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The Role of the Carboxy Terminus in the Folding and Secretion of Proaerolysin

by

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ABSTRACT

Aeromonas spp. secrete proaerolysin into the extracellular environment using the General Secretory Pathway. The protein crosses the inner membrane co-translationally and folds in the periplasm before crossing the outer membrane. There is some evidence that the carboxy terminus of the protein (amino acid residues 427 – 470) plays a role in proaerolysin secretion. Several variants that contained deletions in the carboxy terminus were generated by recombinant PCR. Deletion of amino acid residues 427 – 438 (Δ12) or deletion of residues 427 – 446 (Δ20) led to variants that could not be secreted and were not resistant to degradation by trypsin, suggesting that the deletions resulted in incorrect folding of the proteins. When the carboxy terminal was deleted altogether by inserting stop codons after residue 426 (End426) the protein could not be detected in either the culture supernatants or cells. However, co-expression of this variant with the C-terminal peptide led to some secretion of the protein into the culture supernatant. Furthermore, using alanine scanning mutagenesis I identified an α-helical region within the C-terminal peptide that is very sensitive to change and can adversely affect secretion when mutated.

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LIST OF ABBREVIATIONS

Abbreviation	
A	Alanine
ABC	ATP-binding cassette
Arg	Arginine
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
Bla	β-lactamase
BSA	Bovine serum albumin
C-terminal	Carboxy-terminal
CBD	Cellulose-binding domain
D	Aspartic acid
Da	Daltons
DNA	Deoxyribonucleic acid
E	Glutamic acid
EDTA	Ethylenediaminetetraacetic acid
F	Phenylalanine
Fig	Figure
G	Glycine
GGI	Gonococcal genetic island
GSP	General secretory pathway
HBS	Hepes buffered saline
His	Histidine

Ig	Immunoglobulin
IMC	Intramolecular chaperone
IPTG	Isopropyl-β-D-thiogalactoside
KDa	kiloDalton
L	Leucine
LB	Luria Bertanii
Leu	Leucine
Lys	Lysine
μg	microgram
μg/ml	microgram per milliliter
μl	microliter
μM	micromolar
mM	millimolar
MFP	Membrane fusion protein
N	Asparagine
ng	nanogram
nm	nanometer
N-terminal	Amino-terminal
Oca	Oligomeric coiled coil adhesin
OMP	Outer membrane protein
PA	Proaerolysin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PehA	Polygalacturonase
pg	picogram
Phe	Phenylalanine
PhoA	Alkaline phosphatase
Q	Glutamine
RBS	Ribosome binding site
rpm	revolutions per minute
RNA	Ribonucleic acid
RT	Room temperature
S	Serine
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
S/N	Supernatant
Spp	Species
Tat	Twin arginine translocase
TPS	Two partner system
Tris	Tris-(hydroxymethyl)aminomethane
TSA	Tryptic soy agar
Tween20	Polyoxyethylenesorbitan monolaurate
V	Valine
v/v	volume to volume
Val	Valine
W	Tryptophan

w/v	weight to volume
w/w	weight to weight
wt	Wild-type
Ypk	*Yersinia* protein kinase
Yops	*Yersinia* outer membrane proteins

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DEDICATION

To my grandmother – my source of inspiration.

INTRODUCTION

Gram-negative bacteria, such as *Escherichia coli*, can be divided into four regions: cytoplasm, inner membrane, periplasm and outer membrane. The final three, extracytoplasmic, compartments constitute the bacterial envelope and they are viewed as one structure that separates the cytoplasm from the external environment. Proteins that are to be secreted begin their synthesis within the cytoplasmic compartment and the proteins can cross the bacterial cell envelope by two main methods. Proteins can go from directly from the cytoplasm to the outside of the cell by employing either the type I pathway or the type III pathway. Alternatively, proteins can first cross the inner membrane to go from the cytoplasm to the periplasm and in a second secretion step cross the outer membrane before they are released into the extracellular environment. Either the Sec or Tat pathways are used by bacteria for the translocation of proteins across the cytoplasmic membrane. The terminal branches of the Sec pathway translocate the proteins across the outer membrane. These pathways include the autotransporters, chaperone/usher, type IV pathway, type V pathway and the type II pathway.

SECRETION ACROSS THE INNER MEMBRANE

Sec pathway

The Sec pathway is the dominant route for protein secretion across the bacterial inner membrane. The proteins that are directed to this pathway are synthesized with an N-terminal signal sequence about 20 amino acids long, comprised of a short stretch of positive charges, followed by a hydrophobic region and a proteolytic cleavage site for its

removal during translocation (Von Heijne, 1990). This signal sequence is proteolytically cleaved when the protein crosses the inner membrane, yielding a mature form of the protein, which is then transported to its appropriate extra-cytoplasmic compartment. The process by which proteins are translocated across the inner membrane by this pathway is well understood (de Keyzer *et al.*, 2003).

Sec translocase

The Sec translocation machinery is composed of a heterotrimeric integral membrane protein complex that includes the proteins SecY, SecE and SecG, and a peripherally bound cytosolic ATPase, SecA (Manting and Driessen, 2000). Two other proteins, SecD and SecF, have been identified as accessory membrane proteins whose absence blocks protein translocation (Gardel, *et al.*, 1990). These two proteins form a complex with a protein encoded by a gene, *yajC*, located on the same operon. This complex functions to stabilize the membrane-inserted SecA by interacting with the SecYEG complex (Duong and Wickner, 1997). The *E. coli* cell employs the protein SecB as a molecular chaperone to prevent the misfolding and aggregation of newly synthesized proteins (Fekkes and Driessen, 1999). SecB has an affinity for SecA and binds to the mature part of preproteins and aids in their targeting to the translocase. It is released with the initiation of the translocation process (Fekkes, *et al.*, 1997). The SecA ATPase binds with a low affinity to the lipid bilayer of the cytoplasmic membrane and with a high affinity to SecYEG and is then ready for the high-affinity interaction with SecB/precursor complexes (Hartl, *et al.*, 1990).

The role of the proton motive force in Sec-dependent transport

The binding of signal sequences to the inner membrane and their insertion are altered by the presence of a proton-motive force, which brings about a conformational change (Van Dalen, *et al.*, 1999). Van Dalen, *et al.*, showed that the initiation of translocation across the inner membrane of the protein PhoE was optimized by the conformational change brought about the presence of a proton-motive force. The primary function of the proton motive force is to provide unidirectionality to transport and this is evidenced by the observation that, in the absence of SecA, ATP and the proton motive force, reverse translocation can take place (Driessen, 1992).

SecYEG

SecYEG is thought to oligomerize to form the protein-conducting channel across the inner membrane with SecYE as the minimal constituent of the integral membrane translocation domain (Duong, *et al.*, 1997). Manting and Driessen (2000) proposed a two-step model for formation of the translocase. In the first step, two SecYEG subunits are brought together because of their affinity for membrane-bound SecA. In the second step, the two units are assembled into one active, heterotrimeric SecYEG channel as SecA inserts into the membrane.

When SecA is bound to the SecYEG complex, it undergoes nucleotide-modulated conformational changes (den Blaauwen, *et al.*, 1996) and these are thought to be responsible for the transmembrane movement of the translocating polypeptide. It was shown that a 30-kDa fragment of SecA appears during translocation and that this

fragment is protected from proteolysis by the inner membrane but becomes protease-accessible when the membrane was disrupted by the addition of detergent or after repeated cycles of freeze-thawing (Economou and Wickner, 1994). With the 30-kDa-domain insertion, approximately 20 aminoacyl residues of the preprotein are translocated across the inner membrane. At this point ATP is hydrolyzed at a second ATP site of SecA and this allows the 30-kDa domain to deinsert from the membrane and the whole cycle is repeated.

The twin-arginine translocation (Tat) pathway

Instead of the Sec pathway, some proteins employ the Tat pathway to move across the inner membrane. This system, unlike the Sec system, is able to translocate folded proteins across the cytoplasmic membrane. This was discovered with the study of Tat substrates that contain cofactors. These proteins were seen to acquire their cofactors and adopt their folded conformations while in the cytoplasm (Berks, 1996). However, proteins need not be associated with cofactors to be translocated via the Tat pathway. In *P. aeruginosa* the Tat pathway is required for the export of phospholipases as well as proteins involved in pyoverdine-mediated iron uptake, anaerobic respiration, osmotic stress defense, motility, and biofilm formation (Ochsner, *et al.*, 2002).

The Tat signal peptide

The proteins that are targeted to the Tat pathway have a signal peptide that has a basic amino-terminal n-region, followed by a hydrophobic h-region, and then a hydrophilic c-region containing the recognition site for the enzyme signal peptidase. The signal peptide

contains the conserved amino acid sequence motif (S-R-R-x-F-L-K) at the n-region/h-region boundary where the consecutive arginine residues are almost invariant, the frequency of the other motif residues are greater than 50%, and x is normally a polar amino acid (Stanley, *et al.*, 2000). Recent studies have suggested that the arginine pair in the consensus motif combined with an h-region that is more hydrophilic than in the Sec signal peptide are absolute requirements in targeting to the Tat pathway (Berks, *et al.*, 2000). Interactions between Tat signal peptides and the Sec translocon are prevented because the Tat signal peptides have an h-region that is not very hydrophobic as well as a basic c-region. Just like the Sec signal peptide, the Tat signal peptide is removed as the protein crosses the inner membrane (Santini, *et al.*, 1998).

The *tatA* and *tatE* operons

That *tatA* operon on the *E. coli* chromosome encodes the four genes *tatABCD*, while the *tatE* operon encodes the gene *tatE* (Sargent, *et al.*, 1998). TatA, B and E are all predicted to have a structure in which an N-terminal transmembrane helix is followed by a cytoplasmic domain including a possible amphipathic helical region. Single deletions of the *tatA* or *tatE* genes impair the secretion of Tat-dependent proteins, but in order to block secretion altogether, complete deletion of both genes is required (Sargent, *et al.*, 1998). Secretion is blocked completely by an in-frame deletion in *tatB* (Ize, *et al.*, 2002). Berks, *et al.*, (2000) have shown that some bacteria require, in addition to TatC, just one copy of a TatA/B/E-like protein for a functional Tat system. TatC, of all the Tat components, shows the highest level of amino acid conservation (Allen, *et al.*, 2002).

Tat complexes

Recently, two different studies have described purification of *E. coli* Tat protein complexes (Bolhuis, *et al.*, 2001; Sargent, *et al.*, 2001). However, the purified complexes have very different overall subunit compositions. Bolhuis, *et al.*, described a TatABC complex in which the three Tat proteins are present in an approximately equimolar ratio. Sargent, *et al.*, observed the co-purification of TatA and TatB as a large complex. In this complex TatA is present in a large molar excess over TatB, but the complex lacks the TatC component.

SECRETION ACROSS THE OUTER MEMBRANE

Several pathways for secretion of proteins across the outer membrane have evolved in Gram-negative bacteria (Figure 1). These pathways can be classified into two broad groups: i) pathways that are Sec-independent and capable of transporting unfolded proteins directly from the cytoplasm to the extracellular environment, (type I pathway and type III pathway) and ii) Sec-dependent pathways that form the terminal branches of the general secretory pathway (GSP) and export folded proteins with cleavable amino-terminal sequences (type II pathway, type IV pathway, type V pathway, autotransporters and the chaperone/usher pathway). A brief review of each of the extracellular secretion pathways employed by Gram-negative bacteria follows.

Sec-independent pathways

1. Type I secretion

Type I or ATP-binding cassette (ABC) protein exporters are involved in the secretion of a large number of lipases, proteases and toxins by a wide range of Gram-negative bacteria. Using this pathway, proteins are secreted directly from the cytoplasm across the outer membrane without involving the periplasm. The prototype for type I secretion is the *E. coli* α-hemolysin. The secretion apparatus for this pathway is composed of three proteins: an inner membrane ABC exporter, an inner membrane-anchored protein that spans the periplasm, termed a membrane fusion protein (MFP), and an outer membrane protein (OMP).

Substrates

Substrates for the Type I pathway lack a cleavable N-terminal signal sequence and possess, instead, a C-terminal secretion signal (Binet, *et al.*, 1997). The presence of a C-terminal secretion signal located in the last 60 amino acids was first identified on α-hemolysin using deletions and gene fusions (Mackman, *et al.*, 1986). Similarly, the highly homologous metalloproteases secreted by *E. chrysanthemi* and *S. marcescens* were shown to have C-terminal secretion signals (Ghigo and Wandersman, 1994; Letoffe, *et al.*, 1994). Many type I exoproteins possess glycine-rich repeats, which have been shown to play a role in Ca^{2+} binding and in folding and may be important for the release of the protein from the machinery (Baumann, *et al.*, 1993).

TolC functions as the OMP for α-hemolysin transport

The OMP for hemolysin transport, TolC, assembles as a trimeric complex in the outer membrane and consists of a β-barrel membrane domain with a C-terminal hydrophilic region that extends into the periplasm (Koronakis, *et al.*, 1997). The OMP is thought to function as the outer membrane secretion channel. The crystal structure of the TolC channel shows that it contains three domains: a 12-stranded β-barrel domain spanning the outer membrane, a 12-stranded α-helical barrel domain protruding far into the periplasm, and a mixed α/β domain (equatorial domain) enveloping the midsection of the α-helical barrel (Koronakis, *et al.*, 2000). In a recent study TolC mutants were generated that secreted normal levels of hemolysin although the secreted toxin was less active enzymatically (Vakharia, *et al.*, 2001). Hemolysin is thought to pass through the TolC barrel in a partially unfolded state owing to its relatively large size. Upon its release into the extracellular medium or upon its contact with the target cell, the toxin must refold to gain enzymatic activity. Vakharia, *et al.*, (2002) suggest that a block in translocation occurs in these mutants that may lead to misfolding and/or aggregation of toxin molecules trapped within the TolC barrel. This indicates that the role of TolC entails more than just providing a passage for the hemolysin molecule.

The membrane fusion protein (MFP)

HlyD, the periplasmic MFP component involved in the type I transport of α-hemolysin in *E. coli*, also assembles as a trimer and interacts with both the OMP and ABC exporter (Thanabalu, *et al.*, 1998). An analysis by Johnson and Church (1999) has shown that MFPs typically contain a hydrophobic amino terminus that is believed to span the inner

membrane or to be anchored in the inner membrane by lipid modification of the amino terminus. The bulk of the MFP is thought to extend across the periplasm to contact the OMP and/or the outer membrane itself. The MFP is thought to aid substrate secretion without the need for a periplasmic intermediate by forming a closed bridge or channel across the periplasm or by fusing the inner and outer membranes, allowing direct contact of the ABC exporter and the OMP channel (Thanassi, *et al.*, 2000).

The ABC exporter

The ABC exporter involved in type I secretion belongs to a large family of proteins found in prokaryotes as well as in eukaryotes, which facilitate the transport of a variety of substrates across membranes with the help of ATP (Higgins, 1992). HlyB, the ABC exporter for α-hemolysin, contains an N-terminal transmembrane domain with six to eight predicted transmembrane segments and a cytoplasmic C-terminal nucleotide-binding domain (Wang, *et al.*, 1991).

Two models for the type I secretion of *E. coli* hemolysin

In an attempt to understand the in vivo sequence of events that take place in type I secretion, Thanabalu, *et al.*, (1998) wanted to see in particular whether part or all of the exporter complex of the *E. coli* α-hemolysin secretion system is pre-formed in the absence of the substrate. The authors also wanted to see whether engagement of the substrate induces the bridging contact between the *E. coli* inner and outer membranes and if so, does the bridge remain in place or if it is reversible. In this model, the ABC exporter and MFP associate before substrate binding. Once the substrate binds to this

complex, contact of the MFP with the OMP is triggered. This contact is lost once the substrate is exported. The ABC exporter hydrolyzes ATP, which drives the release of the substrate outside the cell but is not required for substrate binding or assembly of the complex.

A second model for type I secretion is based on work done on *S. marcescens* hemoprotein HasA and *E. chrysanthemi* metalloproteases B and C secretion (Letoffe, *et al.*, 1996). In this model, the substrate first binds to the ABC exporter, which then triggers binding of the MFP. This complex then interacts with the OMP, which allows secretion of the substrate.

The signal sequence of *E. coli* α-hemolysin

Hui and Ling (2002) have tried to determine the functional elements of the *E. coli* α-hemolysin signal sequence. Previous studies by these researchers have identified the last 20 residues of the signal sequence as essential for transport across the outer membrane. They found that an amphiphilic α-helical region in the hemolysin sequence plays a critical role in secretion. Moreover, one region (between residues -16 and -9) in the extreme C-terminal region was shown to have no requirement for primary or secondary structural elements and it can be replaced by almost any combination of amino acids and still retain wild-type secretion competence. Another region within that same extreme C-terminus favors nonpositively charged residues for transport. By describing the remarkable collection of signal sequence variants that can be transported, this study illustrated the versatility of the hemolysin transporter system. Two principles of transport

were illustrated: i) the distinguishing features of hemolysin are contained in the secondary structure and not the primary sequence, and ii) there are multiple features that contribute to secretion (Hui and Ling, 2002).

2. Type III secretion

The type III secretion pathway enables bacteria to inject virulence factors directly into the cytosol of eukaryotic host cells. This pathway has been identified in a number of animal and plant pathogens and the secretion of *Yersinia* outer proteins (Yops) by *Yersinia* spp. represents the prototype.

Substrates

Virulence proteins that are secreted by the type III pathway vary greatly in size, structure and function (Hueck, 1998). Some of these proteins serve as accessory proteins since their function is to aid the secretion and translocation of the actual virulence factors. The *Yersinia* type III secretion system produces 13 Yops. According to Hueck (1998), they can be grouped into 3 categories: i) proteins with direct antihost functions (YopE, H, M, and YpkA), ii) translocatory proteins that are involved in the translocation process (YopB, D, K, and R), and, iii) regulatory proteins mediating the cell-dependent contact induction of *yop* gene expression and Yop secretion (YopN, LcrG, LcrV, and LcrQ).

YopE is a cytotoxin secreted by *Yersinia* spp. The domain responsible for the cytotoxic effect is probably situated in the carboxy-terminal one-third, since a truncated YopE lacking this region does not exhibit cytotoxicity although it is still normally secreted into

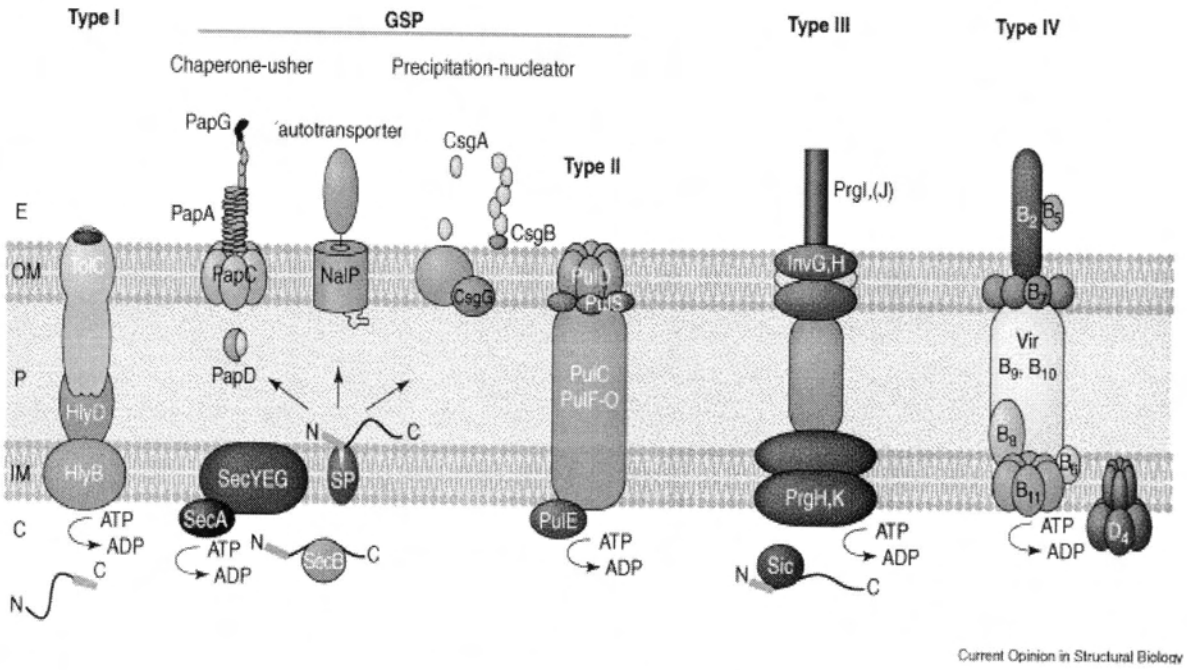


Figure 1. Secretion pathways employed by Gram-negative bacteria (Remaut and Waksman, 2004)

the supernatant (Rosqvist, *et al.*, 1990). YopH has been shown to be a specific and highly active tyrosine phosphatase (Zhang, *et al.*, 1992). It has a highly conserved catalytic domain, and mutation of a conserved cysteine to alanine completely abolishes phosphatase activity (Guan, *et al.*, 1990). YpkA is an autophosphorylating protein kinase with homology to eukaryotic protein kinases (Galyov, *et al.*, 1993). YopM is homologous to the thrombin-binding domain of the α chain of human platelet surface glycoprotein Ib and also to a portion of von Willebrand factor (Leung, *et al.*, 1989), a protein that has been predicted to compete with platelets for thrombin binding. It may inhibit platelet activation *in vivo*, muting the local inflammatory response to the bacteria (Reisner, *et al.*, 1992).

Type III secretion components and the flagellar basal body

Most of the type III components are thought to localize to the inner membrane. Parallels have been drawn between the type III components and those of the flagellar basal body (Hueck, 1998). The flagellar basal body has been shown to span the inner and outer membranes and to provide an anchor for the flagellar filament (Macnab, 1996). The machinery that drives secretion and flagellar assembly by ATP hydrolysis is contained in the cytoplasmic face of the basal body. The flagellin monomers are exported through a central channel within the basal body and filament for assembly at the distal end of the growing flagellum. Electron microscopy has shown the *S. typhimurium* type III structure to be similar to the flagellar basal body, with a hollow projection extending out from the bacterial surface (the needle) instead of a flagellar filament (Kubori, *et al.*, 1998). The

needle structure is probably extended to assemble long pili in all type III systems (Knutton, *et al.*, 1998).

Secretion signals

There are two N-terminal secretion signals for the export of Yop proteins. The first signal lies in the mRNA and is thought to target the RNA-ribosome complex to the type III machinery for coupled translation and secretion. This has been shown to be true for YopQ (Ramamurthi and Schneewind, 2003). The second signal behaves as a binding site for cytoplasmic chaperones (Syc proteins) and possibly targets Yops to the type III machinery for translocation into host cells (Cheng and Schneewind, 1999). A different observation was made in the case of YopE. It has been shown that the N-terminus of YopE is critical for secretion but the sequence of the 5' coding region of yopE mRNA is not (Lloyd, *et al.*, 2001). Infact, a recent study shows that the Yersinia type III secretion system preferentially exports substrates containing amphipathic N-terminal sequences (Lloyd, *et. al.*, 2002).

YopB, YopD and the translocation pore

It has been hypothesized that type-III-secreted proteins form pores in the host cell membrane for translocation purposes. This is based on studies with YopB and YopD, which are type III secretion substrates that are required for translocation of Yop effector proteins into host cells. It was seen that YopB and YopD are both required for pore formation in macrophage membranes, and that both proteins are inserted into liposomes by the type III machinery (Tardy, *et al.*, 1999). Translocation of the effectors in type III

secretion also requires the secreted LcrV protein, which was the first antigen to be associated with virulence of *Yersinia pestis* (Mulder, *et al.*, 1989). This protein interacts with YopB and YopD (Sarker, *et al.*, 1998) and is surface exposed before target cell contact (Pettersson, *et al.*, 1999).

Co-regulation of expression and secretion

Yersinia spp. co-regulate the expression of type III secreted proteins with their secretion (Hueck, 1998). Once the bacteria come into contact with the target cell(s) the secretion channels are opened. One way that the channels are kept shut in the absence of a target cell is by the presence of Ca^{2+}. In addition, YopN is thought to control the polarized secretion and translocation of Yop proteins into eukaryotic cells. However, YopN is not translocated into eukaryotic cells (Boland, *et al.*, 1996). YopN is thought to act as a regulator that blocks type III secretion channels in the presence of Ca^{2+} or the absence of a target cell (Forsberg, *et al.*, 1991).

Sec-dependent pathways

1. The autotransporter secretion system

Proteins that are transported via the autotransporter secretion pathway, a terminal branch of the GSP, include proteases, toxins, adhesins and invasins (Henderson, *et al.*, 1998). The *Neisseria gonorrhoeae* IgA1 protease is the prototypical member of the autotransporter family (Pohlner, *et al.*, 1987). The primary structures of all autotransporters are composed of three domains: the signal sequence, the passenger domain and the translocation unit (Desvaux *et al.*, 2004). The N-terminal signal sequence

enables the protein to be transported to the periplasmic space across the inner membrane via the Sec pathway. Autotransporters gain their diverse effector functions from the passenger domain. The translocation unit is composed of a short linker region having an α-helical secondary structure and a carboxy-terminal β-domain which has a β-barrel secondary structure when embedded in the outer membrane, through which the passenger domain passes to the cell surface (Oliver, *et al.*, 2003). The autotransporters are known to be synthesized in the cytoplasm in a pre-pro-protein form and are translocated into the periplasm in a pro-protein form (Desvaux, *et al.*, 2004). Upon translocation across the outer membrane the pro-protein may be cleaved from the β-barrel domain or it may remain associated with the domain.

The role of the β-domain

Following its release into the periplasm, the pro-protein interacts with the hydrophobic environment of the outer membrane which promotes a spontaneous insertion of the β-domain into the lipid bilayer of the outer membrane by the first and last β-strands of the autotransporter which form hydrogen bonds in an antiparallel manner to close the ring conformation (Henderson *et al.*, 1998). This permits the establishment of a molecular pore. It has been shown that folding of the passenger domain takes place in the periplasm before, or perhaps, simultaneously with, translocation across the outer membrane (Veiga *et al.*, 1999). Evidence that supports the notion that the β-barrel can aid the transport of folded proteins comes from studying the secretion of the IgA1 protease. This molecule has been shown to form an oligomeric complex of ~500 kDa in the shape of a ring-like structure containing a central cavity of ~2 nm (Veiga *et al.*, 2002).

The PD002457 domain – an intramolecular chaperone

Oliver, *et al.*, (2003) have shown in their study of the *Bordetella pertussis* BrkA autotransporter that part of the molecule is an intramolecular chaperone – the PD002475 domain. This domain is thought to play an essential role in the regulation of correct folding of the passenger domain before its insertion. Hence, it can be assumed that the passenger domain is at least partially folded as it moves through the channel formed by the monomeric β-barrel and that the folding, aided by the intramolecular chaperone domain, begins in the direction of the C-terminal domain as the passenger domain emerges on to the bacterial surface.

The role of the linker region

While the β-barrel is important for translocation of the passenger domain, the linker region upstream of the β-domain is also essential for secretion. This linker region is composed of a 21-39 amino-acid, α-helical region preceding the β-domain and is probably involved in the formation of a hairpin structure that leads the secretion of the passenger domain through the channel formed by the β-barrel (Henderson *et al.*, 1998). This linker region and the β-domain together are referred to as the translocation unit. The passenger domain is cleaved from the translocation unit at the bacterial surface. The former is then released into the extracellular environment or remains bound to the cell surface.

2. Chaperone-usher-mediated pathway

The chaperone/usher pathway is also a terminal branch of the GSP, which is responsible for the biogenesis of a superfamily of surface structures associated with pathogenesis (Thanassi, *et al.*, 2002). There are two components involved in the secretion mechanism: a periplasmic chaperone and an outer membrane protein called an usher. The prototypical members of the chaperone/usher pathway are the *pap* and *fim* gene clusters of uropathogenic *E. coli* that code for P and type 1 pili (fimbriae), respectively. Six structural proteins that make up the P pili interact to form a fiber composed of two subassemblies: a 6.8-nm-thick helical rod comprised mainly of PapA and a 2-nm-diameter linear tip fibrillum comprised mainly of PapE (Kuehn, *et al.*, 1992). PapD and PapC are the chaperone and usher for P pili, respectively. Type 1 pili bear the mannose-binding FimH adhesin and two minor components, FimF and FimG, which play important roles in pilus biogenesis (Jones, *et al.*, 1994). The major subunit of the type 1 pilus, FimA, is arranged in a tight, right-handed helical rod. FimC and FimD are the type 1 pilus chaperone and usher, respectively.

The periplasmic chaperone

The periplasmic chaperone consists of two immunoglobulin-like (Ig) domains. It has three main functions: facilitating the folding of pilus subunits, capping their interactive surfaces, and maintaining the subunits in stable conformations. The pilus subunits interact with the chaperone after their translocation across the inner membrane via the Sec pathway. The chaperone recognizes and binds to a conserved C-terminal motif present on each of the pilus subunits. The surface of the subunit possesses a deep groove

that exposes its hydrophobic core. This groove is filled by a G_1 β-strand from the chaperone which facilitates subunit folding via a mechanism termed donor strand complementation (Sauer, *et al.*, 1999). This mechanism involves the chaperone uncapping from a subunit to be coupled with the simultaneous assembly of the subunit into the pilus fiber. Therefore, in the pilus fiber, the N-terminal extension of every subunit completes the Ig fold of its neighboring subunit by occupying the same site previously occupied by the chaperone. The donor strand complementation simultaneously functions to prevent premature pilus formation in the periplasm (Thanassi, 2002).

The outer membrane usher

The periplasmic chaperone-subunit complexes next target the outer membrane usher. There is experimental evidence to show that in the absence of the usher, the complexes accumulate in the periplasm, but, that no pili are assembled or secreted (Valent *et al.*, 1995). Pilus assembly is a self-energized process that takes place at the periplasmic face of the usher (Jacob-Dubuisson, *et al.*, 1994). A process termed donor strand exchange now takes place, whereby the G_1 β-strand of the chaperone is exchanged for the highly conserved N-terminal extension of an incoming subunit (Barnhart, *et al.*, 2000). The mature pilus thus consists of an array of Ig domains, each of which contributes a strand to the fold of the preceding subunit to produce a highly stable organelle. Pilus assembly begins with incorporation of the adhesin, followed by assembly of the tip fibrillum and finally the rod. This process is facilitated by the usher (Dodson, *et al.*, 1993). Thus, chaperone-adhesin complexes from both the P and type 1 pilus systems have been found to bind tightest and fastest to their respective ushers, indicating that the kinetic

partitioning of chaperone-adhesin complexes to the usher is a defining factor in the tip localization of the adhesin (Thanassi, 2002).

The usher channel (~3 nm in diameter) is large enough to allow secretion of folded pilus subunits assembled into a linear fiber such as the tip fibrillum (~2 nm). However, the helical pilus rod (~6.8 nm) is too large to pass through this channel. There is experimental evidence to suggest that P and type 1 pilus rods can be unwound into linear fibers which consist of a linear array of folded subunits small enough to pass through the usher channel and adopt the final helical conformation of the pilus upon reaching the cell surface (Thanassi *et al.*, 1998; Saulino *et al.*, 2000). The coiling of the rod outside the cell has been thought to facilitate the outward translocation of pili (Jacob-Dubuisson, *et al.*, 1994) and this, combined with the targeting affinities of chaperone-subunit complexes for the usher and the binding specificities of subunits for each other, may provide sufficient energy and information for the ordered assembly and secretion of pili across the outer membrane.

3. Type IV secretion

Type IV secretion is a recently discovered secretion pathway that is primarily used to mobilize DNA, either from bacteria to bacteria or from bacteria to eukaryotic cells. The prototype for the type IV transporter family is the VirB system of *Agrobacterium tumifaciens*. This particular system transfers a piece of single-stranded DNA from the bacteria into a plant cell and it exhibits mechanistic details that are very similar to those of conjugation systems (Christie, 1997). A recent review (Ding, *et al.*, 2003) classifies

type IV family members into 3 groups: i) conjugations systems mediating DNA transfer to recipient cells, e.g. *A. tumifaciens* T-DNA transfer system, ii) 'effector translocator' systems that transfer molecules termed effectors to eukaryotic cells during infection, e.g. the virulence mechanism of *A. tumifaciens*, *H. pylori*, and *L. pneumophila*, and iii) 'DNA uptake or release' systems mediating DNA exchange with the milieu, e.g. the gonococcal genetic island (GGI) encoded DNA export system in *N. gonorrhoeae*.

The VirB system of *Agrobacterium tumifaciens*

There are 11 VirB proteins of which 10 (VirB2 – VirB11) associate to form a long pilus that is about 3.8 nm in diameter and an associated transport system that spans the cytoplasm of the cell, the inner membrane, periplasmic space and the outer membrane, to the outside of the cell (Fullner, *et al.*, 1996). VirB1 is processed, followed by the export of its carboxy-terminal portion to the exterior of the cell (Baron, *et al.*, 1997). VirB2 and VirB3 are exporters (Fernandez, *et al.*, 1996), with VirB2 localizing to the surface of the cell (Fullner, *et al.*, 1996). VirB4 is believed to be an integral cytoplasmic membrane protein with two periplasmic domains (Dang, *et al.*, 1997). VirB5, VirB7, Vir8, VirB9 and VirB10 are membrane-associated. Of these proteins, VirB7, VirB8, VirB9 and VirB10 fractionate with both inner and outer membranes (Thorstenson, *et al.*, 1993) and extend into the periplasmic space. VirB11 is believed to be located on the inner side of the cytoplasmic membrane. The existence of VirB proteins in both the inner and outer membrane fractions supports the idea that they form a transport complex that spans both membranes. There is now evidence to suggest that proteins of the VirB transport system form pili (Fullner, *et al.*, 1996). VirB2 is the major pilin subunit and it is thought to

undergo cyclization that may serve to stabilize the pilus structure (Eisenbrandt, *et al.*, 1999). However, it is unknown whether the pili simply mediate contact between the bacterium and the plant cell or whether the pilus might actually serve as a conduit through which proteins and DNA can pass.

VirB4 and VirB11 as energy providers

Two type IV transporter proteins contain nucleotide-binding motifs, indicating that they may form the motor for the transport process. Another role for these proteins would be to serve as a signal for the opening of a gate or channel as a result of kinase activity, or to act as molecular chaperones in the assembly of the transporter or during the transport process itself. VirB4 and VirB11 in the VirB system have been shown to be critical for transport (Rashkova, *et al.*, 1997; Fullner, *et al.*, 1994). VirB4 has ATPase activity while VirB11 has ATPase activity and phosphorylating activity.

Mechanism of translocation

There is still no clear data to suggest the actual mechanism behind transport via the type IV pathway. It has been suggested that transfer via the VirB system takes place in one step, by which the associated proteins and the associated DNA cross both bacterial membranes simultaneously (Christie, 1997).

4. <u>Type V secretion</u>

The type V secretion system family of proteins contains secreted proteins that i) contain all the information required for translocation through the cell envelope and/or require

single accessory factors, and ii) are translocated across the outer membrane via a transmembrane pore formed by a β-barrel (Desvaux, *et al.*, 2004). Characteristics of proteins secreted by this pathway include i) an N-terminal signal sequence for passage across the inner membrane via the Sec pathway, ii) a functional passenger domain that can be surface-exposed or released into the extracellular environment, iii) a linker region that aids in the outer membrane translocation of the passenger domain, and, iv) a C-terminal region that is responsible for formation of a transmembrane pore. This family has two sub-groups: the two-partner system (TPS) and the Oca (oligomeric coiled coil adhesin) family.

The two-partner secretion system

Unlike the autotransporter pathway, in the TPS, the passenger domain (TpsA) and the pore-forming β-domain are translated as two separate proteins (TpsB) (Jacob-Dubuisson, *et al.*, 2001). Translocation of TpsA across the outer membrane takes place via a β-barrel pore formed by TpsB. This β-barrel is probably different from the one associated with the autotransporters as it is predicted to contain 19 amphipathic β-strands in TpsB (Guedin, *et al.*, 2000), as opposed to 14 predicted for the autotransporters (Loveless and Saier, 1997). TpsB is thought to be involved in the maturation of the passenger domain into its active form. The prediction is that the passenger domain leaves the periplasm in an unfolded state and folds at the cell surface as it is translocated through the transporter domain (Guedin, *et al.*, 1998). Jacob-Dubuisson, *et al.*, (2001) have concluded that in TPS the translocation across both membranes seems coupled and that the driving force for translocation across the outer membrane is derived from the free energy of folding.

The Oca family

YadA is the prototype for the Oca family. The members of this family have been described in a recent study as a subfamily of surface-attached oligomeric autotransporters (Roggenkamp, *et al.*, 2003). YadA has six different domains: i) an N-terminal signal sequence, ii) head-D, iii) neck-D, iv) stalk-D, v) linking-R, and, vi) a C-terminal region consisting of only four β-strands. It has been shown that deletion of the C-terminal domain abolishes membrane insertion of YadA (Tamm, *et al.*, 1993) while deletion of the linker region results in the degradation of the whole protein (Roggenkamp, *et al.*, 2003). The C-terminal domain along with the linker region form a β-barrel pore consisting of 12 β-strands after trimerization (Hoiczyk, *et al.*, 2000).

5. Type II secretion

The type II secretion pathway is also known as the main terminal branch of the GSP. A wide variety of Gram-negative bacteria employ this pathway for the secretion of extracellular enzymes and toxins. The prototype for type II secretion is the pullulanase secretion system of *Klebsiella oxytoca* (Pugsley, *et al.*, 1986). The proteins to be secreted undergo a co-translational proteolytic cleaving of the N-terminal signal peptide and fold while being translocated across the inner membrane via the Sec pathway. The mature proteins are then released into the periplasm space. In this compartment they may undergo further modifications, such as disulfide bond formation or subunit assembly, before they are translocated across the outer membrane. The type II secretion apparatus has been shown to be highly specific; not only can it distinguish between proteins to be secreted and resident periplasmic proteins but it can also discriminate between its own

secreted proteins and those introduced from other species (Wong, *et al.*, 1993). It was shown that *A. hydrophila* and *A. salmonicida* is capable of releasing alkaline phosphatase – a protein that is periplasmic in *E. coli*, when fused to portions of proaerolysin.

The secreton

There are between 12 to 16 genes involved in the type II secretion process and they form the type II secretion machinery, otherwise known as the secreton (Sandkvist, 2001). These genes and their gene products have been designated by letters A-O and S. However, in *Pseudomonas* spp. the letters P-Z and A have been used.

The secretin

Protein D is an integral outer membrane protein belonging to the secretin superfamily, whose members are known to form highly stable ring-shaped complexes of 12-14 subunits with central channels ranging from 5-10 nm in diameter, large enough to accommodate folded substrates (Bitter, *et al.*, 1998). The secretin family includes proteins that are required for type IV pilus biogenesis, filamentous phage extrusion, and type III secretion (Genin, *et al.*, 1994). These proteins are present in the outer membrane, where they are thought to form gated secretion pores. Bitter, *et al.*, (1998) have shown that secretins exhibit ion-conducting properties and an oligomeric structure that can be visualized by electron microscopy. Proper outer membrane insertion of protein D is assisted by protein S, a small lipoprotein, in *K. oxytoca* (Daefler, *et al.*, 1997) and *E. chrysanthemi* (Shevchik, *et al.*, 1997), but the requirement of protein S in other species has not been confirmed. The PulDS complex from *K. oxytoca* forms channels in

experimental lipid bilayers and there is strong evidence to suggest channel gating (Brok, *et al.*, 1999).

The C-terminal domain is conserved among the secretins and it is thought to be embedded in the outer membrane, whereas the N-terminus is variable and may be exposed to the periplasm where it interacts with other components of the secretion apparatus. The amino-terminal half of secretins may serve as the channel gate, whereas the carboxy-terminus appears to direct oligomerization and contain the channel-forming activity (Brok, *et al.*, 1999). It was hypothesized that the interaction between the N-terminal domain of protein D and other components of the secretion apparatus or the secreted proteins themselves may induce a conformational change in the C-terminal domain that opens the channel. It was suggested that the secreted *Erwinia chrysanthemi* PelB protein binds directly to protein D during secretion (Shevchik, *et al.*, 1997). OutD is stabilized and protected from proteolysis when co-expressed with PelB in *E. coli*, but any internal deletions in the N-terminal domain of OutD could not protect it from degradation.

Role of Protein B

Protein B is another protein that has been identified in some type II secretion systems and it may be another component that interacts with protein D. It has been shown that, in *E. chrysanthemi*, OutB can be cross-linked into a larger complex, but only if OutD is present (Condemine and Shevchik, 2000). It was also shown that OutB and OutD can stabilize each other and that the overproduction of OutD could complement the secretion defect

observed in an *outB* mutant. Protein B has been suggested to regulate secretion by transducing energy for the opening of the secretion pore in *Aeromonas hydrophila* (Howard, *et al.*, 1996).

The pseudopilins

Most secreton components have been found to be associated with the inner membrane (Russel, 1998). GspG, H, I, and J exhibit limited homology to the type 4 pilus structural subunit, pilin. These 'pseudopilins' undergo processing by the GspO inner membrane protein, a prepilin peptidase interchangeable with the type 4 pilus prepilin peptidase (Strom, *et al.*, 1991). The pseudopilins have been proposed to assemble into a pilus-like structure that spans the periplasm. Sauvonnet, *et al.* (2000) overexpressed the type II secretion genes from *K. oxytoca* in *E. coli* and demonstrated that the PulG protein was able to assemble into long pilus-like bundles. The role of the pilus has been thought to be that of a piston pushing the secreted proteins through the secretion pore (Filloux, *et al.*, 1998). This is thought to occur by extension and retraction of the cytoplasmic membrane-anchored pilus. Based on studies done with the type IV pili, it has been suggested that polymerization and retraction of the pilin-like proteins of the type II secretion apparatus could push the secreted proteins through the secretion pore or open the gated secretion pore to allow for outer membrane translocation of the secreted protein (Wolfgang, *et al.*, 2000).

Brok, *et al.* (1999) and Nouwen, *et al.* (1999) have suggested that the secretins have a tendency to aggregate and form higher-ordered multimers. This suprastucture formation

suggests that pilus extrusion and protein secretion do not need to occur through the same pore structure (Sandkvist, 2001). Sandkvist suggests that several D oligomers composed of 12 individual D proteins might assemble into a higher ordered pore structure in which each oligomer supports the extrusion of a single pilus, and that protein secretion occurs through the central channel formed by these oligomers. Alternatively, the suprapore formed by the D oligomers could hold several pili and the individual D oligomers might then support secretion.

Type II pathway substrates

As stated earlier, a wide variety of proteins are secreted via the type II pathway. There is no obvious homology between the primary amino acid sequences of these proteins. Although the three-dimensional structures of several of these proteins show a relatively high beta-sheet content (Allured, *et al.*, 1986; Parker, *et al.*, 1994; Chapon, *et al.*, 2001), some of the proteins are monomeric (elastase) while others are oligomeric (cholera toxin). Moreover, the secreted proteins have varying functions. These proteins include toxins that act within eukaryotic cells and hydrolytic enzymes with very different substrate specificities acting on proteins, lipids, chitin or complex cell wall structures (Sandkvist, 2001).

Secretion signal

A signal within proteins that directs them towards secretion across the outer membrane is thought to be created as the proteins fold in the periplasmic compartment. This signal may be composed of residues from various parts of the linear sequence and it may only

come together when the protein is correctly folded. Alternatively, the sequence may be linear and may only be recognized by the secretion machinery when displayed on the correctly folded protein (Sandkvist, 2001). The periplasmic protein β-lactamase can be secreted across the outer membrane in *P. aeruginosa* when fused to residues 60 – 120 of exotoxin A (Lu and Lory, 1996). It has also been shown that *E. coli* alkaline phosphatase, another periplasmic protein, can be secreted by *Aeromonas salmonicida* under certain conditions (Wong and Buckley, 1993). Although there is sufficient evidence to suggest that successful secretion via the type II pathway requires folding (Palomaki, *et al.*, 1997; Chapon, *et al.*, 2000), no consensus structure or signal that may target the secreted proteins to the secretion apparatus has been uncovered.

Secretion of pullulanase by *K. oxytoca*

Klebsiella oxytoca (originally known as *Klebsiella pneumoniae*) secretes pullulanase, which is a 14.5-kDa enzyme that catalyzes the hydrolysis of (1→6) α-linkages in starch (Pugsley, *et al.*, 1986). The production of pullulanase is induced when *K. oxytoca* is grown in the presence of maltose, indicating the positive regulation of *pulA* (the structural gene for pullulanase) by the MalT protein – activator of the maltose regulon (Chapon and Raibaud, 1985; Michaelis, *et al.*, 1985). The signal sequence of pullulanase is 19-residues in length and it is followed by a cysteine that becomes the N-terminus of the mature protein. This N-terminal signal peptide is processed by lipoprotein signal peptidase during the translocation of the polypeptide across the inner membrane (Pugsley, *et al.*, 1986). It has been determined that secretion of pullulanase across the outer membrane is dependent upon eight secretion genes that flank *pulA* (d'Enfert and Pugsley, 1989;

d'Enfert, *et al.*, 1989; Pugsley and Reyss, 1990). *Escherichia coli* K-12 is able to secrete pullulanase when transformed with recombinant plasmids carrying *pulA* and flanking DNA from the *K. oxytoca* chromosome (d'Enfert, *et al.*, 1987). Genes upstream of *pulA* are part of an operon which is transcribed in a direction opposite to that of *pulA* and which, like *pulA*, is regulated by the MalT protein (d'Enfert, *et al.*, 1989).

Location of secretion signals in *pulA*

The nucleotide sequence of the *K. oxytoca pulA* gene contains a single open reading frame encoding a 117-kDa precursor polypeptide that is processed by lipoprotein signal peptidase to generate a 116-kDa mature protein (Kornacker and Pugsley, 1989). The signal peptide is the only hydrophobic region in the protein. The central domain of pullulanase, containing eight short sequences, is homologous to sequences found in amylases and isoamylases and it has been shown to be involved in catalytic activity. The nucleotide sequence for the *K. oxytoca pulA* gene shows 90% homology to the *K. aerogenes pulA* gene's nucleotide sequence. One of the areas of non-homology lies at the C-terminal region. The possibility that this region might contain the secretion signal for *K. oxytoca* pullulanase was ruled out based on the fact that *E. coli* carrying pullulanase secretion genes from *K. oxytoca* can also secrete the *K. aerogenes* pullulanase. It was proposed that the well-conserved N-terminal region of the pullulanase polypeptide contains secretion signals as well as a collagen-like, short, variable sequence that separates the secretion signal domain from the conserved catalytic domain, while the poorly conserved C-terminal domain is involved in protecting pullulanase against proteolytic attack. Further studies have shown that the C-terminal 256 amino acids of

PulA are not necessary for pullulanase secretion, as evidenced by the efficient secretion of a PulA – β-lactamase hybrid lacking this region (Sauvonnet, *et al.*, 1995).

By studying PulA – β-lactamase hybrids containing various deletions within *pulA*, two regions, A and B, were identified in pullulanase, which together could promote secretion of β-lactamase (Sauvonnet and Pugsley, 1996). Three possibilities have been considered: i) there are two secretion signals, one each in regions A and B, that complement each other when brought together in the folded pullulanase, ii) the secretion signal is contained in only one of the regions, while the other region only aids in the correct presentation of this signal on the surface of the folded protein, and, iii) regions A and B contain parts of the complete secretion signal which are brought together once the protein is correctly folded. None of these have been proven to be correct.

PulD

PulD is the outer membrane protein (secretin) required for pullulanase secretion (Nouwen, *et al.*, 1999). Secretins are known to be composed of two main domains: the N-domain and the β-domain (Genin and Boucher, 1994). The N-domain is predicted to face the periplasm and is conserved only in secretins from related secretion pathways. The β-domain is predicted to contain several amphipathic transmembrane β strands that are probably embedded in the outer membrane, and this domain is known to be relatively conserved among all secretins. There is usually a serine-and-glycine-rich spacer segment that separates the N-domain from the β-domain. This spacer sequence is absent in PulD. Instead there is a short C-terminal domain, the S domain that binds the pilot outer

membrane-anchored lipoprotein PulS. PulS protects PulD from proteolysis and it is essential for the insertion of PulD into the outer membrane (Hardie, *et al.*, 1996a; Hardie, *et al.*, 1996b).

The association of PulD and PulS forms the Pul secretin complex. This complex forms a ring-shaped structure with a large cavity, much like most other secretins that have been studied (Crago and Koronakis, 1998; Koster, *et al.*, 1997). However, the PulD-PulS complex is different from other secretins due to the presence of radial structures that extend from the ring. Nouwen, *et al.*, (1999), suggested that the complex is composed of 12 subunits of PulD surrounded by 12 subunits of PulS. These authors demonstrated that fusion of proteoliposomes containing the purified PulD-PulS complex with a planar lipid bilayer resulted in the appearance of small, voltage-activated, ion-conducting channels.

A recent model of the PulD-PulS complex describes it as being composed of a ring, 20 nm in diameter and 23 nm thick, that supports a smaller cup-like structure that is 14 nm in diameter and 8.5 nm high (Nouwen, *et al.*, 2000). It is the broader of the two rings that is hypothesized to integrate into the outer membrane. According to this model, the majority of the PulD-PulS complex protrudes so far into the periplasm that the ends of the top rim of the cup would almost touch the inner membrane, and is therefore likely to come into contact with other components of the secretory pathway that are located in the inner membrane.

It was also shown in the study discussed above, that the ends of the rim of the cup fold back into the cavity of the channel. This was proposed to be part of the N-terminal domain of PulD forming the 'plug' in the centre of the ring. Such a 'plug' has been seen in studies of the outer membrane ferrisiderophore transporters FhuA and FepA (Ferguson, *et al.*, 1998; Buchanan, *et al.*, 1999), where the function of the 'plug' is thought to involve regulation of channel opening.

PulE

PulE has been shown to be mainly associated with the cytoplasmic membrane in *E. coli* cells expressing all the genes associated with pullulanase secretion (Possot, *et al.*, 1992). This association is through both hydrophobic and non-hydrophobic interactions. PulE is thought to be anchored to the inner membrane so that its nucleotide-binding site is exposed to the cytoplasm (Possot and Pugsley, 1994). PulE has been shown to share sequence homology with a large number of ATP-binding proteins (Pugsley, 1993) known as membrane-associated ABC proteins, or, traffic ATPases. PulE homologues have been shown to be involved in pilus formation, transformation and conjugation, as well as in extracellular protein secretion (Hobbs and Mattick, 1993). While PulE homologues, like all ABC proteins, contain Walker box A and Walker box B (Walker, *et al.*, 1982), the highly conserved region between the two Walker boxes is what distinguishes them from ABC proteins. PilB, a PulE homologue, has been shown to be involved in the assembly of type IV pili in *P. aeruginosa* (Nunn, *et al.*, 1990). This led to the suggestion that PulE is an ATP-binding protein involved in the assembly of the pullulanase secretion machinery (Possot, *et al.*, 1992; Pugsley and Possot, 1993).

Possot and Pugsley (1994) suggested that PulE interacts with another Pul protein, PulF, in the membrane. It was speculated that PulF, a highly hydrophobic protein, may be equivalent to the integral membrane segment found to be associated with typical ABC proteins (Possot, et al., 1992).

3' end of the *pulC* operon

A 5.1 kb fragment at the 3' end of the *pulC* operon contains five genes (*pulK*, *pulL*, *pulM*, *pulN* and *pulO*) that are required for secretion of pullulanase (Pugsley and Reyss, 1990). *pulO* is the last gene in the operon and it was established that transcription, initiated at the *pulC* promoter, extends to the end of the *pulO* gene. PulL, PulM, PulN, and PulO, each contain at least one long region of high hydrophobicity. These regions, according to Pugsley (1989), could act as signal peptides and/or signal sequence/membrane anchors. It was predicted that these proteins are located in the cell envelope as evidenced by the fact that they are all associated with inner membrane vesicles when expressed from the T7 gene promoter (Pugsley and Reyss, 1990).

Prepilin peptidase activity of PulO

It was suggested that PulO is a peptidase that processes and activates proteins that are required for pullulanase secretion and that have a consensus cleavage site for prepilin peptidase (Nunn and Lory, 1991). The products of three genes in the *pulC-O* operon, *pulG*, *pulI* and *pulJ*, are similar in size to the *P. aeruginosa* pilin and appear to have consensus type IV prepilin peptidase cleavage sites in their signal sequences. It has been

shown that PulO processes PulG, PulH, PulI and PulJ precursors (Pugsley and Dupuy, 1992). Although PulO has been shown to exhibit broad specificity (Dupuy, *et al.*, 1992), its activity, like that of other prepilin peptidases, is dependent on the presence of sequences other than the consensus cleavage sequence in the mature part of the polypeptide.

Role of DsbA in pullulanase secretion

Pullulanase secretion is adversely affected by a mutation in the *dsbA* gene (Pugsley, 1992). DsbA is a disulfide oxidoreductase that catalyzes disulfide bond formation and this function is thought to be crucial for the correct folding of secreted proteins (Hu, *et al.*, 1997). In a recent study it was found that the *dsbA* mutation causes the failure of intramolecular disulfide bond formation in PulK and PulS (Pugsley, *et al.*, 2001). While PulK without a disulfide bond is stable, PulS without a disulfide bond is rapidly degraded. Therefore, the observed secretion defect by the *dsbA* mutation may be explained by the lower ability of the protein to function in secretion (PulK) or by the presence of reduced amounts of protein (PulS). However, the highly unstable PulS was seen to function normally when overproduced, leading to the conclusion that sufficient disulfide-bonded PulS might be formed in the absence of DsbA in the overproduced systems to ensure correct localization and assembly of secretin PulD.

In conclusion, pullulanase is secreted by *K. oxytoca* by the type II secretion pathway and the secreton involved is composed of up to 14 proteins, only two of which are located exclusively in the outer membrane.

Type II secretion in *Aeromonas hydrophila*

A type II pathway is employed by *Aeromonas* spp. to secrete a large number of extracellular proteins, including aerolysin (Howard and Buckley, 1985), proteases (Leung and Stevenson, 1988), amylase (Gobius and Pemberton, 1988), and acyltransferase (Thornton, *et al.*, 1988). Aerolysin is secreted as an inactive protoxin, termed proaerolysin. Proaerolysin has a precursor, called preproaerolysin, which contains a typical 23 amino acid, N-terminal signal sequence that directs its passage across the inner membrane *via* the Sec system. Co-translational proteolytic cleavage of the signal peptide and folding of the protein accompany this. In the periplasm the protein dimerizes and folds before it is translocated across the outer membrane via the main terminal branch of the general secretory pathway (Hardie, *et al.*, 1995). It has been shown that a proton motive force is required for the passage of the toxin across the outer membrane (Wong and Buckley, 1989).

Transposon mutagenesis was used to isolate two mutants of *A. hydrophila* that are unable to secrete aerolysin (Jiang and Howard, 1991). Since the protein is synthesized, but cannot be detected in the culture medium, it was concluded that the genes inactivated by the transposon are required for successful translocation across the outer membrane. One of these mutants is deficient in the proper assembly of outer membrane proteins, while the other contains the same outer membrane proteins as wild type. Genes inactivated by the transposon insertions, when cloned from the wild type, are able to complement the mutations.

***exeC-N* operon**

Analysis of the mutant that is unable to secrete aerolysin and that also had reduced amounts of two of the major outer membrane proteins revealed a 4.1 kb fragment that is capable of complementing the mutation (Jiang and Howard, 1992). This fragment contains two complete genes, *exeE* and *exeF*, along with fragments of two other genes, forming part of an operon. These four genes show substantial similarity with the *K. oxytoca* genes *pulD*, *pulE*, *pulF*, and *pulG*, which, as mentioned earlier, are essential for the successful translocation of pullulanase across the outer membrane.

ExeE was predicted to be a membrane-associated protein, anchored in the cytoplasmic membrane by a single hydrophobic segment (Jiang and Howard, 1992). It contains a nucleotide-binding site and it possesses largely hydrophilic character, lacking a signal sequence. This led to the conclusion that this protein is localized on the cytoplasmic face of the inner membrane. Since ExeE bears sequence homology to PilB and PilT, proteins required for type IV pilin assembly and twitching motility in *P. aeruginosa* (Nunn, *et al.*, 1990; Whitchurch, *et al.*, 1990), and VirB11, a protein involved in DNA transfer in *A. tumifaciens* (Ward, et al., 1988), it was hypothesized that the *exeE* product transduces the energy derived from ATP hydrolysis to other Exe proteins in the membranes that are responsible for translocation of proteins across the outer membrane.

Studies involving the 3' end of the *exe* operon have identified eight more genes, *exeG* to *exeN*, followed by a terminator (Howard, *et al.*, 1993). *exeG*, along with *exeH*, *exeI*, *exeJ*, and *exeK*, were predicted to encode proteins containing a prepilin signal peptide. As

mentioned earlier, the *pul* operon of *K. oxytoca* contains a gene, *pulO*, which encodes a prepilin peptidase. In contrast, the *A. hydrophila exe* operon does not contain a homologue of the prepilin peptidase gene. This has led to the conclusion that in the latter organism the prepilin peptidase is encoded by a gene that is not linked to the secretion operon (Howard, *et al.*, 1993).

Mutations generated by the insertion of a kanamycin resistance gene in the *exeE* gene, the *exeK* gene and a region upstream of the 5' end of a fragment containing *exeE* and *exeF*, have all resulted in deficiencies in two outer membrane proteins as well as in a lack of secretion of aerolysin into the culture medium. The two outer membrane proteins affected by these mutations are similar to the porins LamB and OmpF of *E. coli* (Jeanteur, *et al.*, 1992). It was, therefore, concluded that these mutations prevent the proper assembly of the porin-like proteins.

The secretin ExeD

ExeD has been identified as the secretin for the type II secretion apparatus in *A. hydrophila*, based on the fact that it has been shown to be homologous to the *P. aeruginosa* proteins, XcpQ (Akrim, *et al.*, 1993) and PilQ (Martin, *et al.*, 1993), both of which form large oligomeric rings in the outer membrane. The presence of these secretins, like the secretin PulD for *K. oxytoca*, as mentioned earlier, is common for all type II secretion systems. While the sizes of trimeric porins are large enough to allow the passage of small (~600 Da) hydrophilic compounds to enter or exit the periplasm, the pores generated by type II secretins must accommodate much larger molecules.

***exeAB* operon**

As stated earlier, transposon mutagenesis has revealed two secretion deficient variants of *A. hydrophila* (Jiang and Howard, 1991). These variants differ from one another in that one is not only incapable of secretion but also lacks two outer membrane proteins. The other variant, however, contains all the necessary outer membrane proteins but is, nonetheless, incapable of secreting aerolysin across the outer membrane. This led to the conclusion that the latter mutation affects an operon other than the *exeC-N* operon (Jahagirdar and Howard, 1994). Jahagirdar and Howard showed that this operon contains two genes, one of which, *exeB*, encodes a protein that shares some homology with the *K. oxytoca* PulB (d'Enfert and Pugsley, 1989) and the *E. chrysanthemi* OutB (Condemine, *et al.*, 1992), while the other, *exeA*, encodes a protein containing an ATP-binding site. ExeB also shares sequence and structural similarity with TonB, a protein that performs an energy-dependent gate keeping function for the inward translocation of ligands across the outer membrane (Howard, *et al.*, 1996). Both ExeA and ExeB have been shown to be largely hydrophilic and both lack signal sequences, but both also contain hydrophobic stretches that could serve to anchor them to the inner membrane (Jahagirdar and Howard, 1994).

The possibility that the *exeAB* operon functions in the regulation of the *exeC-N* operon was ruled out based on the fact that, while an insertion mutation in the *exeC-N* operon results in an incorrectly assembled outer membrane, this is not the case with mutations in the *exeAB* operon. It was hypothesized, rather, that in the *exeAB* mutants, the ExeC-N proteins are synthesized but cannot be used by aerolysin for translocation across the outer

membrane. It has been suggested that ExeA and ExeB function on the cytoplasmic side of the inner membrane and that the translocation process of aerolysin begins with the recognition of these two proteins, which then behave as chaperones, causing the toxin to be taken up by the ExeC-N secreton and resulting in its eventual secretion to the extracellular medium (Jahagirdar and Howard, 1994).

A proposed model for type II secretion by *A. hydrophila* involves the ExeAB complex transducing ATP hydrolysis energy and proton motive force energy to the opening of the secretin ExeD in the outer membrane (Schoenhofen, *et al.*, 1998). In fact, it has been shown that ATP is required for the formation of the ExeA-ExeB complex. The complex is not formed when mutations are introduced in the ATP binding site of ExeA. It was proposed that ExeA forms homodimers, based on the fact that similar traffic ATPases containing only one hydrophobic domain invariably dimerize, probably to allow four distinct functional domains (Hyde, *et al.*, 1990) to assemble in the tertiary structure of the protein. This led to the conclusion that the ExeA-ExeB complex must function as a heterotetramer.

It has been shown that overexpression of ExeD can suppress secretion defects seen in *exeAB* mutants (Ast, *et al.*, 2002). In *exeAB* mutants, ExeD is unable to multimerize and it accumulates in the inner membrane. This led to the conclusion that the ExeAB complex is required for the multimerization of ExeD and for its assembly in the outer membrane. Moreover, Ast, *et al.*, showed that when *exeAB* was introduced *in trans* to these mutants, ExeD was converted to the multimeric form and secretion of aerolysin was restored. The

authors found strong evidence for the role of the ExeAB complex in the transport of the secretin to the outer membrane rather than assembly. This is based on the observation that in wild-type cells overexpressing ExeD, larger amounts of the multimer are able to reach the outer membrane than are observed in the *exeAB* mutants overexpressing the secretin.

Role of the C-terminus of proaerolysin in secretion across the outer membrane

It has been demonstrated that aerolysin is released into the extracellular medium as a protoxin that is activated by proteolytic removal of the C-terminal peptide (Howard and Buckley, 1985; van der Goot, *et al.*, 1992). The crystal structure of proaerolysin (Figure 2) shows that the peptide adopts a β-strand-helix-β-strand conformation with the strands hydrogen-bonding onto β-strands located in domain 4 of the toxin (Parker, *et al.*, 1994). Trypsin activates proaerolysin by cutting at Lys-427, chymotrypsin at Arg-429 (van der Goot, *et al.*, 1992) and proteinase K at Val-428 (Burr, 2001). This nicking and the consequent loss of the propeptide leads to conformational rearrangements in Domain 4 due to the exposure of a buried hydrophobic patch and the loss of strands contributing to the existing β-sheet structure (Cabiaux, *et al.*, 1997).

Regions within the C-terminus of a number of proteins have been found to play a role in their folding and translocation across the bacterial outer membrane. It must be reiterated here that a targeting signal could consist of one or more stretches of residues correctly presented on the three-dimensional structure of the protein or it could be formed by a

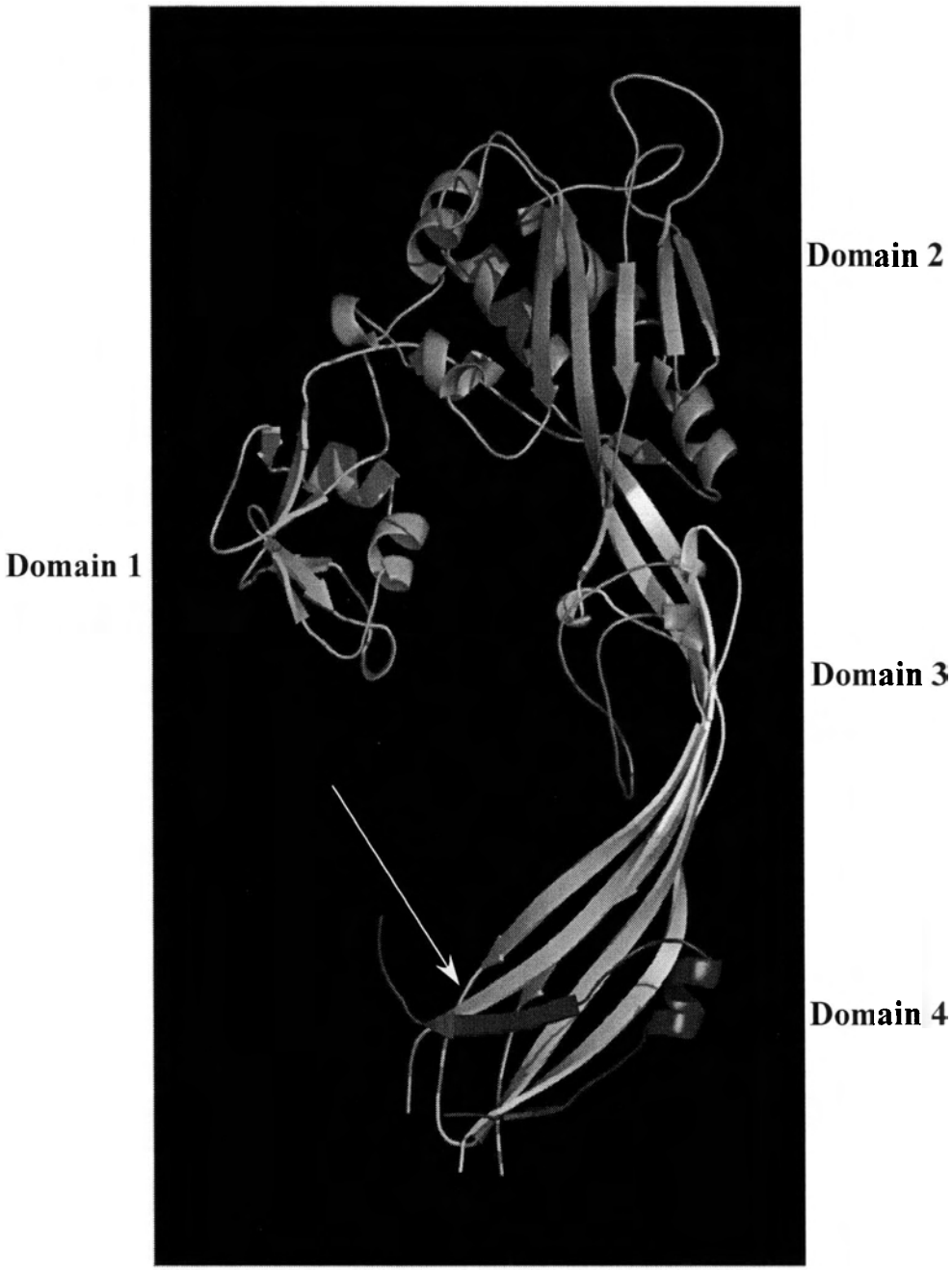


Figure 2. Structure of aerolysin showing the C-terminal peptide.

group of residues brought together by protein folding. A recent study has shown the importance of the extreme C-terminal region of polygalacturonase (PehA) secreted by *Erwinia carotovora* (Palomaki, *et al.*, 2002). PehA has been shown to direct secretion of β-lactamase when fused to it, but when deletions of more than two amino acids from the C-terminus are made the hybrid protein cannot be secreted and it accumulates in the periplasm (Palomaki and Saarilahti, 1995). Furthermore, even single deletions of any of the last seven residues hinder secretion (Palomaki and Saarilahti, 1997). It was concluded that the residues within the C-terminus of this protein play a role in secretion by stabilizing a structure needed for proper exposure of the proposed targeting motif. The C-terminal cellulose-binding domain (CBD) of the cellulase Cel5 (formerly EGZ) secreted by *Erwinia chrysanthemi* has been shown to play a different role in the secretion process. A disulfide bond is present in Cel5, between two cysteine residues within the CBD, which is essential for the successful translocation of the protein across the outer membrane (Bortoli-German, *et al.*, 1994). Mutations within CBD that affect the formation of the disulfide bond result in the formation of molecules that are catalytically active and can bind cellulose but that are not secreted outside the cell.

Previous studies in the Buckley laboratory have attempted to define the importance of the C-terminus in the secretion of proaerolysin (Burr, 2001). It was seen that deletion of the last 20-30 amino acid residues from the C-terminal end resulted in complete loss of secretion (Burr, 2001). Moreover, these variants were incorrectly folded as evidenced by their degradation upon treatment with trypsin. It was concluded that these 20-30 amino

acids in the proaerolysin sequence play an important role in the folding and secretion of proaerolysin.

Aim of this thesis

It has also been shown recently that the C-terminal region of proaerolysin from *A. sobria* plays a role in the secretion and proteolytic stability of the toxin (Nomura, *et al.*, 1999; Nomura, *et al.*, 2000). These authors deleted 1, 2, 3, or 10 amino acid residues from the C-terminal end of the toxin. They found that deletion of more than 3 residues inhibited the appearance of proaerolysin in the culture supernatant while deletion of 10 amino acids blocked secretion completely. Furthermore, the variant with 10 amino acids deleted was degraded in the cells. They concluded that the C-terminus is involved in the formation of a structure that protects proaerolysin from proteolysis in the cell and that variants that are able to form this structure (deletion of 1, 2, or 3 amino acids) can cross the outer membrane. The variant that lacked 10 amino acids was unable to form this stable structure and so was degraded by proteases in the periplasm. However, since the variant that had 3 amino acids deleted was not as efficient as wild type in being translocated across the outer membrane, it was concluded that this variant must form a less stable protoxin compared to wild type. The aim of this thesis was to further illustrate the role of the C-terminus in folding and secretion, extending the observations of Burr (2000) and Nomura, *et al.*, (1999; 2000). This was accomplished by generating deletion variants and creating amino acid substitutions within the C-terminal peptide (residues 427 – 470).

MATERIALS AND METHODS

Media and reagents

Luria Bertanii (LB) medium, pH 7.5, was made according to Sambrook, *et al.*, (1989). LB-Davis medium was made by mixing 9 parts LB medium with 1 part of 10x modified Davis buffer (Ashton, *et al.*, 1980). Glucose was added to culture medium to a final concentration of 0.2% (w/v).

Culture conditions

E. coli strain DH5α, which was used for cloning and propagation, and MM297, which was used as a helper strain for transconjugation, were grown at 37°C in LB media. Kanamycin was added to MM297 cultures only, to a final concentration of 70 μg/ml. The protease deficient strain of *Aeromonas salmonicida*, CB3, was used in all experiments requiring *Aeromonas* spp. unless otherwise mentioned. This mutant strain was generated by Tn5 mutagenesis and it releases little or no protease (Buckley, 1990). All strains are described in Table 1. CB3 was grown at 27°C in LB supplemented with Davis salts (Miller, 1972) and 0.2% glucose (w/v). Rifampicin and kanamycin were added to CB3 cultures to a final concentration of 40 μg/ml each. Ampicillin was added to *E. coli* and to CB3 cultures harboring the broad-host-range expression vector pMMB66HE (Furste, *et al.*, 1986) to a final concentration of 100 μg/ml.

Table 1. Bacterial strains used

Strain	Genotype or description	Source
Aeromonas salmonicida CB3	Rif-1::*Tn5*; deficient in secreted protease; Km^r and Rif^r	This laboratory
Escherichia coli DH5α	*SupE*44 Δ*lac*U169 *rec*A1 *end*A1 *gyrA*96 *thi*-1 *relA*1; Amp^r; used for cloning and propagation	This laboratory
Escherichia coli MM297	Contains plasmid pRK2013. Replicon: ColE1. Vector for *E. coli*. Carries *Tn903*. Contains RK2 transfer genes. Helper plasmid for mobilization of non-self-transmissible plasmids.	This laboratory

Growth conditions

Cultures in liquid media were grown in Erlenmeyer flasks at 250 rpm in a New Brunswick Scientific Controlled Environmental Incubator Shaker (Model G-25) for *A. salmonicida* and 350 rpm in a New Brunswick Scientific Gyrotary Water Bath Shaker (Model G-76) for *E. coli*. Overnight cultures were subcultured 1/100 into fresh media and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1mM to induce proaerolysin expression when cells had entered log phase. Growth was monitored by measuring the optical density at 600 nm (A_{600}) using a CARY 1 Bio UV-Vis spectrophotometer. Generally, cells were taken to have entered log phase when the A_{600} had reached a value of ~0.5.

Expression of proaerolysin

At various time points (0, 2, 4, and 16 h) after induction with IPTG, aliquots of the cell culture were taken and the cells harvested using a ThermoIEC microcentrifuge at 13000 rpm for 2 minutes. The supernatant was saved and the pelleted cells were resuspended in Hepes buffered saline (20 mM Hepes, 0.15 mM NaCl, pH 7.4; HBS). Proaerolysin in the cells and culture supernatants was then determined both by immunoblotting and by hemolytic titre assay.

Electrophoresis and western blotting

Proteins were separated on Invitrogen Life Technologies' NuPAGE Novex 10% Bis-Tris high-performance pre-cast polyacrylamide gels. After separation, proteins were stained with Coomassie brilliant blue or electroblotted onto nitrocellulose membranes. The

membranes were blocked for 1 hour at room temperature or overnight at 4°C in phosphate buffered saline (PBS) containing 0.5% (w/w) Tween 20 (ACP Chemicals) and 0.5% (w/v) skim milk (Difco). Proaerolysin was detected using an anti-aerolysin mouse monoclonal or rabbit polyclonal antibody (Burr, 2001). Membranes were incubated for 1 hour at room temperature with 1:4000 dilutions of the antibody in PBS containing 0.5% Tween 20. This was followed by incubation with the appropriate secondary antibody conjugated to horseradish peroxidase (Amersham Biosciences; Caltag Laboratories). The blots were then developed using enhanced chemiluminescense (Amersham Biosciences) according to the manufacturer's instructions.

Constructing proaerolysin variants

Deletion variants were generated by recombinant PCR (Vallette *et al.*, 1989) using primers designed to make desired changes (Table 2). The PCR products were then digested with the appropriate restriction enzymes (New England Biolabs) and the digested products separated by agarose gel electrophoresis using the method described by Sambrook, *et al.*, (1989). A QIAEX II gel extraction kit (Qiagen) was used, according to the manufacturer's instructions, to purify the digested products, which were then ligated into the cloning vector pTZ18U using T4 DNA ligase (New England Biolabs) for amplification. The resulting clones were sequenced to ensure that the correct mutations had been generated. Sequencing was carried out by the DNA Sequencing Facility, Centre for Biomedical Research, University of Victoria.

Recombinant clones were selected by screening via PCR and plasmids containing the correct mutations were transferred into *A. salmonicida* by conjugation using a filter mating technique described by Haryama, *et al.*, (1980; see below). Alanine and his-tagged variants were generated using QuikChange II Site-Directed Mutagenesis Kits from Stratagene (according to the manufacturer's instructions) using the appropriate primers (Table 3; Table 4).

Restriction digestion

Restriction digestion mixtures contained approximately one unit of restriction enzyme for every μg of DNA and the digestions were carried out in the appropriate NEB buffer supplemented with bovine serum albumin (BSA) where necessary. All digestions were carried out at 37°C for 2 hours.

Ligation

Insert and vector DNA were mixed together in a ratio of approximately 10:1. This was followed by the addition of T4 DNA ligase buffer and T4 DNA ligase (NEB) and the samples were mixed gently. Ligations were carried out at 13°C for 16 hours.

Transformation

E. coli DH5α cells were made competent by the method outlined by Cohen *et al.* (1972). Briefly, cells at log phase were harvested by centrifugation and then resuspended in 1/4 volume cold 100 mM $MgCl_2$. The cells were then repelleted and resuspended in 2 volumes of cold 100 mM $CaCl_2$. The resuspended cells were incubated on ice for

Table 2. Nucleotide sequences of primers used for construction of deletion variants.

Primer	Nucleotide Sequence
Δ12 fwd.[1]	TCGCGGCTGACAGCCAAGGCCTGAGGCT
Δ12 rev.[2]	AGCCTCTGGCCTTGGCTGTCAGCCGCGA
Δ20 fwd.	GCTCGCGGCCGACAGCCTCGATGCGCAAGAGC
Δ20 rev.	GCTCTTGCGCATCGAGGCTGTCGGCCGCGAGC

[1] fwd. indicates forward primers
[2] rev. indicates reverse primers

Table 3. Nucleotide sequences of primers used for alanine scanning mutagenesis.

Primer	Nucleotide Sequence
P466A fwd.[1]	AGCGTCAGCGTGACCGCTGCTGCCAATC
P466A rev.[2]	GATTGGCAGCAGCGGTCACGCTGAGGC
T465A fwd.	AACGTCAGCCTCAGCGTGGCCCCTGCTAATC
T465A rev.	GATTGGCAGCAGGGGCCACGCTGAGGCTGACGTT
V464A fwd.	GTCAGCCTCAGCGCGACCCCTGCTGCCAATC
V464A rev.	GATTGGCAGCAGGGGTCGCGCTGAGGCTGAC
S463A fwd.	CAACGTCAGCCTCGCGGTGACCCCTGC
S463A rev.	GCAGGGGTCACGGCCAGGCTGACGTTG
L462A fwd.	CTTCAACAACGTCAGCGCCAGCGTGACCCCTGC
L462A rev.	GCAGGGGTCACGCTGGCGCTGACGTTGTTGAAG
S461A fwd.	GGCTTCAACAACGTCGCCCTCAGCGTGACCCCTG
S461A rev.	CAGGGGTCACGCTGAGGGCGACGTTGTTGAAGCC
V460A fwd.	GGCTTCAACAACGCCAGCCTCAGCGTGACC
V460A rev.	GGTCACGCTGAGGCTGGCGTTGTTGAAGC
N459A fwd.	CTTGGCTTCAACGCCGTCAGCCTCAGCG
N459A rev.	CGCTGACGCTGACGGCGTTGAAGCCAAG
N458A fwd.	GCTTGGCTTCGCCAACGTCAGCCTCAGCGTG
N458A rev.	CACGCTGAGGCTGACGTTGGCGAAGCCAAGC
F457A fwd.	CTCTCCGGGCTTGGCGCGAACAACGTCAGC
F457A rev.	GCTGACGTTGTTCGCGCCAAGCCCGGAGAG
G456A fwd.	GCTCTCCGGGCTTGCCTTCAACAACGTCAGC
G456A rev.	CGTGACGTTGTTGAAGGCAAGCCCGGAGAGC
L455A fwd.	GAGCTCTCCGGGGCCGGCTTCAACAACGTC
L455A rev.	GACGTTGTTGAAGCCGGCCCCGGAGAGCTC
G454A fwd.	GCAAGAGCTCTCCGCGCTTGGCTTCAACGTC
G454A rev.	GACGTTGAAGCCAAGCGCGGAGAGCTCTTGC
S453A fwd.	GCAAGAGCTCGCCGGGCTTGGCTTCAAC
S453A rev.	GTTGAAGCCAAGCCCGGCGAGCTCTTGC
L452A fwd.	GCGCAAGAGGCCTCCGGGCTTGGCTTCAAC
L452A rev.	GTTGAAGCCAAGCCCGGAGGCCTCTTGCGC
E451A fwd.	CTCGATGCGCAAGCGCTCTCCGGGCTTGGC
E451A rev.	GCCAAGCCCGGAGAGCGCTTGCGCATCGAG
Q450A fwd.	CCGCTCGATGCGGCAGAGCTCTCCGGG
Q450A rev.	CCCGGAGAGCTCTGCCGCATCGAGCGG

[1]fwd. indicates forward primers
[2]rev. indicates reverse primers

Table 4. Nucleotide sequences of primers used for constructing his-tagged variants.

Primer	Nucleotide Sequence
HisCT fwd.[1]	CATCATCATCATCATTAACGGCAGCGCG
HisCT rev.[2]	ATGATGATGATGATGCACGCTGAGG
EndHis 1 fwd.[a]	GCTGCCAATCAACATCATCATTAACGGCAGCGC
EndHis 1 rev.[a]	GCGCTGCCGTTAAATGATGATGTTGATTGGCAGC
EndHis 2 fwd.[a]	GCCAATCAACATCATCATCATCATTAACGGCAGCGC
EndHis 2 rev.[a]	GCGCTGCCGTTAATGATGATGATGATGATGTTGTTAGGC
D435His 1 fwd.[b]	CGTGCTCGCAGTGTGCATCATCATGGTCAAGGCCTGAGG
D435His 1 rev.[b]	CCTCAGGCCTTGACCATGATGATGCACACTGCGAGCACG
D435His 2 fwd.[b]	GTGCATCATCATCATCATCATCTGAGGCTGGAGATC
D435His 2 rev.[b]	GATCTCCAGCCTCAGATGATGATGATGATGATGCAC

[1]fwd. indicates forward primers
[2]rev. indicates reverse primers

a. EndHis was constructed by two rounds of QuikChange, the first round using the EndHis 1 primers and the second round using the EndHis 2 primers.

b. D435His was constructed by two rounds of QuikChange, the first round using the D435His 1 primers and the second round using the D435His 2 primers.

approximately 45 minutes and then pelleted. The cells were resuspended in 1/10 volume cold 100 mM $CaCl_2$ and incubated on ice for approximately 45 minutes before glycerol was added to a final concentration of 15% (v/v). The competent cells were stored at -70°C until use.

Recombinant plasmids were transformed into competent DH5α cells according to the method described by Inoue *et al.* (1990). Competent cells (200 µl aliquots) were incubated with 0.5-1.0 ng of DNA for one hour on ice. The cells were then subjected to heat shock at 42°C for 4 minutes and cooled on ice immediately afterwards for 2 minutes. Following this, 500 µl of LB media (pre-warmed to 37°C) were added to each sample and the cells were incubated for 1 hour at 37°C with mild agitation. 150 µl aliquots were then plated onto LB agar plates containing 50 µg/ml ampicillin and incubated overnight at 37°C.

Polymerase Chain Reaction

In order to generate the deletion variants, Deep Vent DNA Polymerase (New England Biolabs) was used for recombinant PCR. The reactions were carried out in a final volume of 50 µl and contained 0.2 mM deoxynucleoside phosphate (Amersham Biosciences), 0.5 µM forward and reverse primers, 0.1 µg template DNA and Deep Vent in ThermoPol buffer (New England Biolabs).

Taq polymerase was used to screen transformed or transconjugated cells for the proaerolysin insert. A reaction cocktail was prepared in PCR reaction buffer containing

0.2 mM dNTPs, 0.5 μM forward and reverse primers, and *Taq* polymerase. 10-μl samples of the cocktail were aliquoted into 0.2 ml tubes and transformed or transconjugated cells were added using sterile toothpicks.

Transconjugation by the filter mating technique

A filter-mating technique (Harayama, *et al.*, 1980) was used to transfer pMMB66HE plasmids containing the *aerA* mutations of interest into *A. salmonicida* CB3. After the bacterial cultures had reached an OD_{600} of approximately 0.5, the cells were spotted onto 0.45 μm filters in a ratio of 2:1:1, recipient (CB3):helper (MM297):donor (DH5α). The filters were then placed on TSA plates containing 5% horse blood and the plates were incubated at 27°C for approximately 4.5 hours. The filters were then transferred into 5 ml LB media. Ten-fold dilutions were made and plated on TSA-horse blood plates containing rifampicin, kanamycin and ampicillin (40, 40, and 100 μg/ml, respectively). After incubation at 27°C for approximately 48 hours, recombinant clones were selected and screened via PCR using pMMB66HE-fwd. and pMMB66HE-rev. primers (Table 4) for the *aerA* insert.

Hemolytic titre

Proaerolysin titres were determined according to our published procedure (Howard and Buckley, 1985a). The toxin was activated by incubation with 2 μg/ml trypsin for 10 minutes at room temperature. The samples were then diluted two-fold in a 96-well plate using HBS. Washed horse erythrocytes were added to a final concentration of 0.4%. The plate containing the activated sample and erythrocytes was incubated at 37°C and the

degree of cell lysis compared to lysis by known concentrations of proaerolysin over the course of an hour.

Osmotic shock in the presence or absence of trypsin

Osmotic shock was carried out according to the procedure of Willis, *et al.*, (1974). After harvesting, cells were resuspended in sucrose shock solution [20% sucrose (w/v), 33 mM Tris, 1 mM EDTA, pH 7.5]. After five minutes incubation at room temperature (RT), the cells were pelleted, and subjected to osmotic shock by rapidly resuspending them in cold distilled water. The samples were then incubated on ice for five minutes. Finally, the cells were centrifuged to separate the shock fluid (shockate) from the shocked cells. Shocked cells were then resuspended in HBS. In some cases, trypsin (40 μg/ml) was added to the water used for shocking (Burr, *et al.*, 2001) and proteolysis was stopped after 15 minutes by the addition of appropriate amount of trypsin inhibitor (10 X concentration of trypsin).

Quantifying secreted alanine variants

A. salmonicida CB3 cells harbouring the *aerA* gene containing individual alanine mutations were induced with IPTG once they had reached log phase. After a 4-hour induction period, the cells were removed, as described earlier. The proteins in the culture supernatants were separated and electroblotted using the method previously described. The membranes were blocked for 1 hour at room temperature in PBS and Odyssey Blocking Buffer (LI-COR Biosciences). Proaerolysin was detected using IRDye-800-labelled anti-aerolysin monoclonal antibody and the amount of each variant in the culture supernatants was quantified using the Odyssey Infrared Imaging system by extrapolation

on a standard curve that was generated using known concentrations of purified wt proaerolysin. The concentrations used for this standard curve were 24 pg, 12 pg, 6 pg and 3 pg of purified proaerolysin.

RESULTS

INTERNAL DELETIONS IN THE C-TERMINAL END OF PROAEROLYSIN

In an effort to examine the role of the carboxy terminus of proaerolysin in the protoxin's folding and secretion, an internal deletion was previously made in this region. Residues 427 – 432 in the C-terminal end were deleted to generate the variant Δ6 (Burr, 2001). This part of the C-terminus of the protoxin is referred to as the activation sequence since it encompasses sites at which trypsin, chymotrypsin and furin can activate the toxin by cutting (Howard and Buckley, 1985a; van der Goot, *et. al.*, 1992; Abrami, *et al.*, 1998). Burr (2001) showed expression of Δ6 in CB3 resulted in secretion of the variant at wt levels. In order to examine the effect of larger internal deletions within this region on secretion and activity, I used recombinant PCR (Materials and Methods) to generate Δ12 (residues 427 – 438 deleted) and Δ20 (residues 427 – 446 deleted) (Figure 3).

Δ12 does not appear in the culture supernatant

Once sequencing confirmed the deletion of 12 amino acid residues (427 – 438), the *aerA* gene containing the mutation was cloned into the broad-host-range expression vector pMMB66HE. The expression of the gene in *A. salmonicida* CB3 resulted in a protein in the cells that migrated to a position below that of wild type AerA (52 kDa) on an SDS-PAGE gel corresponding to the predicted smaller mass (Figure 4). The results in Figure 4 also show that the protein is not found in the culture supernatant. Somewhat more Δ12 protein than wild type was found in the cells suggesting that the protein is unable to cross the outer membrane like the wild type.

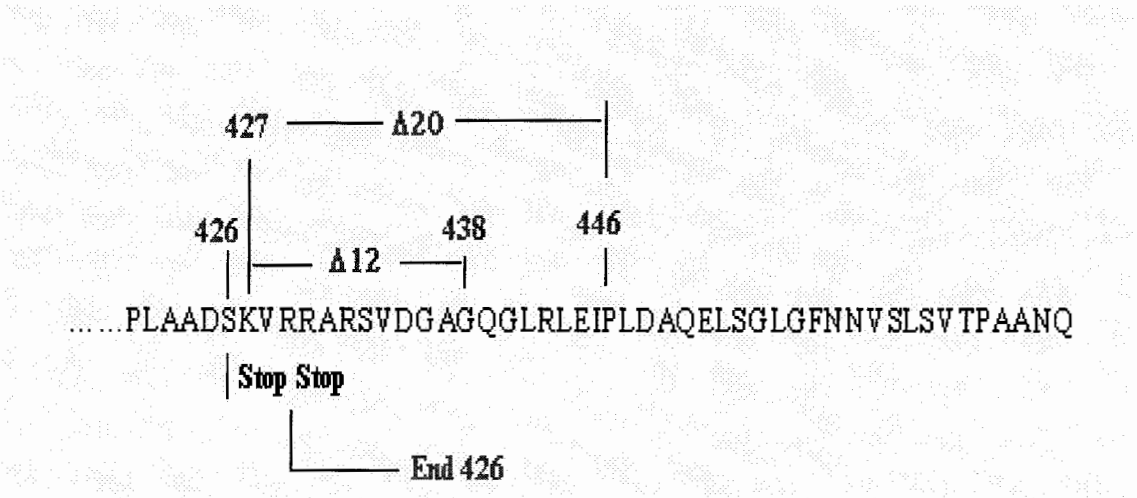


Figure 3. Sequence of the C-terminus of proaerolysin showing the internal deletions and the end variants generated for this study.

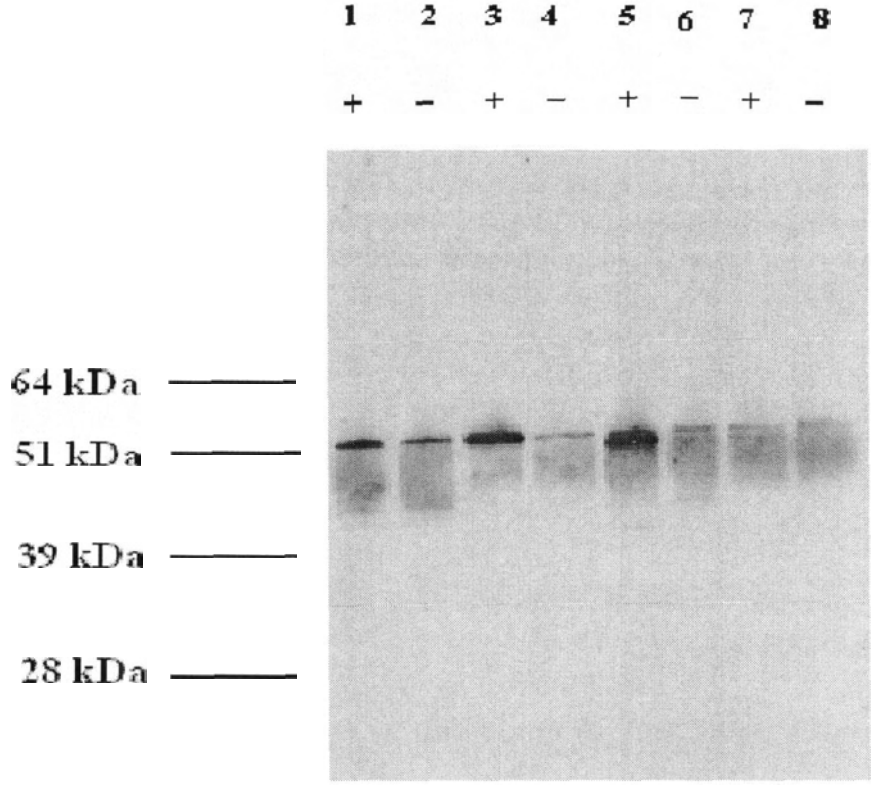


Figure 4. Production of Δ12 by CB3. CB3:Δ12 and CB3:pNB5 cells were induced for 4 hours with 1 mM IPTG. Lanes 1 and 2 correspond to induced (+) and uninduced (-) pNB5 cells while lanes 5 and 6 correspond to induced (+) and uninduced (-) Δ12 cells. Lanes 3 and 4 correspond to induced (+) and uninduced (-) pNB5 culture supernatants while lanes 7 and 8 correspond to induced (+) and uninduced (-) Δ12 culture supernatants. Samples were separated by SDS-PAGE and immunoblotted according to the text.

Δ12 is not correctly folded

Having shown that CB3 cells are incapable of secreting the Δ12 variant, the next step was to see if this was due to a defect in folding, based on a previous study that pointed towards a correlation between folding and secretion of proaerolysin (Burr, 2001). Cells containing this mutation were induced and then osmotically shocked in the presence or absence of 40 μg/ml trypsin. Correctly folded proaerolysin is converted to aerolysin by trypsin, and aerolysin resists further breakdown by the protease (Garland and Buckley, 1988). Improperly folded protein, on the other hand, cannot be correctly processed by trypsin and is degraded to smaller peptides (Burr, 2001). When the cells containing the Δ12 variant were shocked in the absence of trypsin (Figure 5) part of the protein was present in the shock fluid while part remained associated with the shocked cells. When the cells were shocked in the presence of trypsin, faint bands smaller than proaerolysin were observed in the lanes containing the shock fluid and the shocked cells. This may be an indication that Δ12 is not folded properly. It should be noted here that a major degradation product in the shocked cells migrated to a position (~ 45 kDa) that was different than the position for the major degradation product (below 28 kDa) in the shock fluid.

Δ20 does not appear in the culture supernatant

Similar to what was seen for Δ12, CB3 cells expressing the *aerA* gene containing the mutation Δ20 were unable to secrete the variant (Figure 6). Once again, the cells were seen to express a protein that is smaller than pNB5, indicating the successful deletion of

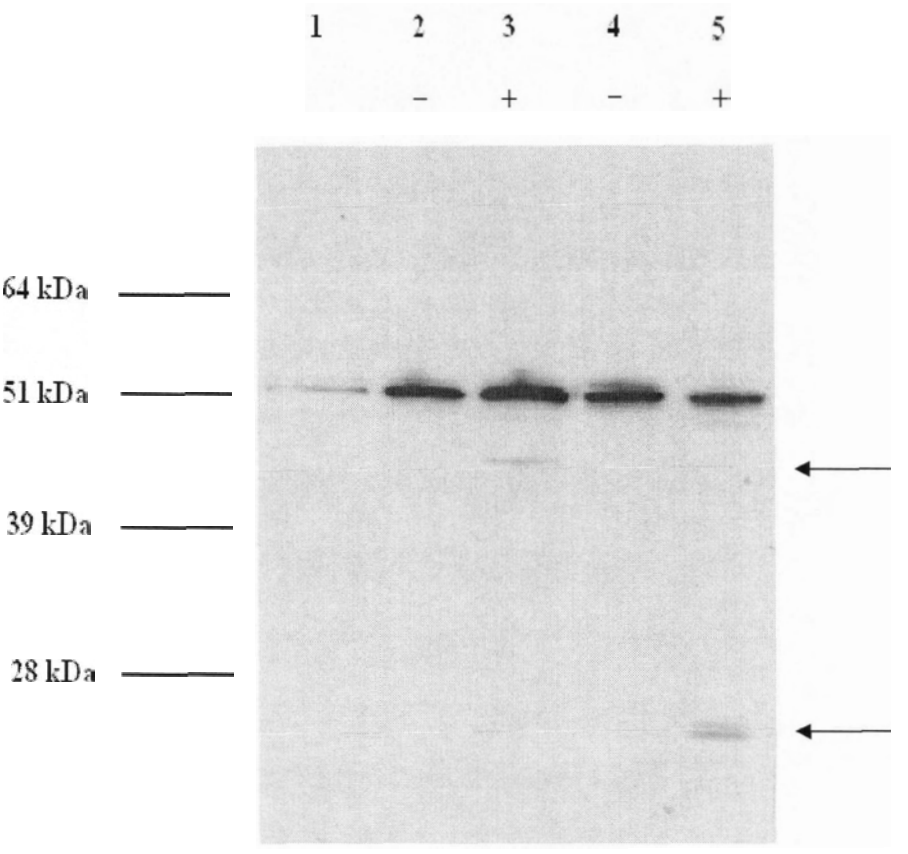


Figure 5. Treatment of Δ12 with trypsin. CB3:Δ12 cells were induced for 4 hours with 1 mM IPTG and then osmotically shocked in the presence (+) or absence (-) of 40 μg/ml trypsin. Lane1, culture supernatant; lanes 2 and 3, shocked cells; lanes 4 and 5, shock fluids. Samples were separated by SDS-PAGE and immunoblotted according to the text. (Arrows indicate degradation products).

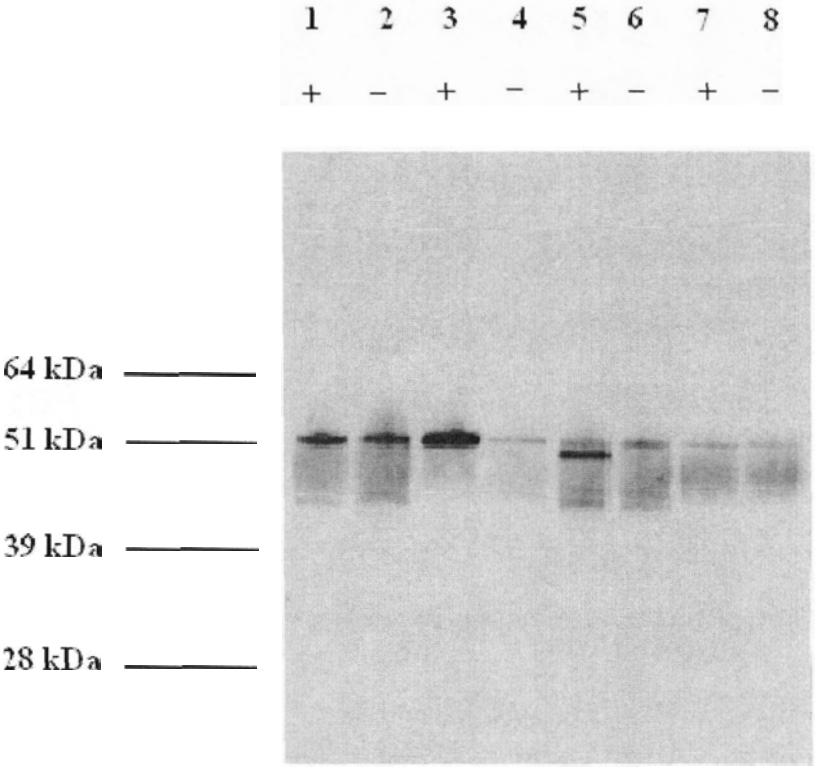


Figure 6. Production of Δ20 by CB3. CB3:Δ20 and CB3:pNB5 cells were induced for 4 hours with 1 mM IPTG. Lanes 1 and 2 correspond to induced (+) and uninduced (-) pNB5 cells while lanes 5 and 6 correspond to induced (+) and uninduced (-) Δ20 cells. Lanes 3 and 4 correspond to induced (+) and uninduced (-) pNB5 culture supernatants while lanes 7 and 8 correspond to induced (+) and uninduced (-) Δ20 culture supernatants. Samples were separated by SDS-PAGE and immunoblotted according to the text.

20 amino acid residues (427 – 446). Once again, none of this variant could be detected in the culture supernatants, unlike wt.

Δ20 is not correctly folded

In order to determine whether the lack of secretion of Δ20 was the result of incorrect folding, CB3 cells harboring the Δ20 mutation were osmotically shocked in the presence or absence of trypsin after induction with 1 mM IPTG for 4 hours. While part of the expressed protein was found in the shock fluid, some of it remained associated with the shocked cells (Figure 7). When cells were shocked in the presence of trypsin a significantly smaller amount of the protein was seen to be present in both shocked cells and shock fluid and the protein was seen to be degraded into smaller peptides. This is a clear indication that deletion of residues 427 – 446 results in an incorrectly folded protein.

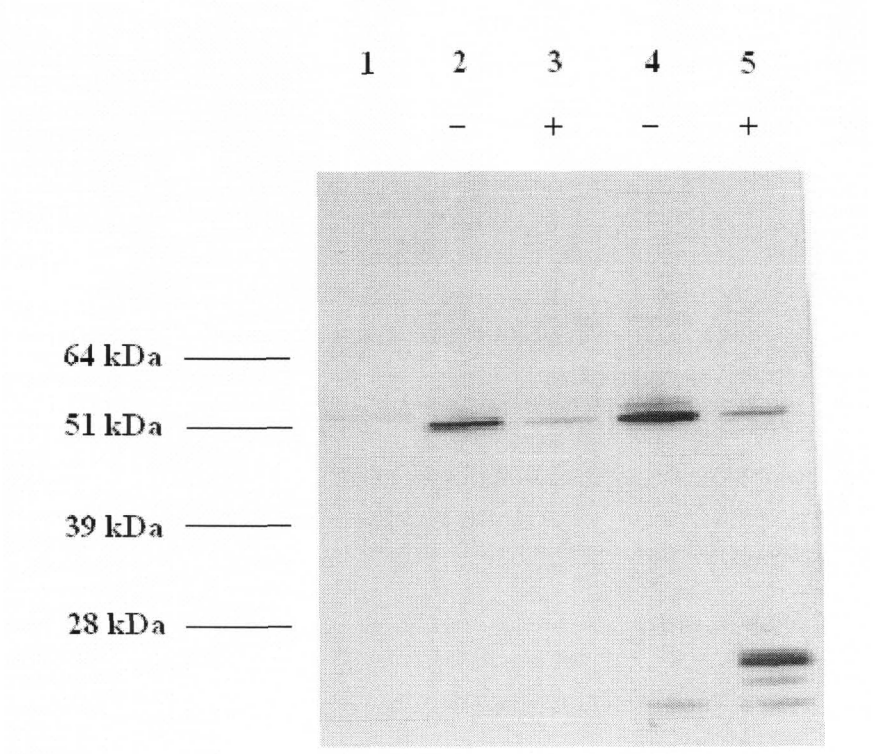


Figure 7. Treatment of Δ20 with trypsin. CB3:Δ20 cells were induced for 4 hours with 1 mM IPTG and then osmotically shocked in the presence (+) or absence (-) of 40 μg/ml trypsin. Lane1, culture supernatant; lanes 2 and 3, shocked cells; lanes 4 and 5, shock fluids. Samples were separated by SDS-PAGE and immunoblotted according to the text.

INTRODUCTION OF HISTIDINES IN THE CARBOXY TERMINUS

Construction of histidine variants

Continuing the strategy of modifying the proaerolysin C-terminus, several constructs were generated that contained six histidines in different regions within the C-terminal end of proaerolysin (Figure 8). The Stratagene QuikChange Site-Directed Mutagenesis Kit was used for the construction of these variants. Two rounds of PCR were applied for the variants EndHis and D435His, each round introducing three histidines.

HCT is secreted like wild-type

The first variant to be studied, HCT, was created by mutating the last six amino acid residues of proaerolysin (465 – 470) to histidines (Figure 8). As described in Materials and Methods, this construct was cloned into the expression vector pMMB66HE, transformed into DH5α and finally transconjugated into *A. salmonicida* CB3. These CB3 cells were induced overnight with 1 mM IPTG. As shown in Figure 9, there was no difference in secretion between CB3:pNB5 and CB3:HCT, indicating that the mutation of residues 465 – 470 of proaerolysin to histidines had no effect on the ability of the bacteria to secrete the variant.

HCT has the same hemolytic activity as wild-type

Having demonstrated that changing the last six amino acids of proaerolysin did not affect secretion of the toxin, it was of interest to examine how such a mutation affects the activity of proaerolysin. Cells carrying this variant were induced overnight with 1 mM

IPTG. Aliquots of culture supernatants obtained from HCT cells were treated with trypsin. In order to determine if trypsin processes HCT like pNB5, trypsin-treated HCT was compared to trypsin-treated pNB5 after being separated by SDS-PAGE and then stained with Coomassie brilliant blue. The results showed that HCT is cut by trypsin once just like pNB5 (Figure 10) and generates a product the same size as aerolysin. A hemolytic titre assay was performed using the trypsin-treated HCT and pNB5 and incubating with horse erythrocytes (see Materials and Methods). The results (Table 5) revealed that there was no difference between the hemolytic activity of HCT and pNB5 when treated with trypsin. This was expected since the protein is processed by trypsin in a manner similar to wt proaerolysin. Furthermore, HCT, just like pNB5, did not exhibit a hemolytic activity when it was not treated with trypsin. Thus, the histidine replacements did not appear to affect the packing of the C-terminus against the rest of the protein.

EndHis and D435His are secreted like wild-type

To further investigate the effect of the introduction of histidines, two more variants, EndHis and D435His, were created. EndHis was constructed by the addition of six histidines between residue 470 and the stop codon while construction of D435His involved the mutation of residues 435 – 440 to histidine (Figure 8). Expression of the *aerA* gene containing either of these mutations in *A. salmonicida* CB3 resulted in secretion of these variants just like wild type (Figure 11). This led to the conclusion that neither the presence of six additional residues (EndHis) nor the mutation of six residues within the C-terminal peptide to histidine (D435His) affected secretion of the toxin.

HCT

PA signal sequence	Residues 1-464	HisHisHisHisHisHis	Stop

EndHis

PA signal sequence	Residues 1-470	HisHisHisHisHisHis	Stop

D435His

PA signal sequence	Residues 1-434	HisHisHisHisHisHis	Residues 441-470	Stop

Figure 8. Schematic representation of proaerolysin showing the regions where residues were replaced with histidines.

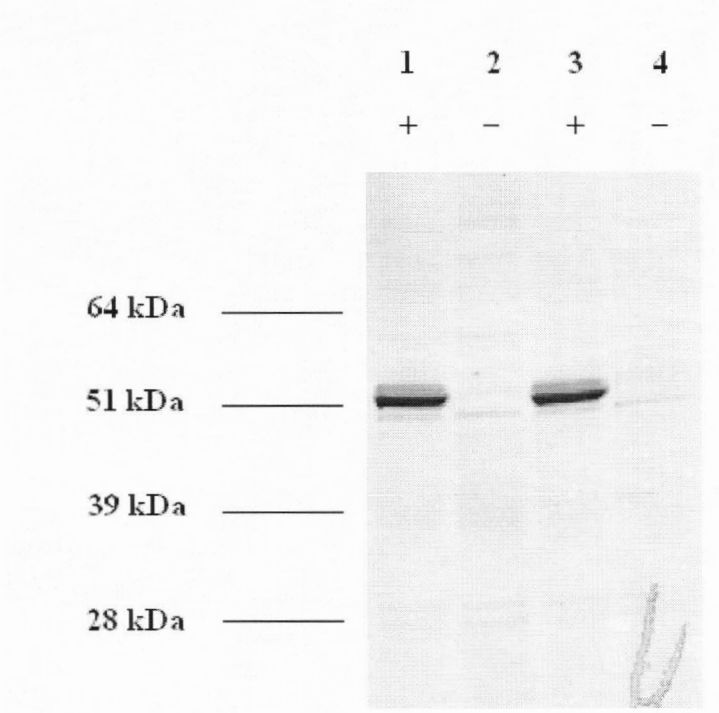


Figure 9. Expression of HCT. CB3:pNB5 and CB3:HCT cells were induced overnight with 1 mM IPTG. Lanes 1 and 2, induced (+) and uninduced (-) HCT culture supernatants; lanes 3 and 4, induced (+) and uninduced (-) pNB5 culture supernatants. Samples were separated by SDS-PAGE and stained with Coomassie blue.

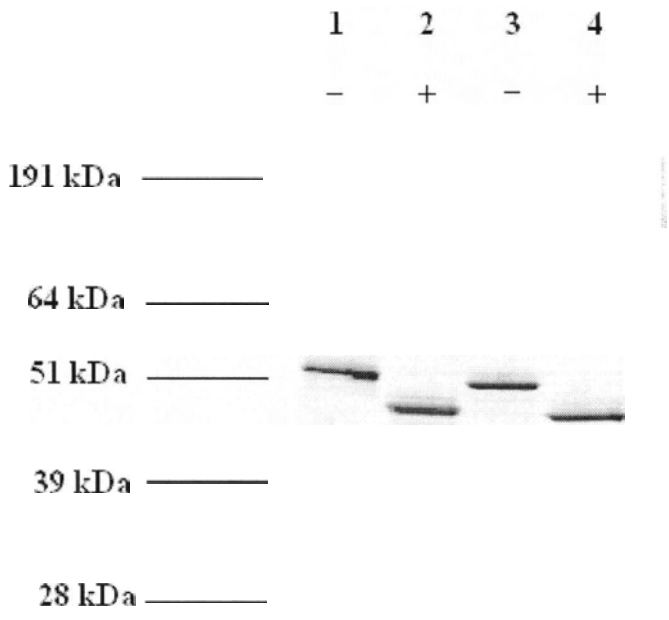


Figure10. Processing of HCT by trypsin. Culture supernatants of CB3:HCT (lanes 1 and 2) and CB3:pNB5 (lanes 3 and 4) cells that had been induced overnight with 1 mM IPTG were incubated with 2 μg/ml trypsin for 10 minutes at RT. Lanes 1 and 3, untreated (-) culture supernatants; lanes 2 and 4, treated (+) culture supernatants. Samples were separated by SDS-PAGE and stained with Coomassie blue.

Table 5. Hemolytic titre of CB3:HCT culture supernatant with or without trypsin.

Culture supernatant	Variant	+/- Trypsin	Hemolytic titre[1]
CB3:HCT	HCT	-	0
CB3:HCT	HCT	+	9
CB3:pNB5	wt	-	0
CB3:pNB5	wt	+	9
N/A	10 µl purified wt proaerolysin[2]	+	9

[1] titre obtained following overnight incubation with 0.4% horse erythrocytes
[2] purified wt proaerolysin used has a concentration of 400 ng/µl
N/A – not applicable

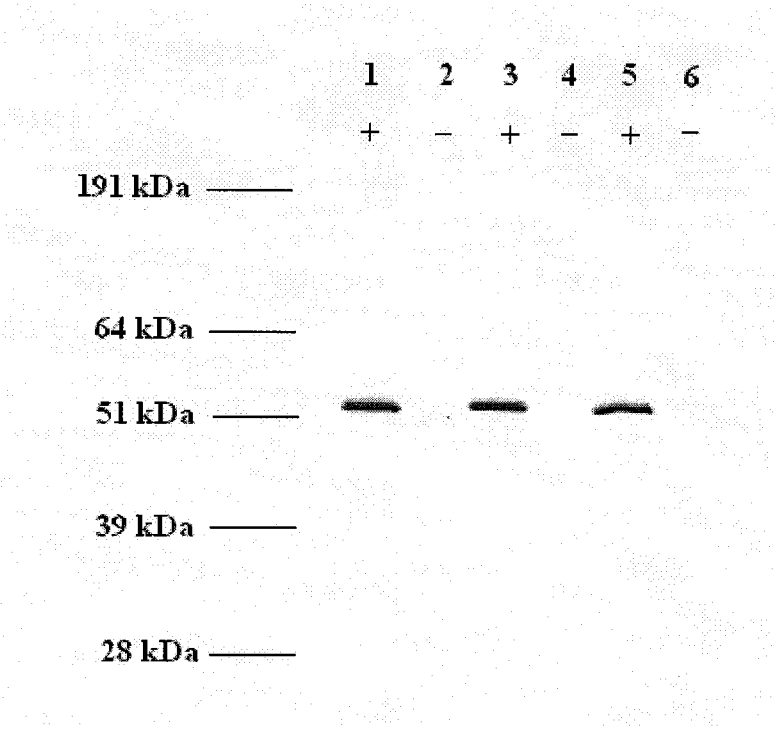


Figure 11. Expression of EndHis and D435His. CB3:pNB5, CB3:EndHis and CB3:D435His cells were induced overnight with 1 mM IPTG. Lanes 1 and 2, induced (+) and uninduced (-) pNB5 culture supernatant; lanes 3 and 4, induced (+) and uninduced (-) EndHis culture supernatant; lanes 5 and 6, induced (+) and uninduced (-) D435His culture supernatant. Samples were separated by SDS-PAGE and stained with Coomassie brilliant blue. Note that EndHis migrates slightly more slowly as it is 6 residues larger than the others.

EndHis and D435His are activated by trypsin

Upon incubation of IPTG-induced culture supernatants of the two histidine variants with trypsin, both variants were cut once, just like pNB5 (Figure 12). A hemolytic assay using trypsin-treated culture supernatants revealed that both variants are as active as pNB5 (Table 6).

Table 6. Hemolytic titre of CB3:EndHis and CB3:D435His culture supernatants with or without trypsin.

Culture supernatant	Variant	+/- Trypsin	Hemolytic titre[1]
CB3:EndHis	EndHis	-	0
CB3:EndHis	EndHis	+	9
CB3:D435His	D435His	-	0
CB3:D435His	D435His	+	9
CB3:pNB5	wt	-	0
CB3:pNB5	wt	+	9
N/A	10 µl purified wt proaerolysin[2]	+	9

[1] titre obtained following 1 hour incubation with 0.4% horse erythrocytes
[2] purified wt proaerolysin used has a concentration of 400 ng/µl
N/A – not applicable

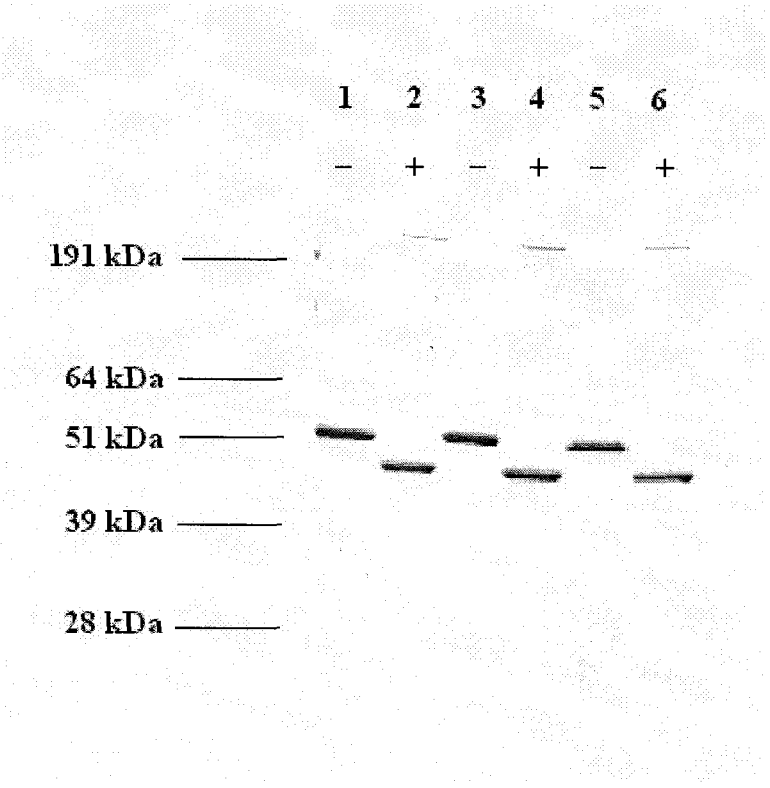


Figure 12. Processing of EndHis and D435His by trypsin. Culture supernatants of CB3:pNB5, CB3:EndHis and CB3:D435His cells that had been induced overnight with 1 mM IPTG were incubated with 2 μg/ml trypsin for 10 minutes at RT. Lanes 1, 3, and 5, untreated (-) samples; lanes 2, 4, and 6, treated (+) samples. Samples were separated by SDS-PAGE and stained with Coomassie blue.

TRUNCATION OF THE C-TERMINAL END OF PROAEROLYSIN

Burr (2001) demonstrated that truncating the C-terminal end of proaerolysin results in incorrect folding of the protein and blocks secretion of the protoxin. Two variants were created: End440 (2 stop codons inserted after residue 440) and End450 (2 stop codons inserted after residue 450). Expression of the *aerA* gene containing either of these mutations in CB3 resulted in the formation of incorrectly folded proteins thereby preventing secretion of the protein. In order to examine the effect of deleting the C-terminal peptide altogether, the variant End426 (2 stop codons inserted after residue 426) was generated. This variant would be identical to activated aerolysin since the stop codon was inserted at the trypsin cut site, and, unlike native proaerolysin, End426 would not require proteolytic activation. Therefore, this mutation was assumed to be lethal for the bacteria itself. To prevent this, an End variant was also created, using an *aerA* gene containing the mutation H132D as a template (End426/H132D). The H132D variant is known to be inactive since it is unable to oligomerize (unpublished data).

End426/H132D cannot be secreted by CB3

The *aerA* genes containing stop codons were cloned into the expression vector pMMB66HE and transconjugated into CB3 cells. These CB3 cells were induced and the culture supernatants and cell lysates were studied by Western blotting. No trace of the variants (should be ~ 51 kDa) was found in either the cell lysate or the culture supernatant (Figure 13). It should be noted here that a faint band appears in Figure 13 in all lanes. This band can be disregarded since it is also present in all lanes for cell samples and culture supernatants from CB3 cells that do not contain an *aerA* insert. It is an

endogenous CB3 protein that cross-reacts with the polyclonal anti-aerolysin antibody used. The difference in intensity of this band seen between samples from CB3:End426/H132D and CB3 cells is due to the fact that they are on two separate gels that were immunoblotted separately. The observation that End426/H132D could not be detected in either the cells or the culture supernatants may indicate that the variant probably gets degraded before it crosses the inner membrane or it gets immediately following its release into the periplasm. It should be noted here that when the same constructs were expressed in DH5α, both End426 and End426/H132D could be detected in the cells (Figure 14). Attention needs to be drawn to the bands that appear in the lanes containing uninduced cells samples in Figure 14. These bands probably indicate some leakage of the promoter.

Co-expression of End426 and End426/H132D with the C-terminal peptide

The above results indicate that the C-terminal region of proaerolysin plays a role in folding or secretion of the protein. While internal deletions within this region led to the expression of incorrectly folded proteins that could not cross the outer membrane, deletion of the C-terminal peptide had an even more profound effect. To see if the C-terminal peptide could affect secretion when co-expressed with End426 and End426/H132D, two variants, End426+C and End426/H132D+C, were created. The DNA for End426 (or End426/H132D) and the DNA encoding the aerolysin signal

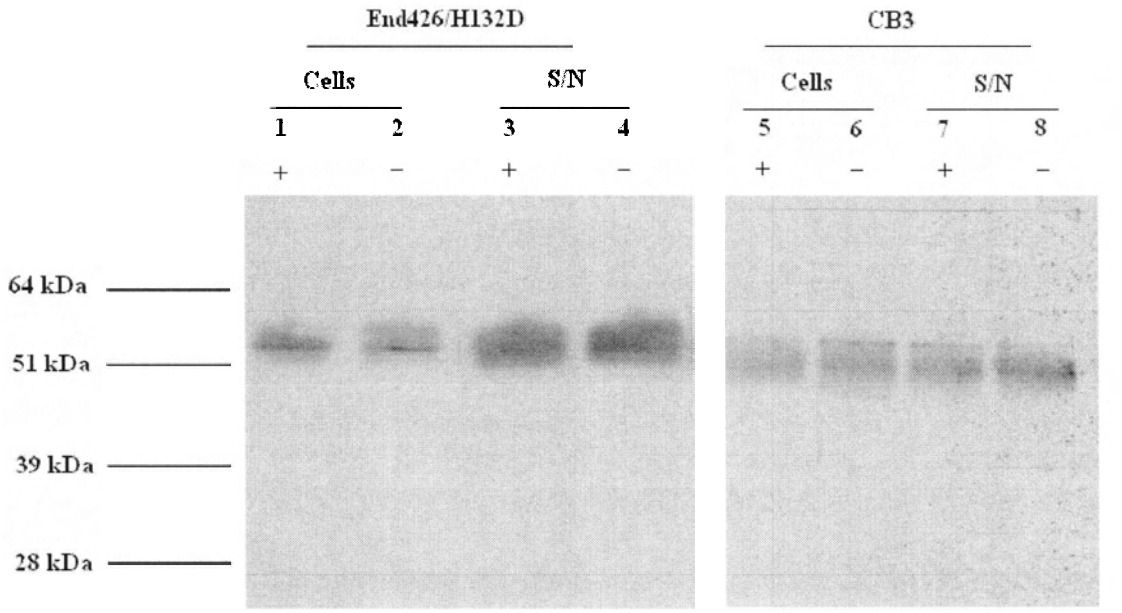


Figure 13. Expression of End426/H132D by CB3. CB3:End426/H132D and CB3 cells were induced for 4 hours with 1 mM IPTG. Lanes contain induced (+) and uninduced (-) cells and culture supernatants (S/N). Samples were separated by SDS-PAGE and immunoblotted as described in the text. Similar results were obtained with End426.

sequence and the C-terminal peptide were isolated from their respective pTZ18U clones and then co-ligated into pTZ18U. The resulting plasmid was reisolated and subcloned into pMMB66HE (Figure 15). Co-expressed constructs contained an inducible *tac* promoter (part of the conjugative expression vector), and each gene was preceded at an appropriate distance by a ribosomal binding site. It should be noted here that the template used as a starting point for these constructs was isolated from *E. coli* cells expressing an *aerA* variant ($aerA^{-loop}$) that lacks a stretch of amino acids at the 5' end of the gene and is unable to form a stem loop structure (Howard and Buckley, 1986). CB3 cells that contain *aerA* variants containing this mutation show a marked increase in protein production.

Secretion of End426+C

CB3 cells harboring End426 and the co-expressed construct End426+C were induced with IPTG and the cells were then separated from the culture supernatant by centrifugation. As before, End426 could not be detected in either the cells or culture supernatants of CB3 cells harboring plasmids containing this mutation. In contrast, co-expression of the truncated proaerolysin variant with the C-terminal peptide resulted in the appearance of some protein in the culture supernatant (Figure 16). This leads to the remarkable conclusion that the C-terminal peptide can facilitate folding or secretion of the rest of the protein even when it is not attached to the protein. Unfortunately, it was not possible to look for the C-terminal peptide itself in the culture supernatant, as an appropriate antibody was not available. These results provide further evidence that the C-terminal end of proaerolysin plays a role in folding or secretion of the toxin.

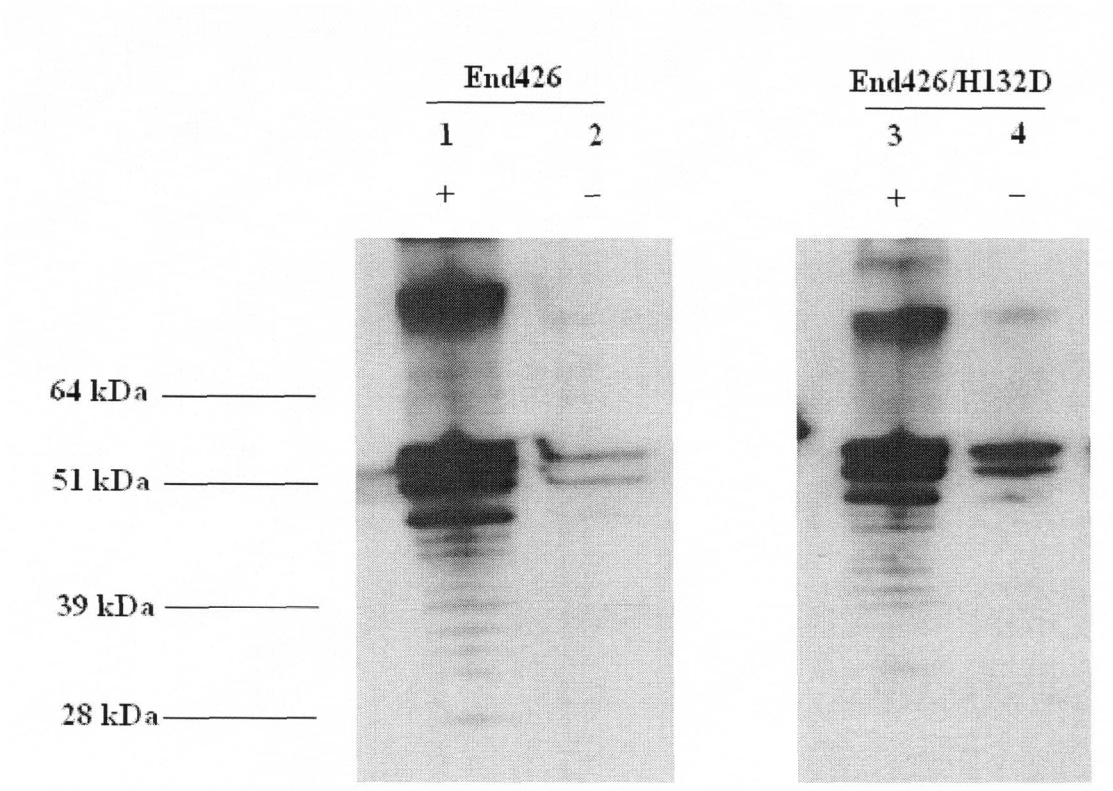


Figure 14. Expression of End426 and End426/H132D in DH5α. DH5α:End426 and DH5α:426/H132D cells were induced for 4 hours with 1 mM IPTG. All lanes contain induced (+) and uninduced (-) cell samples. Samples were separated by SDS-PAGE and immunoblotted according to the text.

End426+C

PA signal Sequence	End426	StopStop	RBS	PA signal sequence	PA C-terminus

End426/H132D+C

PA signal Sequence	End426/H132D	StopStop	RBS	PA signal sequence	PA C-terminus

Figure 15. Schematic representation of the co-expressed constructs of End426 (and End426/H132D) with the C-terminus.
RBS = ribosome binding site

End426+C is inactive

Having shown that co-expression with the C-terminus allows the secretion of a protein corresponding to mature aerolysin, I wanted to see if the secreted protein had any activity. CB3 cells expressing the variant End426+C were induced and the culture supernatants were assayed for hemolytic activity (see Materials and Methods). There was no difference in the hemolytic activity of culture supernatant obtained from CB3:End426+C treated with trypsin or without (Table 7). Furthermore, culture supernatant from CB3:End426+C displayed no more hemolytic activity than culture supernatant from CB3 cells not carrying the *aerA* gene on a plasmid. It was concluded that while co-expression with the C-terminal peptide enables some secretion of aerolysin, it does not result in the formation of a protein capable of forming channels. An explanation for this observation would be that the protein is folded in a conformation that allows it to be recognized by the secretion machinery for translocation across the outer membrane but cannot be activated when treated with the protease trypsin.

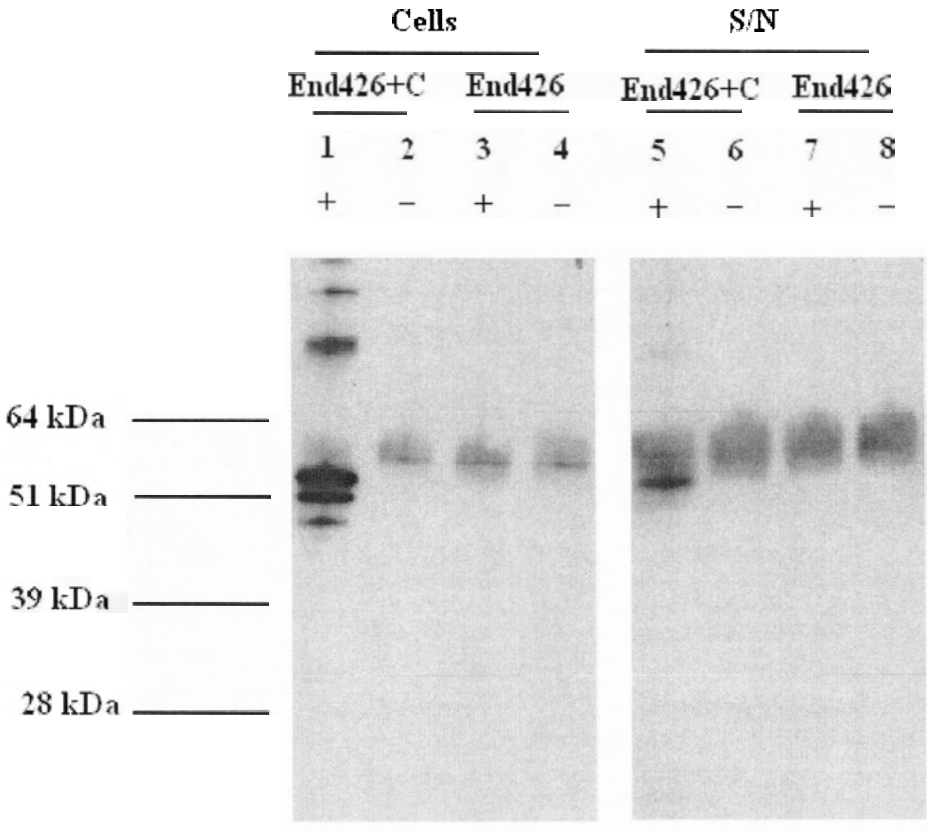


Figure 16. Expression of End426 and End426+C by CB3. CB3:End426 and CB3:End426+C cells induced for 4 hours with 1 mM IPTG. All lanes show induced (+) and uninduced (-) cells and culture supernatants (S/N). Samples were separated by SDS-PAGE and immunoblotted according to the text.

Table 7. Hemolytic titre of CB3:End426+C culture supernatants with or without trypsin.

Culture supernatant	Variant	+/- Trypsin	Hemolytic titre[1]
CB3:426+C	426+C	-	0
CB3:426+C	426+C	+	0
CB3	N/A	-	0
CB3	N/A	+	0
N/A	10 µl of purified wt proaerolysin[2]	+	9

[1] titre obtained following 1 hour incubation with 0.4% horse erythrocytes

[2] purified wt proaerolysin used has a concentration of 400 ng/µl

N/A – not applicable

ALKALINE PHOSPHATASE FUSION

A previous study has shown that *Aeromonas* spp. can secrete an otherwise periplasmic protein, *E. coli* alkaline phosphatase (Wong and Buckley, 1993). Hybrids were generated between portions of *aerA* and full-length *phoA* (gene for alkaline phosphatase). While the PhoA portions of the hybrids could be detected in the culture supernatant, the AerA portion of all three hybrids were degraded before translocation across the outer membrane could occur. The smallest of the three fusions, pAD3, contained the first two amino acids of proaerolysin and the entire *phoA* gene. It was seen that when the culture medium was alkaline the amount of protein secreted into the culture supernatant by *A. salmonicida* CB3 increased with time. However, when glucose was added to the medium, the pH of the medium decreased with respect to time and this resulted in a decrease over time of the amount of PAD3 that was secreted into the culture supernatant. I was interested to see if a fusion between pAD3 and the C-terminal peptide would allow the *aerA* portion of the hybrid (PhoAfusion) to be detected outside the cells and also whether this fusion protein could cross the outer membrane efficiently regardless of the pH of the culture medium.

PhoAfusion is expressed by DH5α cells

Plasmids that had been shown by sequencing to contain the desired fusion were transformed into *E. coli* DH5α cells (see Materials and Methods) and the cells were grown and induced with IPTG. The cells were separated from the culture supernatants by centrifugation and analyzed by western blotting for the presence of both PhoA and proaerolysin. The fusion protein could be detected in the DH5α cells (Figure 17) with the

help of an anti-PhoA antibody and, as expected, migrates to a position higher (~51 kDa) than native PhoA (50 kDa).

PhoAfusion cannot be detected in CB3 cells or culture supernatant

Plasmids containing the desired fusion were transconjugated from DH5α cells into CB3 cells and the cells grown and induced with IPTG. The cells and culture supernatants were separated by centrifugation and analyzed by western blotting for the presence of the fusion protein. An anti-PhoA antibody could not detect the fusion protein in either the cells or the culture supernatant (Figure 18).

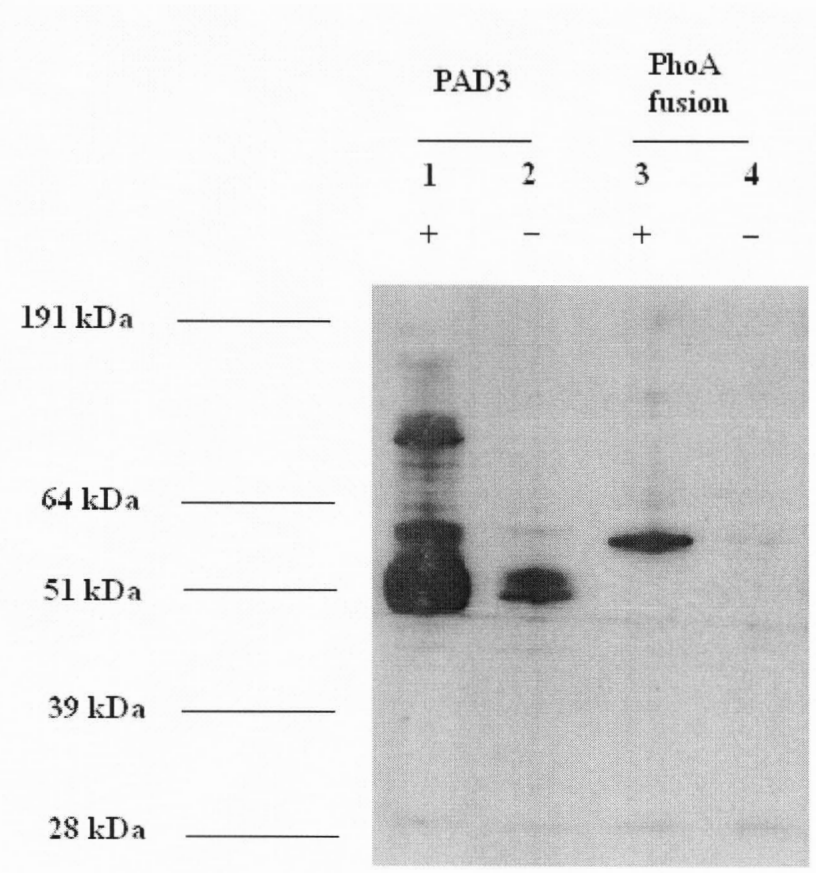


Figure 17. Expression of PAD3 and PhoAfusion by DH5α. DH5α:pAD3 and DH5α:PhoAfusion were induced for 4 hours with 1 mM IPTG. All lanes contain induced (+) and uninduced (-) cell samples. Samples were separated by SDS-PAGE and immunoblotted using anti-PhoA antibodies.

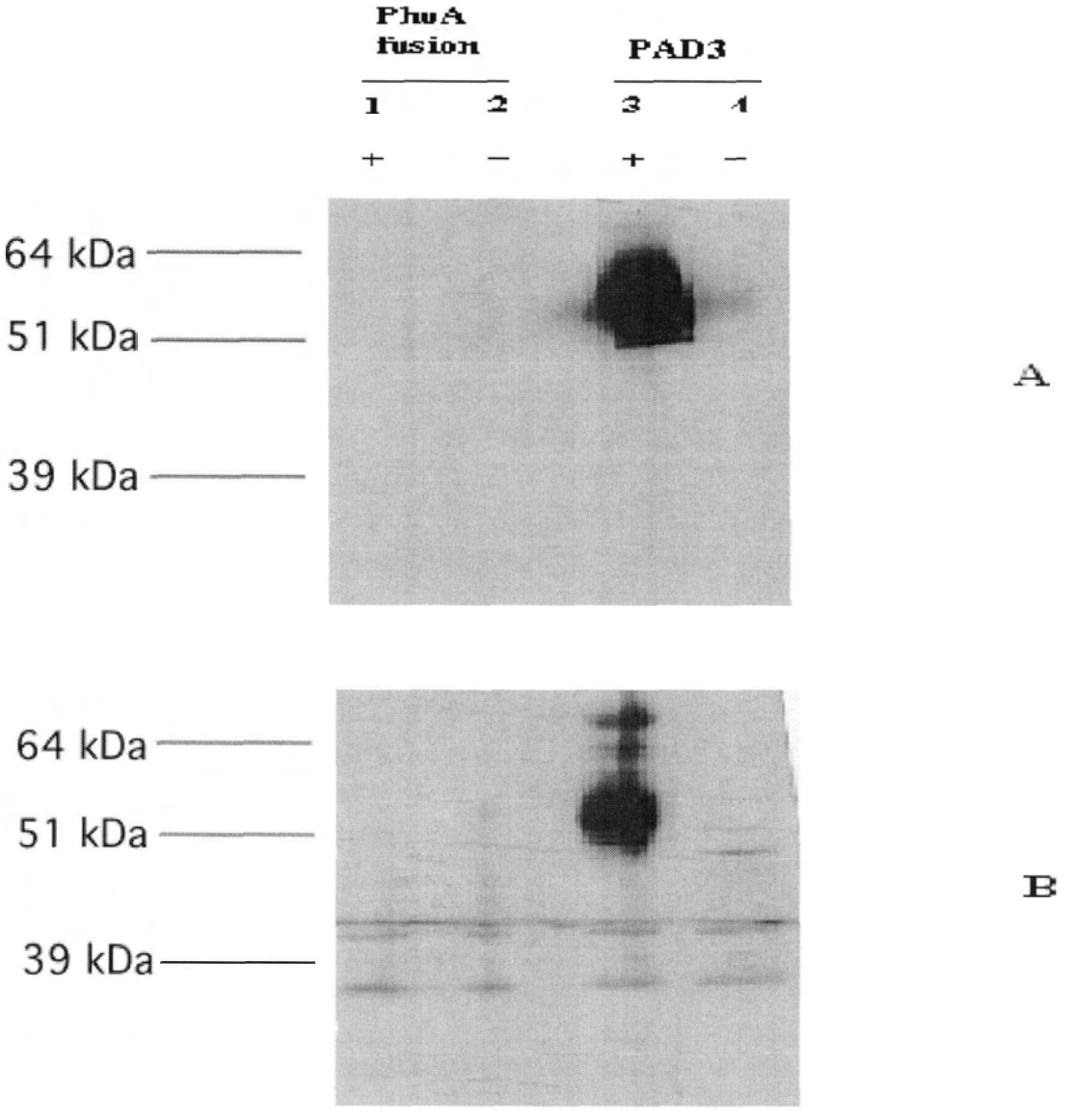


Figure 18. Expression of PhoAfusion in CB3. CB3:pAD3 and CB3:PhoAfusion cells were induced for 4 hours with 1mM IPTG. All lanes in Panel A contain induced (+) and uninduced (-) culture supernatants while Panel B contains the corresponding cells. Samples were separated by SDS-PAGE and immunoblotted using anti-PhoA antibodies.

POINT MUTATIONS THAT AFFECT PROAEROLYSIN SECRETION

It has been shown that a deletion of six residues (Δ6) within the C-terminal region of proaerolysin did not have any effect on folding or secretion (Burr, 2001). Larger internal deletions within this region (Δ12 and Δ20) resulted in incorrectly folded proteins and complete loss of secretion. I have also demonstrated that without the presence of the C-terminal peptide the variant End426 could not be detected either in the cells or in the culture supernatant. Co-expression of End426 with the C-terminal peptide allowed partial secretion of the protein across the outer membrane.

While it is known that proteolytic cleavage of the C-terminal peptide of proaerolysin activates the toxin, the role of this peptide in the secretion of the protein, if any, has not been defined. Some work has been done with the aerolysin from *A. sobria* (Nomura *et al.*, 1999; Nomura *et al.*, 2000), which shows a 68% nucleotide sequence homology to *A. hydrophila* aerolysin at the amino acid level (Figure 19). Nomura and colleagues have shown the last 10 amino acids of the *A. sobria* proaerolysin to be crucial in correct folding and efficient secretion of the protein, since deletion of these residues lead to an unstable form of the protein that cannot be secreted across the outer membrane and is rapidly degraded by periplasmic proteases. As seen from previous experimental evidence, the last 20 – 30 amino acid residues of the *A. hydrophila* proaerolysin are crucial for secretion and folding of the toxin. I was interested in trying to identify a more specific region in the C-terminal end of proaerolysin, which might play a role in secretion and folding.

Alanine scanning mutagenesis

Nomura and colleagues used deletion variants in their studies (Nomura, *et al.*, 1999; Nomura, *et al.*, 2000), and Burr (2001) as well as this study has also shown the significance of deletions within the C-terminal region of proaerolysin. I wanted to see if single amino acid substitutions at the C-terminal end would affect folding or secretion. The amino acid sequence of aerolysin from *A. sobria* shows the last three amino acids from the end to be glutamine, valise and proline. Alignment of this sequence with aerolysin from *A. hydrophila* (Figure 19) identifies the three corresponding amino acids in the latter organism to be glutamine, asparagine and alanine. Nomura and colleagues showed that it required the deletion of all three amino acids to affect folding and secretion. Therefore, using the *A. hydrophila* proaerolysin, the first substitution was made by mutating the proline at position 466 to alanine. Alanine scanning mutagenesis was performed to see if substitutions made in the C-terminal region, starting with residue 466 and moving inwards, would affect folding and secretion. A total of 17 *aerA* constructs containing single alanine substitutions were generated for this purpose (See Materials and Methods).

Construction and expression of the alanine variants

While the internal- and end-deletion variants, described earlier, were generated by a 2-step PCR, the alanine variants were produced using site-directed mutagenesis kits obtained from Stratagene. These kits employ one step to generate point mutations. A restriction digestion with the enzyme DpnI then helps remove any parental non-mutated DNA leaving only the mutated plasmid. The DpnI endonuclease is specific for

methylated and hemimethylated DNA and is used to digest the parental DNA template and to select for the mutation-containing DNA (QuikChange II Instruction Manual). The mutant genes generated were moved into the expression vector pMMB66HE from the cloning vector pTZ18U using the appropriate restriction enzymes, transformed into competent DH5α cells and then transconjugated into *A. salmonicida* CB3 cells. The entire *aerA* insert was sequenced in all clones to check for the correct mutations.

A region within the C-terminus that is affected by alanine mutation

Once the mutant genes were transconjugated into CB3 cells, clones were picked and the presence of the right-sized inserts was checked by PCR (See Materials and Methods for details). Transconjugates that were known to harbor the correctly modified *aerA* genes were induced overnight with 1 mM IPTG and the cells separated from the culture supernatants by centrifugation. The culture supernatants were examined for secreted protein while the cells were assayed for protein that may not have been able to cross the outer membrane. Proteins in the culture supernatants were separated by SDS-PAGE and stained with Coomassie brilliant blue. Mutating any of the residues 466 – 458 to an alanine did not affect secretion of the protein across the outer membrane (results not shown). Based on the intensity of staining, CB3 cells that expressed any of these variants appeared to secrete the 52 kDa toxin into their culture supernatants as efficiently as wild type proaerolysin. However, mutating any of the residues 452 – 457 had a significant effect on appearance of the protein in the culture supernatant (Figure 20). There was a significant difference in the intensity between the amount of protein present in the culture supernatant obtained from CB3 cells harboring the *aerA* gene that contained the alanine

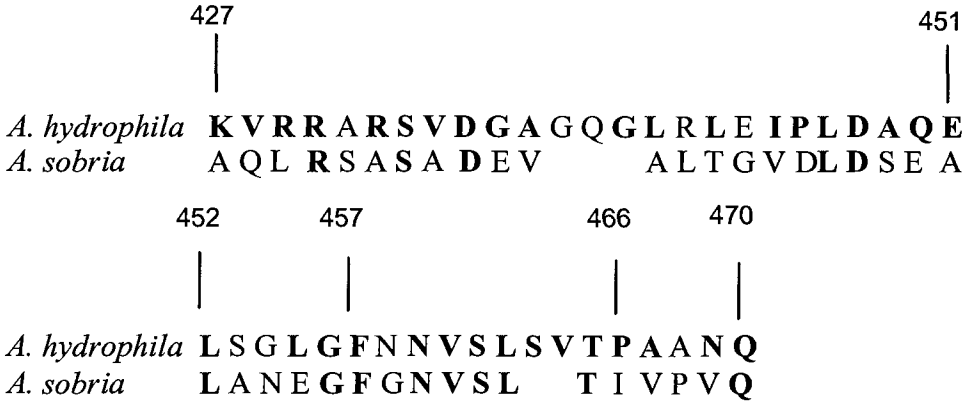


Figure 19. Comparison of C-terminus amino acid sequence of the *A. hydrophila* aerolysin with the *A. sobria* aerolysin. The numbers correspond to the position of the residues in the amino acid sequence of the *A. hydrophila* proaerolysin.

substitution in the region encompassed by amino acids 452 – 457 and wt *aerA*. Moreover, mutating some residues within this region seem to have a greater impact on secretion than others; CB3:F457A and CB3:L452A contain far less protein in the culture supernatant than other variants within this region. The cells that had been separated from the culture supernatants (mentioned above) were then examined. Proteins in the cell samples were separated by SDS-PAGE, and immunoblotted (see Materials and Methods). The results show that when an alanine mutation is made within the region encompassed by residues 452 – 457 there is a greater accumulation of protein within CB3 cells (Figure 21) than when the substitution was made outside that region. Moreover, lanes containing variants that are secreted less than wt AerA all show a degraded product of ~45 kDa.

Quantitating alanine variants

I next made a quantitative comparison of the alanine variants in the culture supernatants. CB3 cells expressing the variants Q450A, E451A, L452A, S453A, L455A, G456A, F457A and N458A and wild-type, pNB5, were induced overnight with 1 mM IPTG. The cells were removed by centrifugation and the proteins in the culture supernatants were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes along with four different concentrations of purified wt proaerolysin (see Materials and Methods). The membranes were blocked for 1 hour with 10 ml Odyssey Blocking Buffer and 10 ml PBS, followed by a 1-hour incubation with a 1:4000 dilution of an IRDye-800-labelled monoclonal anti-aerolysin antibody in PBS containing 0.5% Tween 20. The membranes were then scanned and analyzed using an Odyssey Infrared Imaging system. A standard curve was generated using purified wt proaerolysin (not shown) and the amount of each

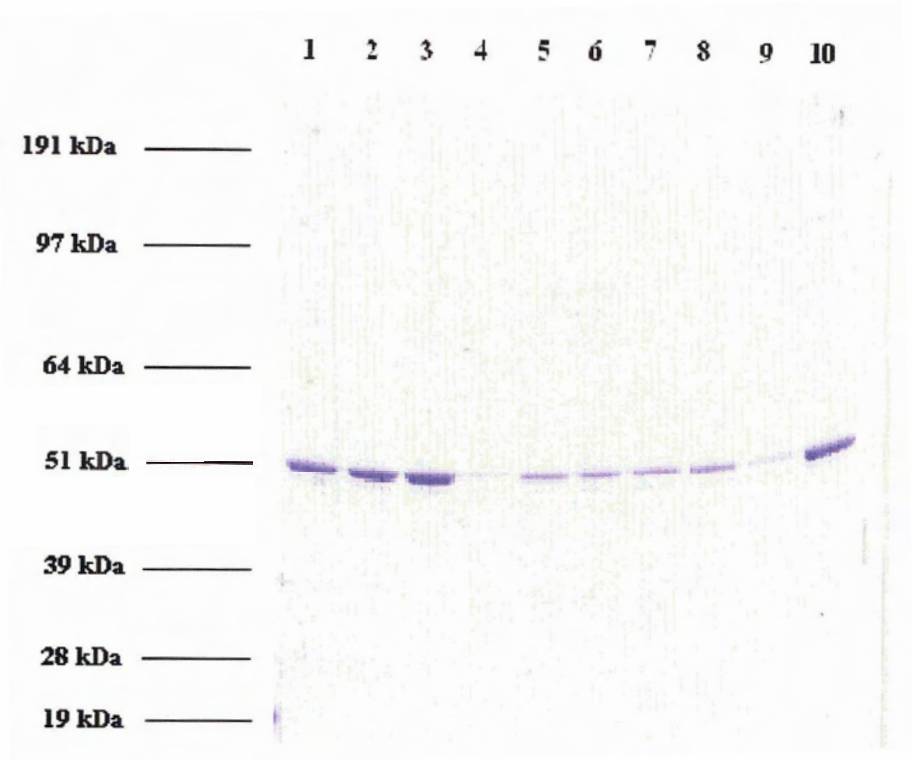


Figure 20. Amounts of alanine variants 452-457 in supernatants of CB3. CB3:V460A (lane 1), CB3:N459A (lane 2), CB3:N458A (lane 3), CB3:F457A (lane 4), CB3:G456A (lane 5), CB3:L455A (lane 6), CB3:G454A (lane 7), CB3:S453A (lane 8), CB3:L452A (lane 9), and CB3:E451A (lane 10) cells were induced overnight with 1 mM IPTG. All lanes show induced culture supernatants. The samples were separated by SDS-PAGE and stained with Coomassie blue.

variant present in the culture supernatant was determined (Figure 22). The results clearly showed that, while N458A and Q450A were secreted in amounts that were similar to the amount of wt secreted by CB3, all other alanine variants were present in smaller amounts in the culture supernatant, with F457A and L452A being most reduced (Table 7). This strengthens the conclusion derived from Figure 20 and Figure 21 that a region within the C-terminal region is important in folding or secretion.

Alanine variants are folded correctly

After establishing that certain point mutations within the C-terminal region reduced the amount of protein that was released from the cells, it was necessary to determine if this was a result of incorrect folding. CB3 cells containing the variants Q450A, E451A, L452A, G456A, F457A and N458A were osmotically shocked in the presence or absence of trypsin (See Materials and Methods). All the variants are cut by trypsin only once (Figure 23). Therefore, it can be concluded that all the variants in this group are correctly folded. It should be noted that there was more of the variants F457A and L452A in the shock fluids compared to the other variants. This can be explained by the fact that the other variants are secreted more efficiently than F457A and L452A (as seen from the quantitative analysis; Figure 22 and Table 8) and so there is less shockable protein in the cells separated from the culture supernatants.

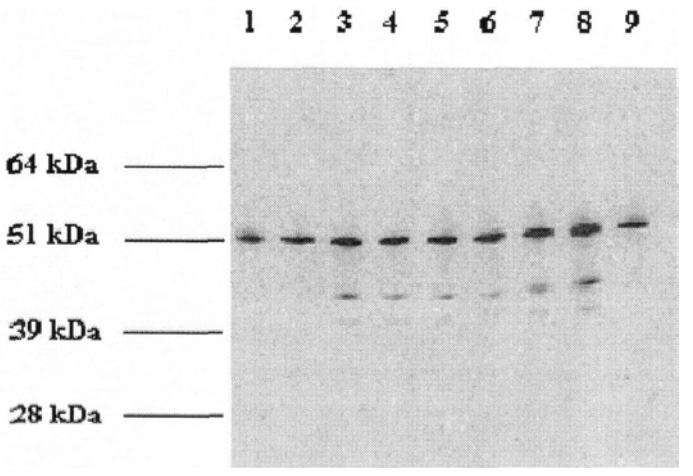


Figure 21. Comparison of alanine variants. CB3:pNB5 (lane 1), CB3:E451A (lane 2), CB3:L452A (lane 3), CB3:S453A (lane 4), CB3:G454A (lane 5), CB3:L455A (lane 6), CB3:G456A (lane 7), CB3:F457A (lane 8) and CB3:N458A (lane 9) cells were induced for 4 hours with 1 mM IPTG. All lanes contain cell samples. Samples were separated by SDS-PAGE and immunoblotted as described in the text.

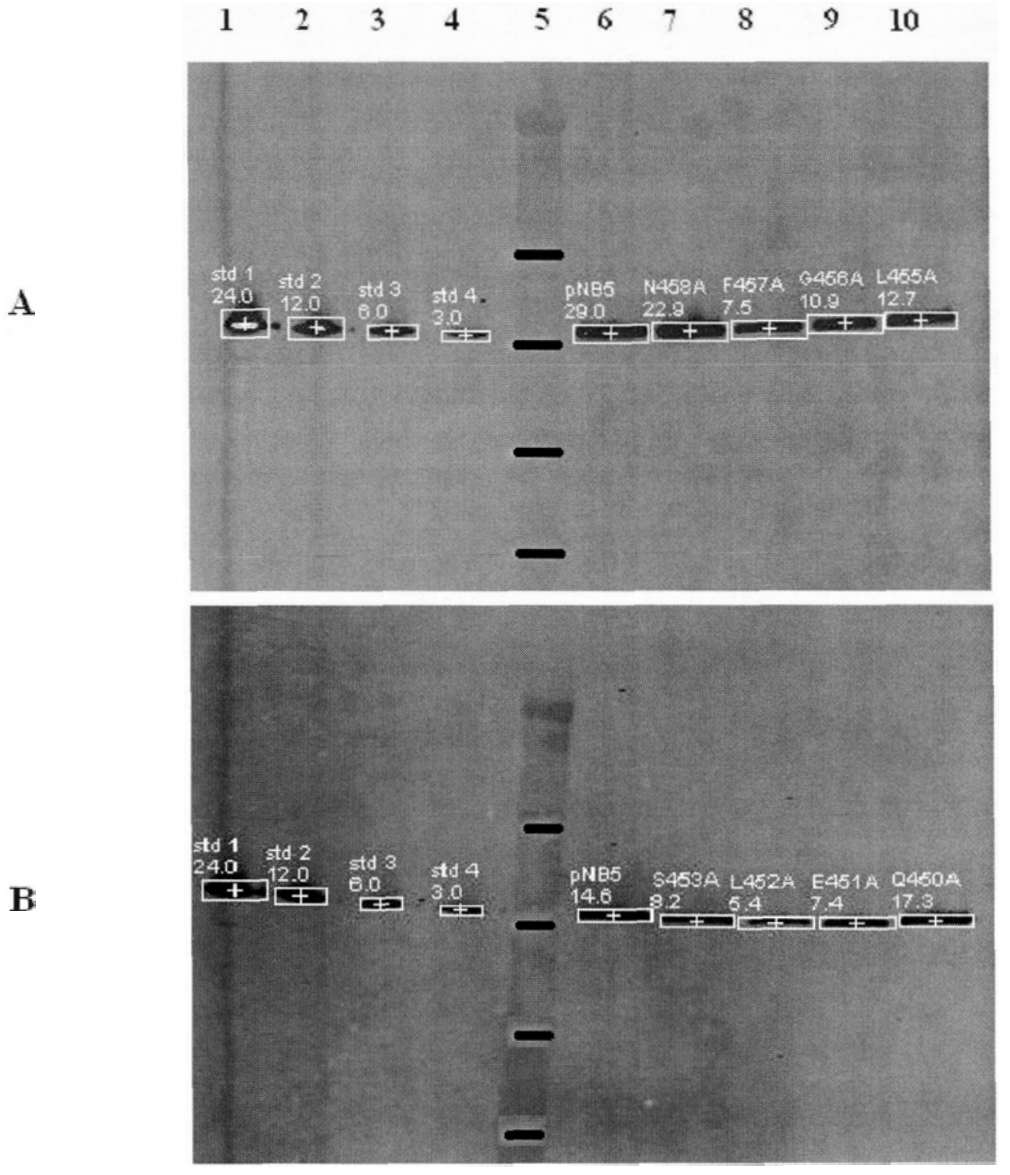


Figure 22. Quantitation of alanine variants. CB3:pNB5 (Lane 6, panels A and B), CB3:N458A (Lane 7, panel A), CB3:F457A (Lane 8, panel A), CB3:G456A (Lane 9, panel A), CB3:L455A (Lane 10, panel A), CB3:S453A (Lane 7, panel B), CB3:L452A (Lane 8, panel B), CB3:E451A (Lane 9, panel B) and CB3:Q450A (Lane 10, panel B) cells were induced overnight with 1 mM IPTG. All lanes contain samples of induced culture supernatants. The samples were separated by SDS-PAGE and electroblotted on to nitrocellulose membranes. Proaerolysin was detected using IRDye-800-labelled anti-aerolysin and quantitated using the Odyssey Infrared Imaging system. Lanes 1 – 4 contain proaerolysin standards at 24, 12, 6 and 3 pg, respectively. Lane 5 contains molecular weight markers (bars indicate, from top, 64, 51, 39 and 28 kDa).

Table 8. Amount of alanine variant present in culture supernatant in comparison to wt. 8A corresponds to Panel A in Figure 22 while 8B corresponds to Panel B in Figure 22.

8A

Variant	Amount present in culture supernatant (picograms)
Wt	29
N458A	22.9
F457A	7.5
G456A	10.9
L455A	12.7

8B

Variant	Amount present in culture supernatant (picograms)
Wt	14.6
S453A	8.2
L452A	5.4
E451A	7.4
Q450A	17.3

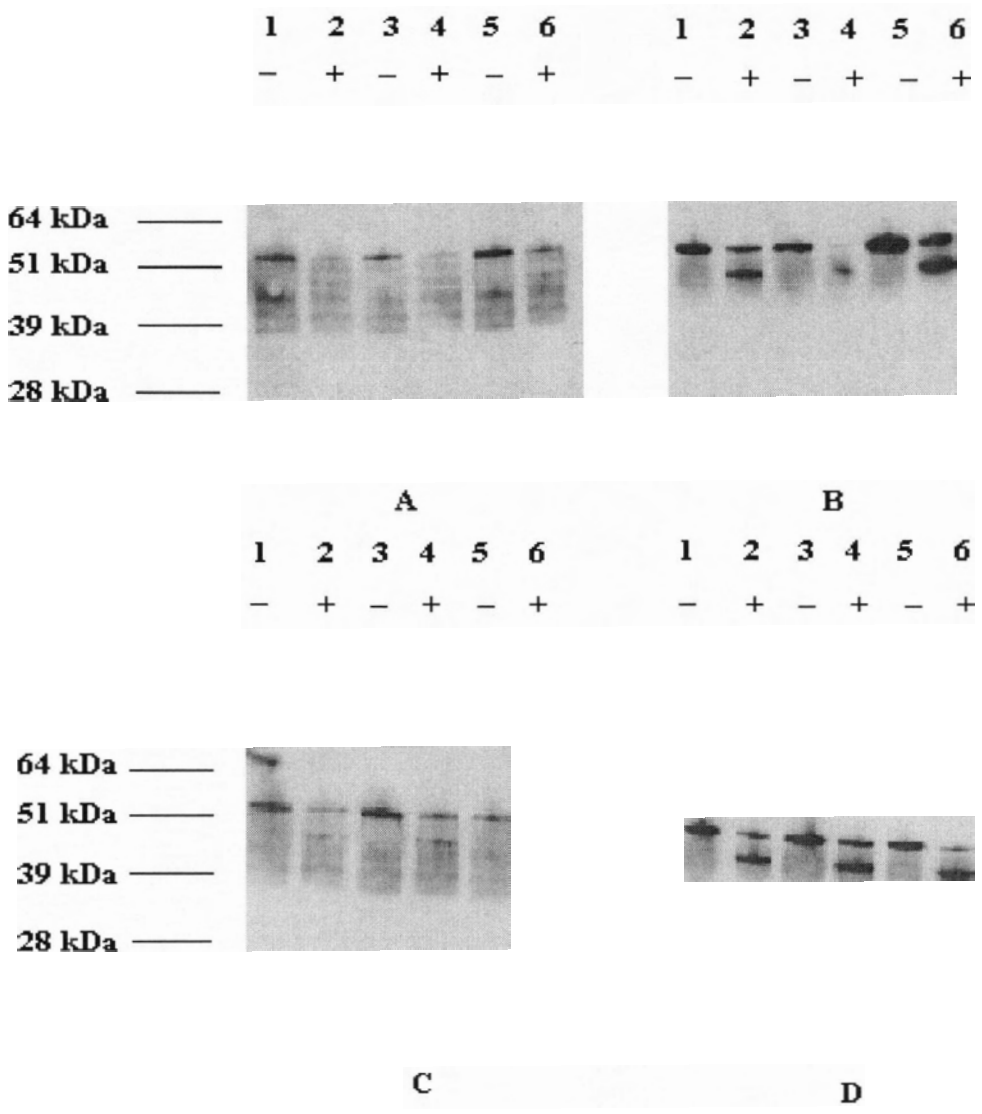


Figure 23. Treatment of alanine variants with trypsin. CB3:Q450A, CB3:E451A, CB3:L452A, CB3:G456A, CB3:F457A, and CB3:N458A cells were induced for 4 hours with 1 mM IPTG and then osmotically shocked in the presence (+) or absence (-) of 40 μg/ml trypsin. Panels A and C contain shocked cells – lanes 1A and 2A, Q450A; lanes 3A and 4A, E451A; lanes 5A and 6A, L452A; lanes 1C and 2C, G456A; lanes 3C and 4C, F457A; lanes 5C and 6C, N458A. Panels B and D contain the corresponding shock fluids. Samples were separated by SDS-PAGE and immunoblotted according to the text.

Overexpression of E451A and L452A

Mutating certain residues within the C-terminal region adversely affected the ability of CB3 cells to translocate the proaerolysin variants across the outer membrane. Some of these variants were less well translocated than others. Culture supernatant obtained from CB3:E451A cells contained more of the protoxin than that obtained from CB3:L452A cells. It was of interest to see what effect, if any, overexpression of these two variants would have on production and secretion of the toxin. In order to accomplish this, E451A and L452A were created using the *aerA* gene containing the mutation *aerA*$^{-loop}$ as a template.

The 5' end of the aerolysin gene contains two inverted repeats upstream of the start site (Howard and Buckley, 1986). The second of these repeats is capable of forming a stem-loop structure, which may affect expression of the aerolysin gene either at the level of transcription or translation. Diep, *et al.*, (1998) constructed an *aerA* mutant in which one half of this stem-loop was removed and replaced with a new sequence. This construct, *aerA*$^{-loop}$, is not able to form the stem loop and cells expressing this variant (CB3:γ123) show a significant increase in the production of the protoxin.

Proaerolysin production by cells expressing AerA^{-loop} was compared to production by cells expressing E451Aγ123 and L452Aγ123 (Figure 24). In a manner similar to CB3:γ123, CB3:E451Aγ123 displayed an increase in the amount of the protein that was recovered in the culture supernatant, as well as accumulation of the toxin within the cells. However, such an increase was not seen for cells expressing L452Aγ123. Moreover,

higher molecular weight bands were seen in the lane containing induced CB3:γ123 and CB3:E451Aγ123 cell samples but not for induced CB3:L452Aγ123 cell samples.

CB3 cells expressing E451Aγ123 and L452Aγ123 were osmotically shocked in the presence or absence of trypsin. Previous studies have shown that when CB3:γ123 is treated in a similar manner, most of the proaerolysin accumulates within the shockable fraction, but a portion of the toxin remains associated with the shocked cells and cannot be released by osmotic shock (Burr, 2001). Similarly, a portion of the variants E451Aγ123 and L452Aγ123 could not be released by osmotic shock and remained associated with the shocked cells (Figure 25).

CB3 cells containing the variants E451Aγ123 and L452Aγ123 were osmotically shocked in the presence or absence of trypsin (see Materials and Methods). Both variants were cut by trypsin once, just like the protein expressed by CB3:γ123 cells. Therefore, it was concluded that neither of the variants was incorrectly folded.

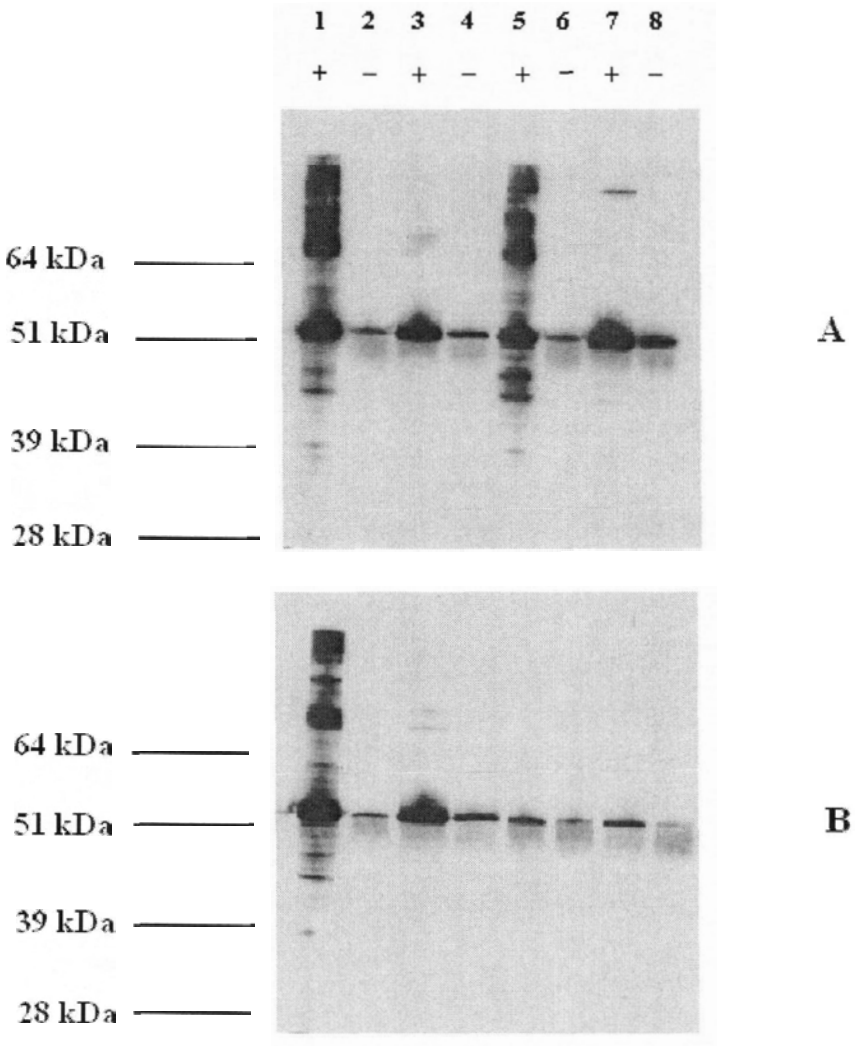


Figure 24. Expression of E451Aγ123 and L452Aγ123. CB3:γ123 (Panels A and B, lanes 1-4), CB3:E451Aγ123 (Panel A, lanes 5-8) and CB3:L452Aγ123 (Panel B, lanes 5-8) cells were induced for 4 hours with 1 mM IPTG. Lanes 1 and 5, induced cells; lanes 2 and 6, uninduced cells; lanes 3 and 7, induced culture supernatants; lanes 4 and 8, uninduced culture supernatants. Samples were separated by SDS-PAGE and immunoblotted according to the text.

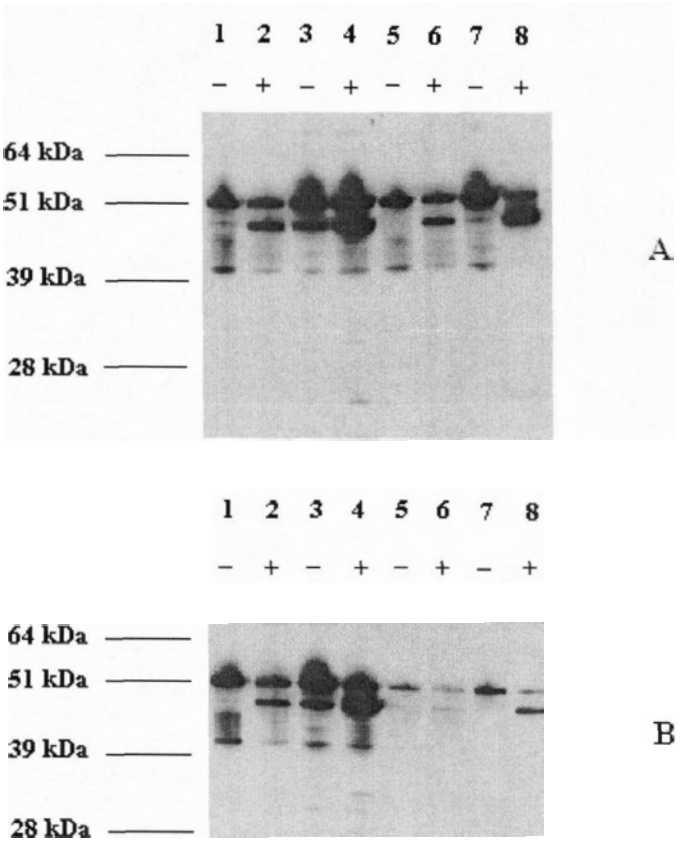


Figure 25. Treatment of E451Aγ123 and L452Aγ123 with trypsin. CB3:γ123, CB3:E451Aγ123 (Panel A) and CB3:L452Aγ123 (Panel B) cells were induced for 4 hours with 1 mM IPTG and then osmotically shocked in the presence (+) or absence (-) of 40 μg/ml trypsin. Lanes 1 and 2, γ123 shocked cells; lanes 3 and 4, γ123 shock fluid; lanes 5 and 6, E451Aγ123 (Panel A) and L452Aγ123 (Panel B) shocked cells; lanes 7 and 8, E451Aγ123 (Panel A) and L452Aγ123 (Panel B) shock fluid. All samples were separated by SDS-PAGE and immunoblotted according to the text.

DISCUSSION

Relationship between folding and secretion

Proteins that are intended for secretion via the type II secretion pathway in Gram-negative bacteria first cross the cytoplasmic membrane in a Sec-dependent manner. This is accompanied or followed by removal of the N-terminal signal peptide that directs the proteins to the Sec machinery. In the periplasm the proteins are folded completely and in this compartment they also gain structure, such as disulfide bonds or subunit assembly, before they are translocated across the outer membrane via the type II secretion apparatus (Sandkvist, 2001). The ability to transport apparently unrelated folded proteins across the outer membrane is a unique feature of the type II secretion pathway.

A link between folding and secretion has been established for a number of proteins. It has been shown that a disulfide bond forms in the cellulose-binding domain (CBD) of the *Erwinia chrysanthemi* cellulase EGZ before the protein is secreted across the outer membrane, indicating that EGZ is in its folded conformation before secretion via the type II machinery (Bortoli-German, *et al.*, 1994). None of the cysteine residues in pullulanase are required for efficient secretion (Sauvonnet and Pugsley, 1998) but it has been shown that disulfide bonds form before the protein crosses the outer membrane (Pugsley, 1992). Proaerolysin has been shown to form intramolecular disulfide bridges in the periplasm before secretion (Hardie, *et al.*, 1995). Proaerolysin dimers composed of disulfide-bonded monomers are able to cross the outer membrane but incorrect disulfide bridge formation inhibits secretion. However, it was also shown that disulfide formation is not required for secretion as evidenced by the fact that even when the protoxin is kept in a reduced form

by β-mercaptoethanol, its release into the culture supernatant is not affected. The *dsba* gene codes for a protein that is a disulfide catalyst. The protein itself has a disulfide bond that is transferred catalytically to folding proteins in the periplasm (Bardwell, 1994). It has been shown that DsbA is required for the efficient secretion of pullulanase by *K. oxytoca* without the need for intramolecular disulfide bond formation (Sauvonnet and Pugsley, 1998).

C-terminal peptide affects folding and secretion

There is now enough evidence, both from this thesis and Burr (2001), to support a correlation between folding and secretion of proaerolysin. The C-terminal region of the protein, in particular, has been demonstrated to play a significant role in the folding and secretion of the toxin across the outer membrane (Burr, 2001). This is quite surprising since the C-terminal end of the protein is the last portion to be synthesized and folding of the protein is presumed to commence as the N-terminal end of the polypeptide chain crosses the inner membrane. Burr (2001) had demonstrated that deletion of the activation sequence (residues 427-430) has no effect on secretion. She also demonstrated that deletion of either 20 or 30 amino acid residues from the C-terminal end of the protein results in incorrectly folded proteins that are not secreted by *A. salmonicida* CB3 cells. The observation that deletions within the C-terminal region affect the ability of CB3 cells to secrete the proteins is explained by the inability of the variants to fold correctly. However, it could not be determined how folding and secretion are related in terms of the C-terminal end of proaerolysin. Changes within the C-terminal region of the protein could prevent it from crossing the inner membrane. Alternatively, the deletions could

result in an incorrectly folded protein that is destroyed by periplasmic proteases. Another possibility is that the protein crosses the inner membrane and folds in a manner that protects it from proteolytic degradation, but that does not allow the correct presentation of the signal that directs it to the type II secretion machinery in the outer membrane, resulting in accumulation within the periplasm.

Results presented in this thesis show that proaerolysin variants containing larger internal deletions (Δ12 and Δ20) were not secreted by CB3 cells (Figs. 4 and 6). It is apparent that these internal deletions did not have any effect on the translation of the protein, since the variants could be detected in the cells by a Western blot. However, the variants were folded incorrectly, as evidenced by their susceptibility to degradation by trypsin (Figs. 5 and 7). It should be noted here that deletion of residues 427 – 438 resulted in a protein (Δ12) which, upon treatment with trypsin (Figure 5), showed less degradation than the protein that was expressed by CB3 cells harboring the variant (Δ20) containing a deletion of residues 427 - 446 (Figure 7). Therefore, it can be concluded that the region encompassed by residues 427 – 446 plays a role in the way the protein folds within the periplasm and therefore affects the stability of the protein for secretion across the outer membrane. Furthermore, the region between residues 438 – 446 is particularly important for stability of the protein, as its deletion resulted in a protein that is more readily degraded by the protease.

It has been proposed that the information that is necessary to target proteins to the secretion machinery is created as the protein folds in the periplasm (Sandkvist, 2001).

This signal could either be composed of residues from various parts of the linear polypeptide chain that come together when the protein is correctly folded, or it could be a linear sequence that is recognized by the secretion apparatus when displayed on the surface of the correctly folded protein. Based on the results I obtained from the generation of the deletion variants in this thesis, it may be possible that the secretion signal lies within the deleted regions.

It has been shown that changing a tryptophan residue (W227) near the middle of the amino acid chain to a leucine or a glycine by site-directed mutagenesis was able to adversely affect the secretion of proaerolysin across the outer membrane (Wong and Buckley, 1991). The resulting variants could not be efficiently secreted into the culture supernatants. These proteins were incorrectly folded, as evidenced by the ability of trypsin to degrade them. No comparable single amino acid mutations have been generated within the C-terminal region.

Erwinia carotovora secretes the enzyme polygalacturonase (PehA) across the outer membrane via the type II pathway (Saarilahti, *et al.*, 1990). Initial studies of this protein showed that fusions made between β-lactamase and portions of mature PehA (PehA-Bla) could not be secreted across the outer membrane, even when 87% of the mature PehA was present in the hybrid protein (Saarilahti, *et al.*, 1992). It was next shown that removal of as many as four amino acid residues from the C-terminal end was enough to significantly reduce the amount of the hybrid protein recovered in the culture supernatant, and that when up to seven residues were deleted, the hybrid was rendered exclusively

cell-bound (Palomaki and Saarilahti, 1995). When site-directed mutagenesis was employed to create amino acid changes to five residues in the C-terminus of PehA, secretion was hindered (Palomaki and Saarilahti, 1997). These studies led the authors to conclude that either the C-terminus of PehA, or residues close to it, could be involved in the formation of the secretion signal necessary to direct the protein to the outer membrane secretion apparatus. This thesis has shown the effect of generating point mutations within the C-terminal region of proaerolysin on folding and secretion.

An α-helical region in the C-terminal region of proaerolysin

In order to determine what effect, if any, amino acid substitutions in the C-terminal region would have on folding and secretion, I replaced a series of amino acid residues with alanine, one at a time. Alanine was chosen because it does not alter the main chain conformation and does not impose extreme electrostatic or steric effects (Morrison and Weiss, 2001). Furthermore, alanine is the most abundant amino acid in proteins and it is found in both buried and exposed positions and in all varieties of secondary structure (Kasturi, *et al.*, 1992). Using this approach I identified a region within the C-terminus of proaerolysin (residues 452 – 457) that was very sensitive to change (Fig. 20). Each of the alanine variants accumulated within the cells (Fig. 21), although all were folded correctly, as evidenced by the inability of trypsin to degrade them (Fig. 23). Mutating Phe-457 and Leu-452 had the most significant effect on secretion. The levels of these variants were more than three times lower than the level of wt proaerolysin in the culture supernatant (Fig. 22).

The crystal structure of proaerolysin (Parker, *et al.*, 1994) indicates that the C-terminal peptide adopts a β-strand-helix-β-strand conformation, and that the helix is formed by residues 449 – 455 (Fig. 26). While Leu-452 is located within this helix, Phe-457 is located outside it (Fig. 27). The aromatic side-chain of Phe-457 is stacked up against the β-strands in domain 4, and this interaction is lost when the residue is mutated to an alanine (Fig. 28). This mutation could possibly disrupt the interaction of that aromatic ring with the β-strands of domain 4, resulting in a protein that cannot be efficiently translocated across the outer membrane. One reason for inefficient secretion of the variant could be incorrect folding. Surprisingly, this is not seen to be the case, since the protein appears to fold correctly, as evidenced by the fact that it is cut only once by trypsin and no further degradation follows. It should be noted that a comparison of the sequence of C-terminal regions of aerolysin from various *Aeromonas* spp. shows that the phenylalanine at position 457 and the leucine at 452 seem to be conserved (Table 9). It would be interesting to see if a similar change in the C-terminal region of proaerolysin from the other species would show similar results.

Histidines in the C-terminal peptide and folding and secretion

Further information on the C-terminal region was obtained from the introduction of histidines in various areas of the C-terminal peptide. Histidine is an amino acid with a positively charged R group and it was anticipated that the introduction of six histidines would interfere with the ability of the protein to fold correctly or directly impede its secretion across the outer membrane. However, the results showed otherwise. Whether the histidines were introduced in a region following the trypsin cut site (D435His) or at

the extreme end of the C-terminus (HCT and EndHis) the protein was secreted with the same efficiency as wt proaerolysin (Figs. 9 and 11). Moreover, all three proteins were cut by trypsin only once, just as the wt protein was (Figs. 10 and 12). Comparison of the hemolytic activity of trypsin-treated culture supernatants obtained from CB3 cells containing any of the three variants with culture supernatant of CB3:pNB5 showed no difference.

Secretion is not possible without the C-terminal peptide

While internal deletions and amino acid substitutions made within the C-terminus of proaerolysin showed that this region of the protein is involved in the folding and secretion of the protein, the most revealing data was obtained when a variant was generated by completely deleting the C-terminal peptide. The variant End426/H132D (the entire C-terminal peptide is deleted) could not be detected either in the periplasm of CB3 cells or the culture supernatant (Fig. 13). Removal of the C-terminal end could conceivably have impaired translation of the variant by CB3 but this was probably not the case, since the protein could be detected within *E. coli* DH5α cells (Fig. 14). Alternatively, the protein may be extremely unstable without its C-terminal peptide and so undergoes proteolytic degradation before it crosses the inner membrane via the Sec machinery and so could not be detected by anti-aerolysin antibodies. This is not a plausible explanation since proaerolysin is cotranslationally translocated across the cytoplasmic membrane and the C-terminal region is the last part of the protein to be translated. Aerolysin from *A. sobria* was also shown to be unstable when variants were generated by deleting amino acid residues from the C-terminal end of the protein

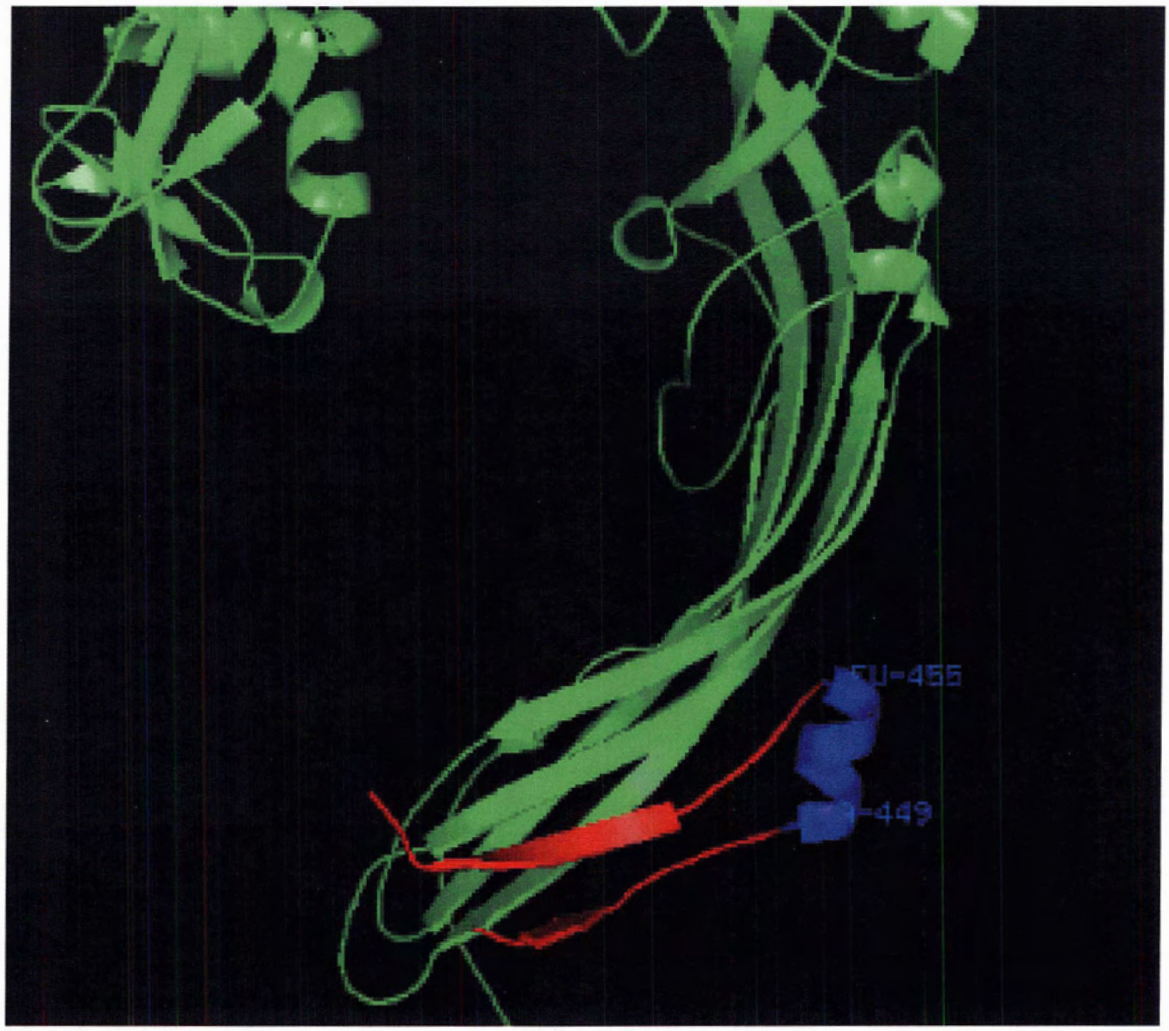


Figure 26. The C-terminus of proaerolysin (in red) showing the α-helix (in blue) composed of residues 449-455.

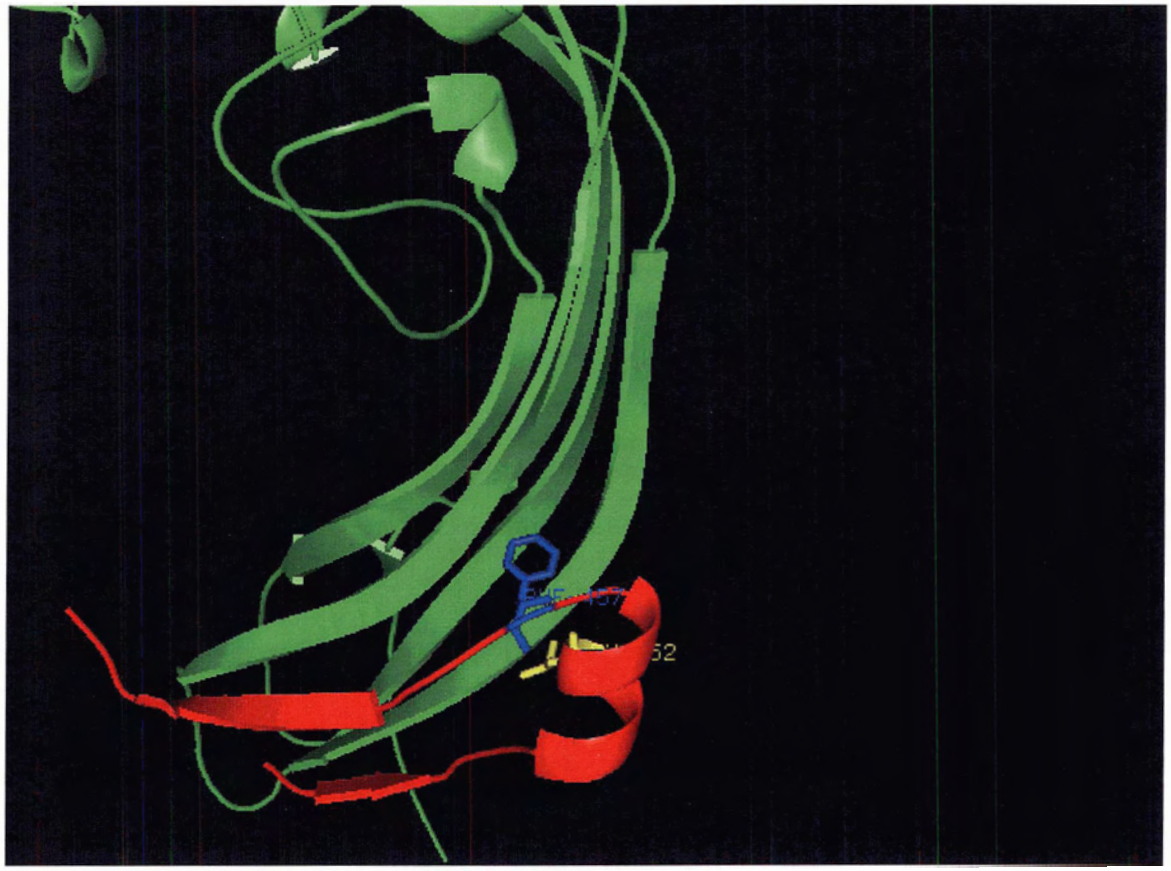
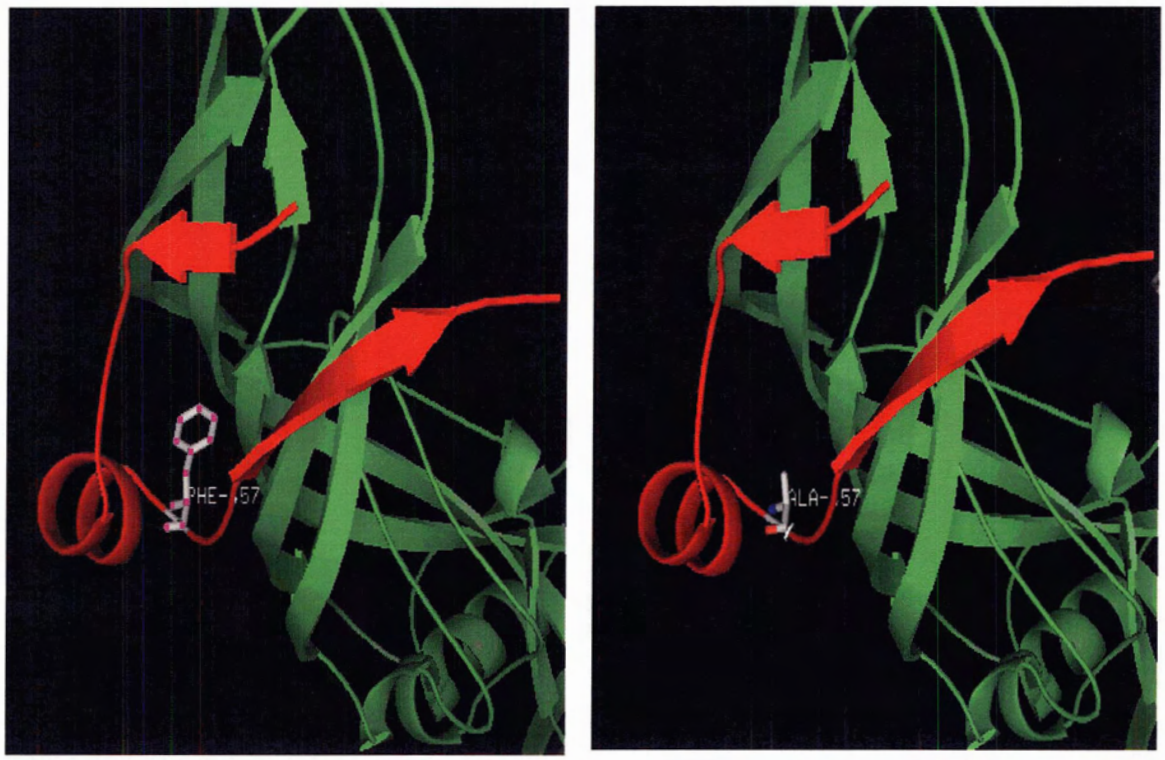


Figure 27. Position of Phe-457 (in blue) and Leu-452 (in yellow) in the C-terminal region (in red).



Wild type proaerolysin F457A

Figure 28. Comparison of wild type proaerolysin with F457A.

Our proaerolysin	KVRRARSVDGAGQGLRLEIPLDAQELSGLGFNNVSLSVTPAANQ
A. hydrophila aerolysin precursor	KVRRARSVDGAGQGLRLEIPLDAQELSGLGFNNVSLSVTPAANQ
A. hydrophila proaerolysin, Chain A	KVRRARSVDGAGQGLRLEIPLDAQELSGLGFNNVSLSVTPAANQ
A. hydrophila hemolysin	KVRRTRSVDGAGQGLRLEIPLDAQELSGLGFSNVSLSVTPAANQ
A. hydrophila aerolysin precursor	KVRRARSVDGAGQGLRLEIPLDREELSGLGFNKSASA-------
A. hydrophila cytolytic enterotoxin	KAPRALSARRGEQGLRLEIPLDAQELSGLGFSNVSLSVTPAANQ
A. hydrophila aerolysin 4 precursor	HSSRARNLSAG-QGLRLEIPLDAQELSGLGFNNVSLSVTPAANQ
A. hydrophila aerolysin 3 precursor	HSSRARNLSAG-QGLRLEIPLDAQELSGLGFNNVSLSVTPAANQ
A. punctuta hemolysin	KVRRARSVDGQTGGLRLEMPLDASELSALGFDNVQIKLEPATDQ
A. enteropelogenes aerolysin precursor	KVRRTRSVDGANTGLKLDIPLDAQELAELGFENVTLSVTPARN-
A. sobria hemolysin	AQLRS---ASADEVALTGVDLDSEALANEGFGNVSLTIVPVQ—
A. sobria aerolysin precursor	AQLRS---ASAEEVALTSVDLDSEALANEGFGNVSLTIVPVQ—
A. salmonicida aerolysin precursor	AQLRS---ASAEEVALTSVDLDSEALANEGFGNVSLTIVPVQ——

Table 9. Comparison of C-terminal sequence of aerolysin from different sources.

(Nomura, *et al.*, 2000). Amazingly, when End426 was co-expressed with the C-terminal peptide, some of the protein was able to cross the outer membrane and could be detected in the culture supernatant (Fig. 16).

The C-terminal peptide as an intramolecular chaperone

Other studies have also shown relationships between propeptides and secretion. Like aerolysin, elastase is secreted by *Pseudomonas aeruginosa*, via the type II secretion pathway, as a proprotein. However, unlike the C-terminal peptide of proaerolysin, the elastase propeptide is cleaved off by autoproteolysis in the periplasm (McIver, *et al.*, 1991; 1993). This cleaved propeptide remains non-covalently associated with mature elastase in the periplasm (Kessler and Safrin, 1988) and it is this propeptide-elastase complex that is transported across the outer membrane (Kessler, *et al.*, 1998). Although there are differences between the fates of proaerolysin and elastase once the proteins are in the bacterial periplasm, it has been shown that like the proaerolysin C-terminal peptide, the N-terminal propeptide of elastase plays a significant role in the folding and secretion of the protein (Braun, *et al.*, 1996). When elastase is expressed without the propeptide, the protein is not secreted into the culture supernatant and the periplasmic protein is degraded over time, indicating incorrect folding. However, when Braun, *et al.*, expressed the propeptide, as a separate polypeptide, in trans with mature elastase active elastase was formed. This has been taken to indicate that the elastase propeptide functions as an intramolecular chaperone.

Intramolecular chaperones (IMCs) were first proposed in studies of prokaryotic and eukaryotic proteases, most of which are synthesized with propeptides, in order to control undesired proteolysis (Wandersman, 1989). It has been proposed that covalently linked propeptides can be regarded as IMCs since they are essential for proper folding of the enzymes to which they are attached (Inouye, 1993; Shinde and Inouye, 1993; Shinde and Inouye, 2000). This thesis has demonstrated the ability of the proaerolysin C-terminal peptide to direct the secretion of the variant lacking the C-terminal region when co-expressed. This would imply that the C-terminal peptide of proaerolysin, like the elastase propeptide, could also function as an IMC.

Since my results appear to show that the C-terminal peptide of proaerolysin could direct the secretion of an otherwise non-secreted variant, End426, across the outer membrane, it was of interest to see if this peptide could direct the secretion of a periplasmic protein, alkaline phosphatase (PhoA), across the outer membrane. PhoA is an *E. coli* periplasmic protein (Reid and Wilson, 1971) that has been used as a reporter enzyme, fused to the N-terminal portions of other proteins in order to detect targeting signals those proteins might contain (Hoffman and Wright, 1985; d'Enfert and Pugsley, 1987; Manoil, *et al.*, 1990; Bina, *et al.*, 1987). Previous studies have shown that when fusions between portions of *aerA* and *phoA* are created, the PhoA portion of the hybrid can be detected outside the cells when expressed in CB3 (Wong and Buckley, 1993). None of the AerA-PhoA fusion proteins could be secreted by the bacteria; all hybrid proteins are reduced to proteins corresponding to PhoA itself. It was proposed that periplasmic proteases of *A. salmonicida* degraded the AerA portion due to an abnormal conformation and its possible

inability to dimerize. In short, CB3 can secrete PhoA under certain conditions. One of these hybrid proteins, PAD3, contained only the first two amino acids of proaerolysin and full-length PhoA. It was seen that CB3 cells harboring pAD3 could only secrete the variant across the outer membrane efficiently under alkaline conditions. As the pH of the culture medium became acidic, the amount of PAD3 in the culture supernatant decreased. In contrast, secretion of native proaerolysin is not affected by pH. I was interested to see if the presence of the C-terminal peptide could direct secretion of PAD3 across the outer membrane regardless of pH of the culture medium. However, the PhoAfusion could not be detected in either the cells or the culture supernatant obtained from CB3 cells harboring the fusion protein (Fig. 17). This was surprising since the fusion protein was expressed by *E. coli* DH5α cells (Fig. 18). Studies with pullulanase have shown that a Pul-PhoA hybrid protein could not be secreted (Kornacker and Pugsley, 1990; Sauvonnet, *et al.*, 1995). It was proposed that this was due to the dimerization of PhoA hybrid in the periplasm. While this might explain the absence of PhoAfusion in the culture supernatant, it does not explain the absence of the hybrid within the cells. Therefore, it will be of interest to do further studies to determine the fate of the hybrid protein in *A. salmonicida* CB3 cells.

SUMMARY

The results presented in this thesis have clearly shown a correlation between folding and secretion of proaerolysin. Variants containing deletions within the C-terminal region of the protein were seen to be folded incorrectly and were, as a result, impaired in their ability to be secreted by *A. salmonicida* CB3 cells.

A region within the C-terminal peptide has been identified that is clearly more sensitive to change than residues in the remainder of the peptide. Introduction of a larger number of histidine residues outside this region did not have any effect on folding or secretion.

FUTURE DIRECTION

It would be interesting to see if the alanine substitutions made within the C-terminal end of proaerolysin from *A. hydrophila* would have a similar effect if the substitutions are made in proaerolysin from other *Aeromonas spp.* Residues that interact with the phenylalanine at position 457 also need to be explored. The α-helix of interest needs to be explored, to see if disruption of the helix would affect folding and secretion.

Further studies need to be done to determine the fate of the End426 variant. Pulse chase experiments can be performed to see if the End426 variant is degraded before it is translocated across the inner membrane or if it gets degraded in the periplasm after crossing the cytoplasmic membrane. It also needs to be seen whether the End426+C

variant that is secreted into the culture supernatant by CB3 cells is stable by shocking CB3 cells containing the mutated aerA gene in the presence of trypsin.

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