to 4 times more ETA than the hypertoxigenic strain PA103.

Arbitrarily primed PCR and Southern blot analysis was performed on the six isolates to determine if they are independent strains since they were isolated from only two patients. With the AP-PCR (Caetano-Anolles (1993)) we could differentiate four amplification patterns among the six strains (data not shown). The three strains from patient FHI (4384, 5154, 5166) share the same amplification pattern (data not shown). PCR product variability was observed with the strains isolated from the second patient FH2, (5552, 5585, 5588) (data not shown). Furthermore, comparison of the amplification products of all six strains obtained from both patients indicated that each patient is colonized with different strains. To confirm this result, Southern hybridization was used on the genomic DNA of each strain. For Southern analysis, the 741 base pair *PstI-NruI* fragment that encodes the highly variable upstream region of the *toxA* gene was used to distinguish each clinical isolates (Ogle et al., 1987). The genomic DNA of each strain was digested with six restriction endonucleases and probed with the upstream *PstI-NruI* fragment. Analysis of the hybridization patterns with the probe-reactive fragment allowed for the identification of five different strains. The restriction fragments identified by Southern hybridization were identical for strain 5154 and 5166. In comparison, the restriction fragments for strain 4384, 5552, 5585 and 5588 were distinguishable. Therefore, isolates 5154 and 5166 represent two clones of the same strain whereas strain 4384, 5552, 5585 and 5588 are unique strains (Table 3).

Table 3: Typing of six CF isolates

Isolate	AP-PCR[a]	*PstI-NruI* probe[b]	Unique strains
4384	**1**	**1**	**1**
5154	**1**	**2**	**2**
5166	**1**	**2**	**2**
5552	**2**	**3**	**3**
5585	**3**	**4**	**4**
5588	**4**	**5**	**5**

a: The AP-PCR method identified four different amplification patterns between the six isolates.

b: Using the upstream *PstI-NruI toxA* probe, five different patterns of hybridization were identified by Southern Blot analysis among the six isolates.

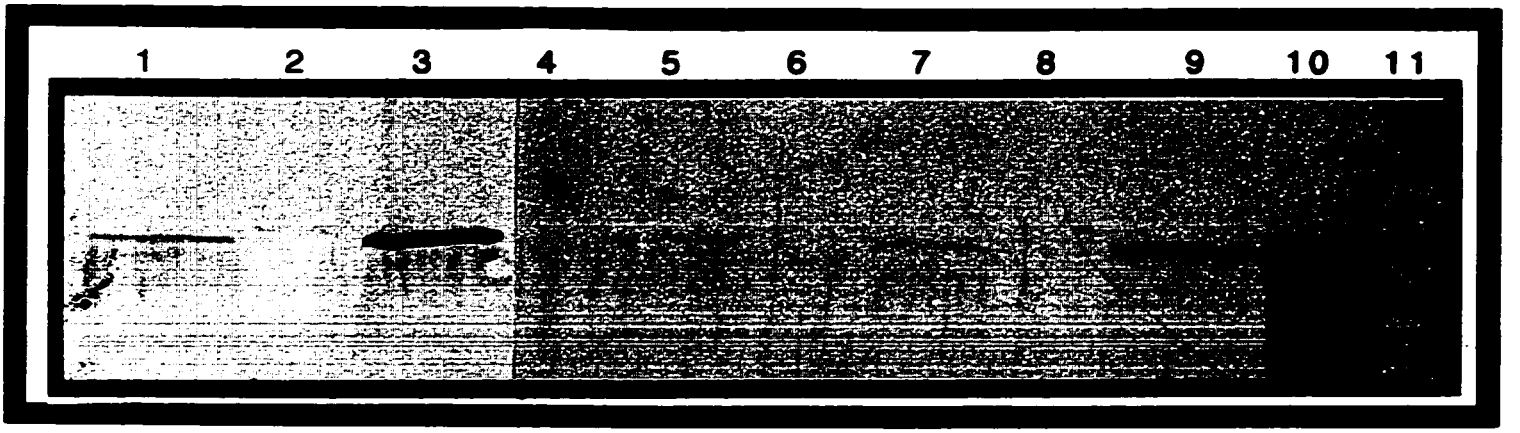
c: The combination of both techniques allowed for the identification of five distinct strains among the six CF isolates.

Since ETA is implicated in the deterioration of the pulmonary function of CF patients, it was of interest to further analyze the production of ADPRT deficient exotoxin A. The most appropriate way to calculate the amounts of ETA produced by the CF isolates would be to measure intact protein rather than using ELISA which measures both intact protein and inactive fragments. To confirm that intact ETA was being produced by the six CF strains with low ADPRT activity, Western Blot analysis using anti-ETA antibodies was performed on the supernatant of all six CF strains previously grown in low iron conditions (Figure 2). It was possible to identify an immunoreactive band of 66 kDa in the supernatants of CF strains 4384, 5166, 5552, 5585 and 5588. This indicates that the ETA protein is expressed and secreted in these five strains. No detectable bands were observed in the supernatant of strain 5154. The lack of an ETA immunoreactive band in this CF isolate may indicate that it produces either an unstable ETA or none at all. However, the amounts of ETA detected by immunoblot analysis do not correlate with the level of ETA previously calculated by Ujack and Rabin (unpublished data). The higher level of ETA detected by the ELISA methodology is probably due to higher levels of protein degradation occurring in CF isolates. Furthermore, it was observed that the levels of ETA produced by strain 4384 and 5585 are comparable to strain PA103.

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

To confirm the molecular basis for the difference in activity of ETA from the six CF isolates compared to that of PA103, we decided to further characterize the production and enzymatic activity of the expressed ETA by one CF isolate, strain 4384. Strain 4384 was chosen for in depth analysis because the level of

Figure 2. Western Blot analysis of secreted proteins of *P. aeruginosa* isolates. All strains were grown overnight in TSBDC. Western blot analysis of the supernatant of each sample was performed using purified anti-ETA antibodies (Olson, unpublished data). Lane 1: CF strain 5166. Lane 2 and 6: inactive laboratory strain PA103*toxA*::Ω. Lane 3: CF strain 4384. Lane 4: prestained Gibco/BRL marker. Lane 5: CF strain 5552. Lane 7: CF strain 5588. Lane 8: CF strain 5154. Lane 9: CF strain 5585. Lane 10: Purified ETA (Swiss Serum Institute). Lane 11: Hypertoxigenic laboratory strain PA103.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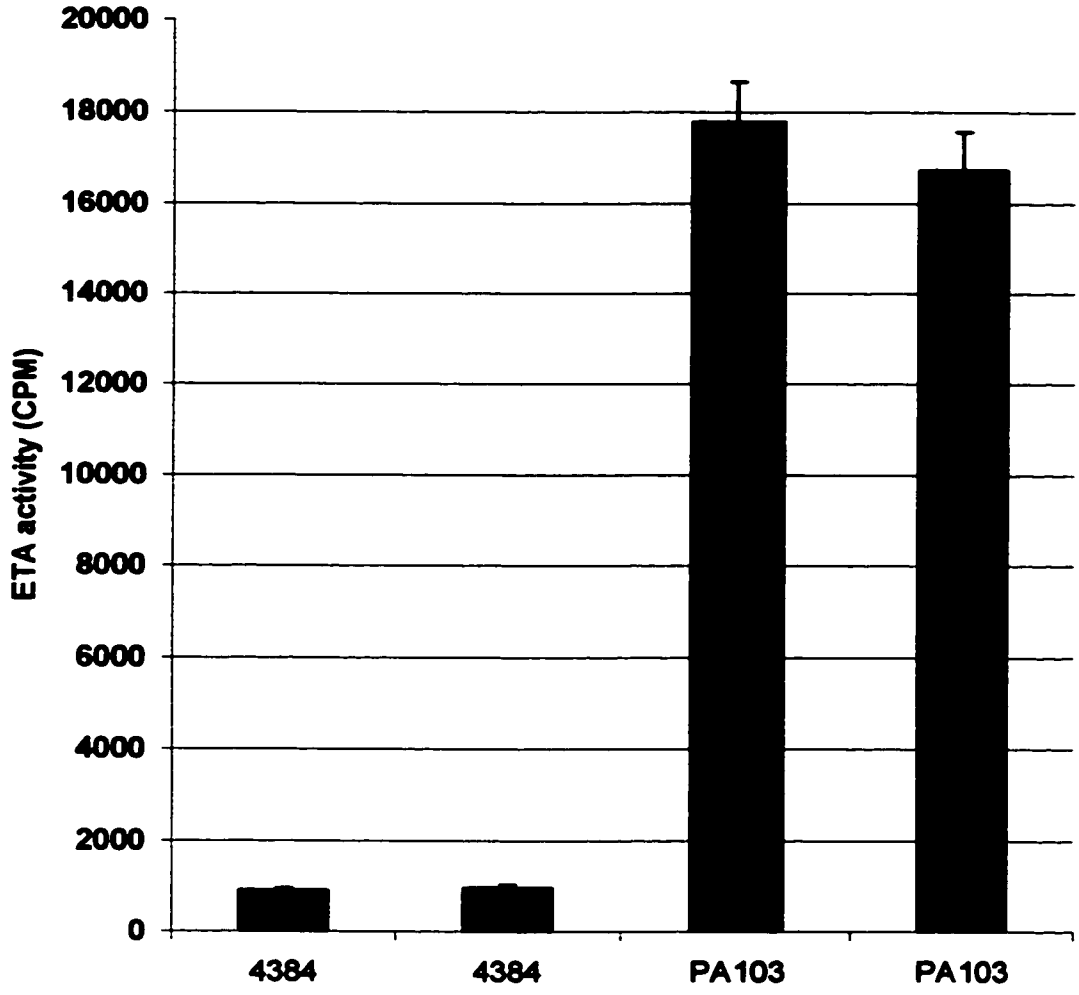
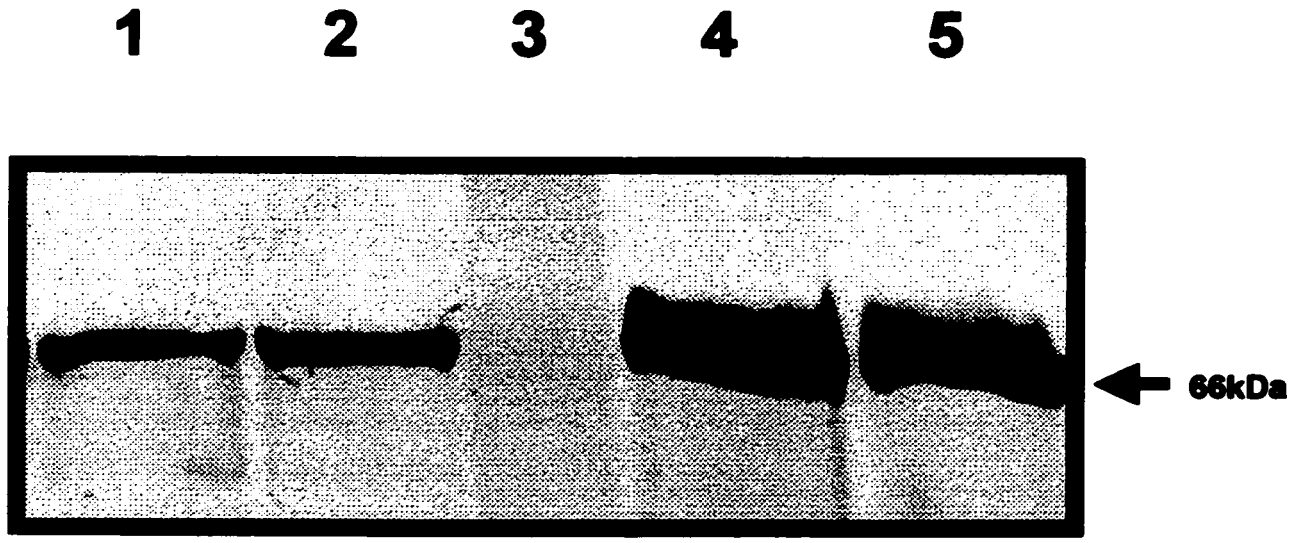
11

transcription of *toxA* is similar to that of the hypertoxigenic strain PA103 (Raivio *et al.*, 1994). In order to make a proper comparison of the difference in ADPRT activity we needed to calculate the specific activity of intact ETA protein from strain PA103 and strain 4384. To analyze the production of ETA from 4384 it was necessary to concentrate the toxin using a microconcentrator filter (Amicon). As a control ETA from strain PA103 was concentrated using the same methodology. ADPRT assays revealed that concentrated ETA from strain 4384 had reduced enzymatic activity compared to ETA from strain PA103 (Figure 3A). Subsequently, Western Blot analysis using anti-ETA antibodies was performed on the concentrated supernatant of strain 4384 and PA103 grown in low iron conditions (Figure 3B). Figure 3B shows that significantly less ETA was concentrated from strain 4384 as compared to strain PA103 even when the two strains were grown under comparable conditions. The results from Figure 3 were used to calculate the specific activity of the two ETA preparations. Briefly, the concentration of ETA from both strains was determined using the Mac Bast software. We initially believed that strain 4384 had no detectable ADPRT activity, however, additional analysis indicated a drasic reduction in ADPRT activity from strain 4384. The specific activity of ETA from PA103 was determined to be 58,898 cpm/μg protein, whereas the specific activity of ETA from strain 4384 was only 5,104 cpm/μg protein. Thus, the concentrated but unpurified ETA produced by strain 4384 had an approximately 12-fold reduction in its enzymatic activity when compared to ETA from strain PA103.

Figure 3. ADPRT activity and Western Blot analysis from strain 4384 and PA103. *Pseudomonas aeruginosa* strains PA103 and 4384 were grown in iron depleted TSBDC to $O.D._{540}$ of 4. (3A) ADPRT assays as described by Chung and Collier (1977) were performed on culture supernatant. Culture supernatant (10μl) was used for strain 4384 and 5 μl for strain PA103. The assays were performed in triplicate and the results averaged. (3B) Subsequently, Western Blot analysis was performed on 10 μl of the same supernatant from each strain using purified anti-ETA antibodies. Lanes 1 and 2 represents 10 μl of supernatants from strain 4384. Lanes 4 and 5 show 10 μl of supernatants from strain PA103. The Benchmark™ prestained marker is shown as a reference separating lanes 2 and 4.


20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
0
ETA activity (CPM)
4384
4384
PA103
PA103

A.



B.

3.3 Cloning and sequencing of *toxA* from clinical isolate 4384.

To determine the nature of the inactivated toxin produced by strain 4384, the entire *toxA* gene from strain 4384 was cloned in pKK223-3 (Figure 4). Approximatively one thousand transformed *E. coli* colonies were screened by colony hybridization and one was found that hybridized strongly with the internal *toxA* probe. This putative clone was saved in glycerol and labeled pCG5. The presence of *toxA* into pKK223-3 was confirmed using Southern Blot analysis (Figure 5). The putative clone pCG5 was digested with *Bam*HI, *Eco*RI and *Eco*RI-*Pst*I and hybridized to the 1.53 kb *Bam*HI internal *toxA* fragment isolated from pMS151. The internal *toxA* probe hybridized with the appropriate sized fragments from pCG5 indicating that the *toxA* gene from strain 4384 had been cloned. Sequence analysis of the *toxA* gene from strain 4384 was performed in order to investigate the possibility of mutations in the enzymatic domain of the ETA protein. In order to facilitate sequencing of the entire *toxA* gene, the *Eco*RI-*Pst*I *toxA* gene from pCG5 was inserted into a pUC12 vector. The resulting plasmid was called pUC12 (*Eco*R1-*Pst*I 4384 *toxA*). Subsequently, smaller fragments that spanned the entire *toxA* coding region were produced using specific restriction endonucleases (Figure 6). All fragments were subcloned into pUC12 and sequenced at the University of Calgary Core DNA Service. Each fragment was sequenced in both directions using the M13 Reverse and Forward commercial primers. Sequence analysis was done using the Blast server and software through NCBI. The nucleotide sequence of 4384 *toxA* gene was compared with the published sequence taken from the *toxA* gene from *P. aeruginosa* strain PA103 (Gray *et al.*, 1984)

It has been previously demonstrated that several amino acids in the enzymatic domain of ETA are essential for ADPRT activity. Chow *et al.* (1989) and Siegall *et al.* (1989) demonstrated that amino acids 400-608, were sufficient

Figure 4. Construction of pCG5: cloning of the *toxA* gene from strain 4384 into pKK223-3 vector. Chromosomal DNA isolated from strain 4384 was digested overnight with *Eco*RI-*Pst*I. The following morning the digested DNA was ligated into the *Eco*RI-*Pst*I site of pKK223-3 and transformed into *E. coli* strain JM109. The putative transformants were picked on LB-Amp100 plates and hybridized with the 1.53 kb *Bam*HI *toxA* DNA fragment. A positive control containing the wild type *toxA* gene from pMS151 was also included on each plate as a positive control. One putative clone labeled pCG5 hybridized strongly with the *toxA* fragment. To confirm the correct construction; pCG5 was further analyzed.

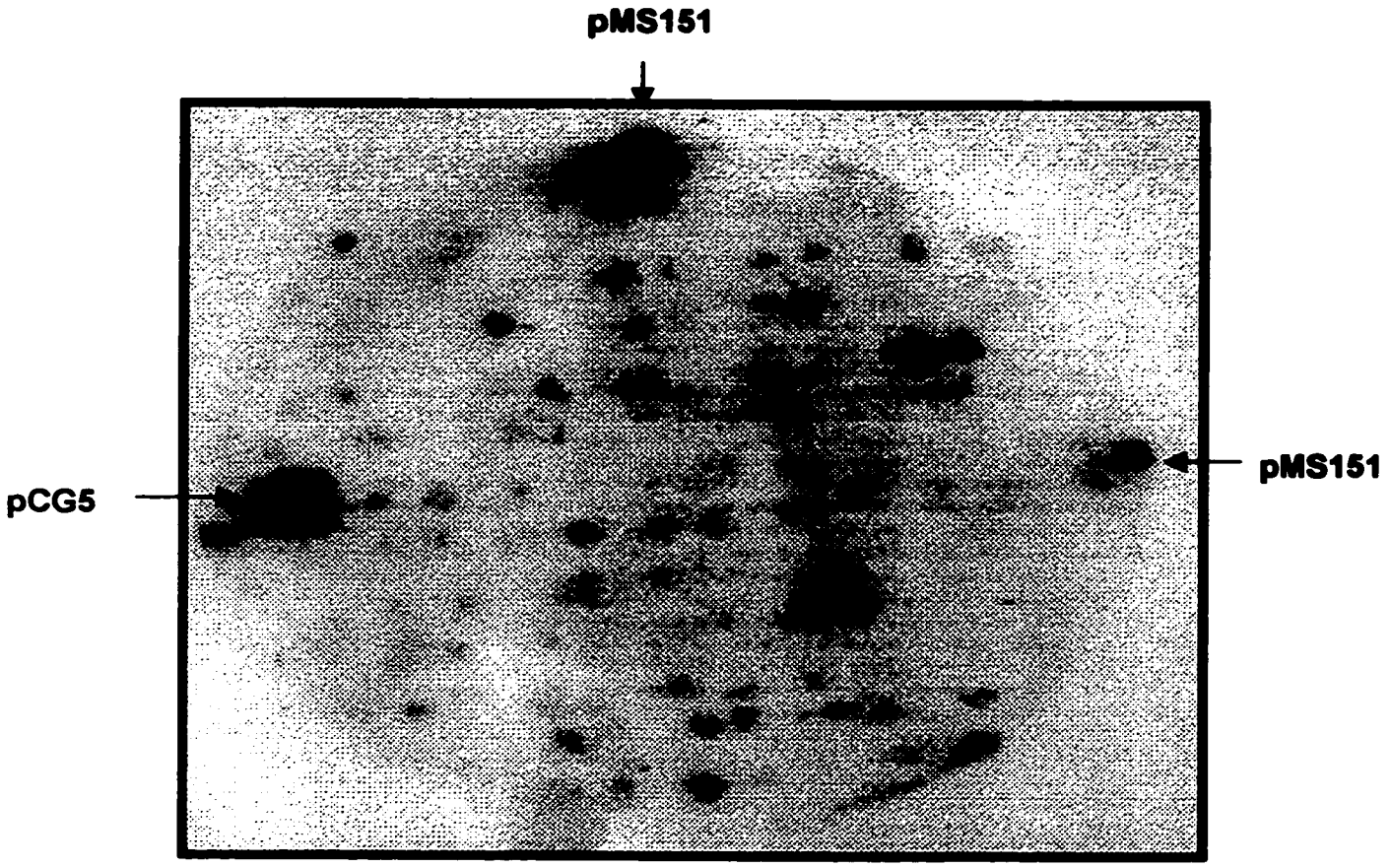

pMS151
pCG5
pMS151

Figure 5. Southern Blot analysis of pCG5 DNA digested with restriction enzymes and hybridized with a 1.53 kb *Bam*HI *toxA* probe. DNA from pCG5 was digested with restriction endonucleases and separated on a 0.8% agarose gel. After electrophoresis, the gel was transferred overnight onto nitrocellulose membrane. Hybridization was performed using the labeled 1.53 kb internal *toxA* probe. Lane 1. Positive control: pMS151 (*Bam*HI). Lane 2: pKK223-3 undigested. Lane 3: pCG5 undigested. Lane 4: pCG5 digested with *Bam*HI. Lane 5: pCG5 digested with *Eco*RI. Lane 6: pCG5 digested with *Eco*RI-*Pst*I. The Blot was visualized using the Fuji phosphoimager.

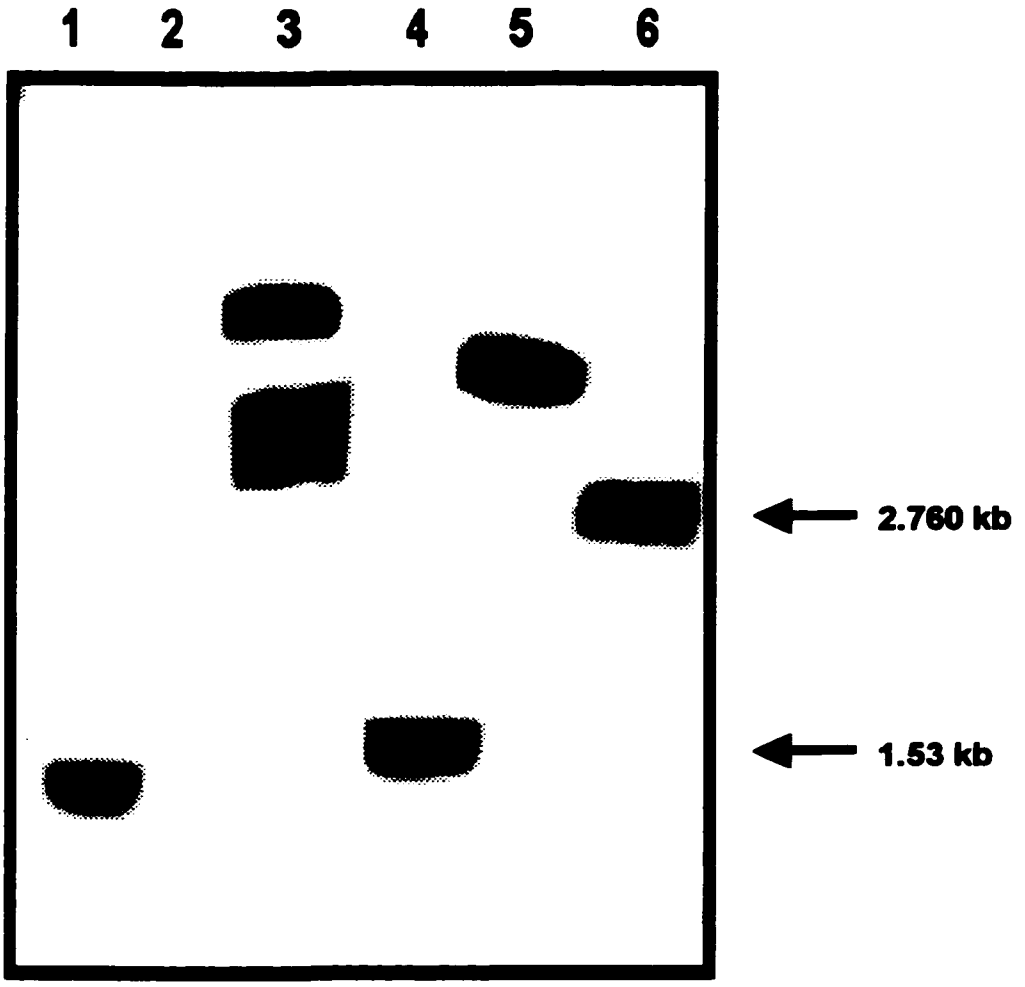
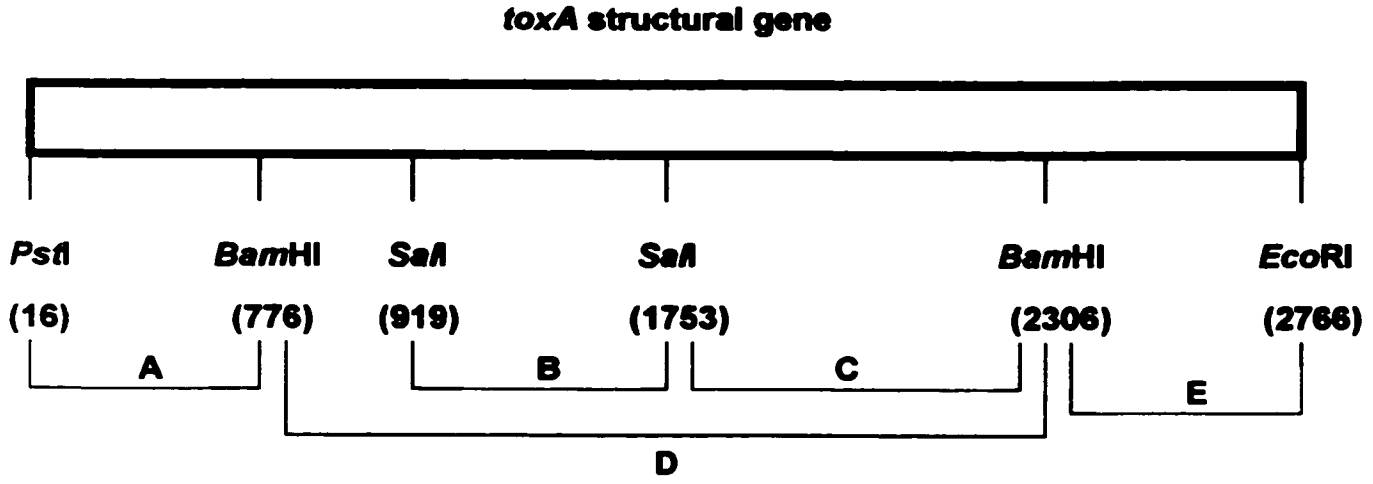

1
2
3
4
5
6
2.760 kb
1.53 kb

Figure 6. Diagrammatic representation showing the location of the different fragments generated from pCG5, by digestion with restriction endonucleases. pCG5 was digested with several restriction enzymes, and the resultant DNA fragments were subcloned into pUC12 and sequenced by The University of Calgary Core DNA services.



A- *Bam*HI-*Pst*I 760 bp fragment

B-*Sal*I 834 bp fragment

C-*Sal*I-*Bam*HI 553 bp fragment

D-*Bam*HI 1530 bp fragment

E-*Bam*HI-*Eco*RI 460 bp fragment

for full enzymatic activity. Therefore, if ADPRT deficient strain 4384 carries inherent mutations in its *toxA* gene responsible for the ADPRT deficient phenotype, they would be located within domain III. Sequence analysis revealed the presence of three mutations in domain III of *toxA* from strain 4384. The first mutation altering Ser-410 to Asn is located outside of the enzyme active site thereby may not affect the interaction of ETA with the reaction substrates, NAD+ and EF-2 (data not shown). The two remaining mutations are located within the active site of ETA and could be involved in catalysis (Figure 7). The first mutation results in a change from a Ala-476 to a Glu. The change of a non-polar amino acid (Ala) for an acidic residue (Glu) may alter the structure of the protein. Interestingly, the mutation is located within an α-helix which is believed to be an important part of the catalytic site (Figure 8). 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. The second mutation, which is at the protein surface, is a substitution of a Gly-515 (non-polar amino acid) for a Ser-515 (polar amino acid) (Figure 8). This amino acid may be essential for protein integrity or be a critical modification site. Neither mutation (position 476 nor 515) had previously been reported to be involved in ADPRT activity. These mutations individually could have an effect on the activity of the protein, or both mutations could act in concert to reduce the ADPRT activity of ETA. The presence of these mutations may affect proper folding of the protein and render the catalytic site inaccessible to the substrate. No further mutations were found in the 4384 *toxA* gene, suggesting that the two mutations found in domain III may account for the observed reduction in ADPRT activity.

To determine which mutation is critical for the enzymatic activity of ETA, an attempt was made to repair the mutations found in active site of the *toxA* gene

of strain 4384 using site-directed mutagenesis. The Altered Sites® II *in vitro* Mutagenesis systems (Promega) was selected since it contain the ability to mutagenize double-stranded template DNA, and can express the mutated gene *in vitro* or *in vivo*. This system exploits antibiotic selection to obtain a high frequency of mutants. The pALTER-1 vector that was used in this study contained two genes for ampicillin and tetracycline selection. However, in this case the ampicillin resistance gene has been inactivated and will be restored during the mutagenesis reaction using an ampicillin repair oligonucleotide. A number of different mutagenic oligonucleotides were designed to repair the two mutations found in the active site of the *toxA* gene of strain 4384, alone or in combination, to the wild type phenotype. Simultaneously, attempts were made to introduce the two mutations found in the active site of the *toxA* gene of strain 4384 into the wild type *toxA* gene. Unfortunately, despite many attempts, neither mutagenic reactions were successful. This could be due to the presence of secondary structure in the cloned *toxA* gene or in the mutagenic oligonucleotides. The presence of secondary structure in the *toxA* gene was previously demonstrated to interfere with the PCR amplification of the entire gene (Dr. R. Merrill, personnal communication). Therefore, to determine which mutations are necessary for the enzymatic activity of ETA an alternative approach should be selected.

Figure 7: Schematic representation of mutations found in the active site of the *toxA* gene from the clinical isolate, strain 4384. The entire *toxA* gene was sequenced, and only two mutations were found in the active site of domain III of ETA. The first mutation is an Ala-476, which is changed for a Glu-476. The second mutation is at Ser-515 and is substitute for a Gly-515.

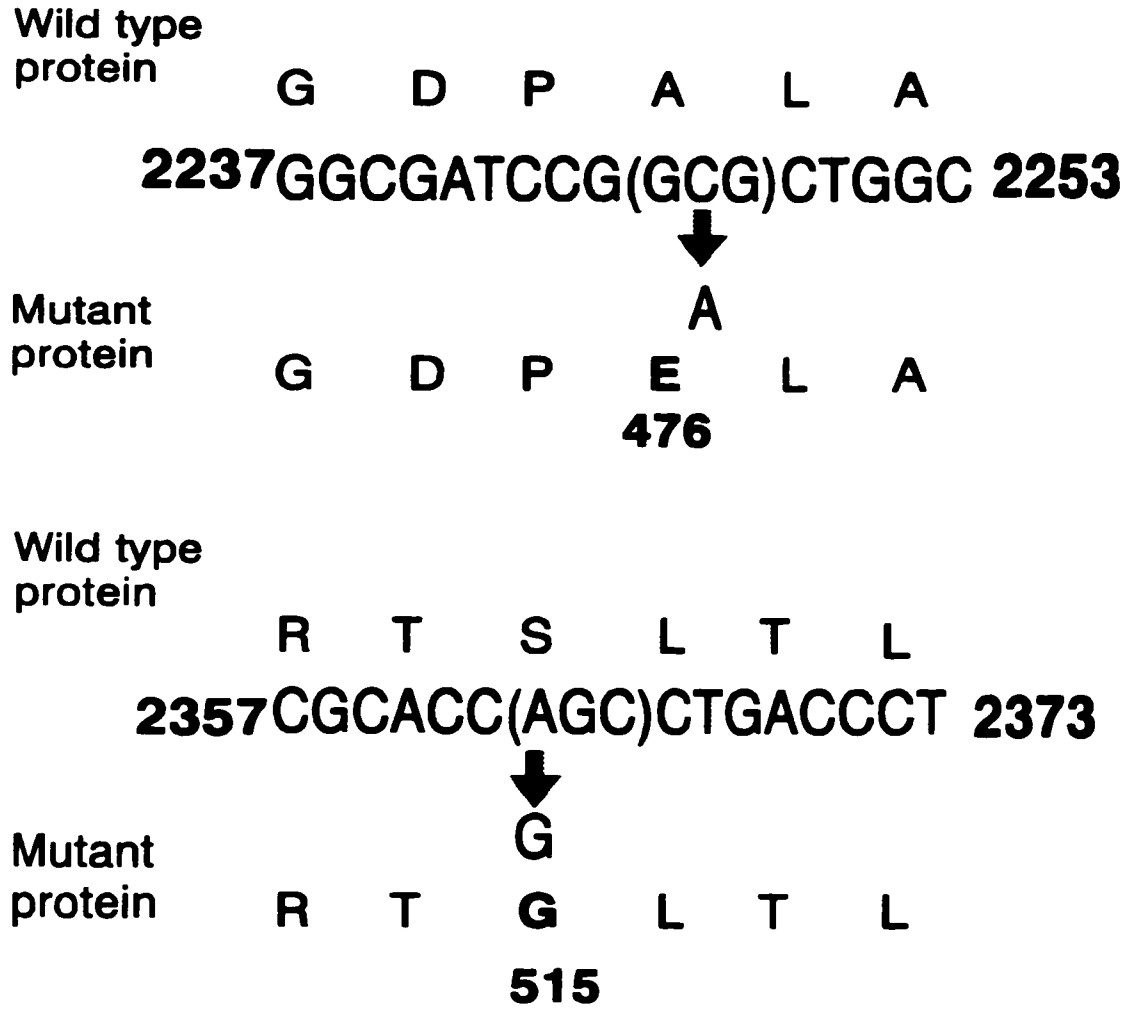
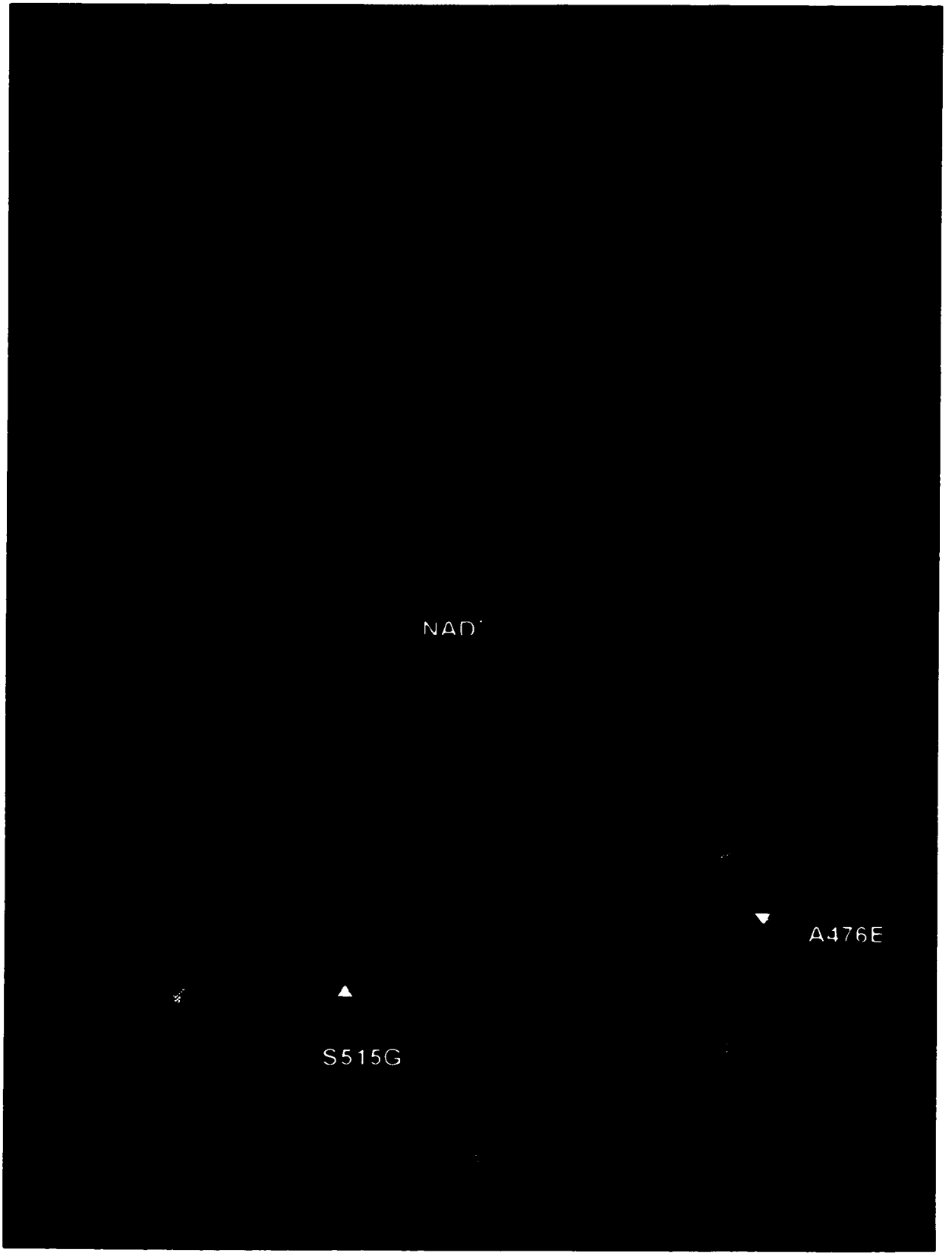

Wild type protein
G D P A L A
2237GGCGATCCG(GCG)CTGGC 2253
A
Mutant protein
G D P E L A
476
Wild type protein
R T S L T L
2357CGCACC(AGC)CTGACCCT 2373
G
Mutant protein
R T G L T L
515

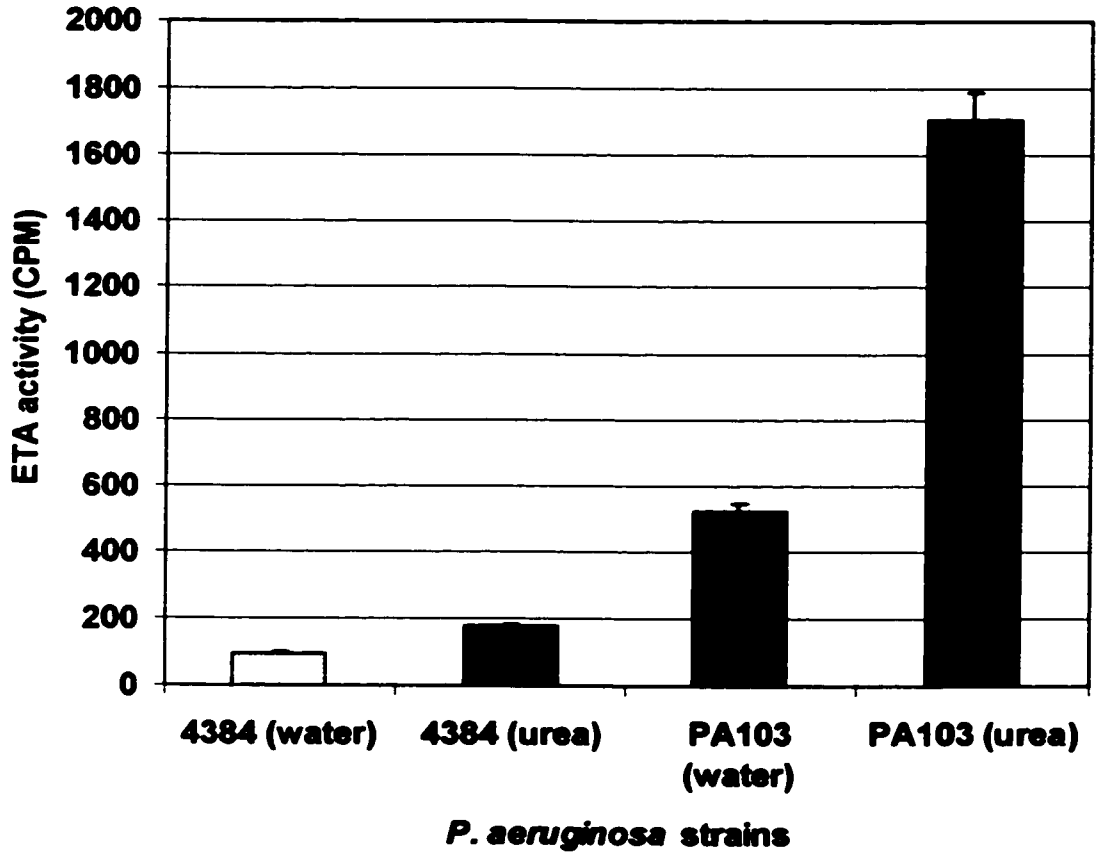
Figure 8. Ribbon diagram of *P. aeruginosa* strain 4384 exotoxin A. According to the domain designations of ETA, residues 476 and 515 are located within the active site of domain III of ETA. The first mutation Glu-476 is designated in orange. The second mutation Gly-515 is labeled in pink. Glu-553 (red color) was found to be critical for enzymatic activity. The NAD^+ substrate molecule is shown in green. The diagram was kindly provided by Dr. A. R. Merrill.


NAD+
A476E
S515G

3.4 Exotoxin A from strain 4384 is efficiently secreted outside the cells

Gray *et al.* (1984) demonstrated that ETA is produced as a 71 kDa protein that contains a 25 amino acid hydrophobic signal peptide that is removed during secretion. ETA is secreted from cells by a two-step mechanism utilizing the generalized secretory apparatus through the inner membrane followed by the xcp machinery of the outer membrane (Gray *et al.*, 1984; Filloux *et al.*, 1990). The identification of two mutations in the enzymatic moiety of domain III of ETA likely explains the observed decrease in ADPRT activity. However, to preclude the possibility of a secretory defect which prevents ETA from being released we decided to determine if the secretory machinery of *P. aeruginosa* strain 4384 was intact. This is a possibility since the ADPRT activity of strain 4384 was calculated from bacterial supernatant and thus only accounts for secreted toxin and not intracellular ETA. Thus, the next step was to measure the intracellular levels of ETA produced by strain 4384. Figure 9 indicates that there is no detectable ADPRT activity inside *P. aeruginosa* strain 4384, even in the presence of urea as an activating agent. In addition, figure 9 also shows that we could detect intracellular ETA in strain PA103. The highest level of enzymatic activity by strain PA103 was obtained when the toxin was activated by addition of urea. Altogether, this experiment demonstrates that a defect in the secretory machinery of strain 4384 is not responsible for the observed reduction of ADPRT activity of 4384 ETA.

Figure 9. Intracellular levels of ETA in strain 4384 and PA103. Strains 4384 and PA103 were grown in low iron TSBDC media. All samples were taken at an $O.D_{540}$ of 4. The cells were pelleted by centrifugation, resuspended in buffer and sonicated three times to lyse the cells to release intracellular stores of ETA. Subsequently, ADPRT assays were performed in triplicate on the cellular contents from each sample (Chung and Collier, 1977). Activation of ETA can be mimicked *in vitro* by treatment of ETA with a reducing agent such as dithiothreitol (DTT) and a denaturing agent such as urea. As a control the denaturing agent was replaced by the same volume of water in one sample for each strain tested.


2000
1800
1600
1400
1200
1000
800
600
400
200
0
ETA activity (CPM)
4384 (water)
4384 (urea)
PA103 (water)
PA103 (urea)
P. aeruginosa strains

3.5 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, strains 4384 and PA103*toxA*::Ω were complemented with the wild type *toxA* on plasmid pMS151-1. PA103*toxA*::Ω serves as a negative control because ETA cannot be expressed due to the insertion of the omega fragment in the *toxA* gene. Figure 10 shows a comparison of the ADP-ribosyl transferase activity from the two complemented strains. Figure 10 shows that even though 4384 was complemented by the wild type *toxA*, it still produced less ADPRT activity than strain PA103*toxA*::Ω. 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. This evidence does not preclude the possibility of an accessory factor in strain 4384 which may inactivate the ETA. Therefore, additional complementation studies were performed to further explore this possibitity. Complementation *in trans* of strain PA103*toxA*::Ω with the wild type *toxA* gene from pMS151-1 allowed expression of a 66 kDa protein which reacted with anti-ETA antibody (Figure 11). Once PA103*toxA*::Ω was complemented *in trans* with pCG (tox-SF) it also produced a 66 kDa protein which reacted with anti-ETA antibodies (Figure 11, Lane 2).

Figure 10. Complementation of strain 4384. The plasmid pMS151-1 (Hamood *et al.*, 1989) which contains the wild type *toxA* gene and the 1.8 kb *Pst*I stabilizing fragment permitting replication in *Pseudomonas* was electroporated into 4384 and PA103*toxA*::Ω and the resulting strains were analyzed for ETA activity. ADPRT activity was determined as described by Chung and Collier (1977) by assaying 10 μl of supernatant from each strain grown in iron depleted TSBDC media to $O.D_{540}$ of 5. Lane 1 represents the ADPRT activity of strain PA103*toxA*::Ω. Lane 2 indicates the enzymatic activity of the complemented stain PA103*toxA*::Ω (pMS151-1). Lane 3 demonstrates the level of activity in strain 4384. Lane 4 represents the ADPRT activity of the strain 4384 complemented with pMS151-1.

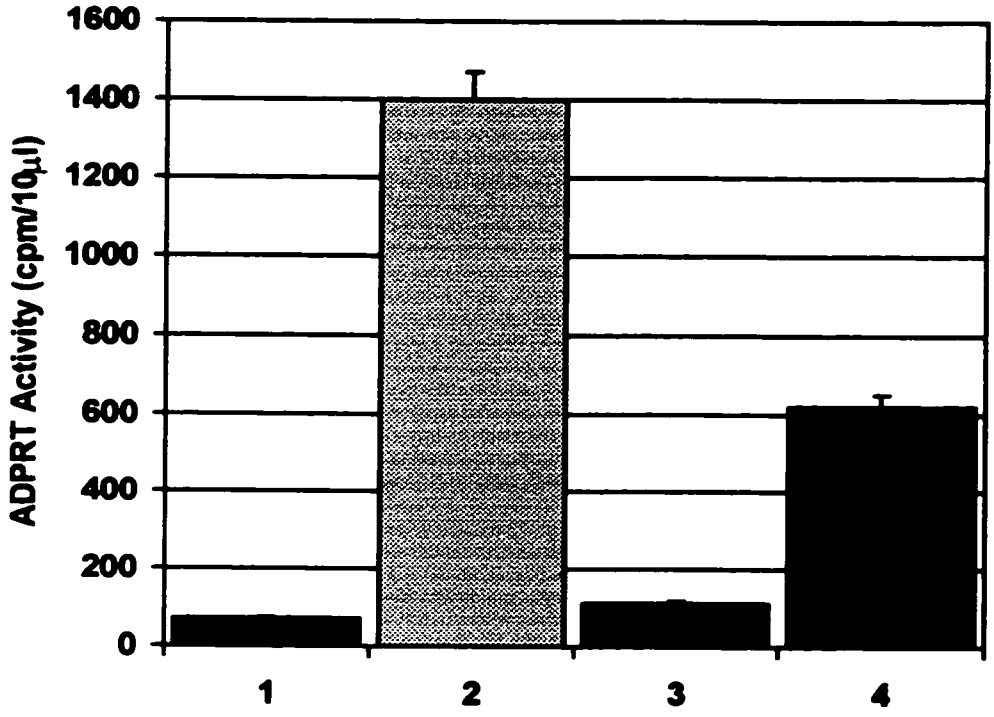

ADPRT Activity (cpm/10µl)
1600
1400
1200
1000
800
600
400
200
0
1
2
3
4

Figure 11. Western blot analysis of supernatant proteins from *P. aeruginosa* strains. All strains were grown in iron depleted TSBDC to an $O.D._{540}$ of 4. Cells were pelleted by centrifugation at 13 000 rpm for 10 minutes and the supernatants collected. Western blot analysis was performed on the supernatants using purified anti-ETA antibodies. Lane 1: strain PA103. Lane 2,9: PA103*toxA*::Ω (pCG (*tox*-SF)). Lane 3: Clinical strain 4384. Lane 4: PA103*toxA*::Ω (pMS151-1). Lane 5,8: PA103*toxA*::Ω. Lane 6: Prestained Gibco/BRL marker. Lane 7: Purified ETA (Swiss Serum Institute).

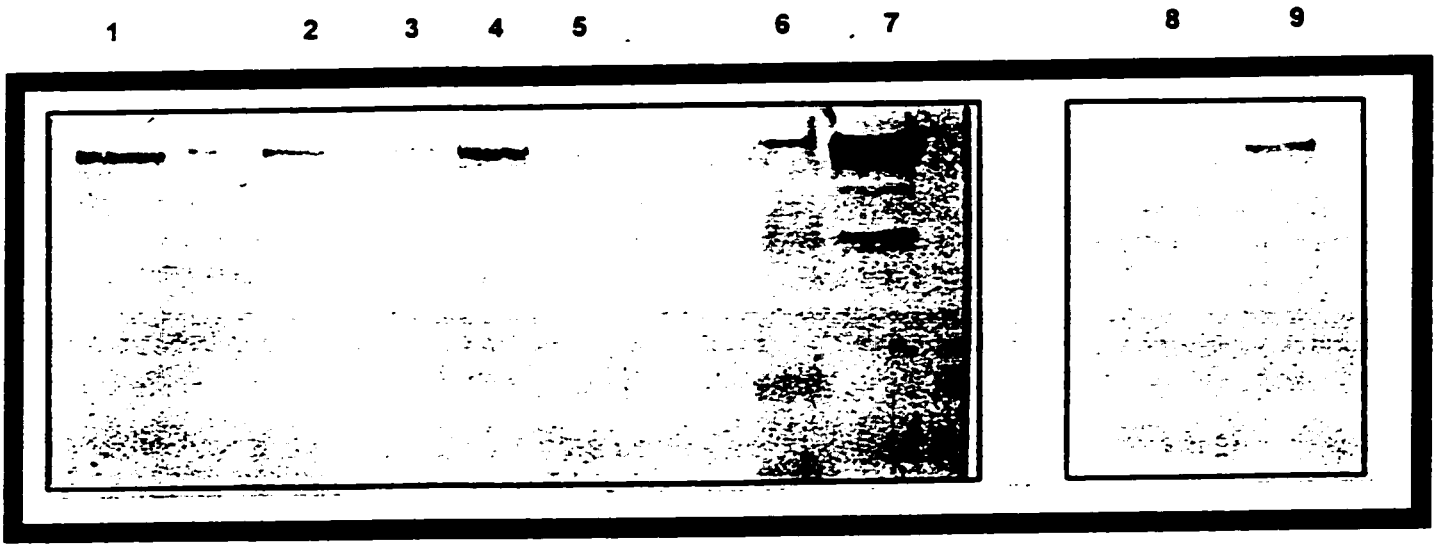

1
2
3
4
5
6
7
8
9

Figure 12 shows a comparison of the ADP-ribosyl transferase activity from the different strains. The two strains with highest ADPRT activity were the hypertoxigenic strain PA103 and PA103*toxA*::Ω complemented with wild type *toxA* from pMS151-1. More importantly, strain PA103*toxA*::Ω complemented with pCG (*tox*-SF) did not produce a toxin with restored ADPRT activity. In addition to this complementation study, a mixed toxin experiment was performed to investigate the possibility of an accessory factor secreted in the supernatant of strain 4384. Supernatants from strains 4384, PA103 and PA103*toxA*::Ω were mixed with purified ETA to look for a reduction in enzymatic activity. If an accessory factor was present in strain 4384 which would post-translationaly modified ETA, a drop in the enzymatic activity of pure ETA would be expected when it was mixed with the supernatant from strain 4384. Each hour post incubation of supernatant with purified ETA, a sample was taken, and the enzymatic activity was determined. When the supernatants of each strain were mixed with the purified ETA, we noticed a drastic increase in enzymatic activity. However, even after long periods of incubation we detected no significant decrease in ADPRT activity in any sample indicative of protein degradation or presence of an accessory modifying factor. After six hours of incubating the supernatant from strain 4384 with purified ETA, the ADPRT activity of the purified toxin remained intact. This study eliminates the possibility of an accessory modifying factor in strain 4384 which acts to modify ETA and reduce the enzymatic activity of the toxin (Figure 13).

Figure 12. Analysis of complementation of *P. aeruginosa* strain PA103*toxA*::Ω with WT and mutant *toxA* through ADPRT assays. pCG5, containing the ADPRT deficient *toxA* from strain 4384 was transformed into PA103*toxA*::Ω and the strain analyzed for ETA activity. To achieve this, the 1.8 kb *Pst*I stabilizing fragment (SF) isolated from PSAK104A was ligated into the dephosphorylated *Pst*I site of pCG5 to allow for replication inside *Pseudomonas*. pMS151-1 containing wild type *toxA* from PA103 was also electroporated into PA103*toxA*::Ω. ADPRT activity was determined by assaying the supernatant of each culture at O. $D._{540}$ of 4 as described by Chung and Collier (1977). Lane 1: PA103*toxA*::Ω. Lane 2: PA103*toxA*::Ω (pMS151-1). Lane 3: PA103*toxA*::Ω (pCG (*tox*-SF)). Lane 4: Strain 4384. Lane 5: Hypertoxigenic strain PA103.

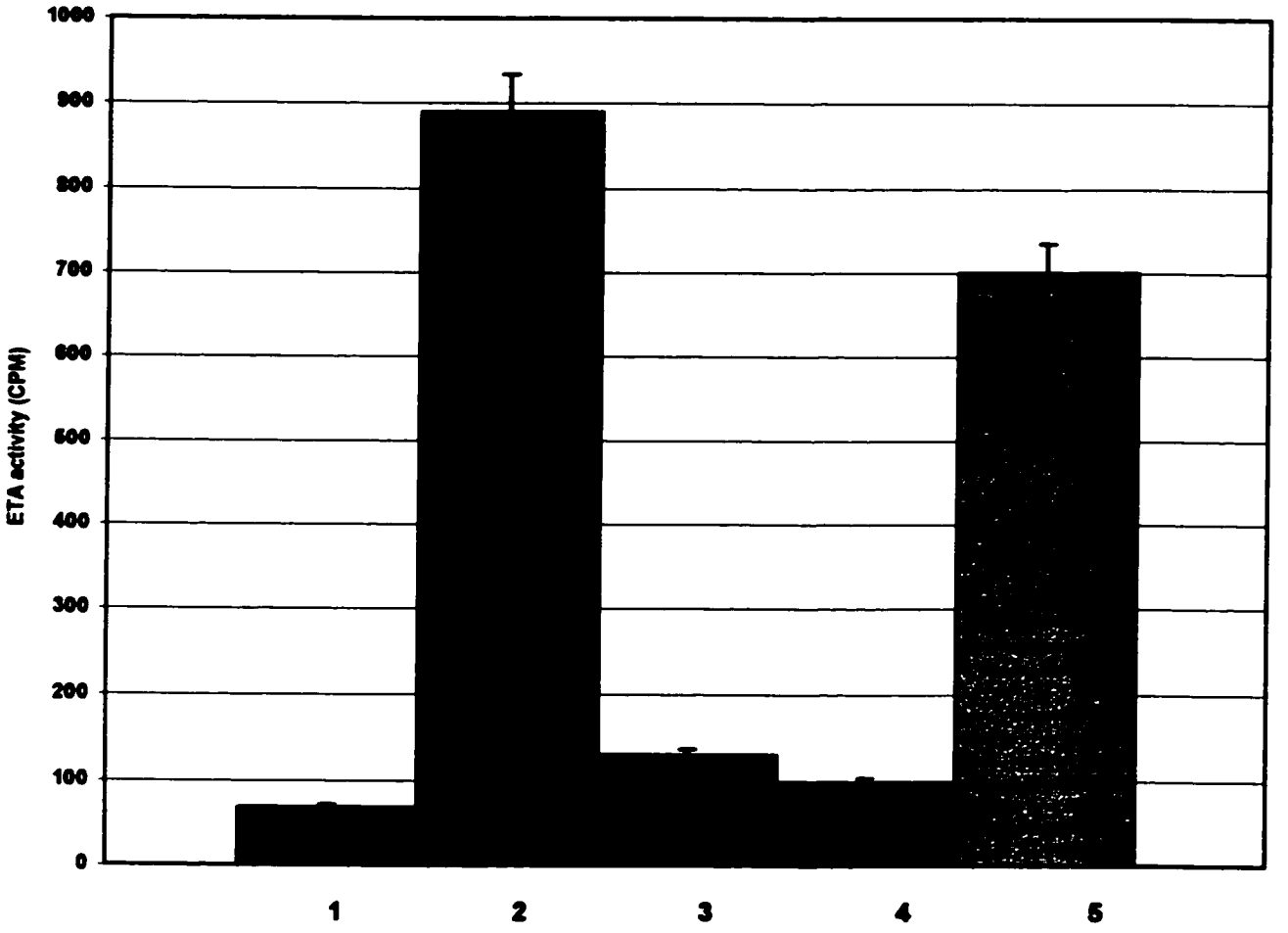
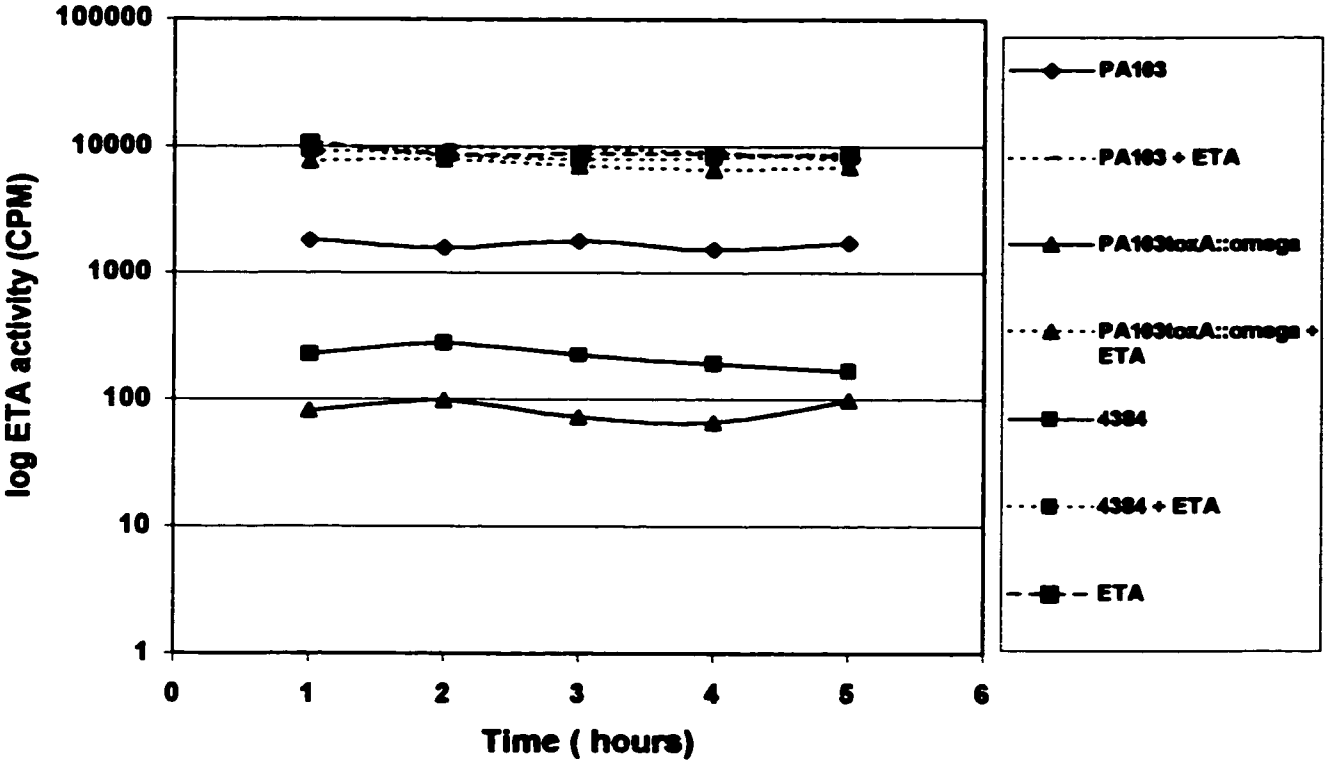

ETA activity (CPM)
1000
900
800
700
600
500
400
300
200
100
0
1
2
3
4
5

Figure 13. ADPRT activity of ETA when mixed with the supernatants from strain 4384 or strain PA103. Supernatants from strain 4384, PA103, PA103*toxA*::Ω were mixed in equal proportions with native ETA (Swiss Serum Institute) and incubated for six hours at 37°C. The enzymatic activity of each sample was then determined using the ADPRT activity assay. The native ETA was used as a control (0.001μg). 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 by itself was used as a positive control for this experiment. Supernatant from strain PA103*toxA*::Ω was also measured by itself or with the native ETA.

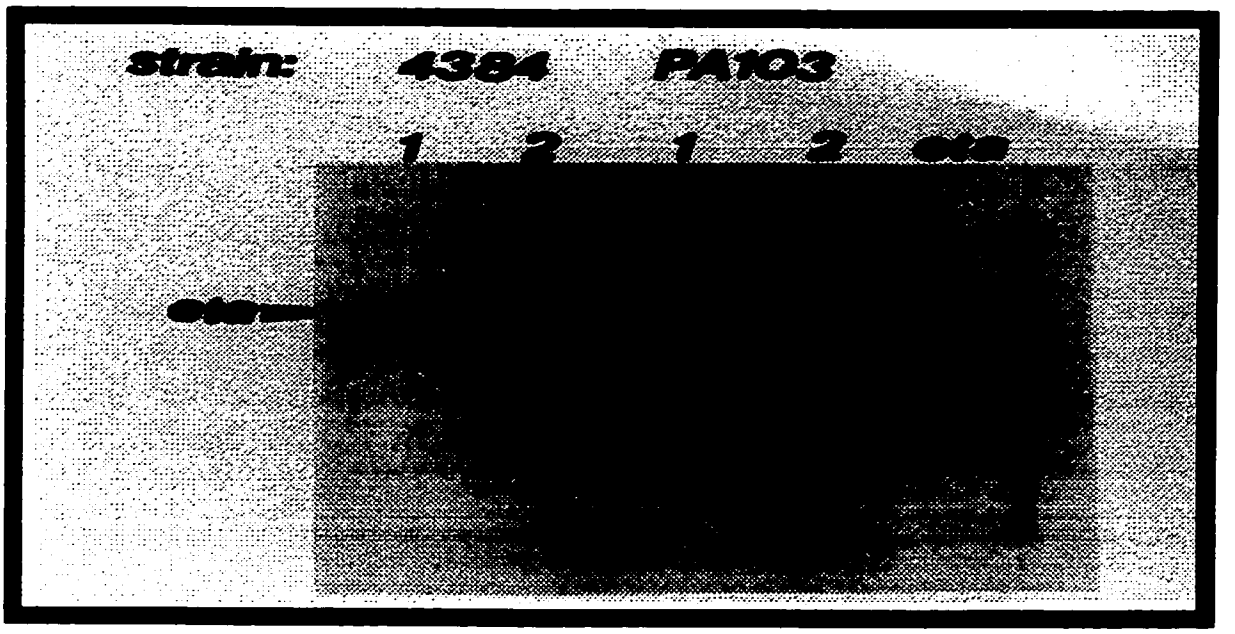

100000
10000
1000
100
10
1
log ETA activity (CPM)
0
1
2
3
4
5
6
Time (hours)
PA103
PA103 + ETA
PA103toxA::omega
PA103toxA::omega + ETA
4384
4384 + ETA
ETA

These experiments taken together demonstrate that the reduced ADPRT activity observed in strain 4384 is an inherent property of 4384 *toxA*.

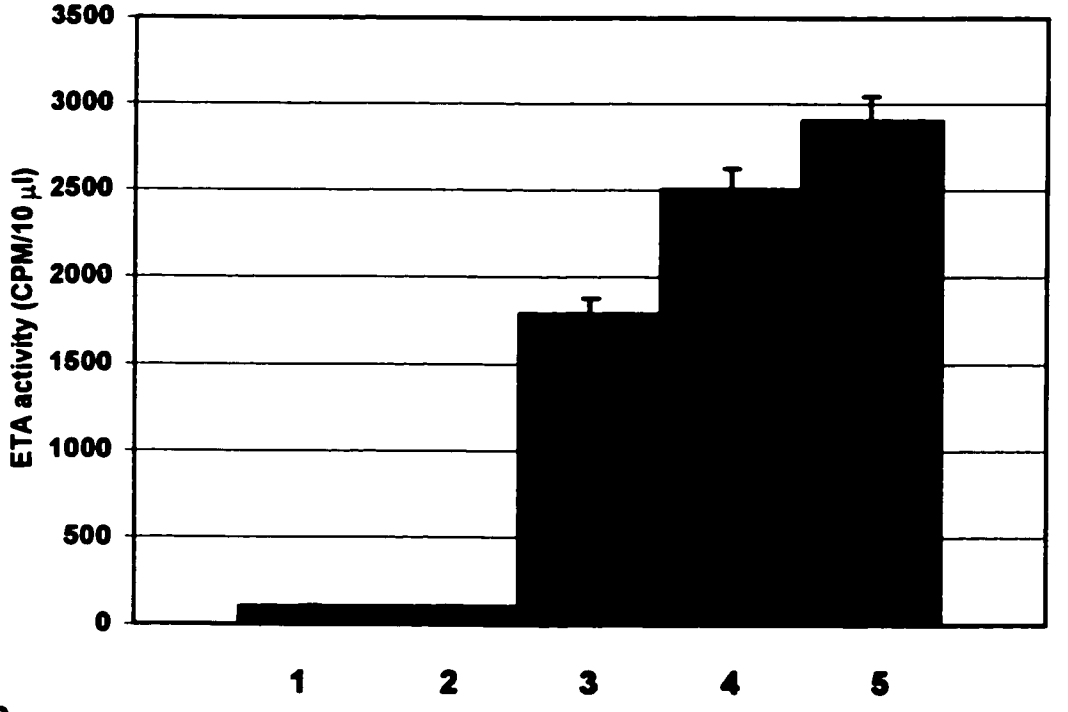
3.6 Comparison of Exotoxin A production in strain 4384 and PA103.

To further explore the decrease in production of ETA by strain 4384 as compared to WT PA103, the enzymatic activity of ETA produced by each strain was investigated. Tracy Raivio, a past PhD student had previously purified ETA from both 4384 and PA103. Western blot analysis, using anti-ETA antibodies, showed that ETA purified from strains 4384 and PA103 are both M_r 66,000 (Figure 14A). Figure 14A 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 (Raivio *et al.*, unpublished data) which are absent in the protease deficient strain PA103. Tracy Raivio was also able to compare the enzymatic activity of the purified toxins to the commercially available purified ETA (Swiss Serum Institute) as demonstrated in Figure 14B. The ADP-ribosyl transferase activity of the two purified ETA preparations from strain 4384 (Lane 1 and 2) is lower than the two preparations from strain PA103 (Lane 3 and 4). The highest level of ADP-ribosyl transferase activity was obtained from the commercially available purified Swiss Serum ETA (Lane 5). The toxins produced by 4384 and PA103 are both 66 kDa, both can be purified using the same methodology and are immunoreactive with the same antibody. Therefore, it can be assumed that both toxins are fundamentally biochemically similar. The ETA from strains 4384 and PA103 were purified by Raivio (unpublished data) using methodology based on the original purification scheme (Liu., 1966). The yield of ETA protein purified by the original scheme was very low. Thus, we decided to repurify more ETA from strains 4384 and PA103 using the same methodology.

Figure 14. Comparison of ETA production and ADPRT activity in strains 4384 and PA103. (A) SDS-PAGE of purified ETA from *P. aeruginosa* strain 4384 and strain PA103. 4384 and PA103 were grown in two different batches of low iron TSBD. All samples were taken at an $O.D._{540}$ of 5. Each sample was electrophoresed on a 10% polyacrylamide gel, blotted to nitrocellulose and stained using anti-ETA antibody. Strain 4384, lanes 1 and 2 represent 10 μl of purified ETA from strain 4384. Strain PA103, Lanes 1 and 2 represent 10 μl of purified ETA from strain PA103. "eta" represents 1 ul of purified ETA from the Swiss Serum Institute. (14B) Subsequently, ADPRT assays were performed on 10 μl of the supernatant from the same samples. In Figure 14B bars 1 and 2 represent the ADPRT assay results from Strain 4384. Bars 3 and 4 represent the ADPRT assay results from Strain PA103 and bar 5 represent the ADPRT assay result from the Purified ETA.


strain:
4384
PA103
1
2
1
2
etx
etx

A.


3500
3000
2500
2000
1500
1000
500
0
ETA activity (CPM/10 µl)
1
2
3
4
5

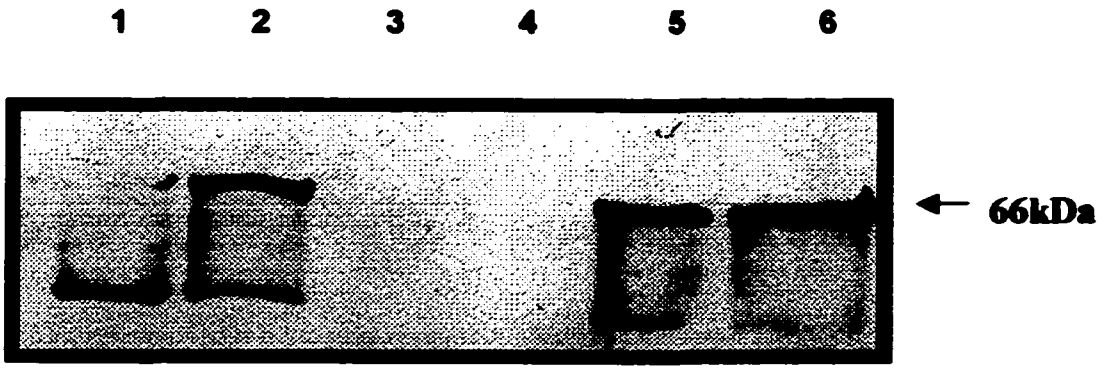
B.

Purification of ETA from both strains would be a useful tool for making our own ETA antibodies and also would allow for further characterization of the enzymatic defect in strain 4384 ETA.

It was previously demonstrated that strain 4384 produced a large amount of proteases which could be responsible for the low level of protein purified from strain 4384. To circumvent this problem, it was decided to purify the toxin from strain PA103 and 4384 in a *P. aeruginosa* isogenic strain; PA103*toxA*::Ω. Strain PA103*toxA*::Ω was selected since ETA cannot be expressed by this strain. The *Eco*RI-*Pvu*II *toxA* gene was isolated from pMS151 and pCG5. Both fragments were successfully cloned into the *Eco*RI-*Sma*I site of pUCSF (data not shown). pUCSF (WT*toxA*) and pUCSF(4384 *toxA*) were electroporated into strain PA103*toxA*::Ω. Western blot analysis using specific anti-ETA antibodies revealed the presence of a 66 kDa immunoreactive band in each sample (Figure 15). However, when the expression of ETA from strain 4384 was compared to strain PA103, a difference in the expression pattern was detected. ETA is produced in strain 4384 at a lower levels than in strain PA103. In addition, the presence of degradation products and loss from the main 66 kDa band in PA103*toxA*::Ω (pUCSF(4384*toxA*)) were detected. This was intriguing since the *toxA* gene from both strains were cloned exactly the same way. This may suggest a more complex regulation of ETA in strain 4384 or an increased susceptibility to degradation. As there was a difference in the expression of ETA from both 4384 and WT in the isogenic strain PA103*toxA*::Ω, an alternative approach to purify the toxin produced by both strains was developed.

The new approach involved the purification and expression of ETA using the QIAgen expression system. This system allows for high levels of expression of recombinant proteins in *E. coli* using a 6xHis Tag.

Figure 15. Western Blot analysis of supernatant proteins from isogenic strain PA103*toxA*::Ω complemented with WT*toxA* or 4384*toxA*. Both plasmids; pUCSF (4384*toxA*) and pUCSF (WTtoxA) were electroporated into *P. aeruginosa* strain PA103*toxA*::Ω. All transformed strains were grown overnight in low iron conditions to maximize ETA production. All cultures were harvested at $O.D._{540}$ of 4.0., 5 or 10 µl of supernatant from each strains was separated by SDS-PAGE. The identification of ETA production was confirmed by Western Blot analysis using ETA specific antibodies. Lane 1 and 2 represents 5 and 10 µl of supernatant from PA103*toxA*::Ω (pUCSF(4384*toxA*)) respectively. Lane 3 and 4 represents 5 and 10 µl of supernatant from negative control strain PA103*toxA*::Ω respectively. Lane 5 and 6 indicate 5 and 10 µl of supernatant from PA103*toxA*::Ω (pUCSF(WT*toxA*)) respectively.


1
2
3
4
5
6
66kDa

The pQE expression vectors contained helpful features such as an optimized inducible promoter consisting of the *E. coli* phage T5 promoter and two lac operator sequences, a translation stop codon in all reading frames and a β-lactamase gene from plasmid pBR322 (Sutcliffe, 1979). This system allows the purification of bacterial proteins in one step under either native or denaturing conditions. The presence of the 6xHis tag at the N-terminal or C-terminal end of the recombinant protein facilitates efficient purification of the protein using a Ni-NTA (nitrilo-tri acetic acid) resin. The NTA ligand contains four chelating sites that are free to interact with metal ions. The addition of the affinity tag to the protein renders it poorly immunogenic. In addition, the 6xHis tag is uncharged at physiological pH, and its presence does not affect protein folding, compartimentalization or secretion. For the purification of ETA from both strains (PA103 and 4384) the type IV constructs were used, which positions the 6xHis tag at the N-terminus of the toxin. The start codon from the *toxA* gene was removed to reduce the level of expressed protein that do not include the 6xHis tag and cannot be isolated. The pQE vector that was appropriate for the purification scheme was pQE-32 since it allows the cloning of the coding region of *toxA* in-frame. The limited cloning sites of the pQE-32 vector necessitated construction of the expression vector in two steps (Figure 16). The first step involved the cloning of the C-terminal end of the *toxA* gene into the pQE-32 vector (Figure 17). The 454 bp C-terminal end of *toxA* from strain 4384 and WT PA103 strain was cloned into the *EcoR*I-*Pst*I site of the pQE-32 vector (Figure 17, Lanes 2 and 8). The second step was the cloning of the 1,530 bp *Bam*HI internal *toxA* fragment into pQE-32 (4384*toxA*-C-terminal end) and pQE-32 (WT*toxA*-C-terminal end). The major problem with this step was the isolation of very few transformants. This may suggest that the *toxA* gene is mildly toxic to *E. coli* cells. After several rounds of transformation, 12 colonies for each strain

were obtained which potentially contained the *Bam*HI insert (1530 bp) (Figure 18). After *Bam*HI digestion, 5 positive clones for *toxA* of strain 4384 and 9 for WT PA103 *toxA* were obtained that produced a 1.53 kb band when digested. To make sure that the internal *Bam*HI toxA band was inserted in the right orientation, all potential positive clones were digested with *EcoR*I and *Kpn*I. If the *toxA* gene is inserted in the right orientation, a 385 bp fragment and a 5081 bp fragment should be obtained. Figure 19 confirms the presence of two positive clones for *toxA* from strain 4384 in lanes 4 and 6. In addition, two positive clones for *toxA* from strain PA103 in lanes 7 and 12 were isolated. The expression vectors were labeled pQE-32 (4384*toxA*) and pQE-32 (WT*toxA*). pQE-32 (WT*toxA*) was screened for its ability to express recombinant ETA with the 6xHis tag at its N-terminus. Initial screening for expression of ETA was performed on a small-scale procedure before the culture conditions to maximize ETA expression were optimized. This procedure determined whether ETA was being expressed in pQE-32 (WT*tox*A). The purification scheme was carried out under denaturing conditions and allowed the isolation of recombinant protein that contained the 6xHis Tag. Original attempts to purify recombinant ETA from *E. coli* strain JM109 were not successful. Subsequently, the k-12 derived *E. coli* strain M15 was selected as the host strain for our expression studies because it contains [pREP4]. The plasmid pREP4 is expressed in multicopy in the pQE system and contains the *lacI* gene which encodes the lac repressor (Farabaugh, 1978). Presence of this plasmid allows a tight regulation of expression from the promoter/operator region. Expression of pQE-32 (WT*toxA*) was induced by the addition of IPTG. Figure 20 shows the expression of ETA from pQE-32 (WT*toxA*) under denaturing conditions. Lane 5-9 revealed an immunoreactive band of 66 kDa. Furthermore, in lanes 5-8, a significant increase in ETA expression post induction with IPTG can be observed. Lane 3 serves as a control and contains

Figure 16. Schematic representation of the approach used to clone the *toxA* gene from strain 4384 and PA103 into the pQE-32 expression vector. Initially, the C-terminal end of *toxA* frpm pMS151 and pCG5 was digested with *Bam*HI-*Eco*RI and ligated into the *Bam*HI-*Pst*I site of the pQE-32 vector with the help of a *Eco*RI-*Pst*I linker. The positive transformants were screened by restriction digestion and the digested products separated by agarose gel electrophoresis. The pQE-32 vectors with the C-terminal ends of each *toxA* were labeled pQE-32 (4384*toxA*-C-terminal end) and pQE-32 (WT*toxA*-C-terminal end). Next, the internal *Bam*HI *toxA* fragment was cloned into the dephosphorylated *Bam*HI site of pQE-32 (4384*toxA*-C-terminal end) and pQE-32 (WT*toxA*-C-terminal end). The orientation of the internal *toxA* fragment was verified by *Eco*RI-*Kpn*I digest. This resulted in the cloning of the *toxA* gene in the correct reading frame downstream of the 6xHis tag.

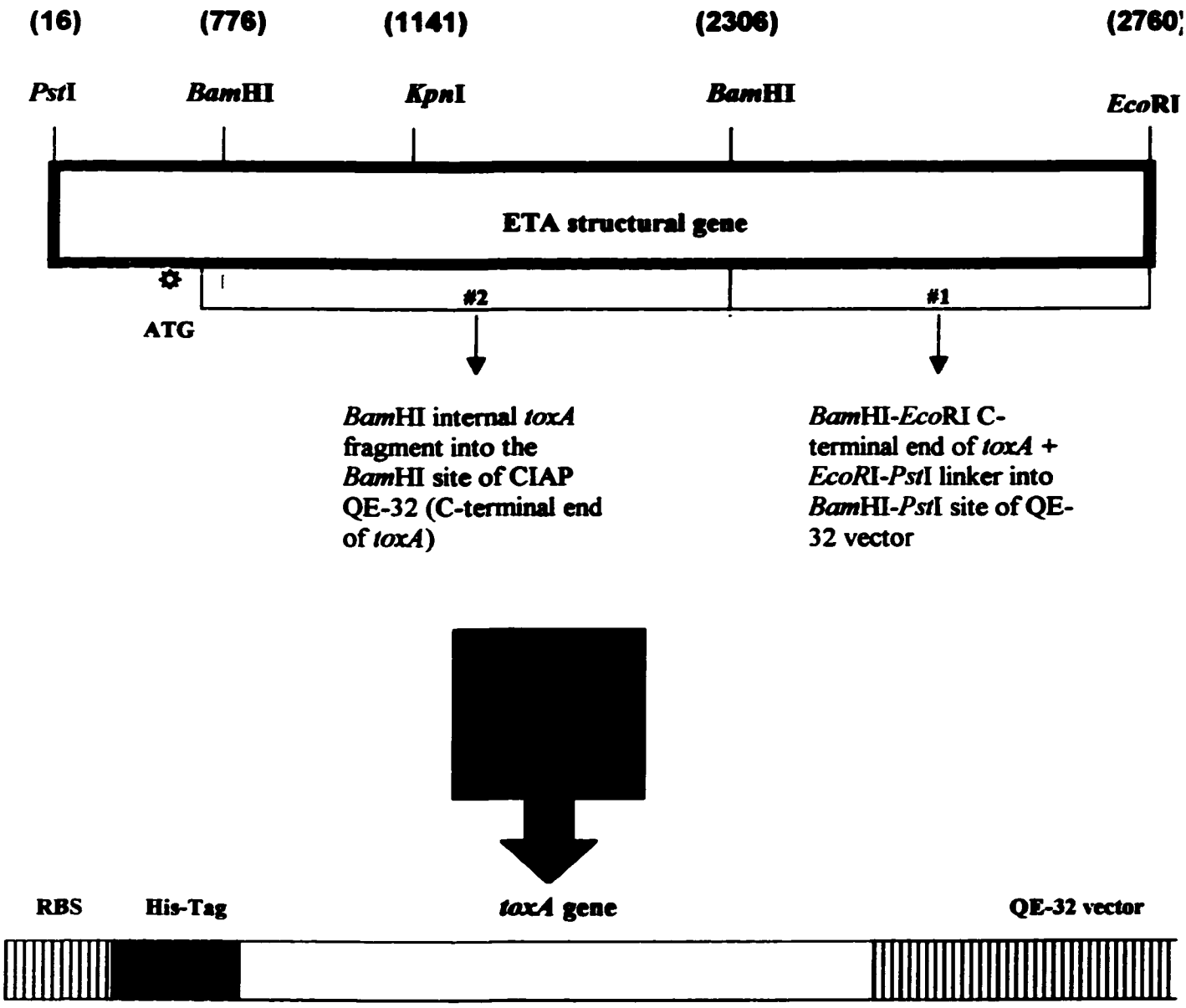

(16)
(776)
(1141)
(2306)
(2760)
PstI
BamHI
KpnI
BamHI
EcoRI
ETA structural gene
ATG
#2
#1
BamHI internal toxA fragment into the BamHI site of CIAP QE-32 (C-terminal end of toxA)
BamHI-EcoRI C-terminal end of toxA + EcoRI-PstI linker into BamHI-PstI site of QE-32 vector
RBS
His-Tag
toxA gene
QE-32 vector

Figure 17. Cloning of the *Bam*HI-*Eco*RI C-terminal end of *toxA* from strain PA103 and 4384. The *toxA* gene from strain 4384 and PA103 was digested with *Bam*HI-*Eco*RI, and the 454 bp fragment was ligated into the *Bam*HI-*Pst*I site of the pQE-32 through the use of a *Eco*RI-*Pst*I linker. The positive transformant were digested with *Bam*HI-*Hind*III to cut out the insert (454 bp) from the pQE-32 vector (3462 bp) and were separated by agarose gel electrophoresis. Panel A contains three putative transformants for the C-terminal end of *toxA* from strain 4384. Lane 1. clone #1 undigested. Lane 2. clone #1 digested with *Bam*HI-*Hind*III. Lane 3. clone #2 undigested. Lane 4. clone #2 digested with *Bam*HI-*Hind*III. Lane 5. clone #3 undigested. Lane 6. clone #3 digested with *Bam*HI-*Hind*III. Panel B contains 4 putative transformants that contained the C-terminal end of the *toxA* gene from pMS151 strain. Lane 7. clone #1 undigested. Lane 8. clone #1 digested with *Bam*HI-*Hind*III. Lane 9. clone #2 undigested. Lane 10. clone #2 digested with *Bam*HI-*Hind*III. Lane 11. clone #3 undigested. Lane 12. clone #3 digested with *Bam*HI-*Hind*III. Lane 13. clone #4 undigested. Lane 14. clone #4 digested with *Bam*HI-*Hind*III. Clone #1 from each strain contains the 454 bp fragment comprising the C-terminal end of toxA. Clone # 1 from panel A was labeled pQE-32 (4384*toxA*-C-terminal end). Clone # 1 for panel B was labeled pQE-32 (WT*toxA*-C-terminal end).

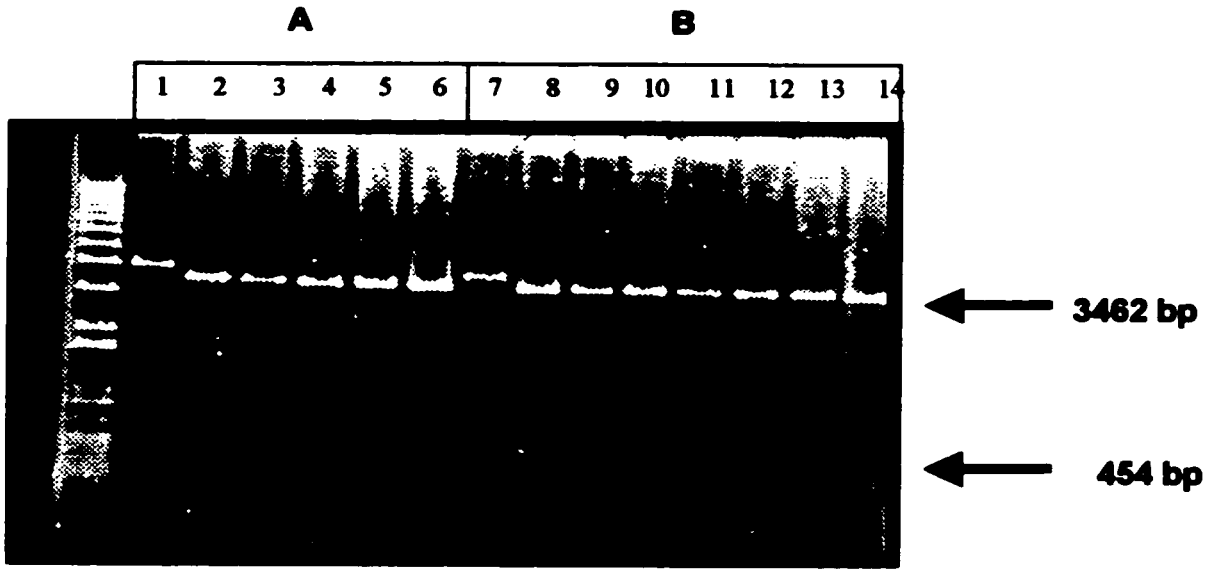

A
B
1 2 3 4 5 6 7 8 9 10 11 12 13 14
3462 bp
454 bp

Figure 18. Screening for correct insertion of the 1.53 kb internal *toxA* *Bam*HI fragment into the *Bam*HI site of pQE-32 (WT*toxA*-C-terminal end) and pQE-32 (4384*toxA*-C-terminal end). Twelve putative clones for each strain believed to contain the 1.530 kb insert were chosen for screening. The DNA of each clone was isolated and digested with *Bam*HI. The digested DNA was then separated by agarose gel electrophoresis. After digestion the clones containing the complete *toxA* gene should contain 2 fragments. A larger fragment of 3.922 kb which includes the vector and the C-teminal end of *toxA* and the smaller fragment of 1.530 kb which contains the internal *Bam*HI *toxA* insert. Lane 1 and 15 contain the 1 kb DNA ladder (Gibco/BRL). Lanes 2 and 16 contain the 1.530 kb *toxA* insert. Lane 3-14 represents the twelve clones isolated with *toxA* from strain 4384. Lane 17-28 represents the twelve putative clones for *toxA* derived from PA103. Plasmids chosen for further investigation are shown in lanes 3,5,10,12,14 for strain 4384 *toxA* and lanes 17,18,19,22,23,24,25,26,28 for WT *toxA*.

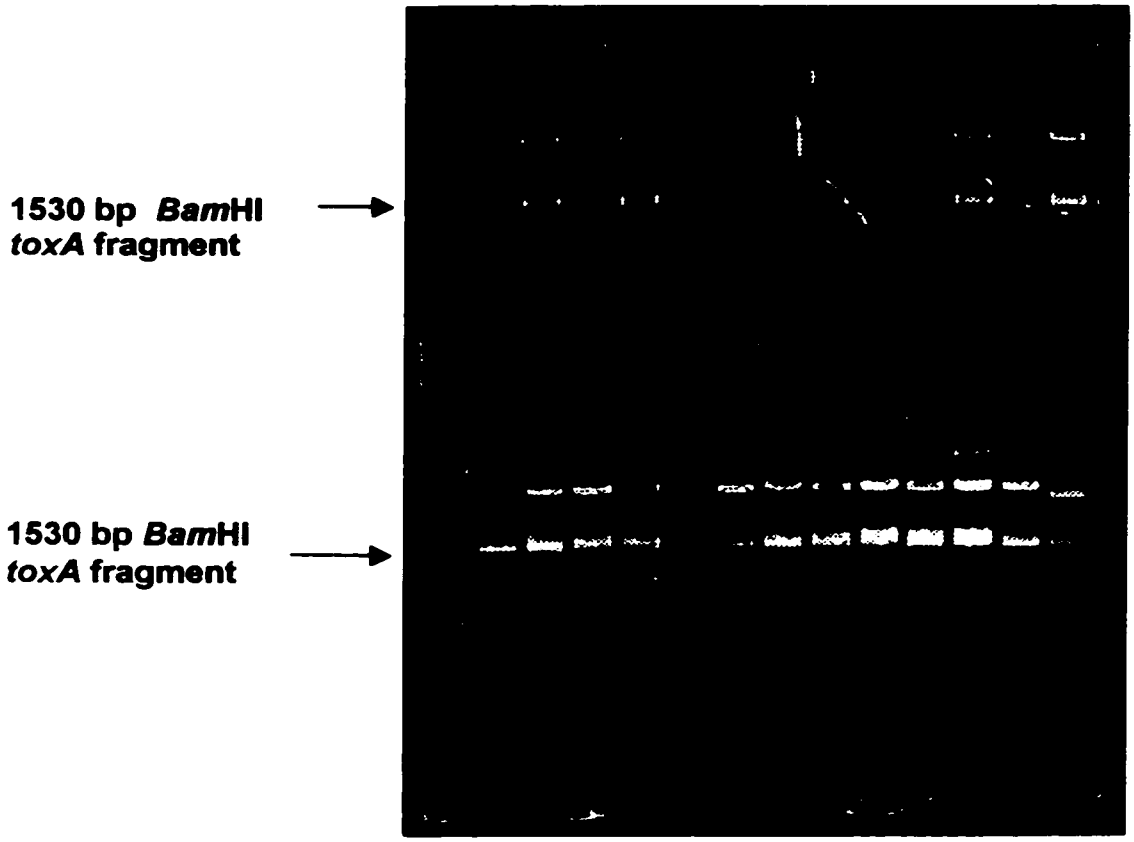

1530 bp *Bam*HI
toxA fragment
1530 bp *Bam*HI
toxA fragment

Figure 19. Analysis of the orientation of the internal *Bam*HI *toxA* gene into pQE-32 (WT*toxA*-C-terminal end) and pQE-32 (4384*toxA*-C-terminal end). Fourteen clones that contain the entire *toxA* gene were isolated. To make sure that the fragment was inserted in the proper reading frame the DNA was digested with *EcoR*I and *Kpn*I. Five clones that contain the *toxA* gene from strain 4384 and 7 clones that contain the *toxA* gene from strain PA103 were selected. If the *Bam*HI fragment is inserted in the correct orientation, the digested DNA should yield a 5081 bp and a 385 bp fragment. Lane 1 represents the DNA ladder. Lanes 2-6 represents the potential pQE-32 (4384*toxA*-C-terminal end) ligated to remaining *toxA* digested with *EcoR*I-*Kpn*I. Lanes 7-12 indicates the pQE-32 (WT*toxA*-C-terminal end) with the remaining *toxA* inserted digested with *EcoR*I-*Kpn*I. Lanes 4 and 6 contain the internal *toxA* gene in the right orientation. Lanes 7 and 12 are also positive for the *Bam*HI *toxA* gene in the proper reading frame. These new clones were labeled pQE-32 (4384*toxA*) and pQE-32 (WT*toxA*).

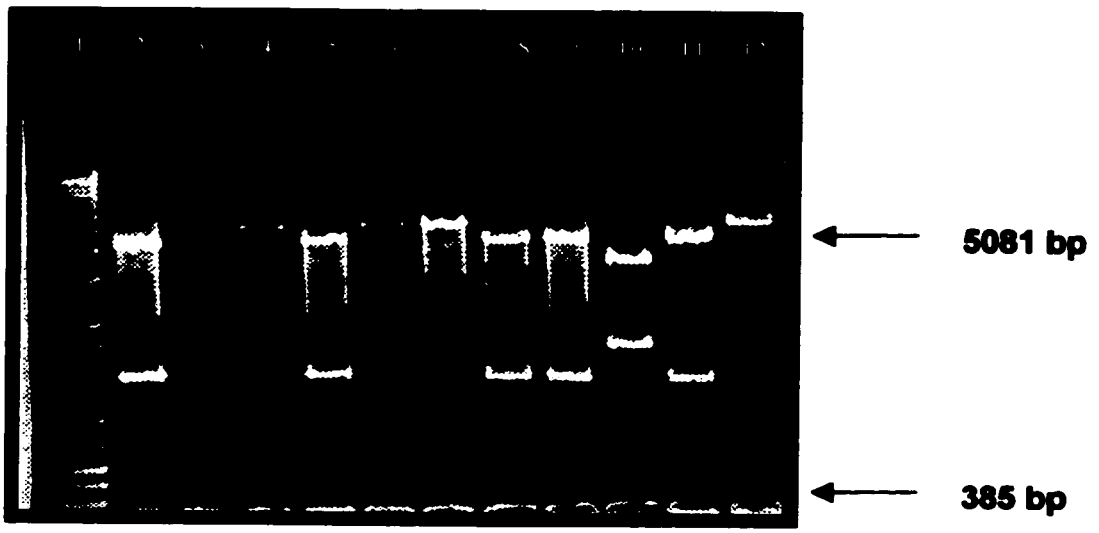
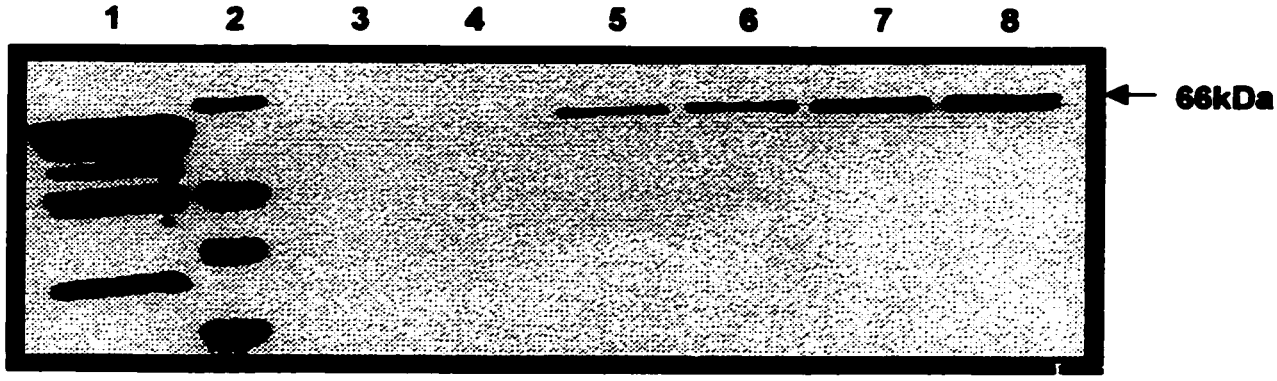

5081 bp
385 bp

Figure 20. Western blot analysis of ETA from pQE-32 (WT*toxA*). The transformed cells were grown overnight in LB supplemented with ampicillin and kanamycin for plasmid maintenance. The expression of ETA was induced 1 hour after inoculation by addition of IPTG. Every hour post induction, 1 ml of culture was transferred to a microcentrifuge tube. The cells were harvested by centrifugation, and the supernatant was discarded. Purification of the ETA protein was done using a rapid screening protocol. Lane 1. Purified ETA (Swiss serum Institute). Lane 2. Prestained Gibco/BRL protein marker. Lane 3. supernatant sample t=1 contains any unbound proteins. Lane 4. ETA expression from pQE-32 (WT*toxA*) before induction t=0. Lane 5. ETA expression from pQE-32 (WT*toxA*) after induction t=1hour. Lane 6. ETA expression t=2 hours. Lane 7. ETA expression t=3 hours. Lane 8. ETA expression t=4hours.


1
2
3
4
5
6
7
8
66kDa

supernatant proteins which have not bound to the Ni/NTA resin. The absence of a 66 kDa immunoreactive band indicative of ETA confirmed that the expressed ETA contains the 6xHis tag and as such binds tightly to the resin. Thus, it was possible to express ETA from pQE-32 (WT*toxA*). However, purification of pQE-32 (WT*toxA*) was performed under denaturing conditions which do not take into consideration the location and constitution of ETA within the cell. Unfortunately, we a small-scale purification of the toxin from pQE-32 (4384*toxA*) was not possible in the time available. However, based on the similarities between the two expression vectors it should be possible to express the toxin from strain 4384 using the same protocol as with pQE-32 (WT*toxA*). Further work has to be done to maximize expression of ETA from both strains.

3.7 Cytotoxicity of ETA derived from strain 4384 and PA103.

Previous studies demonstrated that cytotoxicity of ETA is linked to its ADPRT activity. At this point it had however been demonstrated that 4384 ETA had a 12 fold reduction in ADPRT activity. Thus, it was of interest to determine whether the reduction in enzymatic activity of strain 4384 ETA would affect the cytotoxicity of the toxin. To make sure that the purified ETA from strain 4384 showed the same decrease in ADPRT activity as the concentrated sample from the same strain the specific activity of the purified ETA from strain 4384 was calculated (Figure 14A) using known concentrations of the purified Swiss Serum ETA as a reference (Figure 21). To compare the cytoxicity of native ETA with that of the purified ETA from strain 4384, a colorimetric microtiter assay was performed. The ability of both ETA preparations to intoxicate HeLa cells is illustrated in Figure 22. At low concentrations, both ETA from strain 4384 and Swiss serum ETA killed equivalent numbers of HeLa cells. However, at higher

Figure 21. Concentration of purified ETA from strain 4384 determined by Western blot analysis. The concentration of purified ETA from 4384 was determined by loading 5 and 10μl of ETA purified from strain 4384 and comparing the level to known concentration of the Swiss Serum purified toxin. Lanes 1 and 2 represents 5 and 10 μl of purified ETA from strain 4384. Lane 3 contains the BenchMark™ prestained protein marker. Lanes 3-9 represents varying concentrations of the Swiss Serum ETA (0.5μg, 0.25μg, 0.125μg, 0.0625μg, 0.03125μg, 0.01563μg, 0.0078μg) respectively.

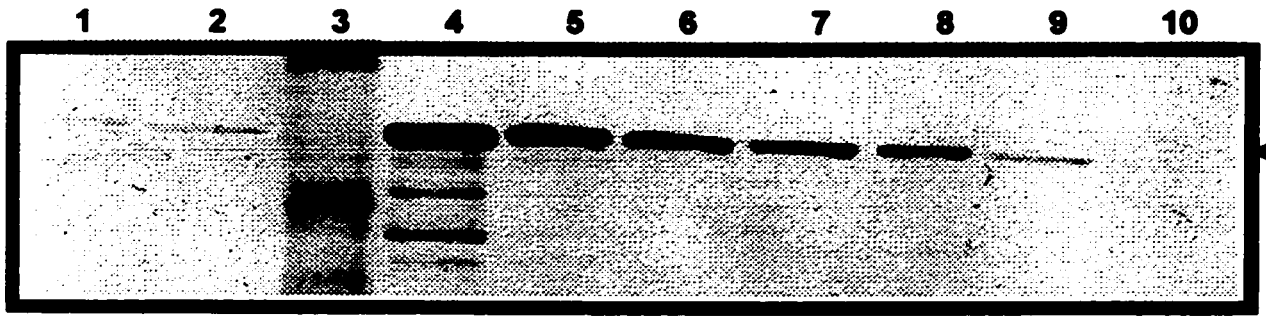
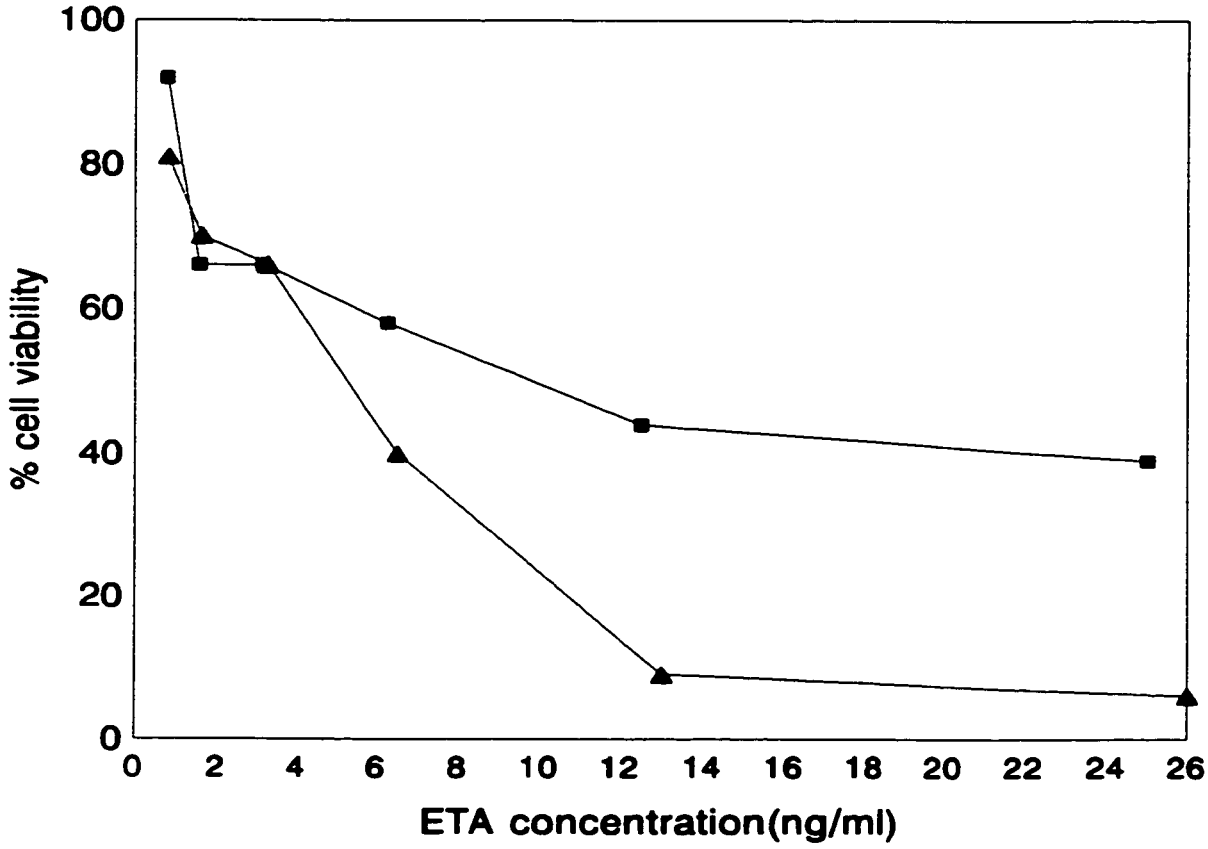

1
2
3
4
5
6
7
8
9
10
66 k

Figure 22. Cytotoxic effects of ETA isolated from strain 4384 and native ETA on HeLa cells. Increasing concentrations of ETA from strain 4384 and Swiss Serum ETA were used to treat HeLa cells in order to compare the cytotoxicity of 4384 to the native ETA protein. Diamonds represents HeLa cells treated with Swiss serum ETA and ovals represents HeLa cells intoxicated with 4384 ETA.


100
80
60
40
20
0
% cell viability
0
2
4
6
8
10
12
14
16
18
20
22
24
26
ETA concentration(ng/ml)

toxin concentrations the percent HeLa cell viability was reduced further when treated with 4384 ETA than with the Swiss Serum ETA. These results suggest that ETA from strain 4384 is slightly more cytotoxic than Swiss Serum ETA. The fact that 4384 ETA proved to be still cytotoxic despite the observed decrease in ADPRT activity may suggest that the cytotoxicity of ETA may be uncoupled from its enzymatic activity.

3.7 Summary.

Analysis of the ADPRT activity of ETA isolated from *P. aeruginosa* CF isolates led to the identification of six CF strains with no detectable enzymatic activity. Further characterization of one of these strains, CF isolate 4384 demonstrated a twelve fold decrease in enzymatic activity when compared to the hypertoxigenic strain PA103. Several studies have demonstrated that strain 4384 does not produce a modifying factor which could be responsible for the observed decrease in ADPRT activity. Sequence analysis of the *toxA* gene from strain 4384 revealed the presence of two mutations in the active site of ETA which may act to compromise the enzymatic moiety of the toxin. Surprisingly, the reduction in ADPRT activity of ETA does not affect the cytotoxicity of the toxin. This may suggest the presence of two independent mechanisms responsible for the toxicity of ETA. Preliminary result indicates that the ETA can be efficiently expressed from a pQE expression vector for small-scale purification.

Chapter 4

Analysis of the reduced ADPRT activity observed among the five *P. aeruginosa* isolates from two chronically infected CF patients.

4.1 Characterization of the altered ADPRT activity observed in five CF isolates.

Previous studies had demonstrated a twelve-fold reduction in ADPRT activity of ETA derived from one CF isolate, strain 4384. The next objective was to determine if strains with altered ADPRT were prevalent among chronically infected CF patients. Five other CF strains also exhibited a strong reduction in ADPRT activity (Table 2). To further characterize the reduction in ADPRT activity in these strains it was decided to determine if this was a common adaptation in CF isolates.

Growth curve analysis in low iron conditions of the CF strains showed variability in the growth rate of each strain (Figure 23). The replication time of two strains, 5552 and 5585 proved extremely slow when compared to strain PA103. Figure 23 shows that strain 5552 and 5585 reached an O. D_{540} of only 1 after almost 15 hours of growth. However, the cell density appears to increase at a more normal pace after this period. Figure 23 also shows that strain PA103 and strain 4384 appear to follow similar growth patterns. To determine if the reduced ADPRT activity observed in the five CF strains was related to the slower rate of growth, ADPRT assays were performed throughout the entire 24 hours growth curve (data not shown). At any given time, no ADPRT activity was detected in the supernatants of each strain. To ensure that there was not a build up of ETA inside each strain owing to a secretory defect, the intracellular levels of ADPRT

Figure 23. Growth curve analysis of the six CF isolates grown in low iron conditions. All strains were grown overnight in low iron conditions. Secondary low iron TSBDC cultures were inoculated to a starting $O.D_{540}$ of 0.02.

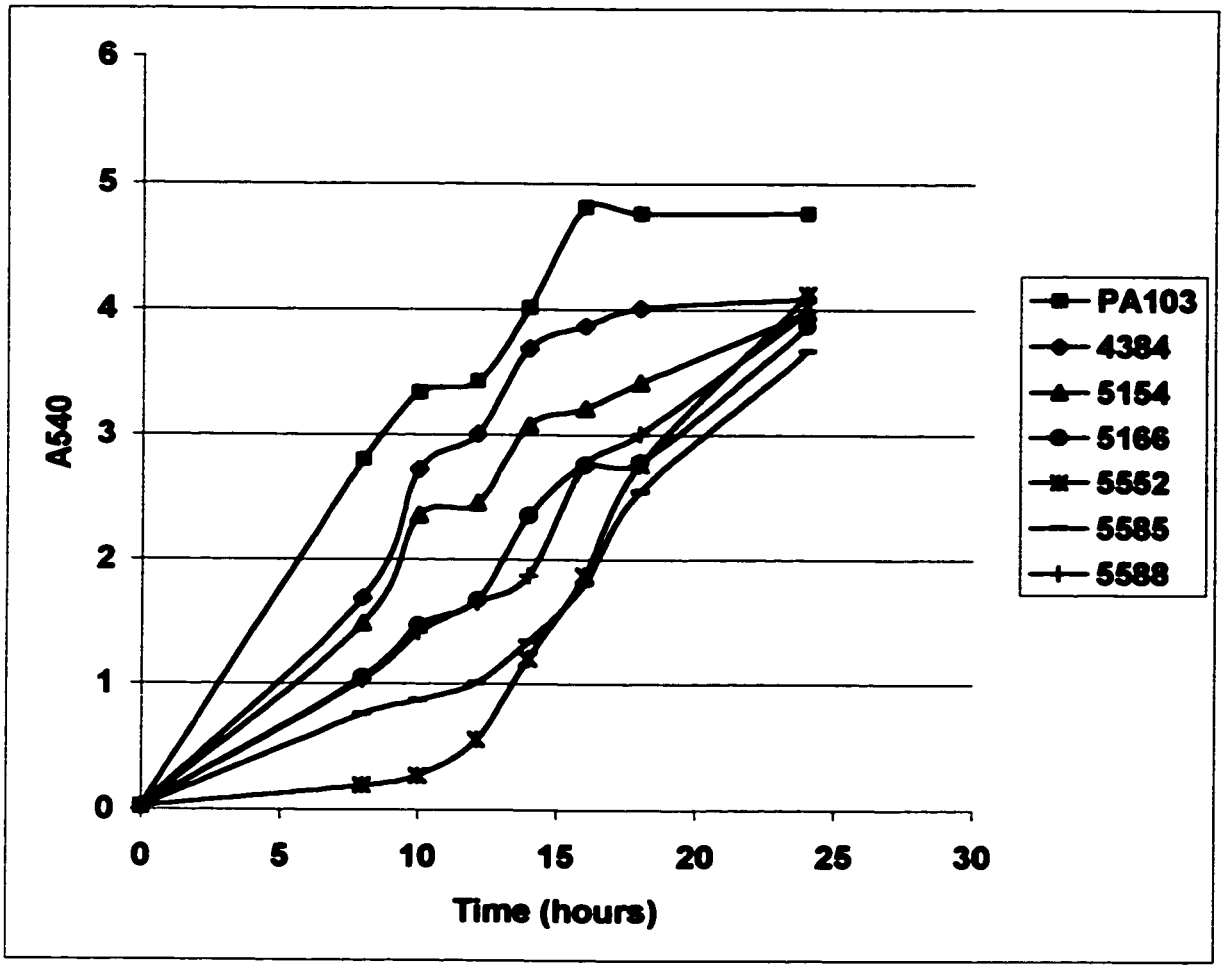
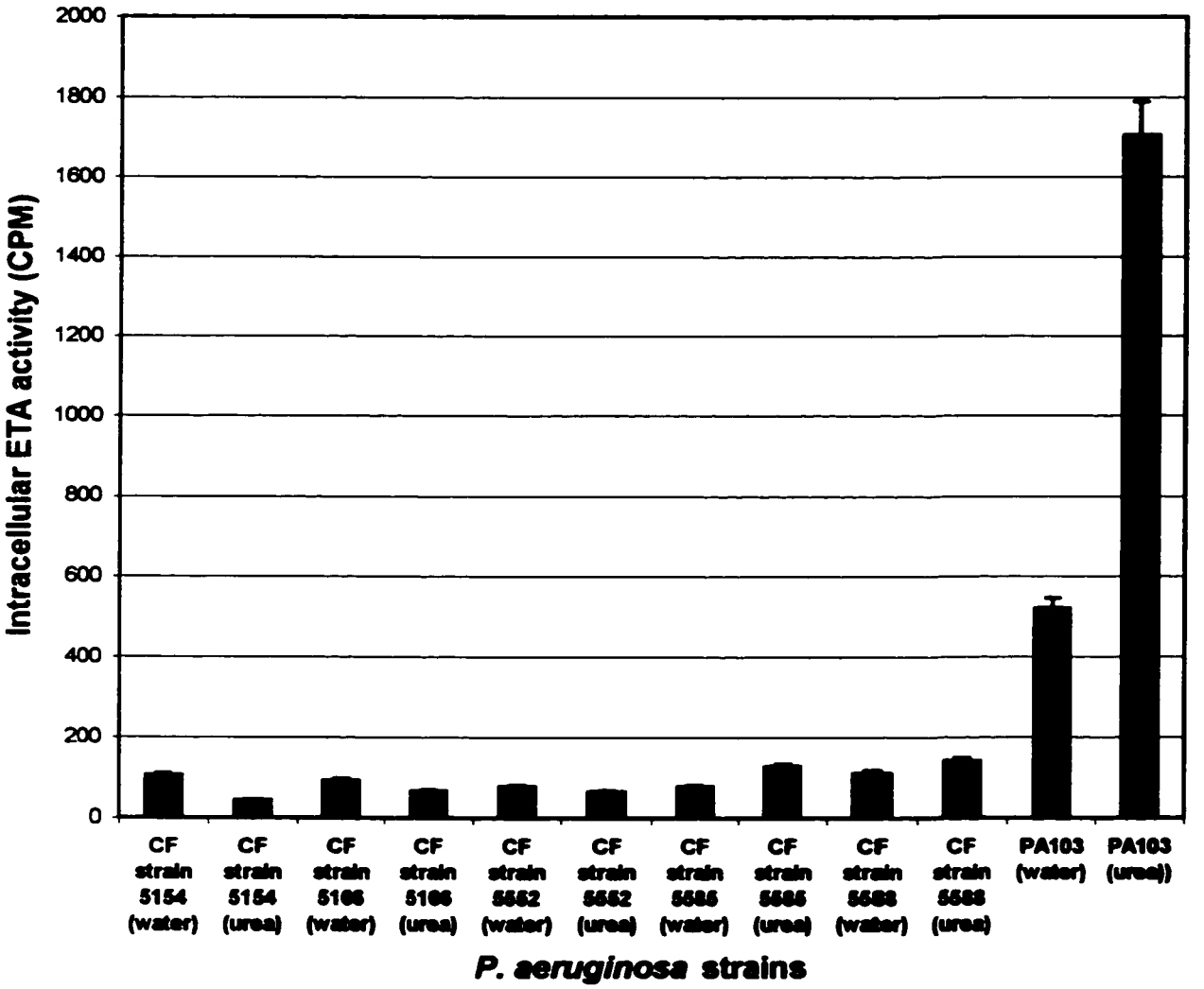

A540
Time (hours)
0
1
2
3
4
5
6
0
5
10
15
20
25
30
PA103
4384
5154
5166
5552
5585
5588

Figure 24. Intracellular level of ADPRT activity among CF isolates. Each strain was grown overnight in 10 ml of TSBDC media. Secondary cultures were inoculated the following day to obtain a starting $O.D_{540}$ of 0.02. Once the culture reached an $O.D_{540}$ of 4, the cells were pelleted by centrifugation and the supernatants discarded. The cell pellets were resuspended in an appropriate volume of 10mM Tris-HCl, 1 mM EDTA. The cells were disrupted by sonication releasing intracellular ETA, and resuspended in buffer solution and assayed for ADPRT activity. Each strain was activated by the addition of urea or water. Each sample was performed in triplicate.


Intracellular ETA activity (CPM)
2000
1800
1600
1400
1200
1000
800
600
400
200
0
CF strain 5154 (water)
CF strain 5154 (urea)
CF strain 5166 (water)
CF strain 5166 (urea)
CF strain 5552 (water)
CF strain 5552 (urea)
CF strain 5585 (water)
CF strain 5585 (urea)
CF strain 5588 (water)
CF strain 5588 (urea)
PA103 (water)
PA103 (urea))
P. aeruginosa strains

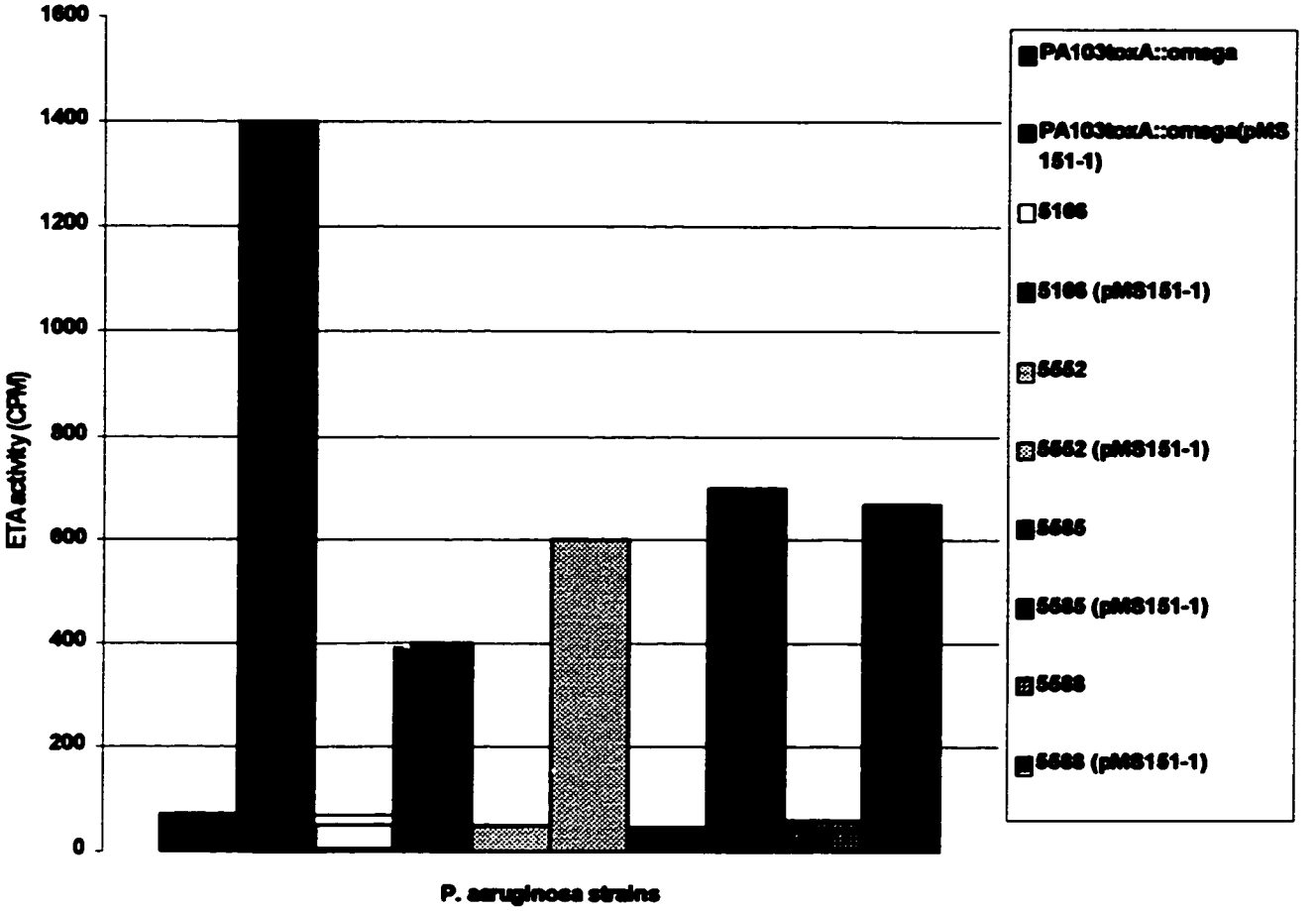
activity for each CF isolate was measured (Figure 24). As it was previously demonstrated for strain 4384, no detectable difference in intracellular ADPRT activity was observed for any of the five CF isolates. This confirms that a defect in the secretory machinery is not the cause of the decrease in ADPRT activity.

Complementation analysis using the wild type *toxA* containing plasmid pMS151-1 was also performed on four of the five CF isolates. Complementation of strain 5154 was hindered by the fact that this strain was highly resistant to carbenicilin. Since the plasmid contains the *bla* gene as the selectable marker this made the screening process impossible. Increasing the concentration of carbenicillin used to select for transformants was attempted but a single colony was not isolated. Figure 25 shows partial complementation of the four CF isolates with pMS151-1. In this figure, the ETA negative strain PA103*toxA*::Ω was complemented *in trans* with the wild type plasmid pMS151-1. Complementation of the four CF strains with pMS151-1 did not restore the ETA activity to the same level as the control strain. These data are similar to the complementation data observed previously with strain 4384. The different levels of complementation achieved in each strain may suggest a difference in the regulation of expression of *toxA* by the CF strains or by differences in secretion patterns between strains.

4.2 Prevalence of mutations in domain III of ETA isolated from five *P. aeruginosa* CF isolates.

To this point, analysis of the five CF strains provided results comparable to what had previously been obtained from the characterization of strain 4384.

Figure 25. Complementation analysis of four CF isolates. The plasmid pMS151-1 was electroporated into strain 5166, 5552, 5585 and 5588. The positive transformants were screened on VBMM supplemented with carbenicillin. The resulting strains were analyzed for ETA activity using the methodology described by Chung and Collier (1977).


1600
1400
1200
1000
800
600
400
200
0
ETA activity (CPM)
P. aeruginosa strains
PA103toxA::omega
PA103toxA::omega(pMS 151-1)
5166
5166 (pMS151-1)
5662
5662 (pMS151-1)
5685
5685 (pMS151-1)
5688
5688 (pMS151-1)

The next objective was to determine if the *toxA* gene from these strains contain mutations which could account for the reduction in ADPRT activity. Previously, sequence analysis of the entire *toxA* gene from strain 4384 showed the presence of three mutations in domain III of ETA. Since no further mutations were identified in the *toxA* gene, it was decided to concentrate on the enzymatic domain of *toxA*. The enzymatic domain of *toxA* was amplified directly from chromosomal DNA using two sets of primers (Figure 26). The first set of primers (KF10-KR12) was used for the amplification of a 358 bp region of the ETA enzymatic domain. The second set of primers (KF6-CL1) was used for the amplification of a 340 bp fragment which included the remaining portion of the enzymatic domain of ETA. For the amplification reaction, the Ultma polymerase which possesses 3' to 5' proofreading ability was used to ensure that the PCR amplification did not generate mutations. It was possible to amplify the 358 bp product from chromosomal DNA isolated from strain 4384 (Figure 27). Next, it was decided to amplify the enzymatic domain of strain 4384 by PCR as a control, to assess whether the mutations identified earlier could be identified by PCR. In addition, it was important to ensure that additional mutations by PCR were not created. As an additional positive control, the same fragment from the plasmid pMS151 containing WT *toxA* was amplified. When the concentration of chromosomal DNA was decreased an amplification product was not detected. In addition, when the chromosomal DNA was substituted with water, this control reaction did not show a similar 358 bp product. The last portion of the enzymatic domain was amplified using the second set of primers (data not shown). The two amplified products were cloned into a T-tailed cloning vector and sequenced. Sequence analysis of the amplified fragments from strain 4384 revealed the

Figure 26. Schematic representation of the PCR amplification of the enzymatic domain of *toxA*. The enzymatic domain of *toxA* was amplified using two sets of primer complementary to the *toxA* DNA sequence. The amplified fragments were then subcloned into PCR 2.1™.

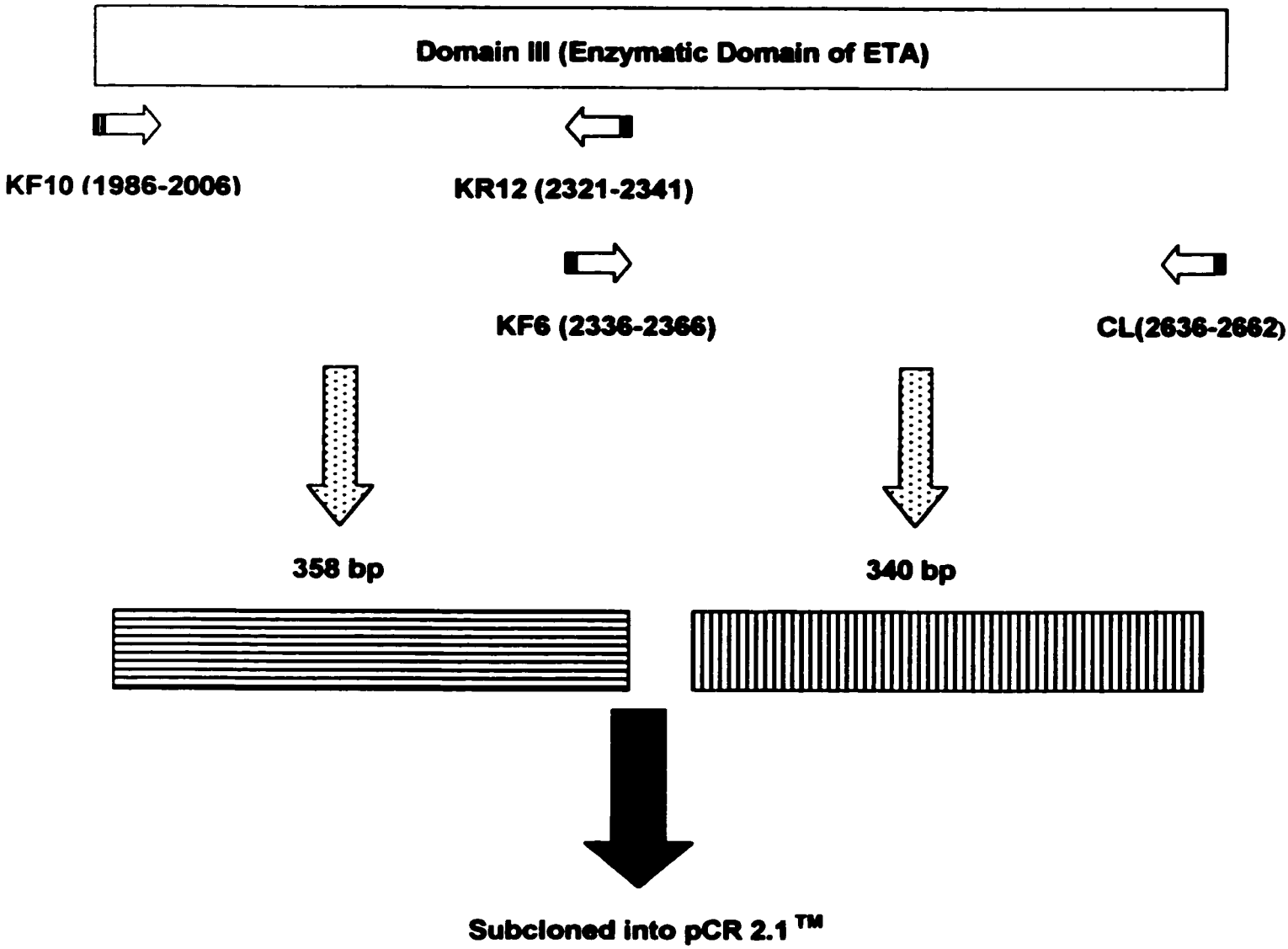

Domain III (Enzymatic Domain of ETA)
KF10 (1986-2006)
KR12 (2321-2341)
KF6 (2336-2366)
CL(2636-2662)
358 bp
340 bp
Subcloned into pCR 2.1 ™

Figure 27. PCR amplification of the 358 bp fragment from the enzymatic domain of *toxA* from CF strain 4384. The first half of the enzymatic domain of *toxA* from strain 4384 was amplified using the KF10-KR12 set of primers. The amplified DNA fragments were visualized by agarose gel electrophoresis. Lane 1. 1 kb DNA ladder (Gibco/BRL). Lane 2. negative control containing water and no template. Lane 3-4 represents the amplified DNA fragment from 1μg of chromosomal DNA isolated from strain 4384. Lane 5 represents the DNA amplified from 1 ng of chromosomal DNA from strain 4384. Lane 6 contains amplified 358bp fragment from the positive control plasmid pMS151.

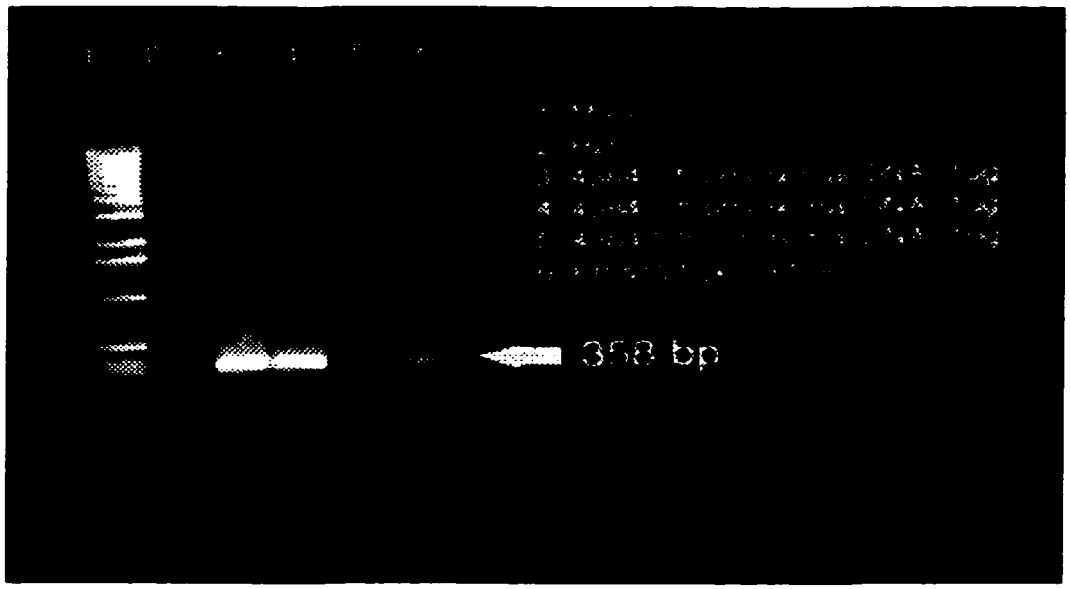
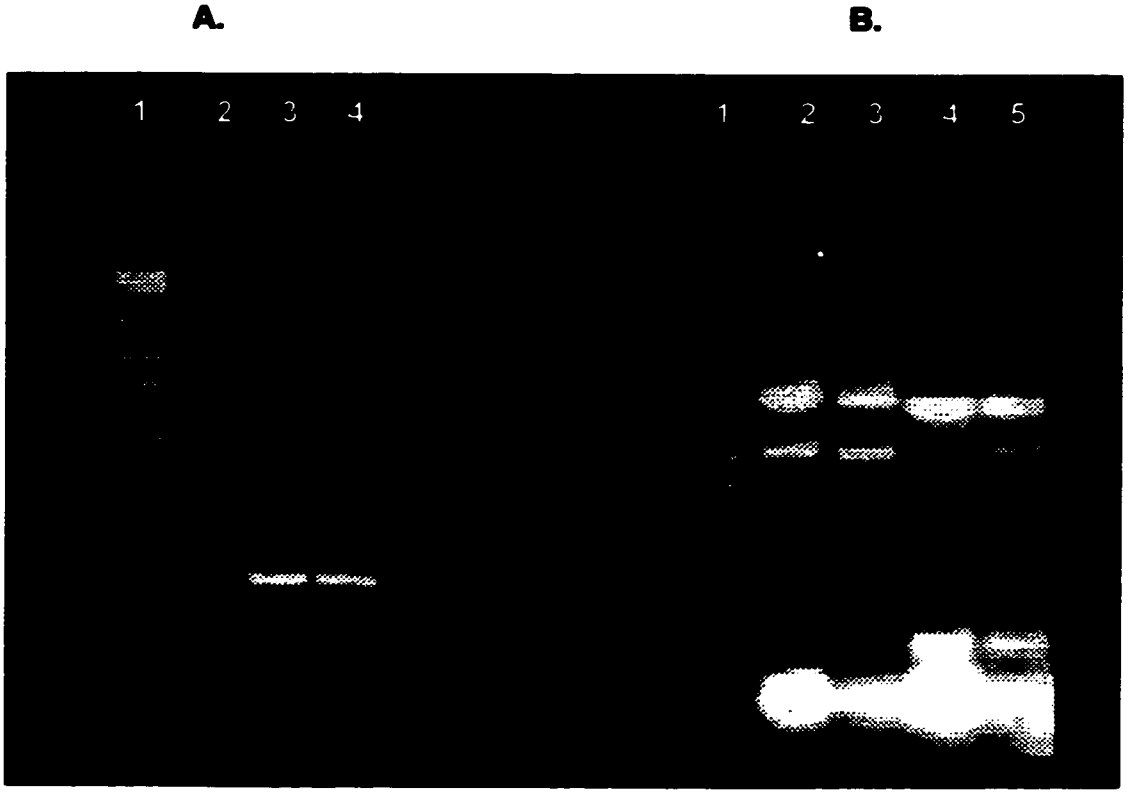
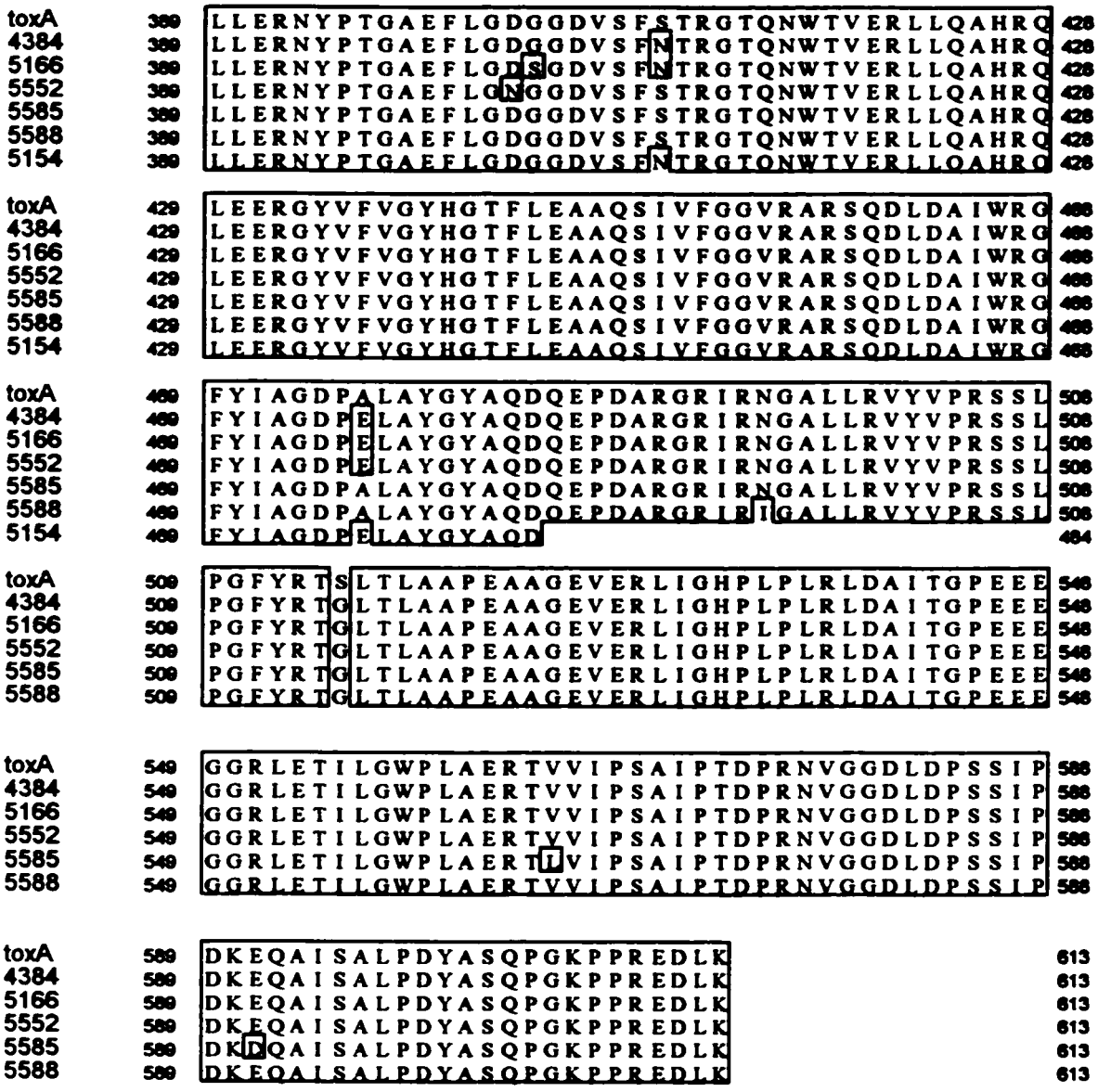

358 bp

Figure 28. Amplification of the 340 bp and 358 bp region of the enzymatic domain of strain 5154. **(A)** The enzymatic domain of *toxA* from strain 5154 was amplified using two sets of primers. The amplified DNA fragments were visualized by agarose gel electrophoresis. Lane 1. DNA ladder. Lane 2. negative control lacking template DNA. Lane 3 represents the 340 bp amplified DNA fragment from 1μg of chromosomal DNA isolated from strain 5154. Lane 4 represents the 358 bp DNA product amplified from 1 μg of chromosomal DNA from strain 5154. (B) Both fragments were cloned into the PCR 2.1™ cloning vector. Lane 1. DNA ladder. Lane 2. PCR 2.1™ 5154*toxA*$^{1986\text{-}2341}$undigested. Lane 3. PCR 2.1™ 5154 *toxA*$^{2336\text{-}2662}$undigested. Lane 4. PCR 2.1™ 5154*toxA*$^{1986\text{-}2341}$digested with *Eco*RI Lane 5. PCR 2.1™ 5154*toxA*$^{2336\text{-}2662}$ digested with *EcoR*I.


A.
B.
1
2
3
4
1
2
3
4
5

same mutations as previously described (Figure 7). No other mutations were generated by PCR amplification. These data indicated that amplification of the enzymatic domain of ETA by PCR could be used to analyze *toxA* genes of the remaining CF strains. Figure 28 shows the successful amplification of the 340 bp and 358 bp fragment from chromosomal DNA isolated from strain 5154 followed by cloning into the PCR 2.1™ vector. Sequence analysis of the enzymatic domain of each strain was performed following amplification and cloning of both fragments for each strain into the T-tailed vector. The DNA encoding the enzymatic moiety of *toxA* from CF strains, 5154, 5166, 5552, 5585 and 5588 were cloned and sequenced in order to investigate the possibility of conserved mutations which could account for the reduced ADPRT activity (Figure 29). The two mutations found in the active site of ETA identified in 4384, Ala-476 to Glu and Ser-515 to Gly, were also present in 5166, 5154 and 5552, but were absent from strains 5585 and 5588. In addition, strain 5154 contained a stop codon at residue 534 which would prematurely truncate the ETA protein in this strain. This explains the apparent absence of ETA demonstrated in Western blotting of 5154 supernatant, as no functional ETA is produced by this strain (Figure 2). Other point mutations were observed throughout the coding region for the enzymatic moiety of *toxA*, however, these were unique to each strain and were not conserved among the five CF isolates examined. Five *P. aeruginosa* environmental isolates, strains 2770, 2850, 2906, 3540 and 3546 were selected to determine if mutations arise only among CF isolates. Amplification of the enzymatic domain of the *toxA* gene of the five strains was performed as previously described. Sequence analysis of the five strains revealed the presence of two conserved mutations in strains 2770, 2850, and 2906 whereas

Figure 29. Schematic representation of mutations found in the enzymatic domain of the *toxA* gene from the six clinical isolates. The enzymatic moiety of *toxA* from 5154, 5166, 5552, 5585 and 5588 were amplified using two different sets of primers. The amplified fragments were then cloned into a T-tailed vector (pCR2.1™) and sequenced by The University of Calgary Core DNA services. Changes in the resultant amino acid composition of the CF isolates ETA are highlighted.


toxA 380 LLERNYPTGAEFLGDGGDVSFSTRGTQNWTVERLLQAHRQ 428
4384 380 LLERNYPTGAEFLGDGGDVSFNTRGTQNWTVERLLQAHRQ 428
5166 380 LLERNYPTGAEFLGDSGDVSFNTRGTQNWTVERLLQAHRQ 428
5552 380 LLERNYPTGAEFLGNGGDVSFSTRGTQNWTVERLLQAHRQ 428
5585 380 LLERNYPTGAEFLGDGGDVSFSTRGTQNWTVERLLQAHRQ 428
5588 380 LLERNYPTGAEFLGDGGDVSFSTRGTQNWTVERLLQAHRQ 428
5154 380 LLERNYPTGAEFLGDGGDVSFNTRGTQNWTVERLLQAHRQ 428
toxA 429 LEERGYVFVGYHGTFLEAAQSIVFGGVRARSQDLDAIWRG 468
4384 429 LEERGYVFVGYHGTFLEAAQSIVFGGVRARSQDLDAIWRG 468
5166 429 LEERGYVFVGYHGTFLEAAQSIVFGGVRARSQDLDAIWRG 468
5552 429 LEERGYVFVGYHGTFLEAAQSIVFGGVRARSQDLDAIWRG 468
5585 429 LEERGYVFVGYHGTFLEAAQSIVFGGVRARSQDLDAIWRG 468
5588 429 LEERGYVFVGYHGTFLEAAQSIVFGGVRARSQDLDAIWRG 468
5154 429 LEERGYVFVGYHGTFLEAAQSIVFGGVRARSQDLDAIWRG 468
toxA 469 FYIAGDPALAYGYAQDQEPDARGRIRNGALLRVYVPRSSL 508
4384 469 FYIAGDPELAYGYAQDQEPDARGRIRNGALLRVYVPRSSL 508
5166 469 FYIAGDPELAYGYAQDQEPDARGRIRNGALLRVYVPRSSL 508
5552 469 FYIAGDPELAYGYAQDQEPDARGRIRNGALLRVYVPRSSL 508
5585 469 FYIAGDPALAYGYAQDQEPDARGRIRNGALLRVYVPRSSL 508
5588 469 FYIAGDPALAYGYAQDQEPDARGRIRIGALLRVYVPRSSL 508
5154 469 FYIAGDPELAYGYAQD 484
toxA 509 PGFYRTSLTLAAPEAAGEVERLIGHPLPLRLDAITGPEEE 548
4384 509 PGFYRTGLTLAAPEAAGEVERLIGHPLPLRLDAITGPEEE 548
5166 509 PGFYRTGLTLAAPEAAGEVERLIGHPLPLRLDAITGPEEE 548
5552 509 PGFYRTGLTLAAPEAAGEVERLIGHPLPLRLDAITGPEEE 548
5585 509 PGFYRTGLTLAAPEAAGEVERLIGHPLPLRLDAITGPEEE 548
5588 509 PGFYRTGLTLAAPEAAGEVERLIGHPLPLRLDAITGPEEE 548
toxA 549 GGRLETILGWPLAERTVVIPSAIPTDPRNVGGDLDPSSIP 588
4384 549 GGRLETILGWPLAERTVVIPSAIPTDPRNVGGDLDPSSIP 588
5166 549 GGRLETILGWPLAERTVVIPSAIPTDPRNVGGDLDPSSIP 588
5552 549 GGRLETILGWPLAERTVVIPSAIPTDPRNVGGDLDPSSIP 588
5585 549 GGRLETILGWPLAERTIVIPSAIPTDPRNVGGDLDPSSIP 588
5588 549 GGRLETILGWPLAERTVVIPSAIPTDPRNVGGDLDPSSIP 588
toxA 589 DKEQAISALPDYASQPGKPPREDLK 613
4384 589 DKEQAISALPDYASQPGKPPREDLK 613
5166 589 DKEQAISALPDYASQPGKPPREDLK 613
5552 589 DKEQAISALPDYASQPGKPPREDLK 613
5585 589 DKDQAISALPDYASQPGKPPREDLK 613
5588 589 DKEQAISALPDYASQPGKPPREDLK 613

no mutation was observed in strains 3540 and 3546 (data not shown). The first mutation is a change of Val-407 for Ile-407. The second mutation is a change of a Ser-515 for a Gly-515. ADP-ribosyl transferase activity assays were performed on each strain and indicated comparable ETA activity in strain 2770 and 2906 to wild type levels. No ETA enzymatic activity was found in strains 2850, 3540 and 3546. Thus, since strain 2770 and 2906 both carried the Gly-515 mutation found in strain 4384 and are still enzymatically active this may suggest that Gly-515 is not critical for the enzymatic activity of ETA. However, it is possible that the combination of both mutations, Gly-515 and Glu-476, cause the loss of ADPRT activity in strain 4384.

Summary.

Five additional CF isolates with reduced ADPRT activity were examined. It was determined that a defect in the secretory machinery was not responsible for the observed decreased in ADPRT activity in these strains. In addition, complementation analysis of four CF strains indicated that no accessory factors are produced by these isolates. Sequence analysis of the enzymatic moiety of each CF strain revealed the presence of mutations in domain III. Some mutations were conserved between each strain, but random mutations were also found in some strains. The presence of mutations in the enzymatic domain of ETA may be responsible for the reduction in ADPRT activity observed in these CF isolates. These results suggest that some CF isolates produce an exotoxin A with reduced enzymatic activity, and this may partially explain the pathogenesis of chronic lung infections due to *P. aeruginosa*. Sequence analysis of the enzymatic domain of five environmental strains demonstrated the presence of two conserved mutations in three of the five strains. However, no random mutations were

observed in those strains suggesting that the presence of mutations in some of the strains may be an acquired phenomenom.

Chapter 5

Discussion

Studies of CF patients have shown that infection by *Pseudomonas aeruginosa* commonly results in colonization of the lung and establishment of a chronic infection. This has a severe impact on patient's prognosis because chronic infections by *P. aeruginosa* leads to accelerated deterioration of pulmonary function (Kerem *et al.,* 1990). Even though considerable progress has been made in CF research, the link between the genetic disease and the associated lung infection that ultimately leads to chronic lung infection and respiratory failure, still remains to be elucidated (Bals *et al.*, 1999). Despite aggressive antibiotic therapies, *P. aeruginosa* pulmonary infections remain the leading cause of morbidity and mortality in CF patients (Hoiby, 1977).

The pathogenesis of *P. aeruginosa* in the lungs of CF patients is complex and certain features have not yet been determined. Chronic lung infections of CF patients with *P. aeruginosa* are characterized by the emergence of *P. aeruginosa* isolates with distinct characteristics (Govan and Harris, 1986). CF isolates are characterized by the conversion to a mucoid phenotype through the overproduction of alginate (Burke *et al.*, 1991; Jaffar-Bandjee *et al.*, 1995; Woods *et al.*, 1986). In addition, the bacteria isolated during the chronic phase of the disease display reduced motility due to the loss of the flagella (Mahenthiralingam *et al.*, 1994) and altered LPS structure (Hancock *et al.*, 1983). Associated with this phase of the lung disease is a gradual rise in the antibody titres to a number

of *P. aeruginosa* virulence factors such as elastase, alkaline protease and exotoxin A (Hollsing *et al.*, 1987; Jaffar-Bandjee *et al.*, 1995; Jagger *et al.*, 1982; Moss *et al.*, 1986). During chronic infections, *P. aeruginosa* elicits a strong inflammatory response that is characterized by increased levels of circulating antibodies and neutrophils at the site of infection (Wheeler *et al.*, 1984).

ETA is a major virulence factor produced by *P. aeruginosa* that inhibits host polypeptide synthesis through its modification of eukaryotic EF-2 (Iglewski and Sadoff, 1977). ETA is cytotoxic to a number of mammalian cells (Middlebrook and Dorland, 1977) and also inhibits granulocytes and macrophage progenitor cell proliferation (Pollack *et al.*, 1978; Stuart *et al.*, 1982). ETA appears to play an important role in the lung infection of CF patients. The importance of this virulence determinant is evident by the high levels of *toxA* transcription by *P. aeruginosa* within the lungs of CF patients (Raivio *et al.*, 1994; Storey *et al.*, 1992). Using polyclonal antibodies against ETA, Jaffar-Bandjee *et al.* (1995) also demonstrated that the production of ETA could be detected in sputa from CF patients. Their data suggested a correlation between acute pulmonary exacerbation and production of ETA (Jaffar-Bandjee *et al.*, 1995). Furthermore, a specific host response against ETA was observed in CF patients as indicated by rising serum antibody titres to ETA (Hollsing *et al.*, 1987; Jagger *et al.*, 1982; Klinger *et al.*, 1978). The importance of ETA was demonstrated by Cross et al. (1980) and Moss et al. (1986) who showed an association between the mortality rate among CF patients and the serum antibody levels to ETA.

These data taken together suggest that ETA is important in the pathogenicity of *P. aeruginosa* in the chronic pulmonary infection associated with CF. However, even though ETA seems to play a role in the pathogenesis of *P. aeruginosa*, its exact role is not well understood.

In this study, the main objective was to examine the role of ETA in CF patients chronically infected with *P. aeruginosa*. Of particular interest were the levels of ETA produced by CF isolates as it had been observed previously that high levels of transcription of *toxA* occurred in the lungs of some patients with CF (Raivio *et al.*, 1994). Thus, two patients who were chronically colonized by *P. aeruginosa* and were suffering from severe respiratory disease were selected for intense study. Based on colony morphology, AP-PCR and Southern Blot analysis, it was possible to isolate five strains of *P. aeruginosa* from the sputum samples of two patients: strains 4384, (5154, 5166), 5552, 5585 and 5588. These results were unexpected since it was previously demonstrated that CF patient can be but are not usually infected with multiple strains. Even though strains 5154 and 5166 were demonstrated to be indistinguishable using these techniques, phenotypic and genotypic variations have been observed.

The production and function of ETA, and the *toxA* from the five CF strains were examined in detail. It was possible to detect ETA in the supernatants from laboratory cultures of five of the six isolates using anti-ETA antibodies and Western blot analysis (Figure 2). The presence of a 66 kDa immunoreactive

band was observed from strains 4384, 5166, 5552, 5585 and 5588. However, it was demonstrated that the level of expression of ETA varies between the strains. Expression of ETA from strain 4384 and 5585 was comparable to that of the hypertoxigenic laboratory strain PA103, whereas less ETA expression was observed from strains 5166, 5552 and 5588. This variation could explain the differences in the levels of transcript accumulation for *toxA* observed between bacterial populations from sputum samples (Raivio, unpublished data). This indicates that ETA expression from *P. aeruginosa* strains may fluctuate between patients or bacterial populations. Despite the differences in the levels of ETA expression, the results suggest that the majority of CF strains are able to produce ETA, albeit at decreased levels. When these isolates were grown in optimal conditions for ETA production, a 18-fold reduction in ADPRT activity was detected in each culture supernatant compared to WT PA103 (Table 2). The apparent lack of ADPRT activity of ETA from supernatants of CF strains that appear to transcribe high levels of *toxA* and express ETA may explain why others have reported low levels of ETA from CF isolates (Woods *et al.*, 1986; Burke *et al.*, 1991).

One strain, 4384 was selected, to further characterize the nature of this enzymatic deficiency and to perform an in depth investigation on the level of ETA expression. Previously, CF isolate 4384 had been shown to transcribe *toxA* at a level similar to the hypertoxigenic strain PA103 (Raivio *et al.*, 1994). In strain 4384, maximal *toxA* transcription occurred in low iron conditions later in the

growth cycle (Raivio, unpublished data). Furthermore the highest point of *toxA* transcription was achieved at an $O.D._{540}$ of 4.0 in strain 4384 which is comparable to strain PA103. The inability to detect high levels of ADPRT activity in the supernatant from strain 4384 was puzzling since it does not correlate with the observed level of *toxA* transcription (data not shown). One possible explanation for this drastic reduction in ADPRT activity could be that the toxin was building up intracellularly as a result of a defect in secretion. However, intracellular ETA from strain 4384 was low (Figure 9). Thus, it is concluded that the secretory machinery of strain 4384 is not responsible for the reduction in enzymatic activity.

The low ADPRT activity from strain 4384 (Table 2) could be due to a high level of protease production by the strain, an inhibitory accessory factor, or it may be an inherent property of the toxin produced. Complementation *in trans* of strain 4384 with the wild type *toxA* containing plasmid pMS151-1 does not completely restore the defect in ADPRT activity of strain 4384 when compared to the ETA deficient strain PA103*toxA*::Ω complemented with the same plasmid (Figure 10). The incomplete complementation of strain 4384 may be due to the high level of proteases produced by this strain acting to degrade the toxin protein (Raivio, unpublished data). Hence, the enzymatic activity of ETA from strain 4384 in a protease deficient and ETA deficient strain PA103*toxA*::Ω was assessed. Figure 11 shows the presence of a 66 kDa immunoreactive band when PA103*toxA*::Ω was complemented *in trans* with pCG (*tox*-SF) which

contains the *toxA* gene from strain 4384. Strain PA103*toxA*::Ω carrying a plasmid with the *toxA* gene from strain 4384 did not produce a toxin with restored ADPRT activity thereby confirming the absence of an accessory factor existing in strain 4384 (Figure 12). In addition, when native ETA was mixed with the supernatant from strain 4384, no decrease in ADPRT activity was observed. Complementation studies (Figure 10-11-12) and mixed toxin studies (Figure 13) showed that neither proteases nor inhibitory accessory factors are responsible for the reduced toxin enzymatic activity of ETA from strain 4384.

An analysis of the specific activity of ETA from strain 4384 suggested that the reduced ADPRT activity may be due to an altered amino acid sequence resulting in a functionally deficient protein. It has been previously shown that amino acids 400-608 in domain III were sufficient for the full enzymatic activity of ETA (Chow *et al.*, 1989, Siegall *et al.*, 1989). Carroll and Collier (1987) identified a critical glutamic acid residue at position 553 of ETA. Their model proposed that the COOH side chain of this residue is close to the NAD^+ molecule and is cleaved during the transfer of the ADP-ribose from NAD^+ to the eukaryotic EF-2. Several studies have also attempted to map the substrate binding site of ETA in order to gain a better understanding of the mechanism of catalysis of ETA. These studies have shown the presence of several residues in the active site of domain III that are critical for the enzymatic activity (Bradhuber *et al.*, 1988; Carroll and Collier, 1987; Han and Galloway, 1995; Wick, 1990; Wick and Iglewski, 1988). The observed reduction in the specific ADPRT activity in strain 4384 may be due

to the presence of mutations within the enzymatic moiety of ETA which could render the toxin partially enzymatically inactive. Sequence analysis of the entire *toxA* gene of 4384 revealed the presence of three mutations in domain III of *toxA*. However, the study was focussed on the two mutations located within the active center of ETA. The first mutation is a change from an Ala-476 to a Glu. This residue is located within an α-helix in domain III of *toxA* which is believed to be an important part of the catalytic site (Domenighini *et al.*, 1994). The presence of a mutation within the α-helix may alter the active site cavity, possibly interfering with substrate binding or transfer of the NAD^+ moiety. The second mutation, which is at the protein surface, is a substitution of Gly-515 for Ser-515. This amino acid may be essential for the protein integrity or may act as a protein modification site. Neither mutation (position 476 nor 515) has previously been reported to be specifically involved in ADPRT activity. Sequence analysis of five environmental strains has demonstrated the presence of two mutations in the enzymatic domain of ETA of three of the strains. The two changes were conserved between these 3 strains and include a change of a Val-408 to an Ile-408 and the substitution of Ser-515 for Gly-515. The change of Ser-515 to Gly-515 was conserved among the CF isolates and three environmental isolates which may indicate the importance of this residue for enzymatic activity. However, two of the three strains have retained enzymatic activity. Based on this data, it seems plausible that Gly-515 is not critical for the enzymatic activity of ETA. However, it is possible that in strain 4384 both mutations may act in concert to reduce the ADPRT activity of the ETA in this strain. Alternatively, the

presence of the two mutations may affect proper folding of the protein and render the catalytic site inaccessible to the substrate.

Several studies have demonstrated that the ADPRT activity of ETA is directly responsible for the inhibition of protein synthesis in eukaryotic cells (Iglewski and Kabat, 1975; Iglewski and Sadoff, 1979). The ADPRT activity of ETA from *P. aeruginosa* was also shown to be critical for the biological effect of the toxin (Douglas and Collier, 1990). The present findings suggest that the specific activity of ETA from concentrated supernatant of strain 4384 (approx. 5500 cpm/ug) was 12-fold lower than that of the toxin from the concentrated supernatant of strain PA103 (approx. 59,000 cpm/ug). As such, it was expected that ETA from strain 4384 would have reduced cytotoxicity to coincide with its reduced ADPRT activity. However, Figure 22 shows that despite the reduced ADPRT activity, the cytotoxic activity of the toxin from strain 4384 was moderately 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 carboxyl terminal end of the protein have also been shown 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. In addition, it is possible that the altered protein can no longer recognize its cellular target and therefore cannot be

internalized or inhibit protein synthesis. However, in this study this question was not addressed. It is also possible that exotoxin A may have either a second cytotoxic activity in domain III or that the cytotoxicity of the toxin is not tightly linked to the ADPRT activity.

That the cytotoxicity of ETA may be independent of its ADPRT is reminiscent of a similar situation that may also be occurring with the other ADPRT toxin produced by *P. aeruginosa*, exoenzyme S. Exoenzyme S is an ADP-ribosyl transferase that transfers the ADP-ribose moiety from NAD to a number of eukaryotic proteins (Iglewski *et al.*, 1978). Exoenzyme S is distinct from ETA by its substrate specificity, including vimentin and members of the G-protein family such as Ras, Rab4 and Rap1A and by its requirement for factor-activating (FAS) exoenzyme S eukaryotic proteins for enzymatic activity *in vitro* (Coburn *et al.*, 1989a; Coburn *et al.*, 1989b; Bette-Bobillo *et al.*, 1998; Coburn *et al.*, 1991). In addition, exoenzyme S differs from ETA since it is translocated directly into mammalian cells by the type three secretion pathway. Exoenzyme S has two immunological related forms, a protein of Mr 49,000 (ExoS) and a protein of Mr 53,000 (ExoT) (Yahr *et al.*, 1996). ExoS has been implicated in the dissemination of the bacteria from the initial infections sites to the bloodstream (Nicas *et al.*, 1985). Interestingly, both forms of this protein induce morphologic changes in tissue culture cells (Vallis *et al.*, 1999). However, ExoT has only 0.2% of the ADPRT activity of ExoS (Yahr *et al.*, 1996). These results suggest that ExoS and ExoT can produce cytopathic effects in tissue culture cells that are

independent of the ADPRT activity. Knight *et al.* (1995) also demonstrated that the enzymatic moiety of ExoS is located within the 222 carboxy-terminal amino acids of the protein. As well, similar to ETA, a critical glutamic acid residue was identified at position 381 and shown to be critical for ADPRT activity (Liu *et al.*, 1996). Further evidence which suggests that ExoS is able to inhibit protein synthesis by two different mechanisms on host cells was provided by Frithz-Lindsten *et al.* (1997). The main objective of their study was to establish whether the ADP-ribosyl transferase activity was important for the cytotoxicity and the antiphagocytic activity of ExoS. Using PCR, the critical glutamic acid residue at position 381 was replaced with an alanine residue creating a full length ExoS with a 2000 fold reduction in ADPRT activity. HeLa cells infected with this mutated version of ExoS showed a cytotoxic response comparable to the wild type strain. This strongly implies that ExoS contains two distinct functional domains or mechanisms to inactivate its target proteins (Frithz-Lindsten *et al.*, 1997).

A similar pattern of reduced ETA ADPRT activity was also observed among five other CF isolates, strains 5154, 5166, 5552, 5585, and 5588. Similar to the observations with strain 4384, the absence of accessory factors or a secretory defect was demonstrated in each of the isolates. To further investigate the reduction in ADPRT activity, the ETA enzymatic moiety of *toxA* (domain III) of the five remaining CF were amplified and sequenced strains to investigate whether the nucleotide and resultant sequences differed from that of the wild

type strain PA103 and from strain 4384. Surprisingly, a number of mutations which could potentially be responsible for the reduced ADPRT activity in the CF strains were identified. Two mutations, Glu-476 and Gly-515, were conserved in strains 4384, 5166, 5585 and 5588 (Figure 29) which suggests that these mutations, by themselves or acting in concert, may play a critical role in the reduction in ADPRT activity. A premature stop codon was identified in strain 5154 resulting in the production of a truncated protein. However, a smaller truncated protein was not detected by Western blot analysis of the supernatant from this strain (Figure 2). The absence of an immunoreactive band may suggest that the truncated protein is unstable and rapidly degraded by proteases or that the polyclonal anti-ETA antibody can no longer detect the expressed truncated protein.

Even though only a small number of isolates have been examined, the results indicate that mutations in *toxA* may be common among CF isolates. The results suggest that the proposed role of exotoxin A in the pathogenesis of CF lung infection may require modifications especially during the chronic phase of the infection. It is likely that during initial infection by *P. aeruginosa*, ETA plays a role in establishment and persistence of the infection. Once the infection becomes chronic, it appears that mutations arise in the colonizing strains. The present research has identified mutations in *toxA*, but mutations may also arise in other genes (Taylor *et al.*, 1992). Previously it has been demonstrated that random mutations occur among numerous loci in CF strains (Boucher *et al.*,

1997; Evans *et al.*, 1994; Taylor *et al.*, 1992). Taylor *et al.* (1992) demonstrated the high occurrence of auxotrophic mutant strains among CF isolates. Auxotrophic mutants of *P. aeruginosa* are found in abundance in the CF lung environment and may have a selective advantage thus permitting their survival (Taylor *et al.*, 1992).

In addition, *P. aeruginosa* strains that are isolated from chronically infected patients are atypical since they often exhibit an LPS phenotype lacking the O-antigen (Lam *et al.*, 1989). Since it is believed that LPS-smooth strains are required for the initial colonization phase of the infection, a mechanism responsible for the loss of the O side chain must exist during the chronic infection. Evans *et al.* (1994) and Romling *et al.* (1994) reported the presence of mutations at the *rfb* locus which encodes the enzyme required for the synthesis of LPS-O polysaccharides. The absence of the O-side–chain antigen among CF isolates may also confer a selective advantage by the evasion of the host response directed towards the O antigen. Furthermore, more recent studies have shown that spontaneous mutations occur in the *algU/algT mucABSD* cluster which leads to conversion to mucoidy in CF isolates (Boucher *et al.*, 1997; Devries and Ohman, 1994). In most cases, the mucoid phenotype is the result of a mutation in the *mucA* gene which codes for a negative regulator of alginate biosynthesis (Boucher *et al.*, 1997). Thus, these findings and the observations of mutations at the *toxA* locus of CF isolates seem to suggest that mutations are commonly found among the CF population.

Most recently, Sokurenko *et al.* (1999) discussed the possibility that pathoadaptive mutations could be responsible for the modification or loss of function of certain genes. This change or loss of function of pre-existing genes would be dependent on the frequency of random mutations and would allow for a selective advantage to the strains to adapt to their niche thereby ensuring persistence. This mechanism of adaptation can be paralleled to the genetic variation observed among *P. aeruginosa* CF isolates that confer increased bacterial pathogenicity. For example, Mahenthiralingam *et al.* (1994) observed that the *P. aeruginosa* strains isolated from chronically infected patients were non-motile. Most surprisingly, isolates taken during early colonization were motile and expressed both flagellin and pilin proteins. It was also shown that the loss of the flagella during the chronic phase of the infection by *P. aeruginosa* confers a 15 fold increase in resistance to nonopsonic phagocytosis (Mahenthiralingam *et al.*, 1994). In addition, mutations in the negative regulator of alginate allow a selective adaptive advantage of *P. aeruginosa* by increasing its resistance to phagocytosis and opsonization. Therefore, it is possible that production of ETA with altered ADPRT activity gives a selective advantage to the bacteria when the patient becomes chronically infected with *P. aeruginosa*.

In the initial infection with *P. aeruginosa* it is likely that ETA activity plays a role in the release of iron and other nutrients by host cells upon cell death. However, in the chronic infections associated with CF, the role of ETA is not

clear. It is possible that in chronic infections, ETA may play a role independent of its ADP-ribosyl transferase activity. Thus, once *P. aeruginosa* is firmly established in the lungs of CF patients, the enzymatic activity of ETA may not be required. One interesting observation from these studies is that the loss of motility, altered LPS structure and overproduction of alginate was rarely observed among environmental isolates and other clinical isolates. This may suggest the presence of an environment signal in the lungs of chronically infected CF patients which would trigger these phenotypic changes. Woods *et al.* (1991) demonstrated, using a chronic rat lung infection model with *P. aeruginosa,* that chronicity elicits the conversion of the non-mucoid to a mucoid phenotype and decreased the production of several virulence determinants. These data taken together imply the presence of an environmental factor associated with *P. aeruginosa* chronic infections.

Sequence analysis of five environmental strains showed the presence of two conserved mutations in three of the strains: 2770, 2850 and 2906. Interestingly, unlike the CF isolates no other random mutations were identified from these strains. How the mutations arose in the CF strains is not clear. However, it is possible that the mutations in the CF strains are related to stresses imposed by rigorous antibiotic chemotherapy characteristic of the common therapy for this disease. Ciprofloxacin is a potent therapeutic agent that is prescribed for the treatment of several gram-negative and gram-positive bacterial infections. Ciprofloxacin is commonly used to reduce the bacterial load in the

lungs of adult CF patients. As a fluoroquinolone, ciprofloxacin induces DNA gyrase, an essential type II bacterial DNA topoisomerase, to cleave the DNA at the site-specific double-strand excision (Gocke, 1991). Even though ciprofloxacin is a potent antibiotic agent, several studies suggest that this compound is a potent bacterial mutagen. Philip *et al.* (1987) reported that an increase in the rate of mutations ocurring in the lactose and galactose operons of *E. coli* was proportional to the use of fluorinated quinolones. Furthermore, reversion of the *hisG428* ochre mutation in *Salmonella typhimurium* was increased by the presence of ciprofloxacin (Clerch *et al.*, 1992). The occurrence of mutations in these genes may be due to the fact that ciprofloxacin induces the error-prone SOS DNA repair system of bacteria. Improvement of the pulmonary function is observed with CF patients that are treated with ciprofloxacin without complete eradication of *P. aeruginosa* (Bryan, 1989). It is possible that after a frequent and prolonged exposure to ciprofloxacin, random mutations arise in the genome of *P. aeruginosa*. In the present study, the prevalence of mutations in the *toxA* gene of six CF strains isolated from the sputum of chronically infected patients was demonstrated. In addition, preliminary evidence demonstrated the presence of similar mutations in the *lasR* gene and the *lasB* gene of CF isolate strain 6106 (Kirkham *et al.*, unpublished data).

At the present time, the specific reason behind the emergence of ETA inactive strains of CF isolates is not known. However, three putative reasons regarding how the presence of ETA with altered ADPRT activity might contribute

to pathogenesis can be postulated. First, the reduction in ADPRT activity may render the organisms less virulent which would be beneficial for the host and the pathogen as they move toward a commensal relationship. Second, the deficient toxin may have no effect on the pathogenicity of the bacteria. Last, the presence of ETA with altered ADPRT activity may increase the virulence of *P. aeruginosa* which would be harmful for the host.

Several studies have demonstrated that during chronic lung infections with *P. aeruginosa,* the levels of virulence factors produced by bacteria are reduced when compared to the production observed in a laboratory setting (Woods *et al.*, 1986). In addition, previous research revealed a high frequency of auxotrophic mutants, non-motile mutants and mucoid producing strains among *P. aeruginosa* clinical isolates (Govan and Harris, 1986; Burke *et al.*, 1991; Woods *et al.*, 1986). This suggests that the presence of mutations in certain genes may confer a selective advantage on strains in the lungs of CF patients (Boucher *et al.*, 1997). Since ETA is one of the most toxic proteins secreted by *P. aeruginosa,* it would be beneficial for bacteria if mutations in the *toxA* gene leave the organism less pathogenic, thereby allowing both the host and the bacteria to survive for a longer period of time. The decrease in virulence may also explain why *P. aeruginosa* lung infections remain restricted to the lungs and do not disseminate into blood or other tissues (Govan and Nelson, 1992).

The second theory is that mutations in domain III of ETA affect the enzymatic activity of the toxin without affecting the virulence of the altered protein. Thus, an alternative activity other than the ADPRT would be present in ETA to inhibit the target cells. Preliminary evidence indicates that despite a 12 fold reduction in its enzymatic activity, ETA from strain 4384 retains comparable cytotoxicity. This implies that in CF lung infections, the virulence of ETA may be due to the intrinsic cytotoxicity of the toxin and less to the enzymatic activity of ETA. It is possible that similar to ExoS, ETA contains two functional domains which interact with the target cells (Frithz-Lindsten *et al.*, 1997).

In addition, there is the possibility that the altered ADPRT activity of ETA increases the virulence of the toxin. An increase in virulence could be advantageous for the bacteria during the chronic infection allowing the release of sequestered nutrients. It is possible that the altered ADPRT activity has an effect on the immune system which might increase the pathogenicity of the strains. This increase in pathogenicity may be due to the hyperproduction of the deficient ETA which could lead to an increase in antibody-antigen complex formation, or by modulating the production of cytokines.

It is possible that the mutations found amongst the six CF strains are not only restricted to the *toxA* gene. This hypothesis is further supported by the fact that other mutations have been found in the *lasR* and *lasB* genes isolated from CF isolate strain 6106 (Kirkham and Storey, unpublished). The mechanism

responsible for the emergence of mutations in the genome of certain isolates may be the presence of mutator strains in the lungs of CF patients. Leclerc *et al.* (1996) demonstrated the prevalence of mutator strains among *E. coli* and *Salmonella enterica* pathogens. In all cases, the mutator phenotype was caused by a defect in the methyl-directed mismatch repair (MMR) system of the pathogen. A defect in the MMR system allows recombination between species. Thus, the bacteria could inherit by horizontal transmission any genes from the reservoir of pathogenic and commensal bacteria that would enhance its survival (Leclerc *et al.*, 1996). The mutator phenotype would be beneficial since it allows a rapid variation of genotype in response to chemotherapeutic agents, or to rapidly alter expressed antigens so as to evade the host immune response. In addition, the mutator phenotype could accelerate adaptive evolution since it would allow bacteria to obtain new genetic information at a time when it is critical for survival and colonization of new hosts (Matic *et al.*, 1997). A characteristic of a mutator population is a high proportion of auxotrophic mutants. These mutants will be characterized by slow growth under a number of conditions. Interestingly, two of our CF isolates, strain 5552 and 5585 have decreased growth rates as compared to the strain PA103 (Figure 23). This observation, and the type of frequency of mutations in the CF isolates, led to the examination of the CF isolates for the presence of mutator strains.

Preliminary evidence from the laboratory demonstrated a high frequency of mutator strains among CF isolates (Azcona *et al.*, unpublished data). The

presence of mutator strains could explain the presence of several mutations found in the *toxA* gene of the six CF isolates. Preliminary data also indicate that strain 4384 is a potential mutator strain (Azcona *et al.*, unpublished data). It is likely that mutator strains would be unstable in the lungs of CF patients. This is due to the hypermutable state which would lead to the rapid accumulation of mutations throughout the genome. The increased levels of mutations in the genome of the bacteria would likely cause mutations in essential housekeeping genes and would be detrimental. However, *P. aeruginosa* may eventually overcome the effect of lethal mutations by aquiring new genetic information. Survival of mutator strains in the lungs of CF patients could potentially introduce a series of mutations into the *P. aeruginosa* population in the lungs. This could be detrimental for the host since it may allow the bacteria to adapt to the host and develop a chronic infection that is refractory to antibiotics.

To date, there is preliminary evidence for the presence of mutator strains in patients chronically infected with *P. aeruginosa*. The link between the occurrence of mutator strains and pathogenicity remains to be elucidated. However, the potential for genetic variation may be advantageous for *P. aeruginosa* to adapt in a changing environment. In summary, this work suggests that *P. aeruginosa* strains producing ETA with reduced ADPRT activity are commonly found among CF isolates. The objective was to determine if the altered ADP-ribosyl transferase activity was due to the presence of mutations in the enzymatic moiety of the toxins. Sequence analysis of the *toxA* gene of six

clinical isolates of *P. aeruginosa* revealed several mutations in the enzymatic domain of the ETA proteins. The location of these mutations has not been previously shown to have an influence on enzymatic activity. An explanation for the reduced enzymatic activity of these toxins may be the presence of potential inhibitory factors produced by the CF isolates. However, complementation analysis of strain 4384 and mixed toxin experiments have eliminated the possibility of an accessory modifying factor in strain 4384. In previous studies the enzymatic activity and cytotoxicity of ETA have been shown to be linked. Thus, it was of interest to determine whether the reduced ADPRT activity observed in strain 4384 resulted in a reduced cytotoxicity of the toxin. In strain 4384, the ETA produced retained cytotoxicity despite a 12 fold reduction in ADPRT activity. These results suggest that at least in some CF isolates, the ETA produced remains cytotoxic despite having a reduced enzymatic activity. The correlation between exotoxin A 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). The present data also suggest that the cytotoxicity of the toxin, rather than the ADPRT activity, may be the more important feature in the chronic phase of CF lung disease. 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 in CF isolates.

Future directions:

The results obtained from this study raise several questions. Initially, six CF isolates that expressed ETA with altered ADPRT activity were isolated. As yet, the mechanism responsible for these mutations is unknown. One possibility is the presence of a triggering factor in the CF lung environment. In this study, the research focussed on isolates from the lungs of CF patients chronically infected with *P. aeruginosa.* To investigate whether the mutations in the *toxA* gene are associated with the chronicity of infections, the prevalence of mutations amongst *P. aeruginosa* isolates from different sources and stages of the disease could be determined. Numerous studies have established that CF patients remain colonized with one or a few strains of *P. aeruginosa* throughout infection (Ogle *et al.*, 1987; Pasloske *et al.*, 1988; Fegan *et al.*, 1991). Therefore, it would be interesting to collect sputum samples from the same CF patient from the initial phase of the infection with *P. aeruginosa* and monitor the occurrence of mutations throughout the lifelong infection.

Previous work by Leclerc *et al.* (1996) determined that defects in the MMR system were responsible for the mutator phenotype in *Salmonella* and *E. coli* strains. In most cases the defect in the MMR system was located in the intergenic region between the *mutS* and *rpoS* genes. Preliminary evidence also demonstrated the presence of mutator strains amongst the *P. aeruginosa* CF populations. It is of great importance to characterize these mutator strains in order to understand their prevalence and significance. It would be interesting to

compare the survival rate of the putative mutator strains in response to environmental and physiological stress conditions with some well-characterized laboratory strains or other CF isolates. If the repair system of the mutator strains is affected, one might expect a rapid decline in the survival of these strains. In addition, whether the MMR system is defective in these mutator strains could be determined by complementation analysis such experiments could also determine if other pathways leading to the mutator phenotype exist in *P. aeruginosa*. Papillation test assays based on reversion to lactose and galactose utilization can also be established in *P. aeruginosa* to determine the mutation rates. Ciprofloxacin was shown to generate mutations in *Salmonella* and *E. coli* by inducing the SOS repair system (Clerch *et al.*, 1991). Due to its prolonged usage in treatment of CF pulmonary exacerbations it would be interesting to examine the role of ciprofloxacin in the formation of mutator strains.

The role that ETA with reduced ADPRT activity plays in the pathogenesis of CF lung infections still remains to be elucidated. In order to study this further the purification of ETA from strain 4384 is required. In this study it was possible to clone the *toxA* gene from strain 4384 and PA103 in an expression vector. The *toxA* gene from strains PA103 and 4384 was cloned into a His-Tag expression vector since it allowed the easy purification of high-levels of bacterial protein. It was also demonstrated that the small-scale purification of ETA from pQE-32 (WT*toxA*) by Western blot analysis was feasible (Figure 20). Thus, the *toxA* gene from strain 4384 should in theory be expressed at similar levels from the

expression vector. However, both purification schemes require optimization for larger scale expression of ETA. Purification of ETA native to both strains may allow for the isolation of ETA-specific antibody that will be useful for future functional studies.

Another exciting result from the present study was the finding that the altered ETA from strain 4384 is cytotoxic. This suggests that ETA, like ExoS, may possess two functional domains for interaction with the host cell. To test this hypothesis, the cytotoxicity of ETA from strain 4384 in different cell lines will have to be measured. It would be also interesting to determine if the cytotoxic effect of 4384 ETA is due to necrosis or apoptosis. In addition, it would be of great interest to compare the LD_{50} of ETA from strain 4384 and strain PA103 in the neutropenic mouse model (Woods *et al.*, 1997). It would be interesting to determine whether an isogenic strain with the altered ETA is more or less virulent than the wild type strain PA103.

Chapter 6

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