Expression and mutagenesis of recombinant cholera toxin A subunit
by Kirsten Louise Vadheim

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology
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Abstract:
Vibrio cholerae causes a severe, potentially life-threatening diarrheal disease which is the result of the intoxication of epithelial cells of the small intestine by the cholera toxin. Cholera, pertussis and E. coli heat labile toxins are all protein exotoxins of the A/B format which exert their intoxicating effects within eucaryotic cells by ADP-ribosylating G proteins involved in the regulation of the adenylate cyclase complex, resulting in the increase of intracellular cAMP. These toxins share two short regions of sequence similarity within the enzymatically active A subunits. Substitution of the amino acid lysine for arginine at position nine in the first region of sequence similarity of pertussis toxin resulted in a protein with no detectable enzymatic activity. These results suggested that this first region of sequence similarity may be implicated in the ADP-ribosyltransferase activity of the toxin.

A previously poorly characterized gene coding for cholera toxin was subcloned, sequenced and expressed in E. coli. A series of site-specific mutations within the first region of sequence similarity was generated by polymerase chain reaction mutagenesis. Assays of the resulting proteins revealed that substitution of lysine for arginine, or arginine for aspartic acid, reduced ADP-ribosyltransferase activity to background levels, as did deletion of the entire region of eight amino acids. Substitution of a glycine for proline at position twelve had no effect on enzymatic activity, however.

Conservative substitutions of particular single amino acids within the first region of sequence similarity of cholera toxin resulted in reduction of enzymatic activity of the mutant cholera toxins to background levels. This corroborates previous studies with pertussis toxin and E. coli heat labile toxin. We conclude that the first region of sequence similarity in these toxins is important for enzymatic activity of the respective proteins, and await the crystallization of cholera toxin for a more detailed understanding of the relationship of these mutagenic studies to NAD binding sites within the toxin.

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by

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This dissertation is dedicated to Marilyn and Roger Vadheim,
whose love and example made it possible.

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ABSTRACT

Vibrio cholerae causes a severe, potentially life-threatening diarrheal disease which is the result of the intoxication of epithelial cells of the small intestine by the cholera toxin. Cholera, pertussis and *E. coli* heat labile toxins are all protein exotoxins of the A/B format which exert their intoxicating effects within eucaryotic cells by ADP-ribosylating G proteins involved in the regulation of the adenylate cyclase complex, resulting in the increase of intracellular cAMP. These toxins share two short regions of sequence similarity within the enzymatically active A subunits. Substitution of the amino acid lysine for arginine at position nine in the first region of sequence similarity of pertussis toxin resulted in a protein with no detectable enzymatic activity. These results suggested that this first region of sequence similarity may be implicated in the ADP-ribosyltransferase activity of the toxin.

A previously poorly characterized gene coding for cholera toxin was subcloned, sequenced and expressed in *E. coli*. A series of site-specific mutations within the first region of sequence similarity was generated by polymerase chain reaction mutagenesis. Assays of the resulting proteins revealed that substitution of lysine for arginine, or arginine for aspartic acid, reduced ADP-ribosyltransferase activity to background levels, as did deletion of the entire region of eight amino acids. Substitution of a glycine for proline at position twelve had no effect on enzymatic activity, however.

Conservative substitutions of particular single amino acids within the first region of sequence similarity of cholera toxin resulted in reduction of enzymatic activity of the mutant cholera toxins to background levels. This corroborates previous studies with pertussis toxin and *E. coli* heat labile toxin. We conclude that the first region of sequence similarity in these toxins is important for enzymatic activity of the respective proteins, and await the crystallization of cholera toxin for a more detailed understanding of the relationship of these mutagenic studies to NAD binding sites within the toxin.

INTRODUCTION

Cholera

History

"I am poured out like water, and all my bones are out of joint:
my heart is like wax; it is melted in the midst of my bowels.
My strength is dried up like a potsherd,
and my tongue cleaveth to my jaws;
And Thou hast brought me into the dust of death."

Psalm 22

Although there is no direct scientific evidence to support the idea, cholera may be one of the many diarrheal diseases that has plagued humanity throughout recorded history. It certainly has been endemic in the Indian subcontinent for centuries. Within the last two centuries seven pandemics have emerged from India, beginning in 1817 and ending with the most recent in 1961-1975. The second pandemic in about 1829 was the first to reach the New World, spreading throughout the whole of North and South America in addition to Russia, Europe and Great Britian. Repeated importations of the

disease brought about the third pandemic in the 1850's, the worst on record. Mortality rates were often 50 - 60% {van Heyningen and Seal, 1983}.

The first breakthroughs in understanding the causes and prevention of cholera occurred in the 1850's. Filippo Pacini reported the etiologic agent of cholera as a tiny curved bacterium, *Vibrio cholerae*, in 1854, and Robert Koch re-discovered it in 1883, naming it *Bacillus virgulus*. The British physician John Snow, by analyzing the water sources of London households, demonstrated that the disease was transmitted by sewage-contaminated water. His suggested preventive measures included personal cleanliness, avoidance of fecal contamination of food and drink, and destruction of the bacteria by cooking food and boiling water {van Heyningen and Seal, 1983, Holmgren and Svennerholm, 1983}.

The last major epidemic in the Western Hemisphere was in 1866-67. Cholera remains endemic in India, Pakistan and Bangladesh, with occasional flareups in these countries and throughout southern and southeast Asia and Africa, but the mortality rate has been reduced dramatically by the development of oral rehydration techniques. Diarrhea remains the leading cause of infant mortality in Third World countries but is more often due to rotavirus, *Escherichia coli* and dysentery bacilli than vibrios. However, in endemic areas cholera continues to cause significant morbidity, with a mortality rate usually less than 1%. Unfortunately, mortality rates still reach 60% in the initial phases of outbreaks in some areas, primarily due to inadequate medical care {van Heyningen and Seal, 1983, Isselbacher *et al.*, 1980, Siddique *et al.*, 1988}.

On January 29, 1991, reports of an outbreak of severe diarrhea in a coastal region of northern Peru reached medical authorities. *Vibrio cholerae* 01, Inaba, biotype El Tor, was isolated from patients' stools. Active surveillance, a national laboratory network and a public information campaign were immediately implemented. Within two weeks of the first reported case of diarrheal disease, 1,859 individuals had been hospitalized with severe gastroenteritis, and 66 died. {Centers for Disease Control, 1991} As of April 26, 1991, there had been 169,255 cases of probable cholera in Peru, 1,007 in Ecuador, 176 in Brazil, 26 in Chile, four in Brazil, and four imported cases in New Jersey {Pan American Health Organization, personal communication}. Clearly, cholera remains a very real threat to millions of people.

Clinical Disease

The most frightening aspects of cholera are its sudden onset and the rapidity with which its victims deteriorate. The incubation period is from six to forty-eight hours. Symptoms begin suddenly with vomiting and diarrhea, usually without any prodrome. Typical rice-water stools continue after the vomiting has ceased, resulting in the loss of 20 to 30 liters of water per day in severe cases. This extreme and rapid dehydration produces the typical picture of cholera: cold, clammy skin, a feeble and often imperceptible pulse, tachypnea, pinched face, sunken eyes, poor skin turgor, and shrivelling of the skin on the hands and feet. The patient is cyanotic, hypotensive and xerostomic. If untreated, severely afflicted patients may die within a few hours of the onset of symptoms. This sudden transformation of a seemingly

normal, healthy individual to an enfeebled, cadaverish victim within a few hours is undoubtedly responsible for the panic that often accompanied the cholera pandemics. The disease usually runs its course in two to seven days, and significant sequelae are rare {van Heyningen and Seal, 1983, Collier and Mekalanos, 1980, and Isselbacher *et al.*, 1980}.

Oral rehydration of a cholera patient was first attempted by a Scottish physician, Thomas Leith, in 1832. He was able to revive the individual, but once rehydrated the patient simply continued to purge and quickly died {van Heyningen and Seal, 1983}. Not until the 1960's was a simple, effective formula for oral rehydration developed. The standard recipe for one liter of rehydration fluid is 20 g glucose, 4.2 g NaCl, 4 g $NaHCO_3$, and 1.8 g KCl.

Treatment usually consists of re-establishing the electrolyte balance by administration of intravenous salt solutions and encouraging the patient to drink the rehydration fluid to replace water lost through diarrhea. Streptomycin or tetracycline will decrease the volume of stool released and may shorten the course of disease by interrupting the multiplication of the bacteria, but antibiotics are of little benefit without simultaneous fluid replacement {Holmgren and Svennerholm, 1983}. Mass chemoprophylaxis, vaccination and quarantine of cholera victims have been proven to be ineffective, and simply divert scarce resources from efforts to adequately treat victims and control further spread of cholera {Centers for Disease Control, 1991}.

Bacteriology and Epidemiology of *Vibrio cholerae*

Vibrio cholerae is an aerobic, Gram negative, curved rod with a single polar flagellum which is responsible for the bacterium's rapid motility {Brock and Madigan, 1988}. In addition to the well-defined cholera toxin (Ctx) proteins, *V. cholerae* elaborates a considerable number of virulence factors, including hemolysins, a neuraminidase, a variety of proteases, hemagglutinins, at least two types of fimbriae, a Shiga-like toxin, and several outer membrane proteins that are regulated along with other known virulence factors and which may be involved in osmoregulation {Holmgren and Svennerholm, 1983, and Taylor, 1989}.

Both O and H somatic antigens are present on *V. cholerae*. All cholera epidemics traced to date have been caused by serogroup O type 1 (O1) bacterial strains. Three antigenic factors, A, B, and C, are used to subdivide the O1 serogroup into the serotypes, shown in Table 1.

Serotype	O Factors
Ogawa	AB
Inaba	AC

Table 1. Serogroup division of cholera vibrios.

Serologic conversion among the serotypes can occur during natural infection; this seems to be related to the appearance of agglutinating antibodies in the serum {Zwadyk *et al.*, 1980}.

Serogroup O1 contains the biotypes cholerae (classical) and El Tor {Zwadyk *et al.*, 1980}. Classical *V. cholerae* was responsible for the first six

pandemics and El Tor vibrios for the seventh. Although both can cause disease, classical strains generally synthesize more toxin {Miller and Mekalanos, 1985}. The two biotypes are differentiated on the basis of physiological properties such as hemolysin production and polymyxin sensitivity {van Heyningen and Seal, 1980}.

The El Tor biotype was named after a quarantine station on the Sinai Peninsula where this strain was first discovered in 1889. El Tor frequently infects without causing disease, and was discovered in pilgrims who had died without any symptoms of cholera. It has a much greater capacity to survive in the environment than classical strains, and has been isolated from aquatic environments and shown to grow in many foods. Because the ratio of inapparent infections to actual cases may be as high as 100 to 1, El Tor can quickly spread within a population and persist easier than the classical vibrios. When the El Tor biotype first appeared in the Celebes, it caused a cholera-like disease with a low case-infection rate but a fatality rate of 50 to 60%, at least as high as classical cholera {van Heyningen and Seal, 1983}.

Nonagglutinable (NAG) or non-cholera vibrios (NCVs) are *V. cholerae* serotypes other than O1. At one time these non-O1 strains were thought to be non-pathogenic, but NAG vibrios are now known to be responsible for up to 5% of the acute diarrheal illness in cholera-endemic areas. Nearly 50 different non-O1 serotypes have been reported so far {Kaper *et al.*, 1981, and Isselbacher *et al.*, 1981}.

All age groups are susceptible to cholera when it spreads into new areas, but in endemic regions it is primarily a disease of childhood. This is demonstrated by the close inverse relationship between the attack rate of

cholera and age. In addition, serum vibriocidal antibody titers increase with the age of individuals in endemic areas {Holmgren and Svennerholm, 1983}.

The primary route of transmission of cholera is through fecally-contaminated drinking water. Because there are a number of significant secondary routes of infection, improvement of drinking water quality alone does not effect major reductions in cholera incidence in endemic areas. Other routes of infection include ingestion of water during bathing, eating contaminated food, and interpersonal contact {Briscoe, 1984}.

Natural reservoirs for cholera outside of endemic regions are poorly understood. A chronic gall bladder carrier state has been observed in elderly convalescent cholera patients, but its relevance to the spread of the disease remains speculative {Isselbacher *et al.* 1980 and 1981}. Comparison of strains involved in a limited epidemic in Louisiana in 1978 with a case in Texas in 1973 and an imported case from Mexico in 1983 showed all three to be identical: biotype El Tor, serotype Inaba, strongly hemolytic, with unique phage sensitivity patterns and with identical *ctx* gene sequences different from those of the pandemic strain being isolated from the rest of the world at that time. This suggests that there may be a strain of *V. cholerae* which has persisted in the U.S. coastal waters for many years, perhaps establishing its own free-living cycle in water {Blake *et al.*, 1983}.

Cholera Toxin

Structure

As early as the 1880s Robert Koch suggested that cholera vibrios produce a toxin which was responsible for the disease. Not until 1959, however, was the existence of an enterotoxin proven and its importance in disease demonstrated {De, 1959, and Holmgren and Svennerholm, 1983}.

Ctx, also known as choleragen {Finkelstein *et al.*, 1964} is a secreted, heat labile protein with a molecular weight of 84,490 {Pearson and Mekalanos, 1982, and Lockman *et al.*, 1984}. This toxin is one of several A/B model toxins where A represents the enzymatically active subunit and B represents the binding subunit. Its subunit composition is AB_5. The *ctx* genes are arranged in a single transcriptional unit with one promoter controlling expression of both subunits (see Figure 1). Some strains, particularly the classical strains, contain multiple copies of the operon in the *V. cholerae* chromosome (Mekalanos 1983).

Cholera Toxin Operon

Figure 1. Genetic organization of the cholera toxin operon.

The A subunit is synthesized as a single polypeptide with a molecular mass of 29,000 kilodaltons (kDa), but is enzymatically active in a proteolytically nicked form which has two disulfide-linked chains, A_1 (23 kDa) and A_2 (6 kDa) {Mekalanos *et al.*, 1983 and Collier and Mekalanos, 1980}. Cleavage occurs at a trypsin-sensitive site immediately preceding two adjacent serine residues, a dipeptide that is unique in the translational product of the *ctx* operon. Although the disulfide bond linking the two chains is easily cleaved by reducing agents, non-covalent forces tend to prevent dissociation of the two subunits under non-denaturing conditions {Lockman *et al.*, 1984}.

The primary translation product of *ctxA* is a 258 amino acid polypeptide with an 18 amino acid hydrophobic signal sequence. The B peptide is composed of 124 amino acids, also has an 18 residue leader sequence and contains one intrachain disulfide bond. The B oligomer (B_5) is highly resistant to dissociation by reducing agents {Mekalanos *et al.*, 1983, Collier and Mekalanos, 1980 and Lockman *et al.*, 1984}.

The *ctx* operon has a characteristic ρ-dependent termination signal. A twenty-five base region of dyad symmetry at the end of the B cistron contains a G + C-rich stem loop region and a poly(T) tail {Mekalanos *et al.*, 1983}.

Regulation

Both *ctxA* and *ctxB* are transcribed from a single promoter preceding the *ctxA* gene, resulting in a polycistronic mRNA transcriptional product which contains one copy each of the *ctxA* and *ctxB* genes. Disparate translational efficiencies appear to account for the increased production of CtxB to achieve a final ratio of five CtxB subunits to one CtxA. Mutations of

the *ctx* operon which placed the *ctxB* product under the control of the *ctxA* gene translational signals resulted in approximately nine-fold less CtxB than usual {Mekalanos *et al.*, 1983}. Comparison of the Shine-Delgarno sequences of *ctxA* and *ctxB* with an *E. coli* consensus sequence also indicated that the *ctxB* sequence is almost identical with the consensus sequence, and therefore possibly translates at a higher efficiency than from the *ctxA* sequence, which differs in several locations {Lockman *et al.*, 1984}.

The *ctx* genes lie in a genetic element with a structure very similar to that of transposons (Figure 2). A 2.7 kilobase (kb) repetitive sequence, RS1, is located adjacent to and upstream of the 4.3 kb core region which contains *ctxAB* and other genes involved in the regulation of cholera toxin {Mekalanos, 1983, Miller and Mekalanos, 1984, DiRita and Mekalanos, 1989}. RS1 also forms the junction between tandem duplications of the *ctx* genetic element and is sometimes found downstream as well, so that the RS1 direct repeats flank the core sequence. RS1 can transpose and is involved in a *recA*-dependent recombination process leading to the duplication or further amplification of tandem repeats of the *ctx* element {Goldberg and Mekalanos, 1986}.

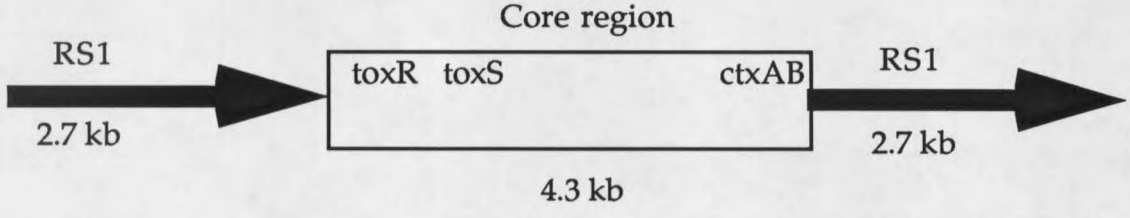

Figure 2. Schematic diagram of the ToxR regulatory region.

Ctx is not produced constitutively in *Vibrio cholerae* but is transcribed when activated by ToxR, a global regulatory protein that is the primary component in the transduction of environmental signals that lead to virulence gene expression {DiRita and Mekalanos, 1989}. The *toxR* gene, which is located upstream from *ctxAB* but within the same core element, encodes a protein of 32.5 kDa {Miller and Mekalanos, 1988}. ToxR is a transmembrane protein with cytoplasmic N-terminal and periplasmic C-terminal regions. The C-terminal domain is responsive to environmental signals such as changes in osmolarity. The N-terminus binds to the DNA sequence TTTTGAT found in multiple, tandem repeats in the region directly upstream from the *ctx* operon, thereby activating transcription from the *ctxAB* promoter. Other genes regulated by ToxR include the cluster *tcpABCDEFG*, which codes for a pilus that is the primary colonization factor of *V. cholerae*, the *acf* operon (*acfABCD*) that codes for an accessory colonization factor, and the *ompT* and *ompU* genes {V. Miller *et al.*, 1989}.

A second regulatory protein, ToxS, is the product of the *toxS* gene, located immediately downstream of the *toxR* gene in the core region of the *ctx* genetic element. The *toxR* gene was originally cloned from the classical Inaba strain 569B, which is a hypertoxinogenic but generally less virulent strain of *V. cholerae*. The 569B chromosome carrries a 1.2 kb deletion in the region where *toxS* is located. ToxS is a 19 kDa transmembrane protein with most of its sequence in the periplasmic space {V. Miller *et al.*, 1989}. It interacts with the C-terminal periplasmic domain of ToxR to confer the $ToxR^+$ state, probably by stabilizing a dimerized form of the ToxR protein {DiRita and Mekalanos, 1991}. ToxR and ToxS do not appear to have the

sensor-regulator relationship found in many other two-component bacterial virulence regulators {J. Miller *et al.*, 1989}.

A third regulatory gene involved in controlling expression of the *V. cholerae* virulence genes, *toxT*, may be a transcriptional activator whose promoter is activated by ToxR. ToxT can activate gene fusions that are dependent on *toxR* in *V. cholerae* but that cannot be activated in *E. coli* by cloned *toxR* alone {DiRita and Mekalanos, 1989}.

Pathophysiological Effects of Ctx

The human small intestine is essentially a hollow tube whose inner surface is covered with a continuous layer of columnar epithelium. This epithelium forms the outer layer of the gut mucosa and is organized into crypts (glands of Lieberkuhn), which penetrate the underlying lamina propria, and villi, which project into the lumen of the gut. Cells of the lower half of the crypts constantly proliferate and gradually migrate as they mature to the tips of the villi, where they are desquamated individually. Because of the rapid rate of cell proliferation and shedding, the entire luminal surface of the intestine is replaced approximately every five days {Junqueria and Carneiro, 1983}.

V. cholerae colonizes, but does not invade, the epithelium of the small intestine and secretes Ctx. The B protomer of the holotoxin binds to the galactosyl - N- acetylgalactosaminyl -(N-acetylneuraminyl) -galactosylglucosyl-ceramide (GM_1) ganglioside on the surface of the gut mucosal cells. GM_1 is comprised of a lipid moiety, ceramide, which is inserted into the lipid matrix of the cell membrane, and an oligosaccharide moiety which is exposed on the

cell surface. It is the oligosaccharide moiety which is responsible for the specific, tight binding of Ctx to the cell membrane {Collier and Mekalanos, 1980 and Holmgren and Svennerholm, 1983}.

Once the holotoxin is bound to GM_1, the A subunit is translocated across the membrane into the cytosol where it catalyzes the cleavage of nicotinamide adenine dinucleotide (NAD). The adenine-diphosphate-ribose (ADP-ribose) portion of NAD is then transferred to a regulatory protein (Gs) of the adenylate cyclase (AC) system. Similarly, pertussis toxin (Ptx) modifies the Gi regulatory protein (see Figure 3).

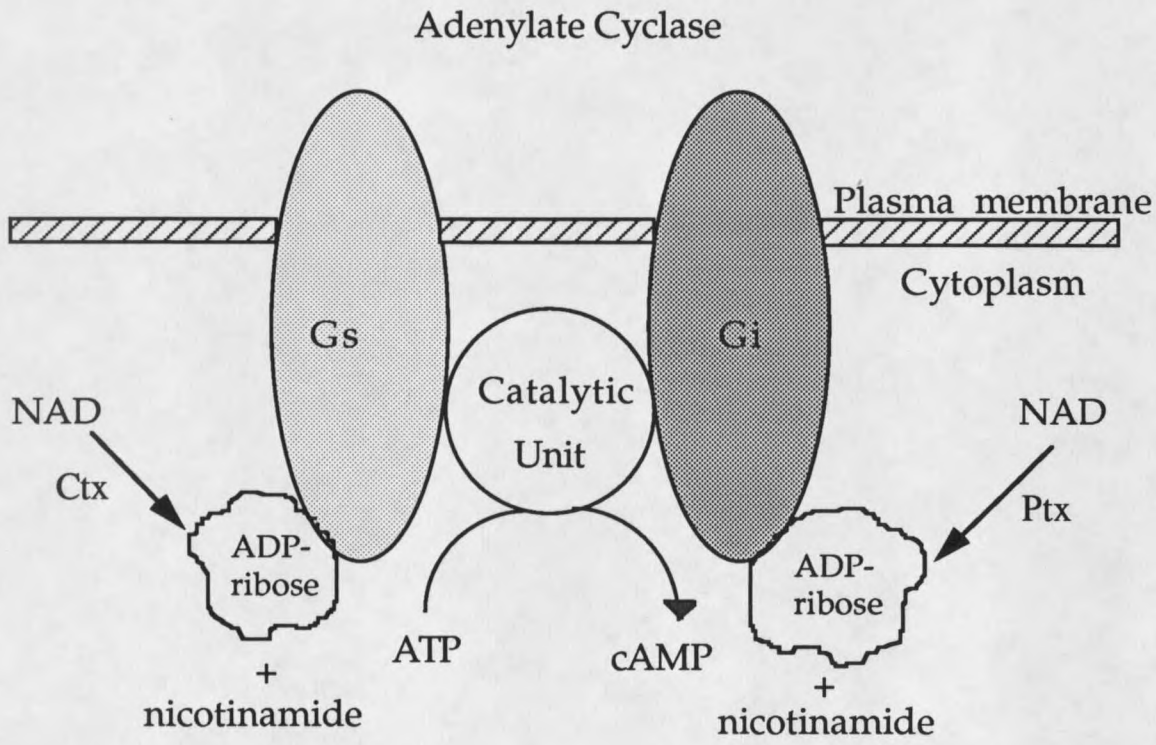

Figure 3. Representation of the adenylate cyclase (AC) complex.

The AC system is composed of stimulatory and inhibitory hormone receptors coupled through guanyl nucleotide-binding proteins (Gs and Gi) to an enzyme catalytic unit that converts ATP to cAMP. Ctx-catalyzed ADP-ribosylation of Gs activates AC, increasing intracellular cAMP concentration {Mekalanos 1983, Holmgren and Svennerholm, 1983 and Tsai *et al.*, 1987}.

Gs and Gi are members of the family of G proteins, a group of guanine-nucleotide binding proteins which also includes the *ras* oncogene products, *ras*-related proteins, and the protein translation initiation and elongation factors {Bobak *et al.*, 1989}. Gs and Gi belong to a subset of the G proteins that couple the activation of cell surface receptors to the regulation of intracellular effectors {Kahn and Gilman, 1986 and Bourne, 1988}. Gs is a heterotrimer composed of α, β and γ subunits. The α subunit is a 45 kDa protein which has two binding sites. One binds the ADP-ribose resulting from the NAD-dependent ADP-ribosyl transferase reaction catalyzed by Ctx, and the other has a high affinity for guanine nucleotides {Gilman, 1984}. As Gs is ADP-ribosylated by Ctx, the affinity of Gsα for the β and γ subunits decreases, so that ADP-ribosylation of Gsα results in an "activated" Gsα which is no longer bound to the β-γ dimer {Tsai *et al.*, 1987 and Kahn and Gilman, 1984}. Activated Gsα binds GTP and then hydrolyzes it, but at such a slow rate (1 molecule/minute) that there exists in the cell a long-lived Gsα·GTP complex which activates the catalyst of AC {Gilman 1984}. ADP-ribosylation prevents GTP hydrolysis, which would inactivate the stimulatory signal to the AC catalyst, so that AC is irreversibly turned on and intracellular cAMP concentration increases {Holmgren and Svennerholm, 1983}.

Increasing the cAMP concentration in crypt and villus cells of the small intestine disrupts the normal ion and fluid movement. Absorption of Na^+ and Cl^- by villus cells is blocked, while crypt cells begin to actively secrete Cl^- and HCO_3^-. As the osmotic equilibrium of the cells is destroyed there is a massive movement of water from the cells to the lumen of the gut, resulting in diarrhea and vomiting {Holmgren and Svennerholm, 1983, and Brock and Madigan, 1988}.

Holotoxin ($CtxAB_5$) binds to the GM_1 receptors irreversibly, so that once cells are poisoned by Ctx they continue to malfunction for their entire lifespan. The small intestine therefore cannot function normally until all Ctx-bound cells have been shed and replaced by normal cells. The epithelial cells of the large intestine are less susceptible to Ctx, but the colon does contribute to the clinical expression of cholera by failing to absorb water normally, and by secreting potassium at increased rates {van Heyningen and Seal, 1983 and Speelman *et al.*, 1986}.

Other factors affecting Ctx activity

Assays of the ADP-ribosylation activity of Ctx have included unspecified "cytosolic factors" added to enhance reactivity. These factors have now been identified as a family of 19 - 20 kDa proteins known as ADP-ribosylating factors, or ARFs, which are found in many types of eukaryotic cells {Bobak *et al.*, 1989}. In the presence of NAD, phospholipids and GTP or a non-hydrolyzable GTP analogue, ARFs appear to interact directly with the toxin in a GTP-dependent manner to enhance its catalytic activity. Both ADP-ribosyltransferase and NAD glycohydrolase activities of CtxA are increased by

ARF {Vaughan *et al.*, 1989}. ARF is also capable of interacting independently with Ctx in other Gsα-independent reactions:

1. auto-ADP-ribosylation of $CtxA_1$
2. ADP-ribosylation of agmatine
3. glycohydrolytic cleavage of NAD
4. ADP-ribosylation of phosphorylase b, serum albumin and α-lactalbumin

Although the function of ARFs in normal cellular metabolism is still unknown, these proteins could be involved in the pathogenesis of cholera by enhancing the catalytic activity of CtxA, thereby increasing the susceptibility of the intestinal cells to the toxin ({Noda *et al.*,1990}.

Similarity to other bacterial toxins

Ctx is one of a number of bacterial protein toxins that catalyze ADP-ribosylation reactions within mammalian cells using NAD as a donor substrate rather than as a coenzyme as in dehydrogenase reactions. Others in this class are *E. coli* heat labile toxin (HLT), pertussis toxin (Ptx), diphtheria toxin (Dtx) and *Pseudomonas aeruginosa* exotoxin A. In addition to their enzymatic similarities, each of these toxins has the A/B subunit structure, consisting of an enzymatically active A subunit and a B subunit which binds to specific cell surface receptors, enabling the A subunit to gain access to the cell's interior.

Ctx is most similar to HLT. Both have AB_5 subunit stoichiometry and the molecular mass values of the holotoxins are very close - 84kDa for Ctx, 86kDa for HLT. Whole A and A_1 subunit molecular weights are also very

similar and the B peptides of both have a molecular mass of 11.5 kDa. Both utilize the ganglioside GM_1 as a cellular binding site, are immunologically cross-reactive and produce the same general clinical symptom: severe diarrhea {Levine *et al.*, 1983}. Ctx and HLT ADP-ribosylate the α subunit of Gs, irreversibly stimulating the AC system {Ueda and Hayaishi, 1985}. The *A* and *B* cistrons of the *ctx* and *elt* operons are 75% (*A*) and 77% (*B*) homologous, resulting in amino acid sequences which are equally similar. The sequence similarity ends with the structural genes, however, as the promoter regions for the *elt* and *ctx* operons are completely different. This is not so surprising since the *elt* operon is located on a plasmid, where it may require more universal transcriptional signals, while the *ctx* operon retains a chromosomal location {Lockman *et al.*, 1984 and Mekalanos *et al.*, 1983}.

The chromosomal location of Ctx is consistent with the increased pathogenicity of *V. cholerae* over *E. coli*. The vibrios elaborate a number of different toxins and virulence factors, most of which are on the chromosome and are coordinately regulated, whereas the HLT and other toxins associated with *E. coli* are plasmid-associated and expressed individually. Because vibrios are capable of establishing a life cycle in fresh water independent of human transmission, they may pose more of an epidemic threat than *E. coli*, which is a human commensal. *E. coli* infections are more often associated with endemic diarrhea or traveler's diarrhea, while even today cholera can quickly become a dangerous epidemic disease whenever water supplies are threatened.

Ptx also shares an AB_5 stoichiometry with Ctx, but the B subunit of Ptx is composed of four dissimilar peptides. Ptx ADP-ribosylates the Gi protein of

the AC complex, uncoupling the inhibitory signal and irreversibly turning on cAMP production. Ctx, Ptx and HLT share two short regions of sequence similarity (Figure 4). Both regions are in the enzymatically active A subunits of the respective toxins.

First Homology Box

Ptx	(8)	Tyr	Arg	Tyr	Asp	Ser	Arg	Pro	Pro	(15)
Ctx	(6)	Tyr	Arg	Ala	Asp	Ser	Arg	Pro	Pro	(13)
HLT	(6)	Tyr	Arg	Ala	Asp	Ser	Arg	Pro	Pro	(13)

Second Homology Box

Ptx	(51)	Val	Ser	Thr	Ser	Ser	Ser	Arg	Arg		(58)
Ctx	(60)	Val	Ser	Thr	Ser	Ile	Ser	Leu	Arg		(67)
HLT	(60)	Val	Ser	Thr	Ser	Ile	Ser	Leu	Arg		(67)

Figure 4. Regions of homology between the A subunits of pertussis, cholera and *E. coli* heat-labile toxins.

These areas of similarity, known as the first and second homology boxes, suggest that these regions may be part of functional domains responsible for the similar catalytic activities of all three toxins {Locht and Keith, 1986}.

Problems addressed by research

Ptx and Ctx ADP-ribosylate two different regulatory proteins within the AC systems of columnar epithelial cells in the upper respiratory tract and the

alimentary tract, respectively. Two short regions of sequence similarity, known colloquially as the first and second homology boxes, between the two toxins have been found in the enzymatically active subunits of each toxin (Locht and Keith, 1986). Alterations of the DNA sequence in the first homology box of Ptx have a dramatic effect on the enzymatic activity, suggesting that this region of sequence similarity may have important functional implications for the ADP-ribosylating activity of these toxins. My goal was to investigate the effect of similar mutations within the first homology box of Ctx to determine whether this region of sequence similarity might correspond with functional attributes of the toxin. This was accomplished by expressing the *ctxA* gene in *E. coli*, sequencing the DNA, and performing site-specific mutagenesis on the region of the *ctxA* gene which codes for the proteins that make up the first homology box.

While pursuing the expression and mutagenesis of Ctx, some interesting results were observed regarding the export of recombinant proteins from the bacterial cytoplasm. These experiments, though not directly related to the biological activity of ADP-ribosylating toxins, shed an interesting light on some of the factors involved in protein export, and will be discussed accordingly.

MATERIALS AND METHODS

Subcloning of *ctxA*

Restriction Endonucleases

All restriction enzymes, except where otherwise indicated, were obtained from Bethesda Research Labs (BRL), Gaithersburg, MD and were used according to manufacturer's directions. DNA digested with these enzymes was analyzed by agarose gel electrophoresis, using 0.8 - 1.5% gels in Tris-borate-EDTA (TBE) buffer. Markers for agarose gels were obtained by mixing lambda phage DNA digested with *Hind*III and ϕX174 DNA digested with *Hae*III with agarose gel loading buffer (BRL, Gaithersburg, MD).

Plasmid Construction

pUC18 Vectors: pPCTX18 and pEC. pRT41 is a pBR322-derived plasmid which was provided by Dr. John Mekalanos of Harvard University. pRT41 carries the entire *ctx* operon from the *V. cholerae* classical serotype strain 569B on a 2.1 kb *EcoR*I-*BamH*I fragment {Mekalanos *et al.*, 1983). pRT41 was digested with *Nde*I to produce a 776-bp fragment encompassing the complete

ctxA gene and the first 54 bases of the *ctxB* gene. The vector pUC18 was digested with *Sma*I and dephosphorylated with calf intestinal phosphatase (CIP, Boehringer Mannheim, Indianapolis, IN). The 5' overhanging ends of the *ctxA* insert were converted to blunt ends by filling in the ends using deoxynucleotide triphosphates and the Klenow fragment of DNA polymerase I [Bethesda Research Labs, (BRL), Gaithersburg, MD]. Ligation of the blunt-ended *ctxA* insert and dephosphorylated vector was accomplished by the addition of T4 DNA ligase and 10X ligase buffer (BRL, Gaithersburg, MD) and incubation at 15° C overnight {Maniatis *et al.*, 1982}. Ligated DNA preparations were transformed into competent *E. coli* RR1 cells by the "supertransformation" procedure {Hanahan, 1983}. Resultant colonies were selected initially by screening for either the presence or absence of β-galactosidase activity as evidenced by blue or white color formation, respectively. Presence of an insert in the cloning site would result in white colonies.

Alkaline lysis plasmid mini-preparations {Maniatis *et al.*, 1982) were performed on overnight cultures grown from 24 white colonies selected from the transformation plates. Purified plasmids were digested with *Pvu*II and *Xba*I to determine the size and orientation of the insert. Two plasmids were found to carry the *ctxA* insert in opposite orientations. The plasmid containing the *ctxA* insert in the proper orientation for expression of the toxin gene was designated pPCTX18 (see appendix for plasmid maps). Both plasmids with the *ctxA* insert were sequenced by the Sanger dideoxy method using the Sequenase kit [United States Biochemical Corp. (USB), Cleveland, Ohio] to confirm the presence of the toxin gene and define the complete DNA

sequence of the fragment derived from the previously incompletely characterized plasmid, pRT41.

Digestion of pPCTX18 with *EcoR*I and *Cla*I produced a 596 bp fragment which contained only the coding region for the A_1 subunit of Ctx. This piece was ligated directly into a pUC18 plasmid previously digested with *EcoR*I and *Acc*I. The resulting plasmid, containing only A1 coding sequences in pUC18, was designated pEC.

pVEX Expression Vector: pCT7 and pCT7r. Plasmid pVEX115f+ was obtained from Dr. Vijay K. Chaudry of the National Cancer Institute, Bethesda, MD. This construct carries the origin of replication from pBR322, the f1(+) origin from pBLUESCRIPT (Stratagene Cloning Systems, LaJolla, CA), the signal sequence from *E. coli* outer membrane protein A (OmpA), and the T7 promoter (ϕ10). Ligation of an insert into the *Nde*I site of this 3.1 kb plasmid produces an in-frame fusion protein with the OmpA signal sequence. Recombinant proteins produced in this system should be exported into the periplasmic space of *E. coli* strain BL21, which expresses the T7 polymerase under the regulation of the lac promoter. Expression of the T7 polymerase is induced by addition of isopropylthio-β-galactoside (IPTG) to the bacterial culture to a final concentration of 1 mM {Studier 1986, Rosenberg 1987}.

pVEX115 was digested with *Nde*I and treated with CIP. The 776 bp *ctxA* fragment was obtained from pRT41 by digestion of this vector with *Nde*I. The *ctxA* fragment was then ligated to the linearized pVEX115 vector. Plasmid constructs from these ligations were transformed into competent Max Efficiency DH5α cells (BRL, Gaithersburg, MD). Colonies that grew on

ampicillin plates were allowed to grow overnight in Luria Broth (LB) containing 50 μg/ml ampicillin (Sigma Chemical Co., St. Louis, MO). Plasmid minipreps were performed using the Miniprep Kit Plus (Pharmacia LKB, Piscataway, NJ), the alkaline lysis miniprep procedure, or the CIRCLEPREP miniprep procedure (Bio101, LaJolla, CA). Purified plasmids were digested with *Nde*I (New England Biolabs) and *Xba*I to determine the size and orientation of the inserts. DNA from these plasmids was sequenced by the Sanger dideoxy method. Constructs in which the *ctxA* fragment was oriented correctly for toxin expression from the T7 promoter were designated pCT7, whereas those carrying the insert in the inverted orientation were designated pCT7r.

The A_1 subunit was digested from pRT41 with an *Nde*I-*Cla*I double digest, and the *Cla*I 5' overhanging ends were filled in by adding only dGTP and dCTP to the reaction with the Klenow fragment. This fragment was then ligated into pVEX115 which had been digested with *Nde*I and *Sma*I. The resulting plasmid, pCA7, carries only the coding region for the A_1 subunit of *ctxA*.

pNPCT Vector. Plasmid pYS3 is a derivative of pVEX115 and was obtained from Dr. Yogendra Singh of the National Institute of Dental Research, Bethesda, MD. It is different from pVEX115 in that it has no signal sequence and the protective antigen (PA) gene of *Bacillus anthracis* is cloned into the *Nde*I site of pYS3. Ligation of any in-frame insert into the *Nde*I site of this 4.4 kb plasmid produces a protein in which the ATG of the CATATG *Nde*I site becomes the translation start site for the gene of interest.

Polymerase chain reaction (PCR) mutagenesis was used to create unique *Nde*I and *Pst*I sites at the 5' and 3' ends, respectively, of the coding region for the mature *ctxA* gene in the pPCTX18 construct. The 780 bp PCR product was digested with *Nde*I and *Pst*I and ligated into the same sites in the pYS3 vector by standard methods. This ligation disabled the PA gene by removing 966 bases from the 5' end of the gene (44% of the complete coding region). The resulting plasmid, pNP-CT, was sequenced to confirm the correct orientation and reading frame.

pCTss vector. pVEX115 was digested with *Bgl*II and *Nde*I to obtain the 145 bp region of DNA including the *ompA* signal sequence and the T7 promoter. The resulting fragment was ligated into the *Bgl*II-*Nde*I sites of pNPCT to achieve the same *ctxA* construct but including an OmpA signal peptide that could aid in export of the resulting protein.

Oligonucleotides

All oligonucleotides, except the M13 reverse and universal primers (USB, Cleveland, Ohio), used for sequencing and mutagenesis were synthesized using a PCRMate Oligonucleotide Synthesizer (Applied Biosystems, Foster City, CA) or a Pharmacia Gene Assembler (Pharmacia LKB Biotechnology, Piscataway, NJ).

DNA Sequencing

All sequencing was performed with the Sanger dideoxy method using either the Sequenase (USB, Cleveland, OH) or T7 kits (Pharmacia-LKB, Piscataway, NJ). All sequencing gels were 6% polyacrylamide (BRL Gel-Mix 6,

BRL, Gaithersburg, MD; Sequagel, National Diagnostics, Manville, NJ; HydroLink, AT Biochem, Malvern, PA). HydroLink is a non-polyacrylamide polymer which is less toxic than polyacrylamide, does not require urea and results in stronger, more flexible gels which are easier to manipulate. The running buffer provided with the gel solution has a pH of 10.7 and separation of the DNA bands is achieved by the establishment of a pH gradient as well as by the sieve effect of the gel polymer. All gels were poured using 0.2 mm wedge or straight spacers. All sequencing was performed using ^{35}S-dATP (New England Nuclear, Boston, MA; specific activity ~1131 Ci/mmol) as the labeled nucleotide. Gels were run at 80 watts, constant power. An IBI Model STS 45 sequencing apparatus (International Biotechnologies Inc., New Haven, CT) and Electrophoresis Constant Power Supply 3000/150 (Pharmacia LKB, Piscataway, NJ) were used for running all gels. Gels were dried on a dual temperature slab gel dryer (Hoefer Scientific Products, San Francisco, CA) and exposed to Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY) overnight at room temperature. Films were developed by a Kodak M35A X-OMAT Automatic Processor .

Expression of Recombinant CtxA

Induction and Preparation of Periplasmic Fractions

All *ctxA* constructs were transformed into *E. coli* strains DH5α and then BL21. DH5α cells were purchased as competent cells from BRL. The BL21 cells, which were obtained from Dr. J. Batra, NCI, NIH, Bethesda, MD, contain a single copy of the T7 gene *1*, which codes for the RNA polymerase, under control of the inducible *lac*UV5 promoter.

Transformed BL21 cells were grown overnight on LB plates containing 50 or 100 μg/ml ampicillin in LB. Colonies taken from these plates the next morning were allowed to grow to an optical density (λ_{600}) of 0.8 or 1.0 in LB plus ampicillin. IPTG was added to the culture to a final concentration of 1 mM and induction allowed to proceed for 90 to 120 minutes. Cells were harvested by centrifugation at 4° C for 20 minutes, resuspended in 20% sucrose solution, pelleted again at 6,000 rpm for 10 minutes, and resuspended in water to create spheroplasts. Periplasmic contents were collected as the supernatant fluid from a final centrifugation and were stored at 4° C or -20° C. Pellets containing the cell lysates were resuspended in water and stored under the same conditions as the periplasmic fractions.

Protein Assays

Protein assays were performed with the Pierce BCA Protein Assay reagent method, using the standard protocol at 37°C according to manufacturer's instructions (Pierce, Rockford, IL).

Protein Gels

Periplasmic fractions and cell lysates from BL21 inductions were analyzed using a Bio-Rad Mini-PROTEAN II electrophoresis chamber (Bio-Rad Laboratories, Melville, NY) and 12% polyacrylamide gels containing sodium dodecyl sulfate (SDS) as a denaturing agent. More recently, samples were applied to pre-cast 4-20% SDS gradient gels supplied by Schleicher & Schuell (Keene, NH) and run in the Profile System electrophoresis chambers (Schleicher & Schuell). Bio-Rad's prestained low range SDS-PAGE standards were applied to each gel and transferred to nitrocellulose membranes as well. For some gels, a gas-stabilized, 30% acrylamide, 0.8% bisacrylamide stock solution called Protogel (National Diagnostics, Manville, NJ) was used as the acrylamide stock solution.

Proteins were visualized by staining with Coomassie blue and de-stained with a methanol-acetic acid solution.

Antibodies

Two monoclonal antibodies were obtained from Dr. James Kenimer of the Center for Biologics, Food and Drug Administration, Bethesda, MD. Monoclonal antibodies CP7-3003F7 (3F7, IgG3) and CP7-3004G6X1 (G6X, IgG1) were produced in response to a synthetic peptide representing amino acids 6 through 17 (the first homology box) of the pertussis toxin. 3F7 showed greater avidity for the CtxA constructs on immunoblots and was therefore used more consistently for detection of the recombinant toxin than G6X.

Polyclonal rabbit antisera generated against CtxA was also used for some immunoblots. This was donated by Dr. Joel Moss, National Heart Lung and Blood Institute, NIH, Bethesda, MD.

Immunoblots

Western blots were performed by the method of Burnette {Burnette, 1981}. Bio-Rad's Mini Trans-Blot module was used for transfer of the electrophoretically separated proteins to nitrocellulose. Monoclonal primary antibodies 3F7 and G6X were used at 1:500 dilutions. Rabbit anti-CtxA antisera was used at a 1:20,000 dilution. The affinity-purified secondary horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit antibodies (ProMega, Madison, WI) were diluted to 1:1000 for adequate detection. Purchased cholera toxin A subunit (CT-A, List Biological Laboratories, Inc., Campbell, CA) was used as a positive control on all Western blots.

Oligonucleotides used for PCR Mutagenesis

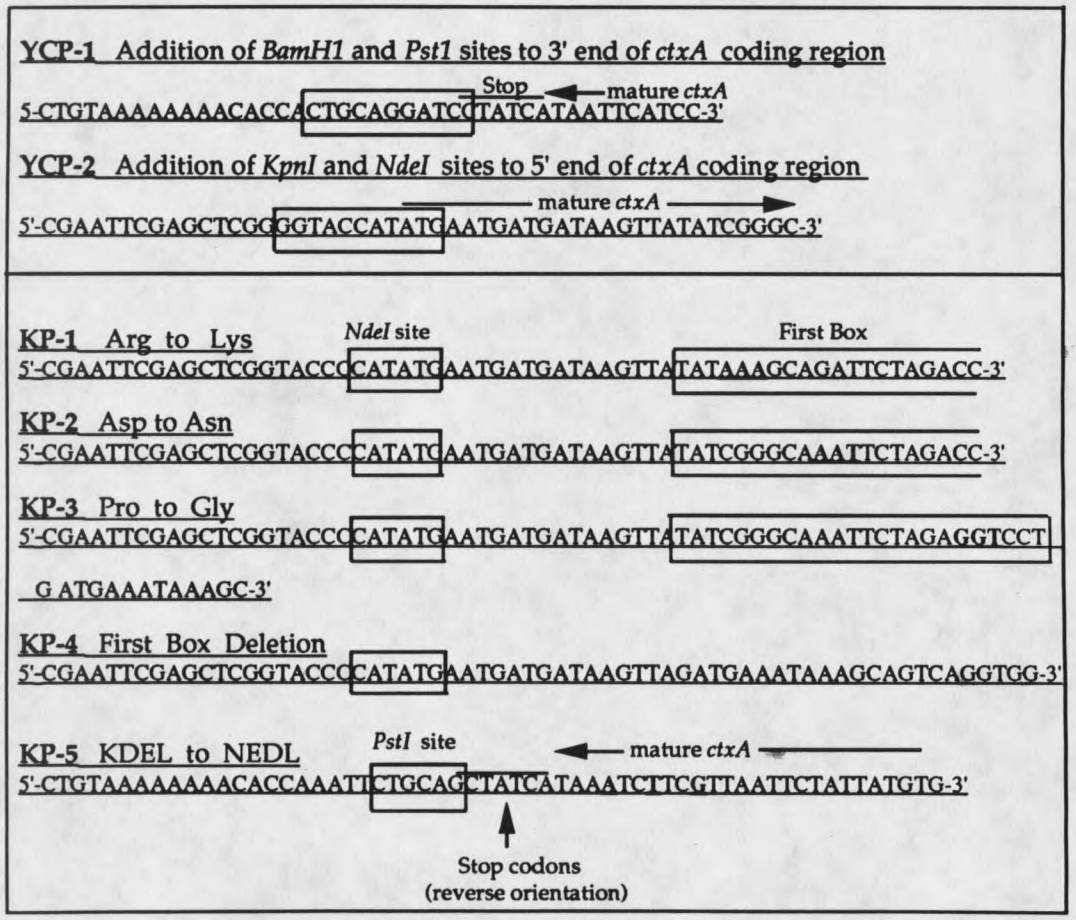

Figure 5. Oligonucleotides used for PCR mutagenesis.

Mutagenesis

Oligonucleotide primers incorporating the desired alterations in specific amino acids were generated on the PCRMate Oligonucleotide Synthesizer (Applied Biosystems, Foster City, CA) with the trityl group left on. Purification of the primers was achieved with oligonucleotide purification cartridges purchased from Applied Biosystems. These oligonucleotides were paired with primers previously used for the generation

of a *Pst*I site in the 3' end of the *ctxA* gene and the paired primers were used to amplify pNPCT DNA, thus creating product DNA with the desired mutations. A Perkin-Elmer/Cetus DNA Thermal Cycler and the GeneAmp Polymerase Chain Reaction (PCR) kit (Perkin-Elmer/Cetus, Norwalk, CT) were used for all amplification reactions. Amplification was allowed to proceed for 30 cycles under the following conditions: samples were denatured at 94° C, primers were annealed at 72° C, and the primer extension reaction was performed at 37° C. Each PCR product was extracted with phenol/chloroform and purified by running on low melting point agarose gels before digestion with *Nde*I and *Pst*I and ligation into pYS3. The mutant constructs were transformed into *E. coli* DH5α, plasmid minipreps were prepared and the plasmid DNA sequenced. Plasmids containing each mutation were then transformed into BL21 cells to test for expression by Western blotting.

Sonication of Cell Lysates

From the procedure used to create spheroplasts (discussed above), the pellets remaining after removal of the supernatant fluid containing the periplasmic fraction were disrupted by sonication using three cycles of 20 seconds each, separated by 30 second incubations on ice. After the last sonication, the lysates were centrifuged for 40 minutes at 10,000 rpm. The pellets were resuspended in 1 ml water and assayed for ADP-ribosylation activity.

Assay of ADP-ribosylation Activity

Agmatine Assay

Periplasmic fractions and cell lysates from expression experiments designed to test recombinant Ctx constructs were assayed for the presence of characteristic toxin ADP-ribosyltransferase activity using the agmatine assay developed by Dr. Joel Moss, NHLBI, NIH, Bethesda, MD {Moss *et al.*, 1976, Tsai *et al.*, 1987}. In this assay, biological activity of cholera toxin was measured by the glycohydrolytic cleavage of the NAD donor substrate into nicotinamide and ADP-ribose, and the subsequent transfer of the ADP-ribose moiety to the acceptor substrate agmatine (1-Amino-4-guanidinobutane; Sigma Chemical Co., St. Louis, MO).

Assays were performed in 300 µl volumes containing 50 mM KPO_4 buffer, pH 7.5, 5 mM $MgCl_2$, 0.2 mM GTP, 20 mM DTT, 10 mM agmatine, 0.1 mg/ml ovalbumin, 1 µCi [(U-^{14}C)adenine]NAD (Amersham Corp., Arlington Heights, IL), sample, and water. The reactions were initiated by the addition of sample and allowed to proceed for one hour at 30° C. Two 100 µl aliquots from each reaction were applied to Bio-Rad AG1 X-2 anion exchange columns and each column was washed with 5 ml water. Effluent was collected directly into scintillation vials. Scintillation fluid (Hydrofluor, National Diagnostics, Manville, NJ) was added and the samples were counted on a liquid scintillation counter. Assays were consistently run with 2 µg purified CT-A as the positive control.

Limited Proteolysis

Purified CT-A, unmutated pNPCT and the Arg to Lys mutation (M1) were subjected to limited trypsin (Sigma) digestion for periods of up to one hour. Proteolysis was inhibited by the addition of phenylmethylsulfonyl fluoride (Sigma) and aliquots of each sample were applied to a polyacrylamide gel. After electrophoretic separation, the proteins were blotted to nitrocellulose and immunoreactive protein detected with a polyclonal anti-CtxA antibody.

Quantitation of Immunoreactive Protein

Dilutions of CT-A and periplasmic fractions of pNPCT and the M9 and M17 mutant constructs were applied to polyacrylamide gels, separated electrophoretically, and immunoblotted as described above. Immunoreactive protein was measured densitometrically.

RESULTS AND DISCUSSION

Sequencing of *ctxA*

The pUC system was originally chosen for subcloning of *ctxA* because it is well characterized, contains a convenient multiple cloning site (MCS) and had been successfully used in our laboratory to express numerous constructs of *B. pertussis* toxin subunits. The 776-bp *Nde*I fragment of pRT42 containing the entire *ctxA* gene was subcloned in frame into pUC18, resulting in the plasmid designated pPCTX18. The *ctxA* DNA and flanking regions of this plasmid were sequenced. Figure 6 shows the nucleotide sequence of the mature CtxA protein aligned with the published sequence of ElTor 2125. The sequence range is from nucleotides 255 to 977 in pPCTX18, which corresponds to nucleotides 570 to 1292 in the ElTor sequence (Mekalanos *et al.*, 1983).

```
              260                  280                  300
            *   *    *   *   *   *    *   *   *   *
  pPCTX18   AATGAT GATAAGTTATATCGGGCAGA TTCTAGACCTCCTGATGAAA
 (ctx 569B) TTACTA CTATTCAATATAGCCCGTCT AAGATCTGGAGGACTACTTT
                |          | 20     |          | 40     |
ElTor 2125  ...... ..................... .....................>
            ^^^^^^ ^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^

                          320                  340                  360
              *   *   *   *    *   *   *   *    *   *   *   *
  pPCTX18   TAAAGCAGTCAGGTGGTCTT ATGCCAAGAGGACAGAGTGA GTACTTTGACCGAGGTACTC
 (ctx 569B) ATTTCGTCAGTCCACCAGAA TACGGTTCTCCTGTCTCACT CATGAAACTGGCTCCATGAG
                 | 60     |          | 80     |          | 100    |
ElTor 2125  ..................... ..................... .....................>
            ^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^
```

Figure 6. DNA Sequence of V. cholerae 569B in pPCTX18.

```
                          380                 400                 420
                     *    *    *    *    *    *    *    *    *    *    *
  pPCTX18    AAATGAATATCAACCTTTAT GATCATGCAAGAGGAACTCA GACGGGATTTGTTAGGCACG
 (ctx 569B)  TTTACTTATAGTTGGAAATA CTAGTACGTTCTCCTTGAGT CTGCCCTAAACAATCCGTGC
                    | 120     |         | 140     |         | 160     |
 ElTor 2125  ..................... ..................... .....................>
             ^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^

                          440                 460                 480
                     *    *    *    *    *    *    *    *    *    *    *
  pPCTX18    ATGATGGATATGTTTCCACC TCAATTAGTTTGAGAAGTGC CCACTTAGTGGGTCAAACTA
 (ctx 569B)  TACTACCTATACAAAGGTGG AGTTAATCAAACTCTTCACG GGTGAATCACCCAGTTTGAT
                    | 180     |         | 200     |         | 220     |
 ElTor 2125  ..................... ..................... .....................>
             ^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^

                          500                 520                 540
                     *    *    *    *    *    *    *    *    *    *    *
  pPCTX18    TATTGTCTGGTCATTCTACT TATTATATATATGTTATAGC CACTGCACCCAACATGTTTA
 (ctx 569B)  ATAACAGACCAGTAAGATGA ATAATATATATACAATATCG GTGACGTGGGTTGTACAAAT
                    | 240     |         | 260     |         | 280     |
 ElTor 2125  ..................... ..................... .....................>
             ^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^

                          560                 580                 600
                     *    *    *    *    *    *    *    *    *    *    *
  pPCTX18    ACGTTAATGATGTATTAGGG GCATACAGTCCTCATCCAGA TGAACAAGAAGTTTCTGCTT
 (ctx 569B)  TGCAATTACTACATAATCCC CGTATGTCAGGAGTAGGTCT ACTTGTTCTTCAAAGACGAA
                    | 300     |         | 320     |         | 340     |
 ElTor 2125  ..................... ..................... .....................>
             ^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^

                          620                 640                 660
                     *    *    *    *    *    *    *    *    *    *    *
  pPCTX18    TAGGTGGGATTCCATACTCC CAAATATATGGATGGTATCG AGTTCATTTTGGGGTGCTTG
 (ctx 569B)  ATCCACCCTAAGGTATGAGG GTTTATATACCTACCATAGC TCAAGTAAAACCCCACGAAC
                    | 360     |         | 380     |         | 400     |
 ElTor 2125  ..................... ..................... .....................>
             ^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^

                          680                 700                 720
                     *    *    *    *    *    *    *    *    *    *    *
  pPCTX18    ATGAACAATTACATCGTAAT AGGGGCTACAGAGATAGATA TTACAGTAACTTAGATATTG
 (ctx 569B)  TACTTGTTAATGTAGCATTA TCCCCGATGTCTCTATCTAT AATGTCATTGAATCTATAAC
                    | 420     |         | 440     |         | 460     |
 ElTor 2125  ..................... ..................... .....................>
             ^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^
```

Figure 6. Continued.

```
                          740                   760                   780
                     *    *    *    *    *    *    *    *    *    *    *
pPCTX18    CTCCAGCAGCAGATGGTTAT GGATTGGCAGGTTTCCCTCC GGAGCATAGAGCTTGGAGGG
(ctx 569B) GAGGTCGTCGTCTACCAATA CCTAACCGTCCAAAGGGAGG CCTCGTATCTCGAACCTCCC
                     | 480     |           | 500     |           | 520     |
ElTor 2125 ..................... ..................... .....................>
           ^^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^^

                          800                   820                   840
                     *    *    *    *    *    *    *    *    *    *    *
pPCTX18    AAGAGCCGTGGATTCATCAT GCGCCGCCGGGTTGTGGGAA TGCTCCAAGATCATCGATCA
(ctx 569B) TTCTCGGCACCTAAGTAGTA CGCGGCGGCCCAACACCCTT ACGAGGTTCTAGTAGCTAGT
                     | 540     |           | 560     |           | 580     |
ElTor 2125 ..................... ..A.................. .....................>
           ^^^^^^^^^^^^^^^^^^^^^ ^^v^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^^

                          860                   880                   900
                     *    *    *    *    *    *    *    *    *    *    *
pPCTX18    GTAATACTTGCGATGAAAAA ACCCAAAGTCTAGGTGTAAA ATTCCTTGACGAATACCAAT
(ctx 569B) CATTATGAACGCTACTTTTT TGGGTTTCAGATCCACATTT TAAGGAACTGCTTATGGTTA
                     | 600     |           | 620     |           | 640     |
ElTor 2125 ..................... ..................... .....................>
           ^^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^^

                          920                   940                   960
                     *    *    *    *    *    *    *    *    *    *    *
pPCTX18    CTAAAGTTAAAAGACAAATA TTTTCAGGCTATCAATCTGA TATTGATACACATAATAGAA
(ctx 569B) GATTTCAATTTTCTGTTTAT AAAAGTCCGATAGTTAGACT ATAACTATGTGTATTATCTT
                     | 660     |           | 680     |           | 700     |
ElTor 2125 ..................... ..................... .....................>
           ^^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^^ ^^^^^^^^^^^^^^^^^^^^^

                     *    *    *
pPCTX18    TTAAGGATGAATTATGA
(ctx 569B) AATTCCTACTTAATACT
                     | 720
ElTor 2125 .................>
           ^^^^^^^^^^^^^^^^^
```

Figure 6. Continued.

The nucleotide sequence of 569B is identical to that of ElTor strains 2125 and 62746 except for a single base change at the pPCTX18 nucleotide number 803. This represents a change in the third position of the codon from the deduced amino acid sequence of GCA in ElTor to GCG in 569B, both of which code for alanine (see Figure 7). As the *ctx* genes of all strains sequenced to date are remarkably similar, it is not surprising to find that the classical 569B strain has a nearly identical sequence with the published ElTor sequences {Lockman *et al.*, 1984, Mekalanos *et al.*, 1983}.

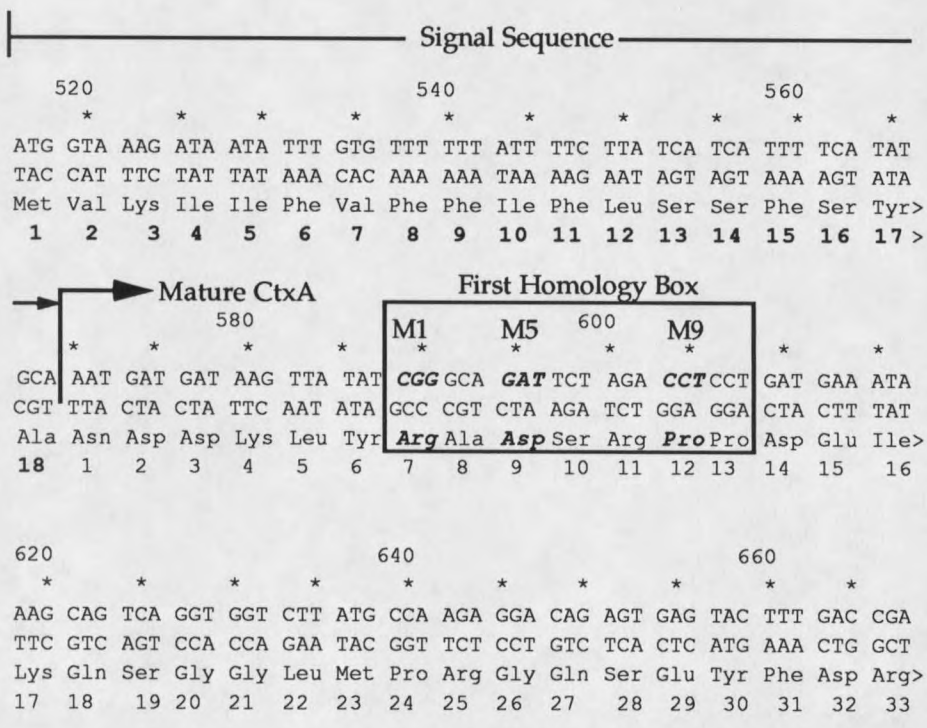

Figure 7. Translated sequence of *V. cholerae* El Tor 2125. Sequence Range: nucleotides 516 to 1292. The signal peptide is numbered in bold, and oligonucleotide-directed mutations are indicated in bold, italicized letters with the mutation designation above.

```
            680                         700
 *      *     *      *      *     *      *      *     *      *
GGT ACT CAA ATG AAT ATC AAC CTT TAT GAT CAT GCA AGA GGA ACT CAG ACG
CCA TGA GTT TAC TTA TAG TTG GAA ATA CTA GTA CGT TCT CCT TGA GTC TGC
Gly Thr Gln Met Asn Ile Asn Leu Tyr Asp His Ala Arg Gly Thr Gln Thr>
 34  35  36  37  38  39  40  41  42  43  44  45  46  47  48  49  50>
```

Second Homology Box

```
720                         740                         760
*     *      *      *      *      *      *      *      *      *      *
GGA TTT GTT AGG CAC GAT GAT GGA TAT GTT TCC ACC TCA ATT AGT TTG AGA
CCT AAA CAA TCC GTG CTA CTA CCT ATA CAA AGG TGG AGT TAA TCA AAC TCT
Gly Phe Val Arg His Asp Asp Gly Tyr Val Ser Thr Ser Ile Ser Leu Arg>
51   52 53  54  55  56  57  58  59  60  61  62  63  64  65  66  67>
```

```
 780                         800                         820
    *      *      *      *      *      *      *      *      *      *
AGT GCC CAC TTA GTG GGT CAA ACT ATA TTG TCT GGT CAT TCT ACT TAT TAT
TCA CGG GTG AAT CAC CCA GTT TGA TAT AAC AGA CCA GTA AGA TGA ATA ATA
Ser Ala His Leu Val Gly Gln Thr Ile Leu Ser Gly His Ser Thr Tyr Tyr>
68  69  70  71   72  73  74  75  76  77  78  79  80  81  82  83  84>
```

```
                    840                         860
    *      *      *      *      *      *      *      *      *      *
ATA TAT GTT ATA GCC ACT GCA CCC AAC ATG TTT AAC GTT AAT GAT GTA TTA
TAT ATA CAA TAT CGG TGA CGT GGG TTG TAC AAA TTG CAA TTA CTA CAT AAT
Ile Tyr Val Ile Ala Thr Ala Pro Asn Met Phe Asn Val Asn Asp Val Leu>
85  86  87  88  89  90  91  92  93  94  95  96  97  98  99  100 101
```

```
       880                         900                         920
  *      *      *      *      *      *      *      *      *      *
GGG GCA TAC AGT CCT CAT CCA GAT GAA CAA GAA GTT TCT GCT TTA GGT GGG
CCC CGT ATG TCA GGA GTA GGT CTA CTT GTT CTT CAA AGA CGA AAT CCA CCC
Gly Ala Tyr Ser Pro His Pro Asp Glu Gln Glu Val Ser Ala Leu Gly Gly>
102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118>
```

```
                   940                         960
 *      *      *      *      *      *      *      *      *      *
ATT CCA TAC TCC CAA ATA TAT GGA TGG TAT CGA GTT CAT TTT GGG GTG CTT
TAA GGT ATG AGG GTT TAT ATA CCT ACC ATA GCT CAA GTA AAA CCC CAC GAA
Ile Pro Tyr Ser Gln Ile Tyr Gly Trp Tyr Arg Val His Phe Gly Val Leu>
119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135>
```

Figure 7. Continued.

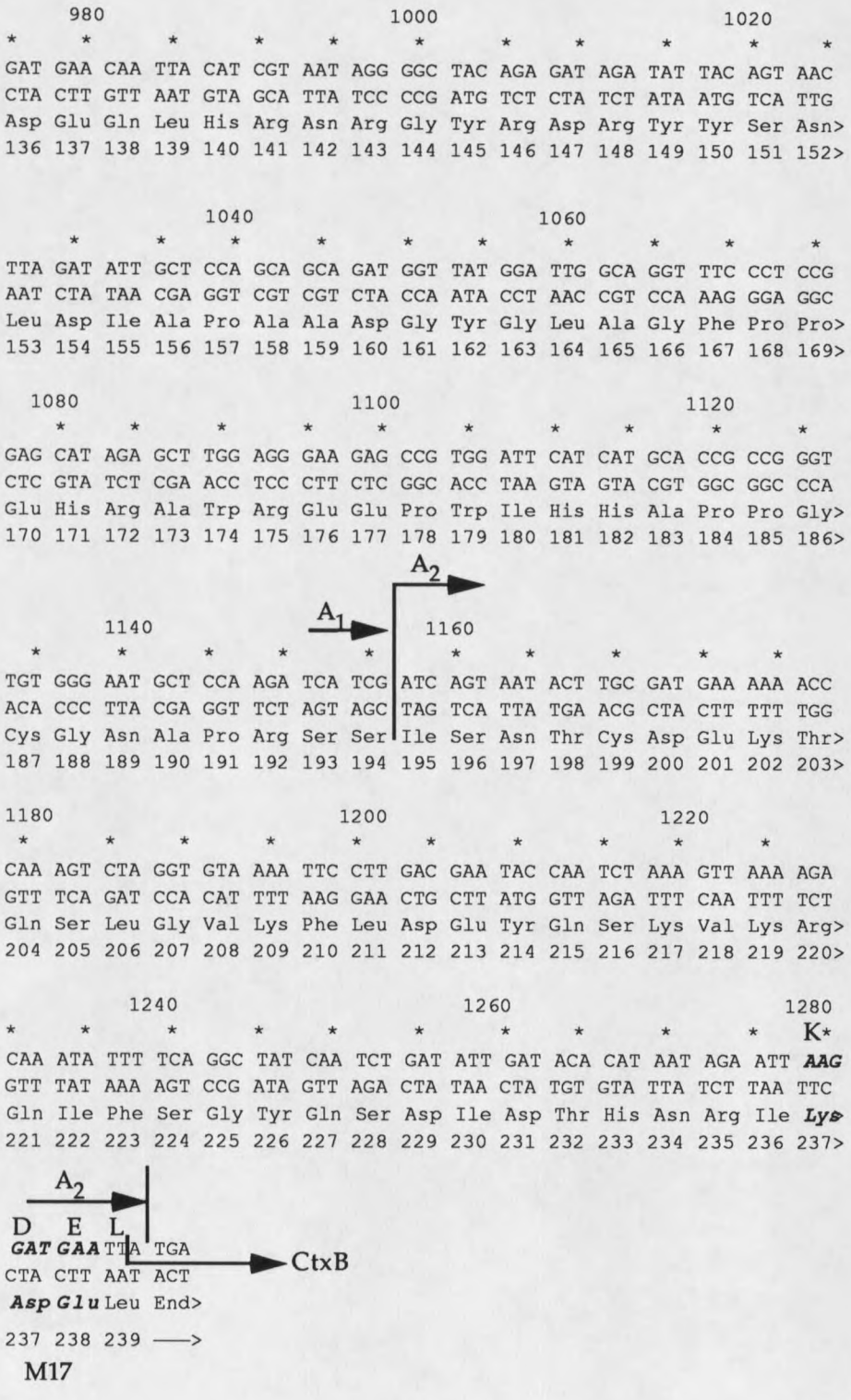

Figure 7. Continued.

Expression

Plasmid vectors were constructed to express either mature cholera toxin A subunit consisting of both A_1 and A_2 or only the mature A1 subunit. In my initial experiments, expression of CtxA could not be detected using pUC18. Other investigators in our laboratory, having difficulty with a gene known to be expressed in pUC, sequenced the MCS upstream of the *Bam*HI site. From their sequencing data, a mutation was found in all of the commercially available pUC18 vectors, except those supplied by Pharmacia. This mutation removed one of two adjacent cytosine residues in the MCS. This change generated a frame shift mutation in which the first ATG, normally the translational start site, was out of frame with the inserted gene. The third codon in the MCS is also an ATG, so a low level of expression could be obtained with some genes from this start site, but as the distance between the Shine-Delgarno sequence and this second ATG is not optimal, expression was considerably lower than expected {Lobet *et al.*, 1989}. Thus it is not surprising that expression was not obtained in my initial experiments using the pUC18 vector.

Ligation of the *Nde*I-digested *ctxA* gene from pRT41 into a pUC18 vector that had an unmutated MCS provided stable expression of CtxA detectable by immunoblotting. Although ADP-ribosylation activity of the recombinant toxin produced from these constructs was detectable by the agmatine assay, there were several disadvantages in continuing to use this vector for expression, *e.g.* CtxA was not exported into the periplasm nor produced in the high yields required to form inclusion bodies, but simply

remained in the cytoplasm. Thus, because of the crude nature of the cytoplasmic fractions, ADP-ribosyltransferase activity of the expressed recombinant toxin fluctuated considerably among different preparations and declined rapidly after the initial assay. Although the recombinant A1 subunit produced from the pEC construct appeared to have considerably more enzyme activity than that produced by pPCTX18 (see Figure 8), it still was not possible to obtain consistent results with the recombinant protein from the crude cytoplasmic fractions.

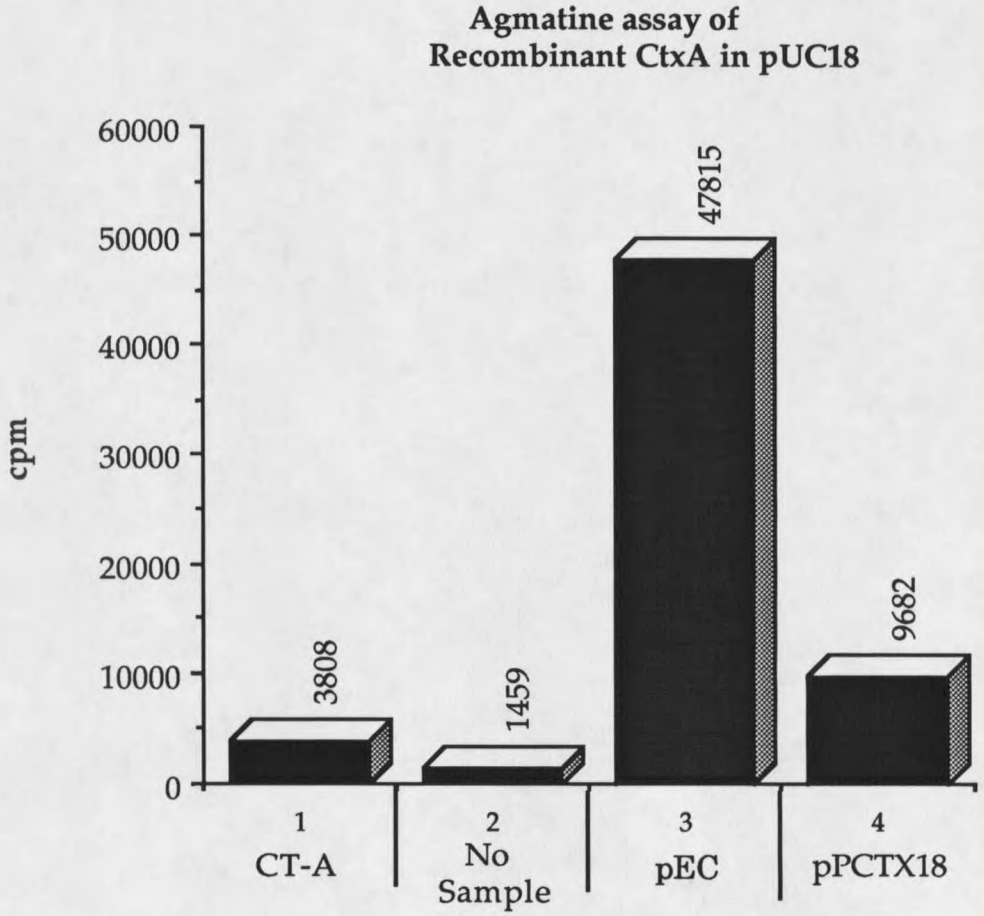

Figure 8. Agmatine assay of ADP-ribosyltransferase activity of CtxA and $CtxA_1$ produced from the pUC18 vector system.

Expression experiments using pUC-derived vectors demonstrated that authentic CtxA could be produced in *E. coli.* However, levels of expression were inadequate to support further experiments relating to mutant construction and corresponding enzyme activity. In order to overcome variable expression levels and stability problems, a decision was made to try various expression systems based on the T7 RNA polymerase. In retrospect, this decision seems most appropriate since Mekalanos *et al.*, have subsequently shown that in *V. cholerae* and in *E. coli* the interaction of upstream TTTTGAT sequences with ToxR is required for high level expression {Taylor, 1989}.

Procaryotic expression systems based on the cloned gene of the bacteriophage T7 polymerase (*gene 1*) have become quite popular because of the absolute specificity of the polymerase for its own promoter, which is not found in *E. coli,* and its unusually efficient rate of mRNA chain initiation and elongation. The T7 system used in my studies relied on the induction of a chromosomal copy of *gene 1* under the control of the *lac*UV5 promoter in *E. coli* strain BL21 {Studier and Moffatt, 1986}. Subcloning of the *ctxA* gene in to this T7 system produced a plasmid known as pNPCT. Although very effective at expressing the *ctxA* gene in BL21 cells, this system is "leaky" in that the very low basal expression levels of T7 polymerase in the uninduced cell may be sufficient to produce detectable amounts of the inserted gene product before induction with IPTG. This leaky phenomenon is problematic only when the gene product is toxic to the cell.

The DNA fragment coding for the A subunit was subcloned into pVEX in both the correct and reversed orientation. In this vector, the inserted gene

is fused to the *E. coli* OmpA signal sequence and expressed under the control of the T7 promoter. This system was selected in an attempt to translocate the expressed protein from the cytoplasm to the periplasmic space, reulting in a cleaner, more stable preparation for testing. However, the pVEX-*ctxA* constructs, pCT7 and pCT7r, did not express detectable levels of CtxA as determined by immunoblotting with polyclonal or monoclonal antibodies. Similarly, the pCA7 construct, which retained only the A_1 coding sequences, did not express $CtxA_1$ at detectable levels. Although it is difficult to explain these results, it seems quite likely that the lack of expression is due to the presence of the OmpA signal sequence, as found in experiments discussed below.

The plasmid pNPCT, constructed from the *ctxA* gene ligated into the *Nde*I site immediately downstream from the T7 promoter in the plasmid pYS3, expressed CtxA at high levels and exported the recombinant toxin into the periplasmic space. These results were very surprising since this construct was designed to express only the mature A subunit without a signal sequence. Addition of the OmpA signal sequence to pNPCT produced the construct pCTss, which was expected to export recombinant CtxA into the periplasm. In an experiment designed to test the effect of the OmpA signal sequence, *E. coli* BL21 cells were transformed with either pNPCT or pCTss and expression of the toxin was induced with IPTG. Proteins in the periplasmic and cytoplasmic fractions were separated by SDS-PAGE and immunoreactive CtxA proteins were identified by immunoblotting with polyclonal anti-CtxA antisera. Comparative expression and cellular compartmentalization of these two recombinant CtxA proteins are shown in Figure 9.

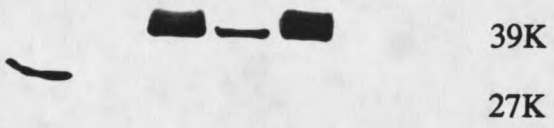

Figure 9. Immunoblot of periplasmic and cytoplasmic fractions of two different recombinant Ctx constructs, pNPCT and pCTss.
Lane 1 - 0.25 μg CT-A
Lane 2 - Periplasmic fraction of BL21/pCTss
Lane 3 - Periplasmic fraction of BL21/pNPCT
Lane 4 - Cell lysate of BL21/pCTss
Lane 5 - Cell lysate of BL21/pNPCT
Lane 6 - Periplasmic fraction of Bl21
Lane 7 - Low MW markers

The immunoblot shown in Figure 9 clearly demonstrated that the plasmid construct pNPCT, carrying only the coding region for the mature CtxA, *i.e.*, lacking a signal sequence, expressed the toxin at a much higher level and efficiently exported it into the periplasmic space. This result was unexpected, but was consistent with the lack of observable expression in the pVEX vector (discussed above), which also carries the OmpA signal sequence under regulation of the T7 promoter. From these experiments, it is clear that

in this expression system, addition of a signal sequence did not enhance expression of recombinant *ctxA*. Attempts to express the toxin in *E. coli* with signal sequences from *V. cholerae* or other *E. coli* genes also have been unsuccessful {C. Locht, personal communication}. Although signal peptides are generally believed to be necessary for efficient translation and translocation of exported proteins, only the early stages of protein secretion appear to be regulated by the signal peptide. The more distal steps of the export process are dependent upon topogenic sequences located within the mature protein {Oliver, 1987}. This export phenomenon is discussed in more detail in experiments involving expression of a CtxA deletion mutant (M13) in the mutagneesis section below (see also Figures 13 and 16 - 19).

To confirm empirical observations related to growth rates of BL21 cells transformed with different plasmid constructs, an experiment was designed to measure the effect of the signal sequence on cell growth. Representative growth curves of BL21 carrying pNPCT and pCTss are shown in Figure 10.

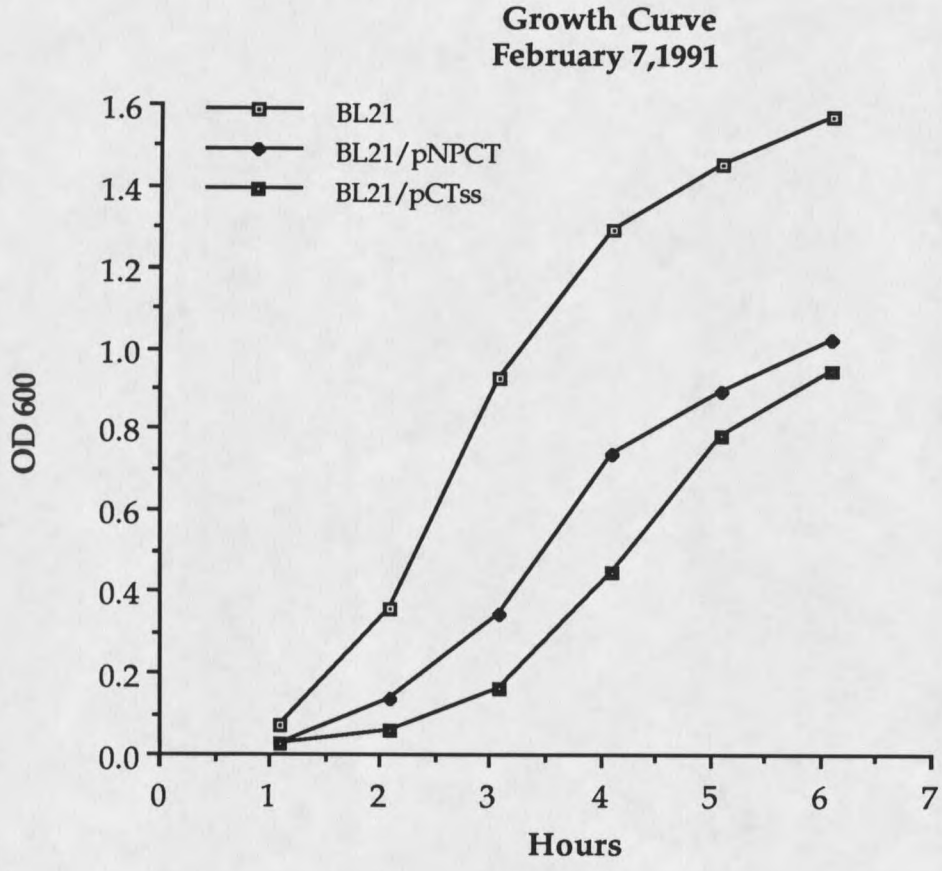

Figure 10. Growth Curve of *E. coli* cells transformed with pNPCT or pCTss or without any plasmid sequence.

As expected, untransformed BL21 cells grew at a faster rate than cultures expressing CtxA with or without the signal sequence because the BL21 cells were grown on media without the antibiotic ampicillin used to maintain the expression plasmid. The cells expressing the CtxA with the signal sequence (pCTss) grew slowest of the three cultures, suggesting that the signal sequence and lack of export of the expressed protein may be inhibiting growth. Time point experiments in which expression of immunoreactive protein was evaluated from the time the cultures were induced with IPTG

indicate that approximately equivalent levels of CtxA are produced whether the culture is induced or not (data not shown). This result is due to the "leakiness" of the T7 polymerase expression inherent in the BL21 cells. This is a problem with plasmids containing target genes that are toxic to the cells, as these plasmids can be very difficult to maintain in this expression system {Studier and Moffatt, 1986}. A fairly high level of background expression did not appear to destabilize the pNPCT construct. However, as a precautionary measure, plasmid stocks were maintained in DH5α, which lacks the T7 polymerase gene, and freshly transformed BL21 cells were used for each induction experiment. If the *ctxA* plasmids were toxic to the cells, I would have expected to see a much greater difference in growth rate between the cells.

Mutagenesis

At the start of this project, I had planned to do the site-specific mutagenesis by subcloning the recombinant CtxA from an optimized pUC18 construct into M13mp18 using a variation of the mutagenesis method described by Eckstein *et al.*, (1985). This approach has been used succcessfully in our laboratory to generate some of the Ptx mutations (Cieplak *et al.*, 1988). As it became apparent that a better expression vector would be required, the technology of PCR mutagenesis improved to the point at which this became the preferred method of mutagenesis. The original subclone of *ctxA* retained two amino acids from the *V. cholerae* signal sequence that were easily removed when the unique *Nde*I and *Pst*I sites were added using synthetic

oligonucleotide primers to facilitate subcloning into the T7 vectors. Using this approach, it was necessary to generate only one new primer for each desired mutant and run the PCR reactions with the previously sequenced *ctxA* DNA as template. Each mutant construct was sequenced to assure that the appropriate mutations had been made and vector-insert junctions had not been altered.

The sequence of the first homology box in CtxA is shown in Table 2 along with the mutation designations corresponding to specific amino acid changes made.

Mutations

Tyr6 Arg7 Ala8 Asp9 Ser10 Arg11 Pro12 Pro13	
M1	Lys
M5	Arg
M9	Gly
M13	← Entire box deleted →
M17	KDEL → NEDL

Table 2. Mutations generated by PCR in recombinant *ctxA*.

The following is a summary of the final mutations generated and the rationale I used for selection of each.

1. Arg_7 to Lys (**M1**) - this is the second amino acid in the region, and, based on results with the Ptx mutations, may be critical for enzymatic activity. As shown in Figure 11, Arg to Lys represents a minimal structural change and maintains the positive charge on the R group at neutral pH.

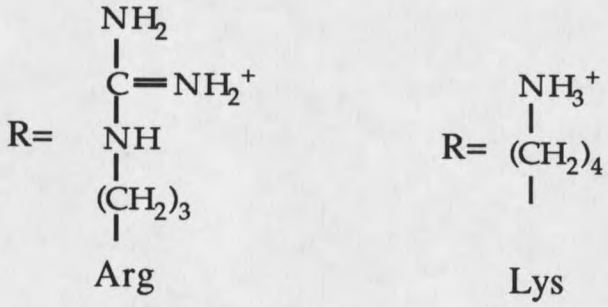

Figure 11. Side chain groups of arginine and lysine

2. Asp_9 to Arg (**M5**) - in the Ptx mutations this Asp was associated with decreased enzymatic activity. We chose to alter the charge from negative to positive.

3. Pro_{12} to Gly (**M9**) - The ring structure of the imino acid proline drastically limits rotation about the N-C^{α} bond, inducing torsional strain on the molecule. Proline residues are often associated with reverse turns or, when adjacent as in this case, may be part of a poly(Pro) helix. In any case, such a drastic structural alteration as Pro to Gly might be expected to produce a significant change in protein structure {Creighton, 1984}.

4. Deletion of first box (**M13**)- This mutation represents a significant alteration to the structure of the N-terminal region of the protein. It would be surprising if this construct had a detectable level of activity.

5. KDEL to NEDL (**M17**) - Recent work by Chaudry *et al.* {1990} suggests that the last four amino acids of the *Pseudomonas* exotoxin, Ctx and HLT may be important for cytotoxicity or be involved in protein export.

To determine the relative level of expression of each of these mutant toxins in comparison to the unmutated pNPCT, proteins from the periplasmic and cytoplasmic fractions of BL21 cultures containing the plasmids pNPCT, the negative control pYS1, and the mutants M1, M5, M9, M13, and M17 were separated by polyacrylamide gel electrophoresis, and either stained with Coomassie Blue or blotted onto nitrocellulose. Figures 12 and 13 show a representative stained gel and immunoblot, respectively, of the periplasmic fractions of all of these constructs.

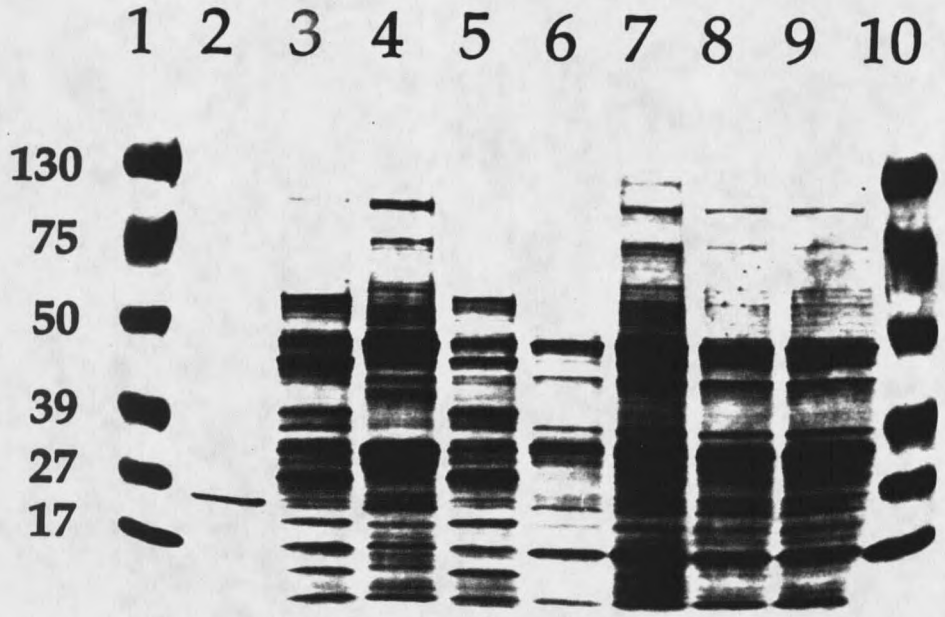

Figure 12. Coomassie Blue-stained SDS-PAGE of periplasmic fractions of CtxA mutants. Lanes 3 through 9 were loaded with 70 μg protein each.
Lane 1 - Bio-Rad prestained low range MW markers
Lane 2 - 0.5 μg CT-A
Lane 3 - pNPCT
Lane 4 - pYS1
Lane 5 - M1
Lane 6 - M5
Lane 7 - M9
Lane 8 - M13
Lane 9 - M17
Lane 10 - Low MW markers

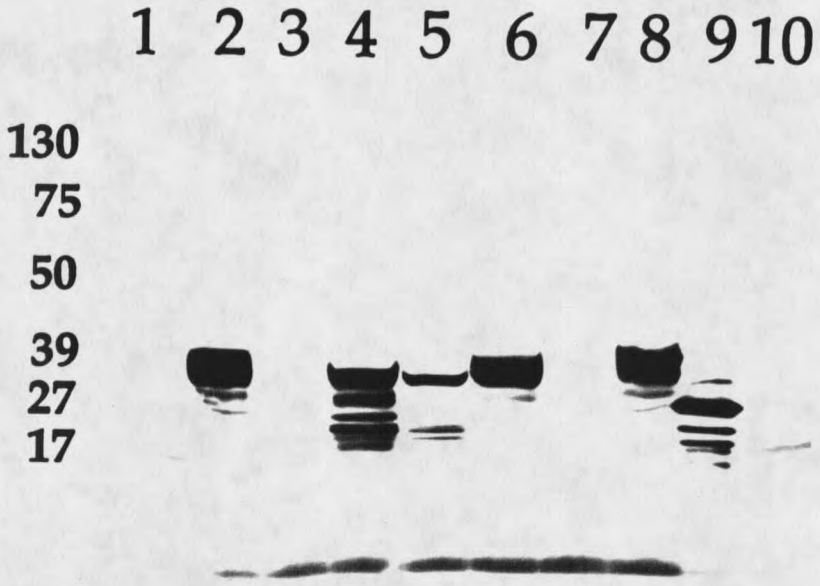

Figure 13. Immunoblot of periplasmic fractions of CtxA mutants using a polyclonal anti-CtxA antibody for detection.
Lanes 2 through 8 contain 50 μg protein each.
Lane 1 - Bio-Rad prestained low range MW markers
Lane 2 - pNPCT
Lane 3 - pYS1
Lane 4 - M1
Lane 5 -M5
Lane 6 - M9
Lane 7 - M13
Lane 8 - M17
Lane 9 - 0.5 μg CT-A
Lane 10 - Low MW markers

Figures 14 and 15 show a representative gel and blot of the cytoplasmic fractions of the BL21/pNPCT, pYS1, M1, M5, M9, M13 and M17 cultures.

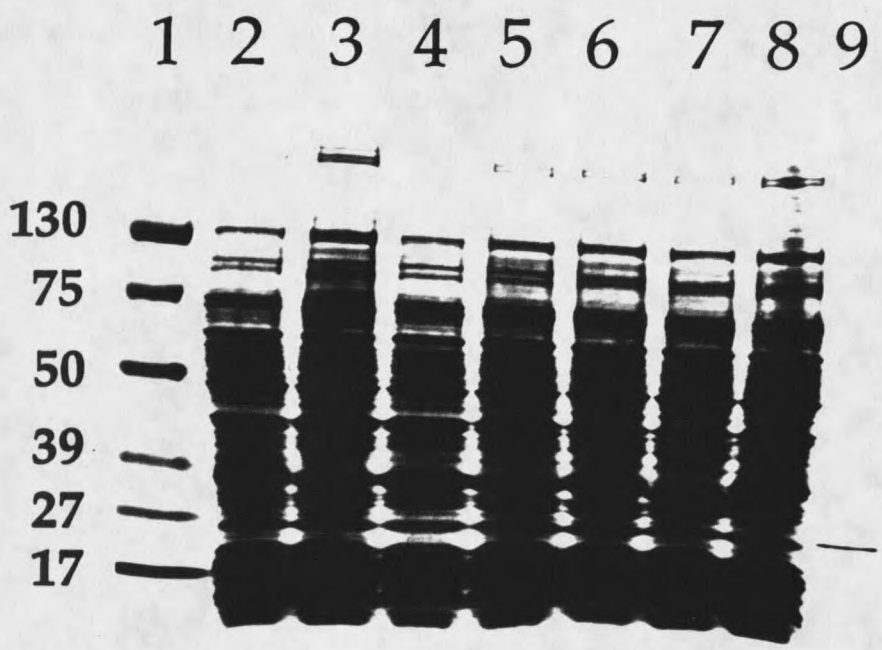

Figure 14. Coomassie Blue-stained SDS-PAGE of cytoplasmic fractions of CtxA mutants. Lanes 2 through 8 each have 25 μg protein.
Lane 1 - Low range MW markers
Lane 2 - pNPCT
Lane 3 - pYS1
Lane 4 - M1
Lane 5 - M5
Lane 6 - M9
Lane 7 - M13
Lane 8 - M17
Lane 9 - 2 μg CT-A

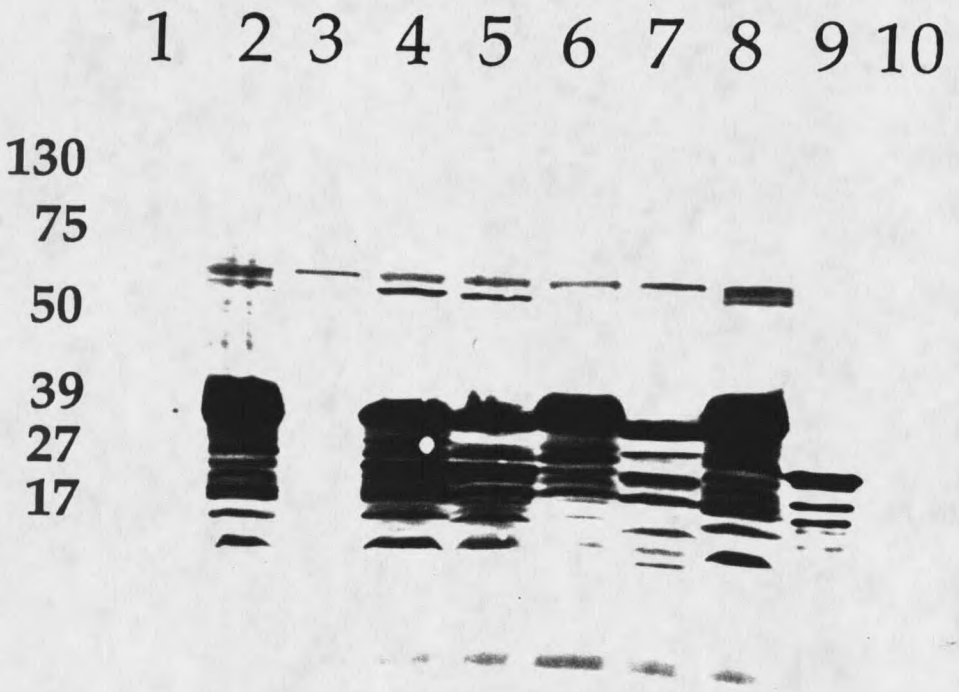

Figure 15. Immunoblot of cytoplasmic fractions of CtxA mutants using a polyclonal anti-CtxA antibody for detection. Lanes 2 through 8 each have 25 μg protein.
Lane 1 - Low range MW markers
Lane 2 - pNPCT
Lane 3 - pYS1
Lane 4 - M1
Lane 5 - M5
Lane 6 - M9
Lane 7 - M13
Lane 8 - M17
Lane 9 - 2 μg CT-A

Comparison of the immunoblots of the periplasmic and cytoplasmic fractions of the CtxA proteins shows that some mutant proteins were exported better than others, and some clearly not at all. pYS1, the negative control, is a parental plasmid which contains all of the features of pYS3 but lacks both *PA* and *ctxA* inserts. As can be seen in Lane 3 of Figure 13, for pYS3 no immunoreactive protein is evident in the periplasmic fraction. The ~65 kDa band in Lane 3 of Figure 15 probably represents a cross-reactive *E. coli* protein, since it is found in all the other lanes as well and since rabbit anti-CtxA antisera was used as the primary antibody for these blots. Some cross-reactive *E. coli*-specific antibody would be expected in the rabbit antiserum.

The blot of the cytoplasmic fractions, Figure 15, clearly shows that all of the CtxA constructs, mutant and non-mutant, express quite well from the T7-derived vector. Quantitation of the immunoreactive protein produced by the mutants will be discussed later, but it is evident from the Western blot that some of the recombinant proteins are produced at higher levels than others.

Lane 7 of Figure 13 shows a complete absence of immunoreactive protein in the periplasmic fraction of the M13 mutant. This protein is different from the other recombinant proteins in that a significant piece of the N-terminal protein, the eight amino acid homology box, was deleted. Since these CtxA proteins were all exported (except M13) without benefit of a known signal sequence, it might be expected that the N-terminal sequence of the mature peptides plays an important role in enhancing or limiting transport through the inner membrane of *E. coli*. Comparison of the N-terminal 20 amino acids of the mature CtxA (pNPCT), the M13 mutant, and the actual CtxA signal sequence (see Figures 15 - 17) show obvious differences

in hydrophobicity, particularly between the actual signal sequence and the pNPCT and M13 constructs. However, it is both the most hydrophobic (the actual *V. cholerae* signal sequence) and the most hydrophilic (pNPCT) sequences which are most efficiently exported in *V. cholerae* and *E. coli*, respectively, while the intermediate M13 construct showed no evidence of transport into the periplasmic space on repeated induction experiments. Clearly, something other than the hydrophobicity of the N-terminal region of the protein is involved in cross-membrane transport. Topogenic sequences have been identified within the mature portion of several *E. coli* proteins, usually appearing as extended stretches of hydrophobic amino acids and often near the N-terminal region of the protein {Oliver, 1987}.

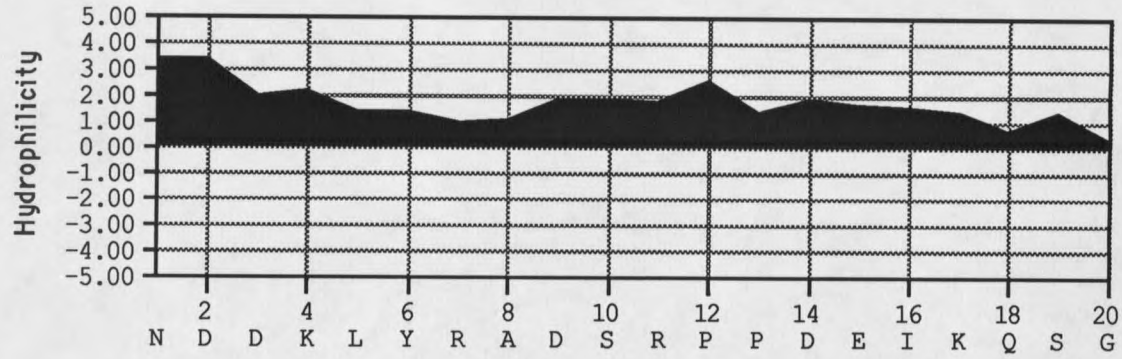

Figure 16. Hydrophobicity plot of the first 20 amino acids of the mature CtxA peptide.

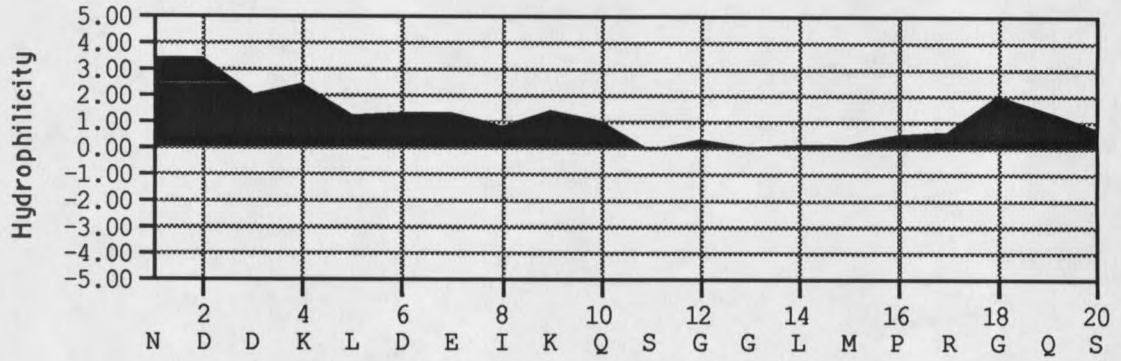

Figure 17. Hydrophobicity plot of the first 20 amino acids of the M13 mutant.

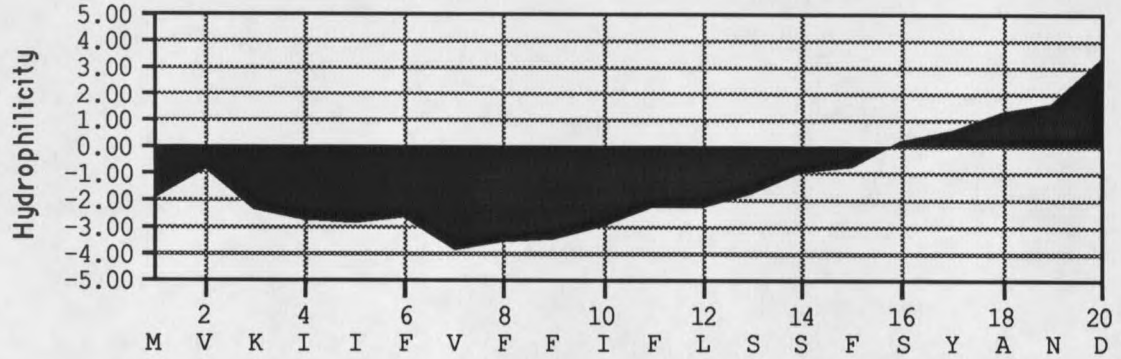

Figure 18. Hydrophobicity plot of CtxA signal sequence (Mekalanos *et al.*, 1983)

Comparison of the OmpA (Figure 19) and CtxA (Figure 18) signal sequences revealed that although there is no DNA homology between the two, both have the three conserved features of typical procaryotic signal peptides: one or two positively charged amino acid residues near the amino terminus, followed by 14 to 20 primarily hydrophobic amino acids, and an AXB consensus processing site (Oliver, 1987). In their natural hosts both Ctx and HLT are exported from the cytoplasm, although due to a more rigid cell wall the *E. coli* toxin is retained in the periplasmic space while cholera holotoxin is translocated into the surrounding medium. It is likely, given the efficiency with which native Ctx is exported by *V. cholerae*, that the bacterium has characteristic mechanisms for rapid processing of the Ctx peptide which are not necessarily contingent upon the presence of a signal peptide. This would suggest that CtxA probably does have some intragenic topogenic sequences which could be responsible for the periplasmic localization of the recombinant proteins expressed in *E. coli*.

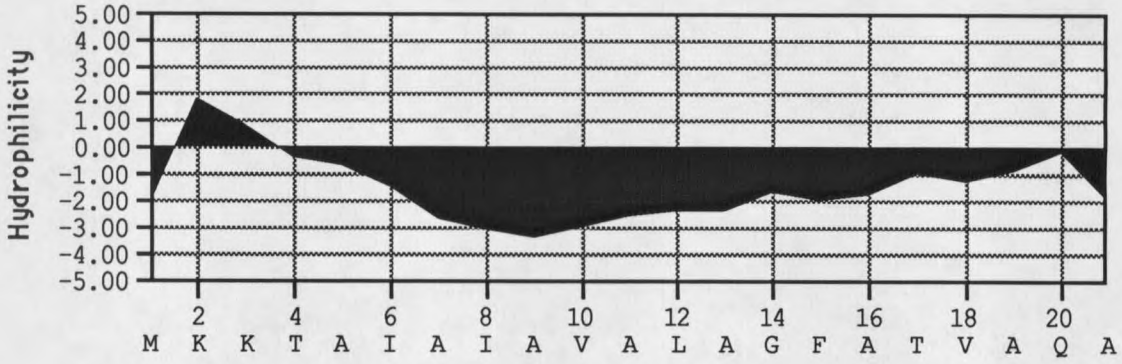

Figure 19. Hydrophobicity plot of the signal sequence of the *E. coli* OmpA protein.

Assays of Biological Activity

The natural substrate for CtxA *in vivo* is Gs, an extremely labile, hydrophobic protein which is very difficult to purify and is unstable unless correctly oriented in an appropriate membrane {Galloway and Van Heyningen, 1987}. Alternate substrates for the toxin such as transducin, a G protein involved in transduction of the visual signal in the eye, or low molecular weight guanidino compounds such as agmatine or L-arginine methyl ester, have been used to assay enzymatic activity of CtxA. Transducin has been used successfully to assay Ptx ADP-ribosylation activity in either the membrane-bound form, as rod outer segments, or as purified Gt {Cieplak *et al.*, 1988}. Other methods include the measurement of the release of bound guanyl nucleotides from pigeon or turkey erythrocyte membranes {Burns *et al.*, 1982, Gill and Meren, 1983}, or a high performance liquid chromatographic method for assay of ADP-ribosylation of L-arginine methyl ester {Larew *et al.*, 1991}.

The agmatine assay measures the glycohydrolytic cleavage of NAD into nicotinamide and ADP-ribose, and the transfer of the ADP-ribose to the agmatine acceptor substrate molecule. In this assay, adenine is uniformly labeled with ^{14}C. Agmatine serves as the acceptor substrate for the cleaved ADP-ribose, and the toxin-catalyzed binding of (^{14}C)ADP-ribose to agmatine is monitored by separation of the reaction mixtures on ion-exchange columns. In the chloride form of the AG 1X resin used in this assay, the counter ion is Cl^-, which is replaced by negative ions in the sample as it flows through the column. The possible reactive components of the agmatine reaction mix

include: NAD, net charge of -1; nicotinamide, no charge; ADP-ribose net charge of -2 charge; agmatine with a +2 charge; and ADP-ribosylated agmatine, net charge of zero. The negatively charged NAD and any available ADP-ribose will therefore bind to the resin, while the uncharged nicotinamide and ^{14}C-labeled ADP-ribosylated agmatine with a net charge of zero will flow through the column in the eluate {Moss *et al.*, 1976, Tsai *et al.*, 1987}.

Biological activity of all recombinant proteins was evaluated by the agmatine assay. The linearity of the assay was established by assaying purified CT-A at a range of concentrations. The glycohydrolytic cleavage of (^{14}C)NAD and the subsequent formation of ADP-ribosylated agmatine was directly proportional to the CT-A concentration (Figure 20).

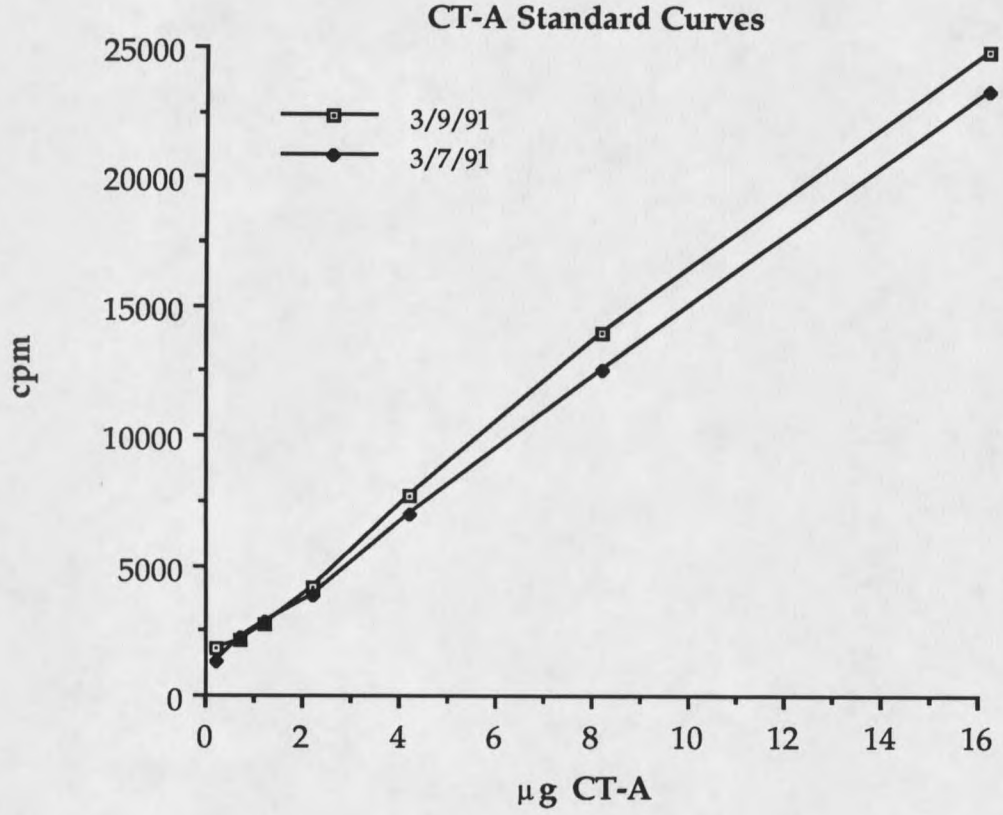

Figure 20. Standard curve of purified CT-A activity as measured by the agmatine assay.

ADP-ribosylation activity of the periplasmic and cytoplasmic fractions of each recombinant toxin were evaluated using the agmatine assay. Figure 21 shows the ADP-ribosyltransferase activity of the periplasmic fractions of three separate induction experiments. Figure 22 represents the ADP-ribosyltransferase activity of the cytoplasmic fractions of the most recent induction experiment.

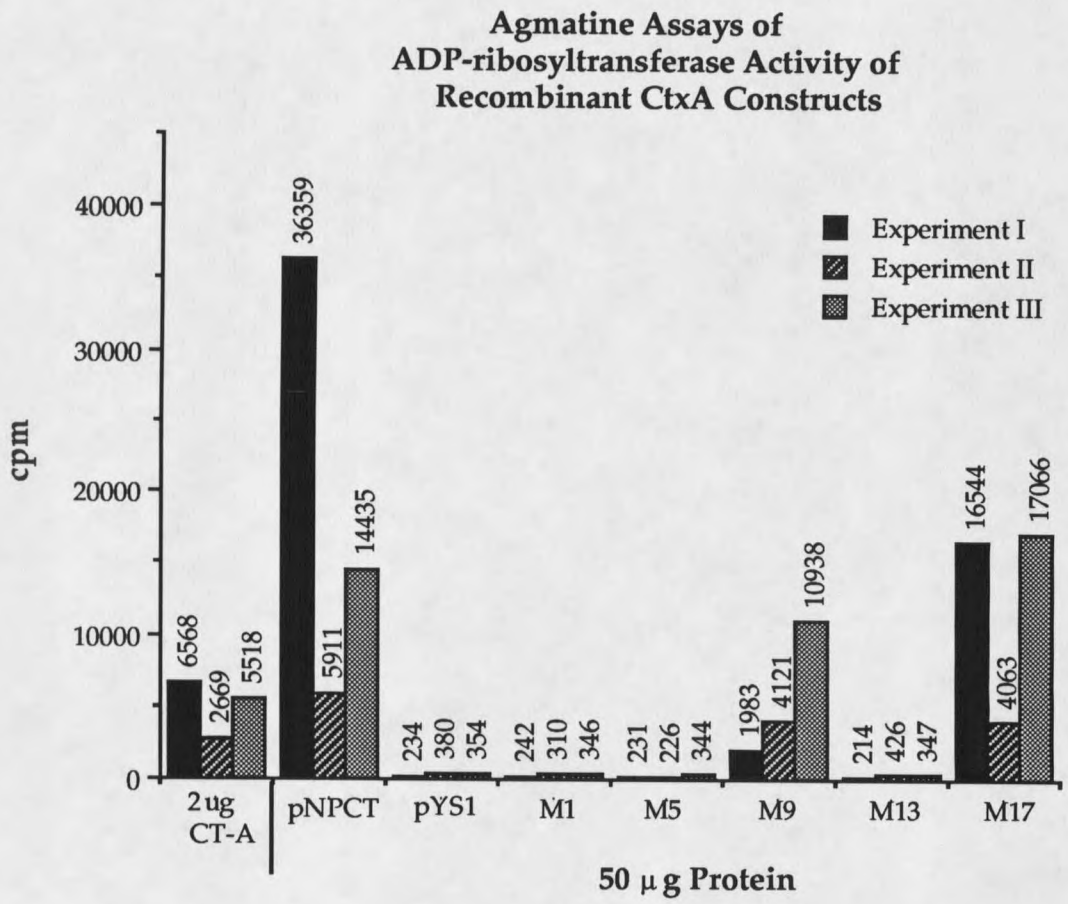

Figure 21. Activity of mutant and non-mutant constructs of recombinant CtxA as measured by the agmatine assay.

As Figure 21 indicates, the various recombinant CtxA proteins display a consistent pattern of differential ADP-ribosylation activity. The unmutated pNPCT plasmid expresses a protein which has more than a ten-fold increase in activity over the background, pYS1. The KDEL mutant, M17, shows almost the same level of activity as pNPCT, and the M9 protein shows somewhat less activity but still more than a three-fold increase over background. These enzymatic activities are relative to test samples containing 50 µg of total protein. Quantitation and equilization of specific CtxA recombinant protein

in these fractions did not significantly change the trend shown in Figure 21. However, comparison of the relative activity of mutant CtxA standardized by the actual amount of CtxA-specific protein in the assays is presented below (see Table 3). The similar activity levels of pNPCT and M17 are not surprising, since the M17 mutation involves a rearrangement of the last four amino acids of the A subunit, a region that is virtually certain to have no impact on enzymatic activity or significant structural changes of the resulting protein based on similar studies with Ptx {Locht *et al.*, 1987}. It was unexpected that the M9 mutation should have as much activity as it does. Substitution of the first proline in a Pro-Pro dipeptide for a glycine represents a potentially fundamental alteration in the secondary structure of the mature protein. Retention of at least half of the activity of the unmutated protein suggests that the Pro-Pro dipeptide is probably not involved in a poly(Pro) helical structure, which can involve as few as two proline residues and which would very likely be disrupted by substitution of a glycine. If that were the case, I would expect to see a significant disruption of the conformation which would probably have a greater effect on the enzymatic activity than is demonstrated with this mutation. Proline and glycine residues are often found associated with reverse turns; it is possible that substitution of a glycine for a proline residue would have no effect on a reverse turn in CtxA {Creighton, 1984}.

The M1, M5 and M13 mutations show no detectable ADP-ribosylation activity on any of the repeated assays. However, in the case of M13, it must be remembered that the activity of only the periplasmic fractions is shown in

Figure 21, and the M13 protein, which has the first homology box deleted, is not exported into the periplasm.

The activity level of the Arg_7 to Lys mutation (M1) is consistent with the reactivity of the same alterations in Ptx {Lobet *et al.*, 1989 and Burnette *et al.*, 1988} and in LT {Cieplak, submitted}. Alteration of the Ptx Arg_9 to any other residue eliminated detectable ADP-ribosylation activity {Lobet *et al.*, 1989}, and it appears that the same is true in LT and Ctx. It seems likely that this conserved arginine residue does play a significant role in enzymatic activity of these ADP-ribosylating toxin.

The M5 protein, containing the substitution of Arg for Asp_9, also consistently demonstrated ADP-ribosylation activity at background levels. Barbieri and Cortina {1988} substituted a serine residue for the aspartic acid at this position in the Ptx S1 molecule and were unable to detect any ADP-ribosyltransferase activity. Thus the lack of activity of the Asp to Arg alteration in CtxA is consistent with similar mutations in Ptx.

Sonicated cell lysates were assayed for ADP-ribosyltransferase activity by the agmatine assay. Figure 22 indicates the relative reactivity of the mutants compared to the unmutated recombinant toxin and purified toxin.

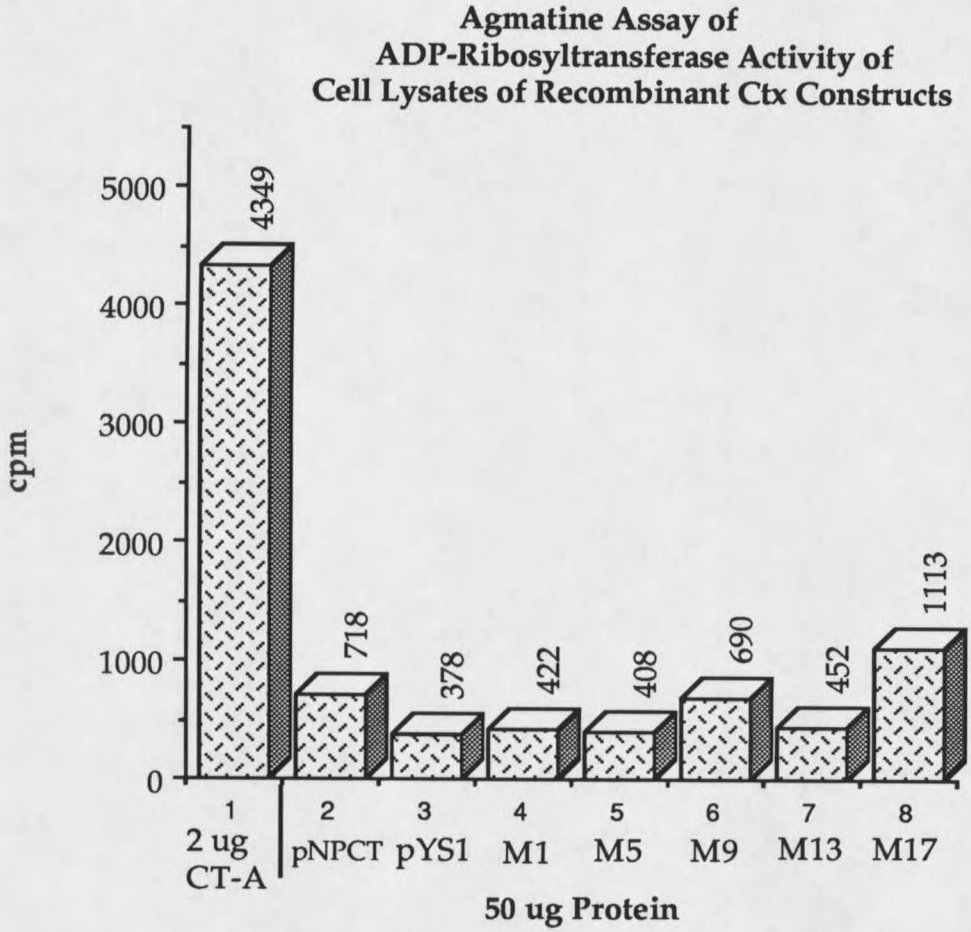

Figure 22. Activity of sonicated cytoplasmic fractions of mutated and non-mutated recombinant CtxA.

Figure 22 shows that ADP-ribosylation activity of the cytoplasmic fractions, while considerably lower than that of the periplasmic fractions, generally demonstrates the same pattern of activity. The general decrease in activity of all samples may be due to degradation of the recombinant proteins by proteases in the cytoplasm of the cells. The immunoblot in Figure 15 does show more breakdown products of the immunoreactive proteins than in Figure 13, the periplasmic fractions. Lower levels of enzymatic activity seen in the M1, M5 and M13 proteins may be partly accounted for by increased

susceptibility to proteolytic degradation in the cytoplasm, and partly due to lower levels of expression. The "inactive" recombinant proteins would therefore have less immunoreactive protein, and therefore potentially less enzymatically active protein, in a given amount of total protein on either an SDS-PAGE gel or agmatine assay. However, clearly all the mutants are expressed at easily detectable levels and the very low levels of ADP-ribosyltransferase activity seen in M1, M5 and M13 cannot be totally or even largely accounted for by lower levels of expression or increased degradation.

Figure 22 also shows that M13, which is not exported into the periplasm, has no activity in the cytoplasmic fraction. Again, this is consistent with published reports of deletion of the first homology box in the S1 subunit of Ptx {Barbieri and Cortina, 1988, Cieplak *et al.*, 1988}.

Repeated assays of the same induction experiments demonstrated that the recombinant proteins from periplasmic fractions were stable for weeks at 4°C or -20°C. The unmutated construct, pNPCT, typically had more than a 10-fold increase in activity over the background level of the negative control, pYS1.

Quantitation of Immunoreactive Protein

Recombinant CtxA proteins were expressed and the enzymatic activity measured in comparison to CT-A purified from *V. cholerae*. Blots and assays were standardized by adding equivalent amounts of total protein to each lane or reaction tube, but it was important to attempt to quantify the amount of recombinant CtxA in each sample. Quantitation of proteins by densitometry

has proven to be a simple and reliable method in recent years, and one particularly well suited to relatively impure protein preparations for which adequate antibody detection is available {Lobet *et al.*, 1989}.

Figure 23 shows the immunoblot that was scanned by a laser densitometer to quantitate the immunoreactive protein produced by the biologically active recombinant constructs. Recombinant proteins M1, M5 and M13 were not scanned because the enzymatic activity of these proteins was below the limits of detection of the agmatine assay. Therefore, it was not possible to relate a quantitated amount of immunoreactive protein with enzymatic activity of zero.

Sample	Total Protein on Gel	Peak Area	μg IR Protein	cpm /μg IR Protein
CT-A	0.05 μg	0.377	0.05	
pNPCT	0.5 μg	0.672	0.089	1,580
M9	0.5 μg	0.355	0.047	2,250
M17	0.5 μg	0.463	0.061	2,740

Table 3. Quantitation of immunoreactive (IR) protein. Data is from Experiment III, Figure 21. Background of 354 cpm was subtracted from the initial values.

The data from Table 3 were used to quantitate the amount of immunoreactive protein in the assays shown in Figure 21. The values listed under Peak Area refer to data obtained from the densitometric scan of the immunoblot shown in Figure 23. The numbers in the last column of Table 3 indicate that both M9 and M17 mutants display a level of ADP-ribosylation

activity about 1.5 to 1.75 times higher than the unmutated pNPCT. As these are crude protein preparations, however, the relative ADP-ribosylation activities listed in Table 3 are the best estimates based on immunoreactive protein. It would be possible to conclude from these data, however, that the Pro to Gly substitution does not decrease the enzymatic activity of recombinant CtxA, but actually increases its activity. The KDEL mutant also does not appear to diminish activity of the recombinant protein. This corroborates the work of Locht *et al.*, {1987} on C-terminal deletions of Ptx S1 subunit and Chaudry *et al.* {1990}, who used C-terminal deletions in the *Pseudomonas* exotoxin to demonstrate that removal of as few as two of the KDEL residues eliminated cytoxicity of the toxin without affecting ADP-ribosylation activity.

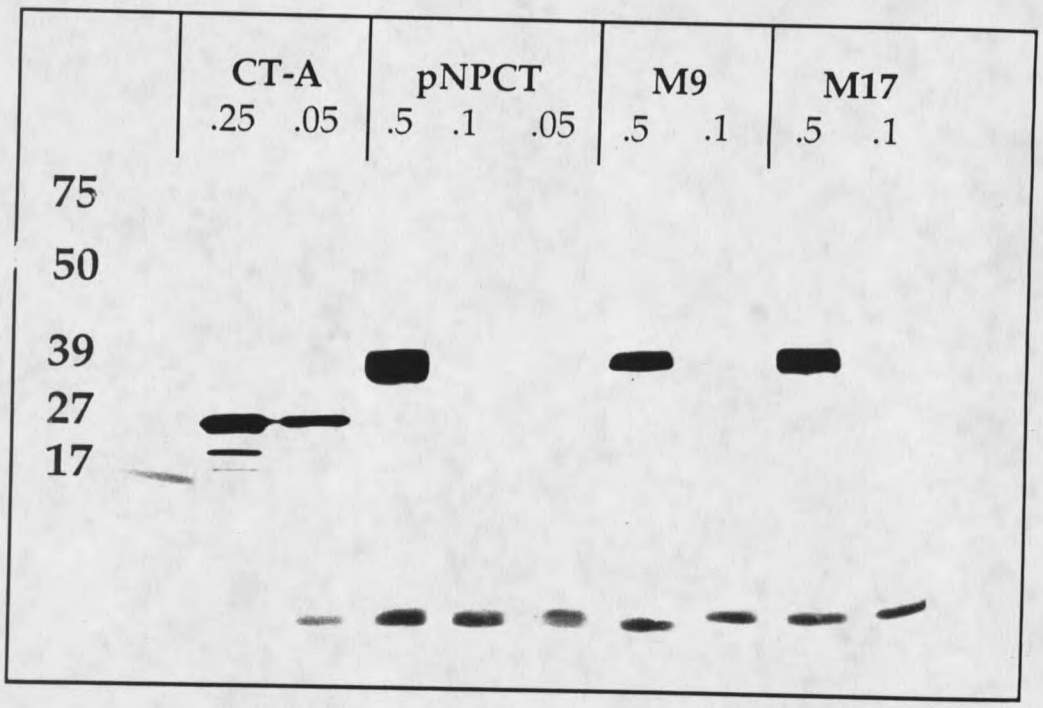

Figure 23. Immunoblot used for quantitation of immunoreactive protein by densitometric scanning.
Lane 1 - Low MW markers
Lane 2 - 0.25 µg CT-A
Lane 3 - 0.05 µg CT-A
Lane 4 - 0.5 µg pNPCT protein
Lane 5 - 0.1 µg pNPCT protein
Lane 6 - 0.05 µg pNPCT protein
Lane 7 - 0.5 µg M9 protein
Lane 8 - 0.1 µg M9 protein
Lane 9 - 0.5 µg M17 protein
Lane 10 - 0.1 µg M17 protein

Limited Proteolysis

When the first and second homology boxes were first identified it seemed likely that such limited sequence homology in toxins which ADP-ribosylated different sites on different G proteins must be important for enzymatic activity. The site-specific mutagenesis studies done by Barbieri and Cortina {1988}, Lobet *et al.*, {1989} and Cieplak *et al* {1988} appeared to confirm the significance of the first homology box and specific residues therein. Subsequent mutagenesis within the Ptx, CtxA and LT molecules has demonstrated, however, that point mutations in many parts of these proteins can have dramatic effects on toxicity {Barbieri *et al.*, 1989, Pizza, *et al.*, 1988, Kaslow *et al.*, 1989}, either because the residues are actually involved in some phase of the catalytic activity of the toxin, or because their alteration induces conformational changes in the protein which inhibit enzymatic activity or prohibits holotoxin formation.

Limited proteolysis of the unmutated CtxA and the Arg to Lys mutation was performed to see if this amino acid substitution might be inducing a conformational change in the recombinant protein.

Figure 24 shows a comparison of immunoreactive proteins from the limited trypsin digests of CT-A and periplasmic fractions of pNPCT and M1, the Arg_7 to Lys mutation.

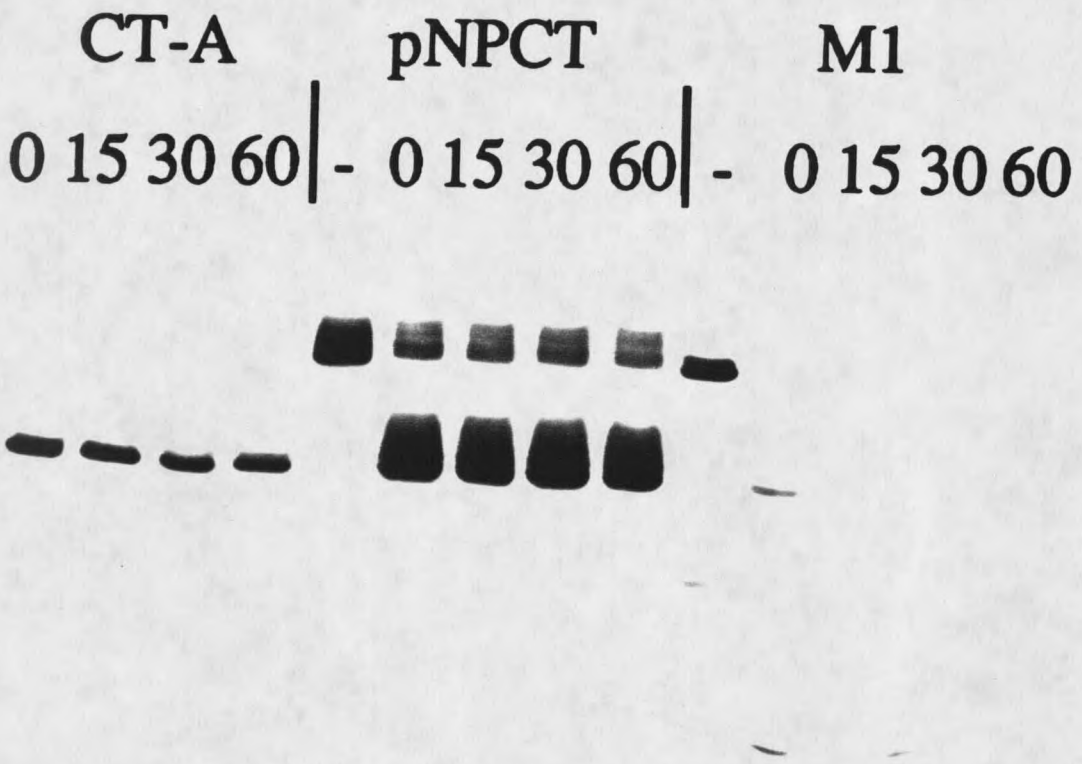

Figure 24. Limited trypsin digestion of purified and recombinant toxin and the Arg to Lys mutation of recombinant toxin.

Trypsin digest of the purified CT-A protein had no effect on the size or number of immunoreactive proteins detected by the polyclonal antibody. This is to be expected as this toxin was purified from *V. cholerae,* which naturally cleaves most of the A subunit into the activated A_1-A_2 conformation. Tryptic digestion of pNPCT cleaved CtxA at the only available trypsin site, between the A_1 and A_2 subunits, so that the resulting protein co-migrates with the purified toxin on SDS-PAGE. Digestion of the M1 protein, in contrast, cuts the mutant into several smaller peptides, indicating that a

number of additional trypsin sites are accessible in this protein that are not available in the wild type or unmutated toxins. This suggests that the Arg to Lys mutation may alter the folding of the mature protein, inducing a conformational change which makes the protein enzymatically inactive.

Results of site-specific mutagenesis studies in the first homology box of Ptx, LT and Ctx are remarkably consistent in demonstrating that any alteration at the Arg_7 position dramatically decreases ADP-ribosylation activity {Cieplak, submitted, Burnette *et al.*, 1988, Lobet *et al.*, 1989}. Alteration of the Asp_9 appears to have similar effects, although this site has not been as well characterized {Barbieri, 1988}. The experiments described here substantiate the role of the first homology box in the catalytic activities of these toxins.

Important catalytic residues have been identified in other ADP-ribosylating toxins, such as Ptx, where Glu-129 has been shown to be the NAD binding site {Barbieri *et al.*, 1989} diphtheria toxin (DT), and *Pseudomonas aeruginosa* exotoxin A (PE). Of these toxins, only Ptx shares any significant sequence homology with Ctx, but particular amino acid residues known to be catalytic in DT, PE and Ptx do have potential equivalents in Ctx. Two glutamic acid residues in CtxA, Glu-110 and/or Glu112, may be equivalent to the Glu-129 of Ptx {Barbieri *et al.*, 1989; Jobling and Holmes, 1991}, which is equivalent to the Glu-148 and Glu-553 residues in DT and EA, respectively {Carroll and Collier, 1984; Carroll and Collier, 1987}. Other residues, such as His-44 and Trp-127, may also be the Ctx equivalents of amino acids involved in NAD binding in DT, EA and Ptx, but conclusive data is not yet available

{Jobling and Holmes, 1991}. Identification of the NAD binding sites of other toxins was accomplished mainly by photolabeling reactions, which have not been successful in Ctx {Galloway *et al.*, 1987}.

Better definition of the specific catalytic sites in Ctx will help to produce an atoxinogenic strain of *V. cholerae* for vaccine production. Efforts to develop A^-B^+ strains have been continuing for several decades, since the role of the cholera toxin in pathogenesis was first demonstrated {De, 1959}. A number of non-toxigenic strains have been identified, but have been unacceptable as vaccine candidates either due to residual diarrheogenic activity or difficulty maintaining the stability or immunogenicity necessary to induce adequate immunity against cholera.

These problems highlight the pathogenic features of *V. cholerae* that make it simultaneously an interesting biological problem and a frustrating epidemiologic phenomenon. *V. cholerae* possesses a large number of virulence factors which are discussed more thoroughly in the Introduction. Nearly all of these appear to be coordinately regulated by the *toxR* locus to respond efficiently to various environmental signals. Elimination of even a primary virulence factor such as the ADP-ribosylation activity of CtxA can reduce the severity of disease or provide less pathogenic organisms to induce immunogenicity, but does not deal with the overall cause of the disease. Perhaps mutagenic alteration of the *toxR* locus to produce genetically detoxified live cholera vibrios may be the only way to develop a truly atoxinogenic strain of *V. cholerae.*

However, the other problem with the pathogenesis of cholera is that the gene coding for the primary virulence factor, *ctxAB*, is located on a

transposon in the *V. cholerae* chromosome which also contains the *toxR* and *toxS* genes, the *zot* toxin gene, and probably other toxins and regulatory factors as well. This transposon is known to be amplified during animal passage of *V. cholerae*, so that passage through the gut appears to select for pathogenicity of the organism or enhances the virulence of these organisms. *V. cholerae* strain 569B, for example, is a hypertoxinogenic mutant which was originally isolated from a nonpathogenic, hypotoxinogenic strain by intestinal passage in rabbits {Mekalanos, 1983}. Whether a genetically engineered non-toxinogenic strain would remain stably non-toxinogenic in the regions of the world where cholera is endemic and a vaccine most important is unknown.

CONCLUSIONS

This dissertation describes the work that I have done over the last four years to contribute to the understanding of bacterial pathogenesis and the development of a better cholera vaccine. I have sequenced an uncharacterized, hypertoxignic strain of *V. cholerae* (569B); successfully expressed an enzymatically active, recombinant cholera toxin in *E. coli*; and genetically detoxified this recombinant protein by eliminating ADP-ribosyltransferase activity with a single amino acid change. The results discussed in this paper confirm experiments with the pertussis and heat labile toxins that specific amino acid residues, particularly substitution of lysine for the first arginine in the first homology box, are involved in catalytic activity of these toxins {Cieplak, submitted, Lobet, *et al.*, 1989, Pizza *et al.*, 1989, Burnette, *et al.*, 1988, and Cieplak, *et al.*, 1988}.

The unexpected translocation into the periplasm of recombinant proteins without signal peptides raises some interesting questions regarding protein export. The rapid export of CtxA from the cytoplasm in *V. cholerae* suggests that there may be topogenic sequences within the mature protein that, along with the signal sequence, facilitate translocation of the toxin. This,

too, contributes to the pathogenesis of the organism, since CtxA is only toxic when attached to the B_5 oligomer and the holotoxin is secreted into the medium. These contributions to the field of cholera pathogenesis will help complete our understanding of the virulence mechanisms of *V. cholerae*, and offer hope for better vaccine development.

Cholera is an ancient disease which continues to cause significant morbidity and mortality in developing areas of the world, even though the pathologic defect in the disease is well known, the cause of cholera well-established, a simple and inexpensive treatment readily available and routes of transmission well documented. The toxin responsible for the disease, an ADP-ribosyltransferase of the A/B subunit type, has been cloned and sequenced {Mekalanos *et al.*, 1983 and Lockman *et al.*, 1984}. Trials of new and improved, but not quite adequate, vaccine strains are continually being carried out, but diarrheal disease remains the primary cause of infant mortality in the world today. New epidemics threaten wherever poverty and overpopulation combine with inadequate sanitation. The very nature of the disease, with its short incubation period, acute onset and rapid dehydrating effect make it difficult to treat successfully with antibiotics. The more we can learn about the pathologic defect in this disease and the precise details of the toxin's ADP-ribosylating activity, the better our chances of developing adequate means of preventing this age-old scourge.

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APPENDIX

PLASMID MAPS

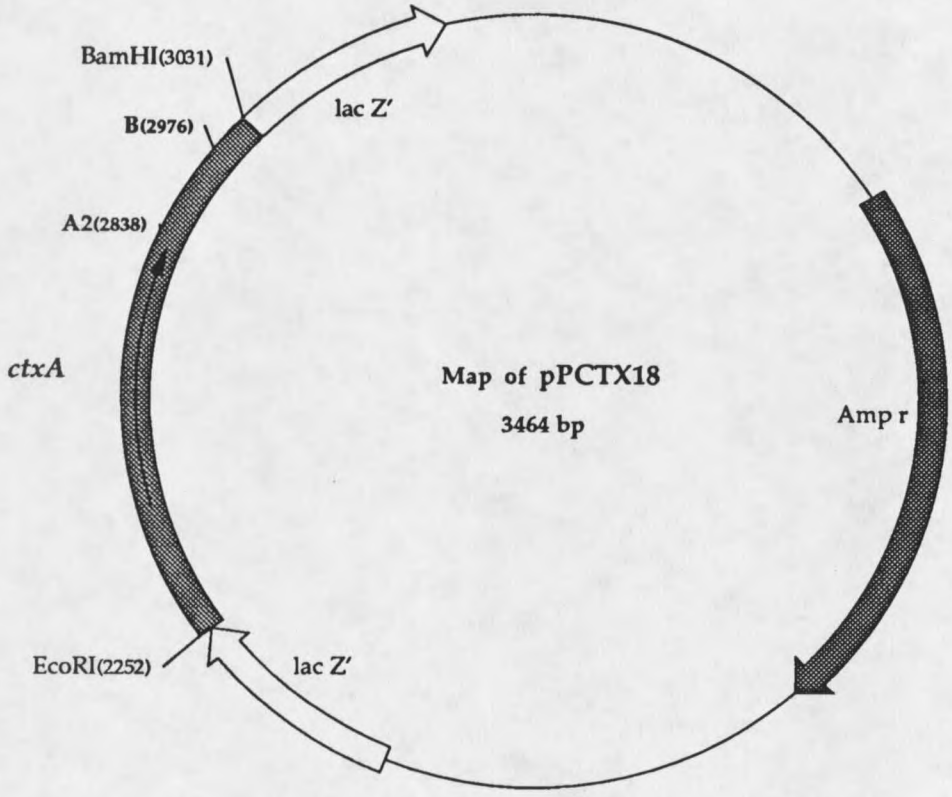

Figure 25. Map of plasmid pPCTX18.

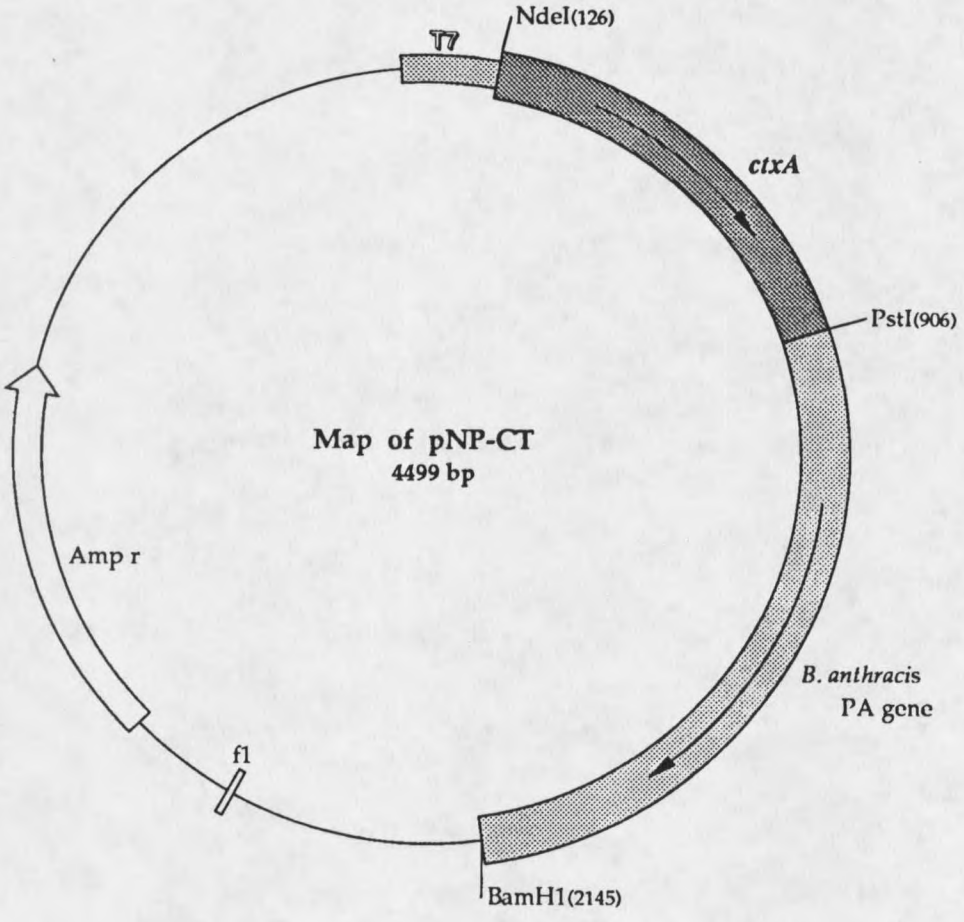

Figure 26. Map of plasmid pNP-CT.