Overexpression and Secretion of Proaerolysin by *Aeromonas salmonicida*.

by

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ABSTRACT

Aerolysin, a pore-forming toxin produced by *Aeromonas* species, is secreted as an inactive protoxin (proaerolysin) *via* the General Secretion Pathway. Recombinant PCR was used to generate proaerolysin variants containing internal deletions in key regions of the protein. This provided a means by which to study the role these protein domains play in proaerolysin secretion. Three proaerolysin variants could not be secreted by *A. salmonicida* and were shown to fold incorrectly within the cell.

Overexpression of proaerolysin by *A. salmonicida* resulted in an increase in toxin secretion into the extracellular medium and an accumulation of toxin within the cells. Further examination carried out using pulse-chase analysis indicated that the cells contain two distinct pools of proaerolysin; one that is rapidly secreted by the cell and a second that is not readily available for secretion. Electron microscopic examination of *A. salmonicida* indicated that intracellular proaerolysin accumulates within an enlarged periplasm. The results obtained suggest that there is a limit to the amount of protein that can be efficiently exported from the cell *via* the General Secretion Pathway.

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LIST OF ABBREVIATIONS

Abbreviation	
A	Alanine
ABC	ATP-binding cassette
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
BSA	Bovine serum albumin
C	Cysteine
cAMP	cyclic adenosine monophosphate
Ci	Curie
C-terminal	Carboxy-terminal
Ctxb	Cholera toxin subunit B
D	Aspartic acid
Da	Daltons
DNase	Deoxyribonuclease
dNTPs	deoxynucleoside triphosphates
DSP	Dithiobis(succinimidylpropionate)
DNA	Deoxyribonucleic acid
E	Glutamic acid
EDTA	Ethylenediaminetetraacetic acid
F	Phenylalanine
Fig	Figure
G	Glycine
GSP	General secretion pathway
HBS	Hepes buffered saline
HEPES	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethane sulfonic acid])
I	Isoleucine
ICDH	Isocitrate dehydrogenase
IPTG	Isopropyl-β-D-thiogalactoside
K	Lysine
kDa	kiloDalton

kv	Kilovolts
LB	Luria Bertanii
L	Leucine
M	Methionine
mRNA	Messenger ribonucleic acid
N	Asparagine
nm	Nanometer
N-terminal	Amino-terminal
OD	Optical density
ON	Overnight
P	Proline
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PT	Pertussis toxin
Q	Glutamine
R	Arginine
RNase	Ribonuclease
rpm	revolutions per minute
RT	Room temperature
RTX	Repeats in toxin
SAC	*Staphylococcus aureus*
scFv	Single chain antibody
SDS	Sodium dodecyl sulfate
SDS PAGE	SDS-polyacrylamide gel electorphoresis
T	Threonine
Tris	Tris-(hydroxymethyl)aminomethane
Triton X-100	t-Octylphenoxypoly-ethoxyethanol
Tween 20	Polyoxyethylenesorbitan monolaurate
V	Valine
Y	Tyrosine

Yops	Yersinia outer membrane proteins
W	Tryptophan
wt	Wild-type
w/v	Weight per volume
w/w	Weight per weight

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DEDICATION

To Mum and Dad

INTRODUCTION

In Gram-negative bacteria, the secretion of proteins from the cytoplasm to the extracellular environment faces two hydrophobic barriers - the cytoplasmic or inner membrane and the outer membrane. At least five different systems have evolved by which bacteria transport proteins across these barriers. These systems may entail passage of the two bacterial membranes simultaneously. In this way, proteins are transported directly from the cytoplasm into the extracellular milieu, thereby omitting a periplasmic intermediate. Alternatively, protein secretion systems may involve passage through the membranes in two distinct steps. Such two-step secretion involves transport of the substrate protein across the inner membrane *via* the well-characterized Sec system (recently reviewed by Manting and Driessen, 2000). Accordingly, two-step secretion is often termed Sec-dependent secretion. Conversely, one-step secretion systems, which do not utilize the Sec machinery, are referred to as Sec-independent. The purpose of this literature review is to familiarize the reader with the five major secretion systems found within the Gram-negative bacteria.

SEC-INDEPENDENT SECRETION

Type I Secretion

The first Type I secretion system to be identified was that of the *Escherichia coli* α-haemolysin (Mackman and Holland, 1984). Since that time, Type 1 secretion has been found to be responsible for the secretion of numerous other proteins in a variety of bacteria. Examples include the *Pseudomonas aeruginosa* alkaline protease (AprA;

Duong *et al.*, 1992), *Erwinia chrysanthemi* metalloprotease (PrtA; Letoffe *et al.*, 1990), *Serratia marcescens* metalloprotease (PrtSM; Letoffe *et al.*, 1991), and *Caulobacter crescentus* surface layer protein (RsaA; Awram and Smit, 1998). Still, secretion of the *E. coli* haemolysin remains the prototype for Type I secretion and as such, will be discussed here.

α-Haemolysin (HlyA) is a 110 kDa protein produced by uropathogenic *E. coli* species. It is a member of the 'repeats in toxin' or RTX class of proteins, so named because of the presence of a repetitive nine amino acid sequence rich in glycine and aspartate residues (reviewed by Coote, 1992). HlyA, produced as a nontoxic prohaemolysin, is activated by the addition of a fatty-acyl group carried out by the acyltransferase HlyC (Issartel *et al.*, 1991). However, this acylation is not required for the secretion of the toxin (Nicaud *et al.*, 1985).

The Signal Sequence

Early studies of the α-haemolysin toxin indicated that the protein does not contain a typical amino-terminal (N-terminal) signal sequence (Felmlee *et al.*, 1985), a signal that could act as a tag identifying the protein for export. Instead, studies carried out by Gray and colleagues (1986) found the carboxy-terminus (C-terminus) of the toxin was critical to its secretion. Indeed the removal of only 27 amino acids from this end of the protein reduced its secretion to less than 6% of that of the wild type protein (Gray *et al.*, 1986), although the haemolytic activity of the protein still remained. Subsequent studies involving deletions and point mutations within the C-terminus of the protein have found the region required for secretion of the haemolysin lies within the last 40 to 50 amino

acid residues (Stanley *et al.*, 1991; Kenny *et al.*, 1992; Kenny *et al.*, 1994). Additional experiments performed by Koronakis *et al.* (1989) involving secretion of a short C-terminal peptide indicated that the haemolysin signal is not removed during export.

While the signal for haemolysin secretion has been mapped to the C-terminal domain, the nature of the signal remains unclear. Comparison of the C-terminal domain from *E. coli* α-haemolysin with those from analogous haemolysins produced by *Proteus vulgaris* and *Morganella morganii* reveals very little primary sequence homology (Koronakis *et al.*, 1989). Similar results were found upon comparison of *E. chrysanthemi* proteases and *E. coli* haemolysin (Letoffe *et al.*, 1990). However, subsequent analyses of these proteins and other related haemolysins and proteases secreted by Type I systems have led to the conclusion that there are structural features present in the C-terminal domain that are common to many members of this family (Stanley *et al.*, 1991; Kenny *et al.*, 1992). These include a flexible amphipathic helix, a cluster of charged residues, and a hydrophobic sequence at the extreme C-terminus that is rich in hydroxylated residues (Stanley *et al.*, 1991; Kenny *et al.*, 1992). There appear to be critical residues within these structures that are essential for α-haemolysin secretion, namely E-978, D-990, D-1009. It is proposed that these residues form contact sites with the translocator proteins thereby promoting haemolysin secretion (Kenny *et al.*, 1992).

The Secretion Machinery

The machinery responsible for carrying out Type I secretion consists of only three proteins: a protein containing an ATP-binding cassette (ABC), a membrane fusion protein (Dinh *et al.*, 1994) and an outer membrane protein (Paulsen *et al.*, 1997). In the

case of *E. coli* α-haemolysin, these proteins are termed HlyB, HlyD, and TolC respectively.

The HlyB protein is a member of the ABC superfamily, proteins that are responsible for the transfer of a variety of solutes both into and out of the cell (reviewed by Higgins, 1992). The protein contains a cytoplasmic ABC domain harbouring the ATP-binding sites (Walker boxes A and B) fused to an integral membrane domain (Koronakis *et al.*, 1995). As part of the haemolysin translocator, HlyB plays a role in the recognition of the haemolysin secretion signal and hydrolyses ATP as a means to provide energy for the toxin's secretion (Binet and Wandersman, 1995; Koronakis *et al.*, 1995).

HlyD is anchored to the inner membrane by a single transmembrane domain; its large C-terminal domain is thought to span the periplasmic space (Schulein *et al.*, 1992). In this way it is able to interact with both HlyB and TolC (located in the outer membrane). Findings by Thanabalu and colleagues (1998) have indicated that HlyB and HlyD interact with each other in the absence of haemolysin, thereby forming a complex within the inner membrane. Subsequent binding of the toxin to the HlyB/HlyD complex then triggers an association between HlyD and TolC, forming a transmembrane channel.

For its part, TolC consists of a β-barrel domain anchored in the outer membrane and a hydrophilic C-terminal domain that extends into the periplasm. The recently solved crystal structure of TolC (Koronakis *et al.*, 2000) has confirmed a previous hypothesis that TolC forms trimers in the outer membrane (Koronakis *et al.*, 1997). In doing so, the three TolC molecules come together to form a continuous channel that spans the outer membrane as well as the periplasmic space. This channel, approximately 14 nm in length, is sealed at the periplasmic end by a set of coiled helices. Koronakis and

colleagues (2000) suggest that the recruitment of TolC to the HlyA/HlyB complex in the inner membrane (the result of substrate binding to the complex) triggers rotation of the coiled helices thereby opening this end of the channel. The net result of these events is the formation of a conduit that connects the cytoplasm to the external environment. Once the substrate HlyA has passed through the channel, the machinery disassembles and the three components (TolC, HlyB, and HlyD) return to their resting states (Thanabalu *et al.*, 1998).

TYPE III SECRETION

Type III secretion, also termed contact-dependent secretion, provides another means by which cells can secrete proteins across the cell envelope in a single step. The secreted proteins are either released into the extracellular environment or injected directly into the cytosol of a eukaryotic or target cell. While type III systems have been found in a number of bacteria including *Pseudomonas* (Yahr *et al.*, 1996), *Salmonella* (Collazo *et al.*, 1997), *Shigella* (Menard *et al.*, 1996), *Bordetella* (Yuk *et al.*, 1998), and *Escherichia* species (Jarvis *et al.*, 1995), perhaps the best characterized are in the pathogenic *Yersinia*, namely *Y. entercolitica*, *Y. pestis* and *Y. pseudotuberculosis*. In these organisms, a Type III system is responsible for the secretion of 12 Yersinia outer membrane proteins or Yops (Michiels *et al.*, 1990) so named for their initial detection in *Yersinia* outer membrane preparations (Bolin, *et al.*, 1985).

The Yops can be grouped into three categories based on their functions (Hueck, 1998). The first category represents a group of effector proteins. When injected into the cytosol of target cells, these proteins (YopE, YopH, YopM, YopO, YopP, and YopT)

adversely affect the functions of the cell. For example, YopE causes disruption of the microfilament structure of the host cells (Rosqvist *et al.*, 1991) whereas YopH inhibits phagocytosis in neutrophils and macrophages (Ruckdeschel *et al.*, 1996). The second category is comprised of the Yop translocators, proteins that play a role in the translocation of the effector Yops into the target cells. Finally, YopN is in a category by itself, playing a role in the regulation of Yop secretion.

The Yop Secretion Signal

Secretion of the Yops was first described by Michiels *et al.* (1990) who established that it did not involve cleavage of an N-terminal signal sequence nor did it involve recognition of a C-terminal domain on the substrate protein. However, further studies by Sory *et al.* (1995) did indicate that the first 15 and 17 amino acid residues of YopE and YopH respectively were involved in the secretion of these proteins. Subsequent examination of the N-terminal regions of different Yops did not reveal any common amino acid sequence that could act as a potential secretion signal. In 1997, Anderson and Schneewind demonstrated that systematically changing codons within the N-terminal region of YopE and YopN did not affect the secretion of either protein. Furthermore, frameshift mutations that modified the peptide sequence in the N-terminal region produced altered polypeptides that were still secreted. This led to their suggestion that the secretion signal is actually found within the 5′ end of the mRNA.

While the mRNA signal is involved in secretion of the Yops, it is not the only secretion signal in this system. In 1997, Cheng *et al.* demonstrated that a second secretion signal is present in at least some of the Yops. In their experiments, these

researchers found that fusion of residues 15 to 220 of YopE to a reporter protein resulted in secretion of the hybrid protein (albeit at low levels), thereby indicating that the N-terminal signal present within the first 15 codons of YopE is not required for all YopE secretion. Cheng and colleagues suggested that a second secretion signal exists within YopE. They were able to map this signal to amino acids 15 to 100, a region that corresponds to the binding site of a small chaperone protein, SycE (Specific Yop Chaperone) and they demonstrated that the chaperone must be bound to this domain if the signal is to be successful. It is thought that YopH, YopN, and YopT also possess this second signal (Cornelis, 1998) since chaperones specific for each of these proteins have been identified, namely SycH, SycN, and SycT.

The Secretion Machinery

The Type III secretion apparatus found within pathogenic *Yersinia* is comprised of the Ysc proteins (Yersinia secretion complex). Together, these proteins form a continuous channel across the inner and outer membranes of the bacterial cell through which the Yops are secreted. *Yersinia* carrying mutations in any of the *ysc* genes lose the ability to translocate the effector Yops (Cornelis, 1998). While the functions of the majority of these Yops are unknown, potential roles for a few of them have been determined. For example, YscN, which contains an ATP-binding site is thought to provide energy for the secretion process (Woestyn *et al.*, 1994). YscJ is thought to connect the inner and outer membranes of the cell in a manner similar to that of its counterpart found in *P. syringae* (Cornelis, 2000).

The YscC protein displays sequence homology to a family of outer membrane proteins termed secretins (Genin and Boucher, 1994). These proteins are involved in the transport of large molecules across the outer membrane in several diverse pathways including Type II secretion (see below), DNA uptake, and the release of filamentous phage. Because of this homology, YscC is thought to play a central role in the movement of Yops across the outer membrane. Like other secretins, YscC has been shown to form large multimeric complexes in the outer membrane (Plano *et al.*, 1995). Visualization of these complexes by electron microscopy has indicated that they are ring-shaped structures approximately 20 nm in diameter with an apparent central pore measuring 5 nm in diameter (Koster *et al.*, 1997). Insertion of YscC into the outer membrane requires the presence of the lipoprotein YscW (Koster *et al.*, 1997).

Other components of the secretion machinery, YscO, -Q, -R, -S, -T, -U, and -V, as well as the YscN and YscJ proteins previously mentioned, display sequence homology to proteins found within the bacterial flagellum. Specifically, these homologous proteins comprise the MS ring, and the C ring of the basal body, as well as the flagellar ATPase (Cornelis, 2000). For this reason, it is thought that the Ysc components assemble into a complex that is structurally similar to the flagellum. Such as assumption has been supported by direct examination of the Type III secretion apparati of *Salmonella typhimurium* and *Shigella flexneri* by electron microscopy (Kubori *et al.*, 1998; Tamano *et al.*, 2000). Each apparatus resembles a syringe or needle, consisting of a thin rod that is supported by a wider base. The base contains two lower rings that interact with the inner membrane, as well as two upper rings that interact with both the peptidoglycan and

the outer membrane. The rod or needle projects outwards from the cell. Such a complex has yet to be visualized in *Yersinia* species.

Translocator Yops

While the Ysc proteins are responsible for movement of the Yops across the bacterial membranes, translocation of the effector Yops across the membrane of a eukaryotic cell is mediated by YopB and YopD, the translocator Yops. Early studies carried out by Hakansson *et al.* in 1993 indicated that YopB and YopD are distinct from the remaining Yops in that they contain hydrophobic domains. This suggested that these Yops are able act as transmembrane proteins. In addition, the C-terminus of YopD was shown to contain an amphipathic domain. This is an interesting observation as amphipathic helices have previously been shown to be important features of antimicrobial peptides, molecules capable of forming channels in artificial membranes (Boman, 1991). Similarly, YopB has be found to share moderate sequence homology to another group of proteins, the RTX family (see above) comprised of proteins which are also capable of forming pores in target membranes. These results suggested YopB and YopD might be capable of forming pores in cell membranes.

Since these early studies, several other studies have confirmed the ability of YopB and YopD to form channels in the membranes of target cells. *Y. pestis* has been shown to lyse sheep erythrocytes, a characteristic that is dependent upon direct contact between the bacterium and the erythrocyte and upon the presence of YopB (Hakansson *et al.*, 1996). Infection of macrophages with *Y. enterocolitica* renders them permeable to small dyes (Neyt and Cornelis, 1999); a YopB and YopD dependent process. As well, YopB and

YopD are required for *Y. entercolitica* to induce channel formation in artificial lipid membranes (Tardy *et al.*, 1999).

Other studies have shown that YopB and YopD are essential for the translocation of the effector Yops into target cells. A key study published by Sory and Cornelis in 1994 reported the creation of hybrid proteins consisting of the N-terminal domains of various Yops fused to the catalytic domain of calmodulin-dependent adenylate cyclase. Infection of eukaryotic cells with *Y. entercolitica* expressing these hybrid proteins resulted in the accumulation of cyclic adenosine monophosphate (cAMP) within the target cells. As there was no calmodium present in either the culture medium or in the bacterial cell, the accumulation of cAMP seen in the eukaryotic cells indicated the presence of the fusion protein within the target cell. Such accumulation of cAMP within the target cell only occurred in the presence of YopB and YopD. These data coupled with observations that YopB and YopD are able to form pores in the membranes of target cells suggest that pore formation by YopB and YopD is related to effector translocation. YopB and YopD are thought to form a channel in the membrane of the target cell through which the effector Yops pass.

YopN

YopN, found on the surface of *Yersinia* (Forsberg *et al.*, 1991) has been implicated in regulating Yop translocation. Forsberg and colleagues (1991) have found that under high Ca^{2+} conditions, conditions that usually prevent Yop secretion, *yopN* mutants secreted large quantities of Yops into the culture medium. Other studies conducted by Rosqvist *et al.* (1994) and Persson *et al.* (1995) examined the location of

YopE and YopH after contact between *Y. pseudotuberculosis* and HeLa cells. Their results indicated that when expressed by wild type *Y. pseudotuberculosis*, these effector Yops were found in the cytosol of the target cells. However, when expressed in *yopN* mutants, the majority of the YopE and YopH proteins were found within the culture supernatant. It is now thought that YopN may have a role in regulating the opening and closing of the secretion channel in response to host factors.

SEC-DEPENDENT SECRETION

The Sec System

The Sec secretion system is found within members of the Gram-negative and Gram-positive bacteria. This review however, will deal with the Sec system found within *E. coli*. In this and in other Gram-negative bacteria, the Sec system is responsible for export of proteins across the inner membrane and into the periplasmic space. The Sec system is well characterized and a great deal is known regarding the functions of its components. Briefly, this system includes seven Sec proteins, the Lep protease and YajC, a protein whose function remains unknown. SecY, SecE, and SecG are integral inner membrane proteins. Recent evidence suggests that four SecYEG trimers come together to form a channel or 'translocase' through the inner membrane (Manting *et al.*, 2000). Peripherally associated with the SecYEG complex on the cytoplasmic side, is the SecA ATPase (Hartl *et al.*, 1990; Oliver , 1993). This protein, which is also found free in the cytoplasm, plays a pivotal role in the translocation of secretory proteins. Another cytoplamsic protein, SecB, acts as a chaperone to the secretory protein. SecD and SecF, both integral inner membrane proteins with large periplasmic domains, form a complex

with YajC. Finally, the Lep protein is responsible for the removal of the signal sequence found on all proteins that are secreted by this pathway. The following is a brief overview of the process that leads to translocation of a secreted protein across the inner membrane *via* the Sec system.

Targeting

Proteins exported by the Sec system contain a 20 - 30 amino acid signal sequence or leader peptide that is located at the extreme N-terminus of the protein. While there is no primary sequence similarity among the leader peptides of secreted proteins, all are composed of a stretch of positively charged amino acids followed by a hydrophobic core (Randall and Hardy, 1989). In addition, the C-terminal end of the leader peptide contains a proteolytic cleavage site that enables it to be removed from the substrate protein following its passage across the inner membrane. Proteins that contain a signal sequence are termed preproteins. The signal sequence carries out two important functions. First, it slows the folding rate of the secreted protein (Park *et al.*, 1988) and in doing so, enables binding of the cytosolic chaperone SecB to the mature protein domain. Second, the signal sequence recognizes and binds to SecA. This binding is required for efficient transfer of the preprotein to the translocase present in the inner membrane (Fekkes *et al.*, 1998).

Binding of the preprotein to SecB occurs as the polypeptide chain is formed on the ribosome (Kumamoto and Francetic, 1993) and it prevents further folding of the protein. Thus, SecB maintains the preprotein in a translocation-competent state (Driessen *et al.*, 1998). However, SecB is also responsible for targeting the preprotein to

membrane-associated SecA (Hartl *et al.*, 1990). As SecB binds to the mature domain of the preprotein, the signal sequence is free to recognize and bind SecA . Binding of the signal sequence to SecA in turn stimulates binding of SecB to SecA. Upon binding to SecA, SecB releases the preprotein, effectively delivering it to SecA. Despite the fact that the signal itself is able to recognize and bind SecA, disruption of the SecA/SecB interaction by removal of the SecB binding site on SecA or alternatively, by introducing mutations in the SecA binding site on SecB, abolishes translocation of the secretory protein (Fekkes *et al.*, 1998). This result indicates that the binding of SecB to SecA is critical for effective translocation of the preprotein.

Translocation

SecB is released from the SecB/SecA/preprotein complex upon binding of ATP to one of the two ATP-binding sites present on SecA (Fekkes *et al.*, 1997). At the same time, the energy of ATP binding drives the insertion of SecA and approximately 25 amino acids of the preprotein into the inner membrane. This membrane insertion is thought to occur at the SecA/Y interface (Scotti *et al.*, 2000) and is stabilized by SecD and SecF (Economu *et al.*, 1995). Hydrolysis of ATP then results in the release of the presecretory protein from SecA. Deinsertion of SecA from the membrane can occur upon the binding and hydrolysis of another molecule of ATP at the second ATP-binding site on the SecA molecule. After deinsertion, SecA is able to rebind the substrate protein, allowing translocation of another segment of approximately 25 amino acids. In this way, repeated cycles of ATP binding and hydrolysis thread the precursor protein through the SecYEG translocase. SecD and SecF are thought to assist in this process by preventing

backward movement of the preprotein (Duong and Wickner, 1997). The proton motive force across the inner membrane has also been shown to drive further translocation of the precursor protein through the inner membrane (Schiebel *et al.*, 1991; Driessen, 1992). While the mechanism by which this happens remains unclear, it is dependent upon SecD and SecF (Arkowitz and Wickner, 1994).

Release

As the substrate protein is translocated through the SecYEG translocase, the proteolytic cleavage site found between the leader peptide and the mature protein domain becomes exposed on the periplasmic side of the inner membrane. This enables cleavage of the leader peptide by the Lep protein (Paetzel *et al.*, 1998). Once this has occurred, the mature protein is no longer attached to the membrane and is free to fold, taking on secondary and tertiary structure. As antibodies against SecD have been shown to prevent the release of translocated proteins in spheroplasts, it is assumed that the SecDF-YajC complex may also play a role in the release of the substrate protein (Matsuyama *et al.*, 1993). It has been suggested that this complex is also involved in closing the translocase once the secretory protein has been released into the periplasm, thereby preventing the nonspecific movement of other molecules through the membrane channel, although such a role has recently been suggested for SecA (Manting *et al.*, 2000).

Once translocation across the inner membrane is complete, the substrate protein may remain in the periplasm, may be incorporated into the outer membrane or may be secreted across the outer membrane and released into the extracellular environment. The

latter occurs *via* one of several branches of the General Secretion Pathway or GSP. Three such branches are discussed below.

The Autotransporters

The term autotransporters is used to describe a group of proteins that do not require accessory proteins for passage from the periplasm to the extracellular environment. Instead, all the information need for such transit is contained within the proteins themselves. Examples of such proteins include the *Neisseria gonorrhoeae* IgA1 protease (Pohlner *et al.*, 1987), the vacuolating toxin of *Helicobacter pylori* (Schmitt *et al.*, 1994), and the EspC protein produced by enteropathogenic *E. coli* (Stein *et al.*, 1996).

The majority of the information presently available on autotransporters has come from studies involving the *N. gonorrhoeae* IgA1 protease, a protein responsible for the cleavage of the mucosal immunoglobulin A1 (Halter *et al.*, 1984). Like all autotransporters, IgA1 protease is composed of three domains. The first domain, found at the N-terminus of the protein, contains a 27 amino acid signal sequence required for secretion across the inner membrane using the Sec system. The second domain comprises the internal passenger domain or α-domain. It is the passenger domain that is ultimately transported across the outer membrane of the bacterial cell. This transport is mediated by the third domain termed the β-domain, found within the C-terminus of the protein.

Initially, the β-domain inserts into the membrane forming β-barrel structure (Klauser *et al.*, 1993). This generates a hydrophobic coat that contacts the membrane, and a closed hydrophilic interior (Jose *et al.*, 1995). With the aid of an essential linker

region found between the passenger and β-domains, the passenger domain is translocated through the pore structure to the cell surface. The passenger domain of IgA1 protease then mediates its own release into the extracellular environment by autocatalysing its cleavage from the C-terminus; the β-domain remains embedded in the outer membrane (Pohlner *et al.*, 1987). Other autotransporters may be released from the β-domain by independent surface-associated proteases, for example cleavage of the IcsA protein (responsible for actin polymerization) of *S. flexneri* is carried out by SopA, a homologue of the outer membrane protease OmpT (Shere *et al.*, 1997). Likewise in *E. coli*, OmpT is required for the release of recombinant IgA1 polyproteins synthesized in the absence of IgA1 protease activity (Klauser *et al.*, 1992). Still other autotransporters may be retained at the cell surface, for example the Hsr protein of *Helicobacter mustelae* (O'Toole *et al.*, 1994).

Folding of the Autotransporter

A comparison of *Neiserria* IgA1 protease with other autotransporters has indicated that all these proteins contain a number of common features. Among these are a low cysteine content (IgA1 protease contains only two cysteine residues) and the inability to form extensive disulphide loops within the passenger domain (Jose *et al.*, 1995). These two features are thought to be critical for the translocation of the autotransporters, as large disulphide loops could potentially impede passage of the passenger domain through the pore created by the β-domain (Jose *et al.*, 1995).

Such a suggestion has been supported by findings carried out with a hybrid protein in which the B subunit of the *Vibrio cholera* toxin (CtxB) was fused to the β-

domain of IgA1 protease (Jose *et al.*, 1996). CtxB was chosen as the passenger in this study because when expressed in *E. coli*, it is transported into the periplasm but not into the extracellular milieu. In addition, two cysteine residues found within CtxB are able to form a disulphide bridge, thus creating a large loop in the protein. However, the creation of this disulphide bridge will only happen in the presence of the DsbA, a periplasmic enzyme that facilitates the disulphide bond formation (Bardwell *et al.*, 1991). The results generated by these studies indicated that the formation of disulphide bonds within the passenger domain inhibited passage of the hybrid through the outer membrane. In contrast, when such disulphide bonds were unable to form, as in a *dsbA* mutant, secretion of the fusion protein across the outer membrane was uninhibited, suggesting that secretion through the autotransporter pore can only occur when the protein is unfolded.

While these results were in agreement with earlier findings of a study carried out with IgA1-CtxB fusions (Klauser *et al.*, 1992), they are in contrast with more recent findings produced by Veiga and colleagues (Veiga *et al.*, 1999). These researchers studied the secretion of a single-chain antibody (scFv) fused to the β-domain of IgA1 protease. As binding of scFv to target antigens is dependent upon the formation of disulphide bridges within the antibody (Veiga *et al.*, 1999), the use of this fusion enabled analysis of the relationship between secretion and protein folding. The results of the study indicated that the active and therefore folded scFv fusion was successfully translocated across the outer membrane, albeit at a 3-fold lower efficiency than that of the inactive or unfolded protein. In light of these results, the authors suggest that autotransporters need not be completely unfolded for secretion to occur. They further suggest that, as CtxB is known to form pentamers in the periplasm (Hirst and Holmgren,

1987), it is possible that the IgA1-CtxB fusion too may oligomerize. They raise the possibility that such oligomerization and not disulphide bond formation *per se* may have inhibited the secretion of the IgA1-CtxB hybrid reported in the previous studies.

The state of an autotransporter during secretion, whether folded or unfolded, raises important questions. For example, if autotransporters do pass through the outer membrane in an unfolded state, how do they travel through the periplasm without being degraded by periplasmic proteases? How do they escape folding promoted by periplasmic chaperones and if they do, how do they fold outside the cell in the absence of such chaperones (Veiga *et al.*, 1999)? On the other hand, if proteins are at least partially folded before they are secreted, how can they pass through the relatively small pores formed by the β-domain and what provides the energy for such an event? These are questions that future studies of autotransporters will need to address.

Secretion of the Pertussis Toxin - A Unique Type IV Secretion System

The Type IV secretion system is particularly interesting in that it has been shown to be responsible for the secretion of both protein and DNA substrates. The prototype for this system is the *vir* operon of *Agrobacterium tumefaciens*, which governs the transfer of tumour-inducing DNA, or T-DNA, from the bacterium into the nucleus of plant cells (Berger and Christie, 1994). Homologous systems have been found in conjugative plasmids where they are responsible for the transfer of the plasmid during bacterial conjugation. An example of such a system is the Tra system of plasmid pKM101. Protein substrates for the Type IV system have been identified in many pathogenic bacteria including *Legionella pneumophila* (Vogel *et al.*, 1998), *Helicobacter pylori*

(Odenbreit *et al.*, 2000), *Brucella* species (O'Callaghan *et al.*, 1999) and *Bordetella pertussis* (Weiss *et al.*, 1993; Covacci and Rappuoli, 1993) Of these examples, the Type IV secretion system of *Bordetella pertussis* is the best characterized.

Type IV secretion in *B. pertussis* mediates the secretion of pertussis toxin (PT). PT is an oligomeric toxin composed of five subunits (S1 - S5) that are non-covalently linked. The protein is a member of the A-B class of bacterial toxins. The A domain, the enzymaticaly active part of the toxin, contains the S1 subunit, while the B domain, responsible for binding to receptors on the target cell, is made up of subunits S2 - S5. Genetic studies by two groups have shown that each toxin subunit contains a typical N-terminal leader peptide, suggesting that the subunits are directed across the inner membrane *via* the Sec system (Locht and Keith, 1986; Nicosia *et al.*, 1986). This finding is surprising as the homologous Vir and Tra systems mentioned above export their respective DNA substrates directly from the cytoplasm, not the periplasm, suggesting that secretion of PT represents a unique branch of the GSP.

Following export *via* the Sec system, the PT subunits present in the periplasm assemble to form the holotoxin. The S1 subunit associates with a B oligomer made up of one copy of S2, S3, and S5, and two copies of S4. Farizo and colleagues (2000) have reported that neither the S1 subunit nor the B oligomer is efficiently secreted from the cell in the absence of the other. This is consistent with findings by Pizza *et al.* (1990) that indicate the secretion of the B oligomer is greatly reduced in the absence of the S1 subunit. In addition, findings by Nicosia and Rappuoli (1987) demonstrated that secretion of a mutant lacking the S3 subunit is completely abolished.

The *ptl* Genes

The first report of a gene product directly involved in transfer of PT across the outer membrane came in 1993 when Covacci and Rappuoli identified a protein that was essential for the secretion of PT from the periplasm into the extracellular environment (Covacci and Rappuoli, 1993). The protein, termed PtlC (Pertussis toxin liberation), was shown to display sequence homology to VirB4, a protein encoded by the *vir* operon of *A. tumefaciens*. Other findings published near this time (Weiss *et al.*, 1993) identified seven other genes linked to *ptlC*. These were termed *ptlA*, *-B*, *-D*, *-E*, *-F*, *-G*, and *-H*. Six of these genes, *ptlB - H*, were also shown to have sequence homology to members of the *vir* operon, specifically to *virB3*, *-6*, *-8*, *-9*, *-10*, and *-11*. An additional gene involved in the secretion of PT has since been identified by Farizo *et al.* (1996). The identification of this gene, *ptlI*, which is homologous to *virB7*, has brought the total number of genes involved in PT secretion to nine.

The Ptl Proteins

While the genes involved in transport of PT across the outer membrane have been identified, the roles the products of these genes play in PT secretion remain unknown. The Ptl proteins may form a channel or gate across the outer membrane through which the PT can pass. Consistent with this hypothesis are findings that demonstrate PtlC, PltE, PtlF, PtlG, and PtlI are found within the total membrane fraction of the cell while membrane-spanning domains have been found within PtlD (Johnson and Burns, 1994).

Studies of PtlH and PtlC carried out by Burns (1995) have shown that both of these proteins contain nucleotide-binding sites. In addition, the introduction of a point

mutation within the nucleotide-binding site of PtlC inhibits PT secretion (Cook *et al.*, 1999). While these findings suggest that nucleotide-binding domains are important in the secretion process, further work is needed to determine their role. It could be that PtlC and PtlH operate as ATPases, thereby providing the energy required for secretion. Alternatively, they could act as kinases to signal the opening of a gate or channel within the outer membrane (Cook *et al.*, 1999).

While a number of genes involved in the secretion of PT have been identified, the process remains poorly understood. No signal sequence that might direct the PT to the Ptl secretion system has been identified and the role of the Ptl proteins is not yet known. As homologues of the Type IV secretion system are being identified in an increasing number of bacteria, further characterization of this system is eagerly awaited.

Type II Secretion

Perhaps the most widespread branch of the GSP is the Main Terminal Branch (Pugsley, 1993). This branch, also referred to as Type II secretion, has been found among many members of the Gram-negative bacteria including *Pseudomonas*, *Vibrio*, *Erwinia*, and *Aeromonas* species. The prototype for Type II secretion is the lipoprotein pullulanase secretion system of *Klebsiella oxytoca* (d'Enfert *et al.*, 1987a, b).

In *K. oxytoca*, transfer of pullulanase across the outer membrane is governed by the products of 15 *pul* genes (*pulB*, *pulS*, and the members of the *pulC-O* operon; Pugsley *et al.*, 1990). Extensive studies carried out in the Pugsley laboratory have examined the effects of mutations in each of the *pul* genes on pullulanase secretion in *E. coli* (Possot *et al.*, 2000). The results have shown that three of the Pul proteins, PulB, PulH, and PulN,

are not essential for pullulanase secretion. The remaining Pul proteins were shown to be required for efficient pullulanase secretion, as mutations in the corresponding genes reduced pullulanase secretion to 10 - 20% of wild type levels (Possot *et al.*, 2000).

PulC

While all but three of the Pul proteins have been shown to be required for pullulanase secretion, the roles that many of these proteins play in the secretion process remain unclear. For example, several potential roles have been assigned to PulC. Subcellular fractionation techniques have indicated that PulC is found in association with both the inner and outer bacterial membranes (Possot *et al.*, 1999) suggesting that this protein may function as a bridge between the membranes. Another study has suggested that PulC plays a role in pullulanase recognition (Guilvout *et al.*, 1999). This idea was based on the finding that all Pul proteins, with the exception of PulC can be replaced with the corresponding protein from the Type II secretion system of *E. chrysanthemi* without the loss of pullulanase secretion (Lindberg *et al.*, 1996). Still further studies have found that chemical cross-linking of PulC results in the formation of a 110 kDa product (Possot *et al.*, 1999). As the appearance of this product is dependent on the presence of PulD it is thought that PulC and PulD are closely associated. In light of this data, Possot and colleagues have proposed that PulC controls the opening and closing of the outer membrane channel formed by PulD (see below; Possot *et al.*, 2000).

PulE

PulE is a peripheral cytoplasmic membrane protein (Possot and Pugsley, 1994). Early studies of PulE indicated the protein contains two nucleotide-binding motifs, namely Walker boxes A and B (Possot *et al.*, 1992). Site directed mutagenesis of conserved residues within these motifs has shown that Walker box A is essential for pullulanase secretion, while Walker box B is not necessary (Possot and Pugsley, 1994). Despite the presence of an essential nucleotide-binding motif, PulE has not been shown to possess ATPase activity. However, these studies have been hampered by the fact that PulE aggregates upon removal from the cytoplasmic membrane or when it is extracted from cells that do not produce any other Pul proteins (Possot and Pugsley, 1994).

The Outer Membrane Pore - PulD

Surprisingly, out of the 15 Pul proteins, PulD is the only integral outer membrane protein (d'Enfert *et al.*, 1989). As such, PulD is a likely candidate to form a channel or gate in the outer membrane through which a secreted protein could pass. This idea is further supported by the fact that PulD and its equivalents are members of the secretin superfamily. As mentioned previously, the secretins are involved in transport of macromolecules across the outer membrane. Further support for PulD to form a channel or pore in the outer membrane comes from direct electron microscopic examination of the protein. This study has revealed the presence of two stacked rings that encircle a central channel (Nouwen *et al.*, 2000). Furthermore, a study carried out by Nouwen and colleagues involving the reconstitution of purified PulD-PulS complexes (see below) into

proteoliposomes has demonstrated that these complexes form ion-conducting channels in artificial lipid bilayers (Nouwen *et al.*, 1999).

PulS

For its part, PulS is a lipoprotein that is peripherally associated with the periplasmic face of the outer membrane (d'Enfert and Pugsley, 1989). It is responsible for protecting PulD from proteolytic degradation and is required for the correct localization of PulD in the outer membrane (Hardie *et al.*, 1996a and b).

The Pseudopilins - PulG - PulJ

Four of the *pul* genes, *pulG - pulJ* are predicted to encode pseudopilin proteins, so named because of their similarity to pilin, the building blocks of type IV pili. Many groups have suggested that these proteins may assemble into a pilus-like structure that spans the periplasm. This structure may then function as a scaffold for the assembly of the secretion apparatus (Pugsley, 1993) or may conduct substrate proteins up to the outer membrane (Russel, 1998). While such a complex has never been observed, recent findings by Sauvonnet and colleagues have provided evidence that suggests the pseudopilins are able to form such a pilus-like structure (Sauvonnet *et al.*, 2000). In their experiments, these authors expressed the *pul* genes on a high copy plasmid in *E. coli*. The result was the formation of pilus-like bundles projecting from the surface of the cells. Immunogold labeling of these projections indicated that they were made up of the pseudopilin PulG. However, when the *pul* genes were integrated into the *E. coli*

chromosome, no such pilus structures were observed. As a result, the role of the pseudopilins *in vivo* remains unclear.

The Prepilin Peptidase - PulO

The pseudopilins undergo processing at the type IV prepilin peptidase cut site (GFXXXE, where X is a hydrophobic amino acid) found near their N-termini. Following cleavage between the G and F residues, the F residue is N-methylated. In the pullulanase secretion system, cleavage and subsequent methylation of the pseudopilins is carried out by the Pul protein, PulO (Pugsley and Dupuy, 1992; Pugsley, 1993). However, in other Type II secretion systems, the protein responsible for processing of the pseudopilins is not encoded for by the secretion operon. For example, in *P. aeruginosa* the pseudopilins are processed by PilD, a protein encoded for by the type IV pilus biogenesis gene cluster *pilABCD* (Strom *et al.*, 1991) and responsible for processing of the type IV pilin.

Interaction between the Pul Proteins

While relatively little is known about how the Pul proteins work together to translocate pullulanase across the outer membrane, several Pul proteins have been shown to interact with each other. In their studies of *pul* mutants, Possot and colleagues found that the protein PulE is unstable in the absence of PulL (Possot *et al.*, 2000). PulL was in turn found to be unstable in the absence of PulM. Further experiments involving chemical cross-linking and immunoprecipitation found that PulE, PulL, and PulM interact directly with one another forming a core complex that is associated with the inner membrane (Possot *et al.*, 2000). [Similar interactions between the PulE, PulL, and PulM

homologues of *P. aeruginosa*, *E. chrysanthemi* and *V. cholera* have also been observed (Michel *et al.*, 1998; Ball *et al.*, 1999; Py *et al.*, 1999; Sandkvist *et al.*, 1999).] As crosslinking of the PulE/PulL/PulM heterotrimer requires the presence of PulG, it is likely that this protein associates with the core complex (Possot *et al.*, 2000). PulC is also thought to be associated with this complex as it can be immunoprecipitated with antibodies against PulM (Possot *et al.*, 2000). A possible association between the core complex and PulC would facilitate an indirect association between the core complex and both PulD and PulS.

Secretion of Proaerolysin

In *Aeromonas*, the Main Terminal Branch of the GSP is involved in the secretion of the lipolytic enzyme GCAT (Brumlik *et al.*, 1997), and the channel-forming toxin aerolysin (Jiang and Howard, 1992). Of these examples, aerolysin secretion is the best characterized. Aerolysin is secreted from the bacterium as an inactive protoxin, termed proaerolysin. The protoxin is activated upon proteolytic removal of approximately 40 amino acids from the C-terminal end of the protein (Howard and Buckley, 1985a). Aerolysin is then able to form seven-membered oligomers capable of producing channels in the membranes of target cells (Wilmsen *et al.*, 1992).

Early studies of proaerolysin secretion found that the proaerolysin precursor, preproaerolysin, contains a typical 23 amino acid signal sequence that directs its passage across the inner membrane *via* the Sec system (Howard and Buckley, 1985b). As the toxin crosses this membrane, the signal peptide is released and the resulting proaerolysin is released into the periplasmic space (Howard and Buckley, 1985b). In the periplasm,

proaerolysin folds and dimerizes before it is translocated across the outer membrane *via* the Main Terminal Branch (Hardie *et al.*, 1995).

The *exeC-N* Operon

In 1992, Jiang and Howard published the molecular characterization of the *exe* loci (extracellular export; Jiang and Howard, 1992) found within the genome of *Aeromonas hydrophila.* Transposon insertion into the *exe* loci results in the periplasmic accumulation of proteins that would otherwise be transported into the extracellular environment (Jiang and Howard, 1992). The authors were able to identify four open reading frames within this region. Analysis of these reading frames indicated they shared extensive sequence homology with genes involved in the secretion of pullulanase by *K. oxytoca*, namely *pulD*, *pulE*, *pulF*, and *pulG*. These findings were followed by the identification of seven more genes involved in protein secretion and outer membrane assembly (Howard *et al.*, 1993). Like the *exe* genes identified previously, the newly identified genes, *exeH* - *exeN*, demonstrated sequence homology with the corresponding *pul* genes. A subsequent study published by Karlyshev and MacIntyre (1995) identified the *exe* gene cluster in *A. salmonicida.* Included in their findings was the complete nucleotide sequence of the *exeC* gene. This gene was shown to contain homology to *pulC* of *K. oxytoca* and also to *outC* present in *E. chrysanthemi* and *E. carotovora* (Karlyshev and MacIntyre, 1995).

The Prepilin Peptidase - TapD

While the Exe proteins, ExeG - ExeJ were shown to contain N-terminal prepilin signal peptides (Howard *et al.*, 1993), analysis of the *exe* operon failed to identify a homologue of the prepilin peptidase gene whose product would be responsible for the processing of the pseudopilins. A later study carried out by Pepe and colleagues revealed that such a protein is in fact not linked to the secretion operon (Pepe *et al.*, 1996). In these studies, an *A. hydrophila* genomic library was transferred into a *P. aeruginosa pilD* mutant defective for Type IV pilus biogenesis. (As previously mentioned, PilD is a bifunctional enzyme that processes components of the GSP as well as Type IV prepilin.) The authors demonstrated that the *pilD* mutation was complemented by a homologous gene produced by *A. hydrophila* termed *tapD* (Pepe *et al.*, 1996). Further characterization of *tapD* indicated it is a part of the *tapABCD* gene cluster, homologous to the Type IV pilus biogenesis genes of *P. aeruginosa* (*pilABCD*; Pepe et al., 1996). Like its *P. aeruginosa* homologue PilD, TapD was shown to posses both N-methyltransferase and endopeptidase activities. Finally, Pepe *et al.* (1996) indicated that TapD it is required for extracellular secretion of aerolysin. It is now thought that TapD is responsible for the processing of the Exe pseudopilins, a hypothesis supported by the observation that processing of ExeG in *E. coli* is greatly enhanced in the presence of PilD (Howard *et al.*, 1993).

ExeA and ExeB

In 1994, Jahagirdar and Howard reported the presence of two additional *exe* genes that were not linked to the *exeC-N* operon. These authors identified the *exeAB* operon

encoding the ExeA and ExeB proteins found within the inner membrane (Jahagirdar and Howard, 1994). Subsequent examination of ExeA has revealed that it contains potential ATP-binding sites (Jahagirdar and Howard, 1994; Howard *et al.*, 1996). It is thought that ExeA may hydrolyze ATP, providing energy for the secretion of proaerolysin, however such activity has yet to be demonstrated (Schoenhofen *et al.*, 1998). Interestingly, no *exeA* homologue has been identified in any other functional Type II secretion system.

Howard and colleagues (Schoenhofen *et al.*, 1998) have provided evidence that demonstrates ExeB shares both sequence and topological similarity to TonB, a protein that couples the electrochemical gradient across the cytoplasmic membrane to active transport across the outer membrane (Postle, 1993). Further characterization of ExeA and ExeB by Schoenhofen *et al.* (1998) led to their proposal that an ExeA-ExeB complex present in the inner membrane is able to transduce energy from ATP hydrolysis and perhaps energy from the proton motive force to the opening of ExeD in the outer membrane. This model is consistent with findings from the Buckley laboratory that indicate proaerolysin secretion from the periplasm to the extracellular environment requires ATP and the proton motive force (Wong and Buckley, 1989; Letellier *et al.*, 1997).

The Aim of this Thesis

The purpose of the studies described in this thesis was to further examine the secretion of the aerolysin protoxin. Extensive studies carried out in the Buckley laboratory had already examined the effect of more than 50 individual point mutations on proaerolysin secretion. Suprisingly, only a handful of these mutations adversely affect

the toxin's secretion. Included in this small group, is a single amino acid residue that is critical to the protein's transport across the outer membrane and to the activity of the toxin (Wong and Buckley, 1991). Wong and Buckley have demonstrated that replacing W-227 with either F, G or L residues prevented release of proaerolysin into the culture supernatant and decreased the haemolytic activity of the toxin. These findings led to their suggestion that the region containing W-227 is critical for insertion of proaerolysin into the outer membrane as well as into the membrane of target cells.

I was interested in examining the potential role that other regions of the protein might play in proaerolysin's secretion across the outer membrane. To this end, recombinant PCR was used to create proaerolysin variants containing internal deletions in these regions. The ability of *A. salmonicida* to secrete these variants was then examined. I was also interested to learn how increased proaerolysin expression affects the secretion process. With this aim in mind, the secretion of proaerolysin produced by an overexpression system was examined.

MATERIALS AND METHODS

MATERIALS

Tryptone, yeast extract, tryptic soy agar, and granulated agar used in growth media were obtained from Difco. Dulbecco's modified Eagle's medium was supplied by Gibco. Methionine assay medium and skim milk powder were also from Difco. Thiamine was purchased from Calbiochem. Isopropyl-β-D-thiogalactoside (IPTG) was from Rose Scientific. Methanol was a product of Caledon. Acetic acid and trichloroacetic acid (TCA) were acquired from Anachemia. Chloroform was purchased from ACP and acetone was obtained from BDH. Sodium dodecyl sulphate (SDS) and polyoxyethylene-20sorbitan monolaurate (Tween-20) were acquired from Fisher Scientific. Tris-(hydroxymethyl)aminomethane was obtained from ACP, whereas all remaining salts were BDH products. *Staphylococcus aureus* cells containing Protein A were obtained from Calbiochem as was CENTA used in β-lactamase detection. L-^{35}S Methionine (>1000 Ci/mmol) and Amplify were from Amersham Pharmacia Biotech. Dithiobis(succinimidylpropionate) (DSP) used in cross-linking experiments was produced by Pearce.

Acrylamide used in making polyacrylamide gels was obtained from BDH whereas N,N'-methylene-bisacrylamide was purchased from Bio-Rad. Agarose, molecular weight standards and nitrocellulose membranes were also Bio-Rad products. Secondary antibodies (anti-rabbit and anti-mouse) conjugated to horseradish peroxidase were from Amersham Life Science. Gold-conjugate anti-rabbit antibodies were produced by British

Biocell. X-OMAT LS and BioMax MR films were from Kodak. Enhanced chemiluminesence reagents were from NEN Life Science.

Primers used to create aerolysin variants were constructed by Gibco BRL. All enzymes and buffers used in restriction digests, DNA ligations, and polymerase chain reactions (PCR) were obtained from Amersham Pharmacia Biotech.

Outdated human blood used in aerolysin detection was donated by the Royal Jubilee Hospital, Victoria, BC. All remaining reagents were from Sigma.

METHODS

Culture Conditions

Escherichia coli strains DH5α (used in cloning and plasmid propagation) and MM297 (helper strain for transconjugation) were grown at 37°C in Luria Bertanii (LB) media. Kanamycin was added to MM297 cultures to a final concentration of 70 μg/ml. *Aeromonas salmonicida* strain CB3 (a protease deficient strain; Buckley, 1990) was grown at 27°C in Luria Bertanii (LB) media supplemented with Davis salts (Miller, 1972) and 0.2% glucose (w/v), and containing 40 μg/ml rifampicin and 40 μg/ml kanamycin. Ampicillin was added to cultures harbouring the pMMB66HE expression vector to a final concentration of 100 μg/ml. Growth in liquid media was carried out in Erlenmeyer flasks in New Brunswick gyratory shakers with mild agitation (250 rpm for *A. salmonicida* and 350 rpm for *E. coli*). Overnight cultures were subcultured 1/100 into fresh media and proaerolysin expression was induced with IPTG once the cells had entered log phase.

Cell Fractionation

Osmotic Shock

Osmotic shock was carried out according to the procedure of Willis *et al.* (1974). Cells were harvested and 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 distilled H_20 containing 0.3mM $MgCl_2$, and 1 mM phenanthroline. The samples were then incubated on ice for two minutes. Finally, the cells were centrifuged to separate the shock fluid (shockate) from the shocked cells. Shocked cells were then resuspended in Hepes buferred saline (HBS; 20 mM Hepes, 0.15 M NaCl, pH 7.4) containing 1 mM phenanthroline. If required, shocked cells were frozen (-70°C) and thawed (+37°C) three times to release their intracellular contents.

Lysozyme/EDTA Treatment

Treatment with lysozyme/EDTA involved resuspending harvested cells in 20% sucrose (w/v), 33 mM Tris, 1 mM EDTA, 70 μg/ml lysozyme, pH 7.5. The samples were then incubated at RT for 15 minutes before being centrifuged to remove the lysozyme solution. The cells were shocked in ice-cold H_2O containing 1 mM phenanthroline and finally centrifuged to separate the supernatant and cells.

Chloroform Extraction

Chloroform extraction, cell fractionation using polymixin B, and Triton X-100 extraction were all performed according to the methods of Thorstenson *et al.* (1997).

During chloroform extraction, cells were harvested and resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). An equal volume of chloroform was added, and cells were vortexed briefly. The samples were then diluted 10 fold with TE buffer and incubated on ice for 30 minutes. Finally, the cells were pelleted by centrifugation.

Polymixin B Treatment

Fractionating cells with polymixin B was performed by resuspending cells in 0.5 M sucrose, 0.2 M Tris (pH 8.0), 0.5 mM EDTA, and 2 μg/ml polymixin B. The samples were incubated on ice for 30 minutes and then centrifuged to recover the supernatant fraction.

Triton X-100 Extraction

Triton X-100 extraction first involved harvesting induced cells and resuspending them in TEX buffer (50 mM Tris, 3 mM EDTA, and 0.025% Triton X-100, pH 8.0). The samples were incubated on ice for 30 minutes, pelleted, then washed with 1 volume of TEX buffer. The supernatants were then pooled.

Sucrose Density Gradient Ultracentrifugation

Cells were harvested and resuspended in 10% sucrose, 20 mM Hepes, 0.15 mM NaCl, 10 μg/ml DNase, 10 μg/ml RNase, pH 7.4. Following this, the cells were passed through a prechilled French pressure cell three times at a pressure of 1100 kg/ cm^3. The resulting lysate was layered on top of a sucrose density gradient (10 - 60%; w/w) and ultracentrifuged at 39000 rpm in a Beckman L8-70M ultracentrifuge using a SW-41 rotor

for 16 hours. After this time, 1 ml fractions were collected from the top of the gradient for further analysis. Inner and outer membrane fractions were identified according to their buoyant density (Osborn and Munson, 1974)

Proaerolysin Secretion

Two hours following induction with 0.1 mM IPTG, CB3:γ123 cells were harvested by centrifugation in a Beckman model J2-21 centrifuge for 10 minutes and washed once in LB media. The cells were then resuspended in fresh media containing 0.1 mM IPTG and 120 μg/ml chloramphenicol. Incubation was continued at 27°C. At indicated time intervals, samples were taken and the cells were osmotically shocked as described above. The presence of proaerolysin in the shock fluid, and shocked cells was then assayed by both immunoblotting and haemolytic titre assay (see below).

Pulse-chase Analysis

Pulse-chase

CB3:γ123 cells that had been induced for 2 hours with 0.1 mM IPTG were harvested and resuspended in Met$^-$ media composed of M9 minimal medium (Miller, 1972) containing 1 mM $MgSO_4$, 1 mg/ml thiamine, 0.2% glucose, 0.3% methionine assay medium. IPTG was added to a final concentration of 0.1 mM. The cells were then labeled by the addition of ^{35}S-methionine to a final concentration of 500 μCi/ml. After 2 minutes incubation at 27°C, unlabelled methionine was added to 1 mg/ml and the cells were immediately microfuged and resuspended in fresh LB media containing 1 x Davis salts, 0.2% glucose and 0.1 mM IPTG. Five minutes following this resuspension,

chloramphenicol was added to the culture to a final concentration of 120 μg/ml. Samples (150 μl) were taken at indicated times and the cells were osmotically shocked as described above. Shocked cells were then resuspended in 150 μl Met^- media.

Immunoprecipitation

Immunoprecipitation of shockate, shocked cells and culture supernatants was carried out according to the method described by Ito *et al.* (1981). Bovine serum albumin (BSA) was added to all samples to a final concentration of 0.2 mg/ml. The samples were then mixed with an equal volume of ice-cold 20% trichloroacetic acid and incubated on ice for at least 15 minutes. After this time, the samples were centrifuged and the precipitates were washed with ice-cold acetone. The samples were then dried in a vacuum dessicator before resuspension in SDS buffer (1% SDS, 50 mM Tris-HCl, 1 mM EDTA, 1 mM PMSF, 15 mM iodoacetamide, pH 8.0). The samples, now in 1% SDS, were heated in a boiling water bath for 2 minutes before being diluted in 4 volumes of Triton buffer (1.25 % TritonX-100, 190 mM NaCl, 1 mM phenylmethylsulfonyfluoride (PMSF), 6 mM EDTA, 15 mM iodoacetamide, 60 mM Tris-HCl, pH 7.4). Following this, 20 μl of a 10% suspension (w/v) of *Staphylococcus aureus* (SAC) cells containing surface Protein A was added to all samples. These mixtures were incubated end-over-end at 4°C for 2 hours before centrifugation for 15 minutes to pellet the SAC cells. The supernatants were then transferred to new eppendorf tubes and 3 μl anti-aerolysin antisera were added to each sample. The samples were incubated end-over-end at 4°C overnight (ON). Following this incubation, 20 μl of prepared SAC cells [washed twice in phosphate buffered saline (PBS; 10 mM NaH_2PO_4, 0.15 M NaCl, pH 7.4) followed by 15

minutes incubation in PBS containing 30 mg/ml BSA] were added to each sample. The samples were then incubated for 1 hour at 4°C (end-over-end). The SAC cells were harvested and washed twice with cold Triton buffer and once in 20 mM Tris-HCl, pH 7.4. After this, the cells were resuspended in 30 µl of non-reducing sample buffer and heated in a boiling water bath for 2 minutes. Finally the SAC cells were pelleted by centrifugation for 2 minutes and the supernatants were separated by SDS gel electrophoresis as described below. After electrophoresis, the polyacrylamide gels were incubated in 7% acetic acid/ 30% methanol for 30 minutes with mild agitation and then soaked in Amplify for 30 minutes. The gels were then dried in a Bio-Rad slab dryer at 60°C before being placed next to BioMax MR film in a film cassette. The cassette was then placed in a -70°C freezer for up to 2 weeks before the film was developed.

Electron Microscopy

Sample Preparation

CB3:pNB5 and CB3:γ123 cells that had been induced with 1mM IPTG were harvested in 2 ml Eppendorf tubes. The cells were then fixed in primary glutaraldehyde fixative containing 2.5% glutaraldehyde, 150 mM NaCl, 200mM NaH_2PO_4, pH 7.4. Fixation continued for 1 hour at RT. After this time, the cells were rinsed in 0.3 M NaCl, 200mM NaH_2PO_4, pH 7.4 prior to post-fixation in 1% OsO_4, 0.3 M NaCl, 100mM NaH_2PO_4, pH 7.4. Postfixation was carried out for 1 hour on ice. The cells were then dehydrated in a graded series of ethanol washes (50 - 100%) followed by 3 washings in propylene oxide. The samples were then placed in a 1:1 mixture of propylene oxide and Epon and incubated ON at RT. Following this, the resin was replaced with a 1:3 mixture

of propylene oxide and Epon and incubated for 7 hours. Finally, the cells were placed into pure Epon resin and cured in a 60°C oven for 3 days. The resulting blocks were sectioned on an ultramicrotome using glass knives and picked up onto 200-mesh copper grids. Thin sections were stained with 2% uranyl acetate and counterstained with 0.1% lead citrate. Samples were examined with a Hitachi 7000 transmission electron microscope using an accelerating voltage of 75 kV.

Preparation of CB3:γ123 cells for immunogold labeling was carried out in a manner similar to that described above, however the following changes were made. The cells were induced in 0.1 mM IPTG and fixed in primary fixative containing 0.1% glutaraldehyde, 4% paraformaldehyde, 150 mM NaCl, 200mM NaH_2PO_4, pH 7.4. Fixation continued for 1 hour on ice. Post fixation was then carried out using 1% OsO_4, 150 mM NaCl, 200mM NaH_2PO_4, pH 7.4. Following dehydration, the cells were embedded in Epon/Araldite resin. Thin sections (60 - 90 nm) were cut on an ultramicrotome using glass knives and picked up onto formvar-coated nickel grids.

Immunogold Labeling

Sections were etched for 30 minutes using 1% sodium metaperiodate. Blocking was then carried out for 15 minutes in fetal calf serum diluted 1/20 into 0.5% BSA and 0.05% Tween-20 in PBS. Sections were labeled for 1 hour at RT with an anti-aerolysin polyclonal antisera diluted 1/1000 in BSA-Tween-PBS. Labeling with the secondary antibody, a 10 nm gold-conjugated goat anti-rabbit IgG diluted 1/50, followed. After 1 hour, the sections were postfixed in primary glutaraldehyde fixative for 15 minutes. The sections were stained with 2% aqueous uranyl acetate and counterstained with 0.1% lead

citrate. Samples were examined with a Hitachi 7000 transmission electron microscope using an accelerating voltage of 75 kV.

Chemical Cross-linking

CB3:γ123 cells were first prepared for osmotic shock as described above. Following incubation in sucrose shock solution, the cells were pelleted, and subjected to osmotic shock by rapidly resuspending them in 20 mM Hepes-NaOH, pH 7.5 containing 0.25 mM DSP dissolved in dimethylsulphoxide. The samples were incubated for 30 minutes on ice before being pelleted by centrifugation. Shocked cells were resuspended in HBS and the cross-linking reaction was quenched by the addition of Tris-HCl (pH 7.4) to both the shock fluid and the shocked cells (final concentration of 0.1 M). The samples were incubated an additional 15 minutes on ice and after this time, non-reducing sample buffer was added to each. Finally, the samples were heated in a boiling water bath for 3 minutes.

Sample Preparation for Mass Spectroscopy

A 320 μg/ml sample of the proaerolysin variant A300C/T253C (Rossjohn *et al.*, 1998) was treated with 0.47 units of immobilized proteinase K and incubated end-over-end for 25 minutes at 37°C. After this time, PMSF was added to a final concentration of 1 mM. The sample was then centrifuged for 5 minutes at 4°C to pellet the protease. The supernatant was then collected and the protein was analyzed by mass spectroscopy. Mass spectroscopy was carried out by Borealis Biosciences Inc., Ontario.

Cell Viability Assay

T-lymphomas (EL4 cells) were grown in Dulbecco's modified Eagle's medium to a density of 1 x 10^6 cells/ml. The cell suspension was then dispensed into 96-well microtitre plates (100 µl suspension per well) and incubated with 20 µl of protoxin at the indicated concentrations. Following one hour incubation at 37°C and in 5% CO_2, cell viability was assessed by the addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium and phenazine methosulphate to final concentrations of 333 µg/ml and 7.7µg/ml respectively. Incubation continued for 4 hours before the absorbance of each sample was measured at 490 nm.

Proaerolysin Assays

Haemolytic Titre

Proaerolysin titres were determined according to our published procedure (Howard and Buckley, 1985a). Briefly, the toxin was activated by incubation with 2 µg/ml trypsin for 10 minutes at RT. After this time, two-fold serial dilutions were made in 96 well plates. Washed human erythrocytes were then added to each well (to a final concentration of 0.4%) and the plates were incubated for one hour at 37°C. The degree of cell lysis was then measured and compared to that of known quantities of proaerolysin. Where indicated, the amount of proaerolysin present in a sample was determined using the following relationships: 6 µg of proaerolysin generates a titre of 10 wells. An increase in titre of one well corresponds to a doubling of the proaerolysin concentration.

Turbidity Assay

Proaerolysin samples (110 μg/ml) were treated with 2 μg/ml trypsin for 10 minutes at RT. The proteolytic reaction was then arrested by the addition of PMSF to a final concentration of 1 mM. The samples were diluted 1:6 in HBS before a 50 μl aliquot was added to plastic cuvettes containing 2 ml of a 0.8% suspension of human erythrocytes. The optical density of the suspensions at 600 nm was monitored using a Varian Cary 1 spectrophotometer.

Enzyme Assays

β-Lactamase Assay

The β-lactamase activity in cell extracts was assayed spectrophotometrically using the synthetic substrate CENTA according to the method of Jones *et al.* (1982). Cell extracts (20 μl) were added to 1 ml aliquots of 0.1 mM CENTA in 0.05M phosphate buffer (pH 7.0) and the change in absorbance at 405 nm was measured.

Isocitrate dehydrogenase Assay

Isocitrate dehydrogenase (ICDH) activity was determined as described by Smith *et al.* (1987). Cell fractions (420 μl) were incubated with 500 μl of 100 mM potassium phosphate (pH 8.0), 20 μl of 250 mM $MgCl_2$, 10 μl of 100 mM isocitrate (pH 7.0) and 50 μl of 8 mM nicotidamide adenine dinucleotide phosphate. The increase in absorbance at 340 nm was then measured.

Electrophoresis and Western Blotting

Proteins were separated on 12% acrylamide slabs by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Neville (1971). Once separated, the proteins were either stained with Coomassie brilliant blue or electroblotted onto nitrocellulose membranes. These membranes were blocked ON at 4°C in PBS containing 0.5% Tween-20 and 0.5% skim milk. In order to detect proaerolysin, the membranes were incubated with either rabbit polyclonal anti-aerolysin antisera or mouse monoclonal anti-aerolysin antibody (1:4000 dilution in PBS containing 0.5% Tween 20, 1 hour at RT), followed by incubation with a corresponding secondary antibody horseradish peroxidase conjugate. The blots were then developed by enhanced chemiluminescense according to the manufacturer's instructions.

Construction of Proaerolysin Variants

Construction of aerolysin variants employed the use of recombinant PCR (Vallette *et al.*, 1988) carried out using primers containing the desired change (see Table 1). The resulting PCR products were digested using appropriate restriction enzymes (see below) and the digested fragments were separated by agarose gel electrophoresis using the method described by Sambrook *et al.*, (1989). The desired products were purified using QIAEX II Gel Extraction Kits according to the manufacturer's instructions. Purified products were ligated into the cloning vector pTZ18u for amplification (for ligation conditions see below). DNA sequencing was then carried out to ensure the correct mutations had been made. Sequencing was carried out by Joanne Whitehead, University of Victoria. Following this, the insert was isolated from the cloning vector

Table 1. Nucleotide sequence of primers used to create proaerolysin variants.

Primer	**Nucleotide Sequence**
Δloop-fwd[1]	gcgagaaggccgcgtcccagaacgggggctc
Δloop-rev[2]	gggacgcggccttctcgctcaggccatag
End440-fwd	cgctggtcaaggctaatagctgaggctggag
End440-rev	ctccagcctcagctattagccttgaccagcg
End450-fwd	gctcgatgcgcaataataggagctctccgggc
End450-rev	gcccggagagctcctattattgcgcatcgagc
Act$^-$-fwd	tcgcggctgacagcagtgtggacggcgctg
Act$^-$-rev	cagcgccgtccacactgctgtcagccgcga
66HE-fwd	catcggctcgtataatg
66HE-rev	ttaatctgtatcaggctg

[1]fwd indicates forward primers

[2]rev indicates reverse primers

and subcloned into the broad-host-range plasmid pMMB66HE (Furste *et al.*, 1986). Recombinant clones were transferred into *Aeromonas salmonicida* by conjugation using the filter-mating technique described by Harayama *et al.* (1980; see below).

Polymerase Chain Reaction

Recombinant PCR used to generate proaerolysin variants was carried out using the *pfu* polymerase. The reactions were carried out in a final volume of 50 µl and contained 0.2 mM deoxynucleoside triphosphate (dNTPs), 0.5 µM forward and reverse primers, 0.1 µg template DNA and 2.5 units cloned *pfu* polymerase in *pfu* Reaction Buffer [20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, and 0.1 mg/ml BSA].

Screening transformed cells for the proaerolysin insert was performed by PCR using the *Taq* polymerase. A cocktail was prepared in PCR reaction buffer [50 mM KCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl (pH 9.0)] containing 0.2 mM dNTPs, 0.5 µM forward and reverse primers and 5 units of *Taq* polymerase. Ten µl samples of this cocktail were aliquoted into 0.2 ml tubes and transformed cells were added using sterile toothpicks.

Restriction Digests

Restriction digests were carried out in Pharmacia One-Phor-All buffer [10 mM Tris-acetate (pH 7.5), 10 mM Mg-acetate, and 50 mM K-acetate] containing approximately one unit of restriction enzyme for every µg of DNA. All digestions were carried out at 37°C for 90 minutes.

DNA Ligations

Insert and vector DNA were mixed together in a ratio of approximately 5:1 and heated at 45°C for 15 minutes. After this time, the samples were diluted in One-Phor All buffer and ATP was added to a final concentration of 1 mM for cohesive-end ligations or 0.5 mM for blunt-end ligations. Following this, 11 units of T4 DNA ligase were added to each sample and the samples were mixed gently. Ligations were carried out at 13°C for either 4 hours (cohesive-end ligations) or 16 hours (blunt-end ligations).

Transformation

E. coli DH5α cells were made competent using the $CaCl_2$ wash method described by Cohen *et al.* (1972). Cells in log-phase (OD_{600} = 0.4 - 0.7) were harvested by centrifugation and washed in 1/4 volume of cold 100 mM $MgCl_2$. The cells were pelleted again and resuspended in two volumes of cold 100 mM $CaCl_2$. The cells were then incubated on ice for approximately 45 minutes. After this time, the cells were centrifuged and resuspended in 1/10 volume of 100 mM $CaCl_2$. Incubation continued for an additional 45 minutes before the addition of glycerol to a final concentration of 15%. Competent cells were then stored at -70°C until use.

Transformation of recombinant plasmids into competent *E. coli* cells was carried out according to the method of Inoue *et al.* (1990). Competent cells (200 µl aliquots) were incubated with 0.5 - 10 ng of DNA for one hour on ice. After this time, the cells were subjected to heat shock at 42°C for 4 minutes. The cells were quickly transferred back onto ice and left to stand for 5 minutes. Following this, 500 µl of LB media were added to each sample and the cells were incubated for 1 hour at 37°C with mild agitation.

Aliquots (150 µl) were then plated onto LB agar containing 50 µg/ml ampicillin. These plates were incubated ON at 37°C.

Filter-mating

The filter-mating technique described by Harayama *et al.* (1980) was used to transfer pMMB66HE plasmids containing the novel *aerA* mutation in *A. salmonicida* CB3. Bacterial cultures were grown to an OD_{600} of approximately 0.5. The cells were then chilled on ice before being spotted onto 0.45 µm filters in a ratio of 2:1:1 recipient (CB3): helper (MM297): donor (DH5α). The filters were then placed onto tryptic soy agar plates containing 12.5% human blood. These plates were incubated at 27°C for 4 hours after which time the filters were placed into 5 ml of LB media. Ten-fold serial dilutions were made and plated on tryptic soy agar-blood plates containing rifampicin, kanamycin, and ampicillin (40, 40, and 100 µg/ml respectively). Recombinant clones were picked after approximately 16 hours incubation at 27°C and the primers 66HE-fwd and 66HE-rev (see Table 1) were used to screen for the *aerA* insert as described above.

Reproducibility

Representative results are presented in this thesis. All experiments were repeated a minimum of three times.

RESULTS

SECRETION OF PROAEROLYSIN VARIANTS

Removal of the loop found within Domain Three

In an effort to examine the role that specific regions of proaerolysin play in the toxin's secretion, deletions were made in the aerolysin gene, *aerA*. The first such mutation, termed Δloop, was constructed with an internal deletion of amino acid residues 239 - 265. The crystal structure of proaerolysin has indicated that this region forms a narrow loop that lies behind a five-stranded antiparallel β-sheet (Parker *et al.*, 1994; Fig. 1). Previous studies carried out in the Buckley laboratory have indicated that during the oligomerization process, this loop moves away from the underlying β-sheet (Rossjohn *et al.*, 1998). While this movement is critical to the formation of an active aerolysin oligomer (Rossjohn *et al.*, 1998), the net result of such movement is unclear. It may be that movement of the loop allows the underlying β-sheet to make critical oligomeric contacts. Alternatively, the loop may play a role similar to that of analogous loop structures found within *Bacillus anthracis* anthrax toxin and *Staphylococcus aureus* α–toxin. In these toxins, the flexible loop is directly involved in membrane insertion (Petosa *et al.*, 1997; Song *et al.*, 1996). If the loop found within proaerolysin is involved in membrane insertion, then it may also be involved in proaerolysin insertion into the outer membrane during the secretion process. It was thought that construction of the Δloop variant might provide the answers to some of these questions.

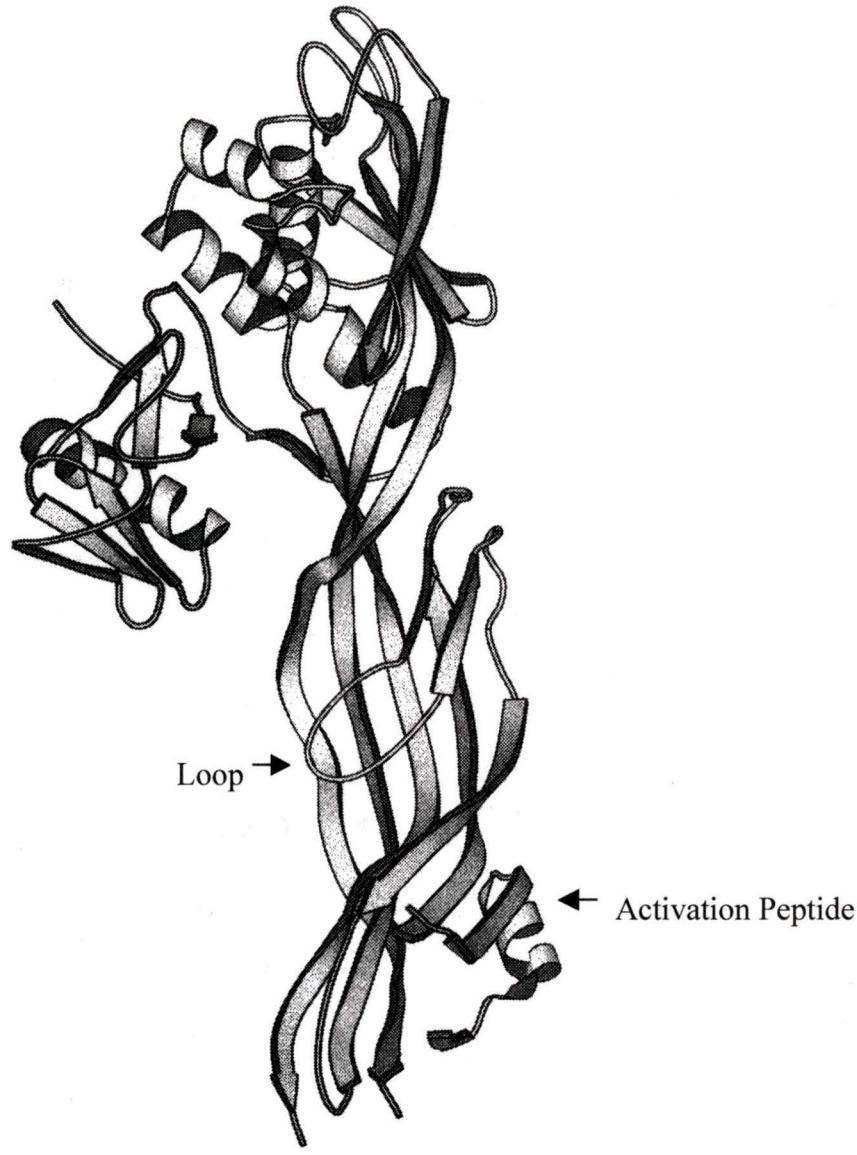


Figure 1. **Ribbon structure of proaerolysin.** Cartoon representation of the proaerolysin monomer. Construction of the Δloop, End440, and End450 variants involved deletions in the loop or activation peptide (indicated) as described in the text.

The Δloop variant is not secreted

Construction of the Δloop variant involved replacement of residues 239-265 with a single alanine residue, inserted to form a bridge between K-238 and A-266. Following construction of this mutation in *aerA*, the mutant gene was cloned into the broad-host-range vector pMMB66HE (Furste *et al.*, 1986); the resulting plasmid was named pSB1. This plasmid was then transferred into *A. salmonicida* CB3 (Buckley, 1990) for expression. CB3:pSB1 cells were induced for 4 hours with 1 mM IPTG to see if the mutant protein was expressed. As can be seen in Fig. 2, an approximately 49 kDa protein that reacts with antiaerolysin antisera is present in the induced cells but not in the uninduced cells thereby indicating the Δloop variant was expressed by *A. salmonicida* CB3. In Fig. 3, culture supernatants collected from the induced CB3:pSB1 cells are compared to those collected from CB3:pNB5 cells, cells that express the wild-type (wt) proaerolysin gene. The results displayed in the figure clearly indicate that CB3:pNB5 cells are able to secrete wt proaerolysin into the culture supernatant. While only a small quantity of proaerolysin can be seen the culture supernatant of CB3:pNB5 cells following 2 hours induction, appreciable amounts are were detected following 4 hours induction. Still more proaerolysin can be seen following 20 hours induction. In contrast, supernatant collected from CB3:pSB1 cells did not contain any Δloop variant even after as much as 20 hours induction with 1 mM IPTG. This result indicates that the loop variant can not be secreted.

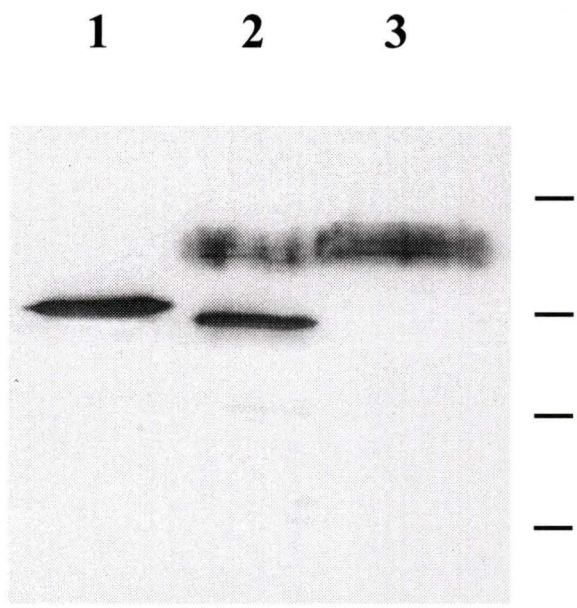


Figure 2. **Expression of the Δloop variant.** CB3:pSB1 cells were induced for 4 hours with 1 mM IPTG. Lane 1, purified proaerolysin; lane 2, induced cells; lane 3, uninduced cells. Samples were separated by SDS-PAGE and immunoblotted according to Materials and Methods. Bars indicate the locations of the molecular mass markers (from top, 80.0, 52.5, 34.9, and 29.9 kDa).

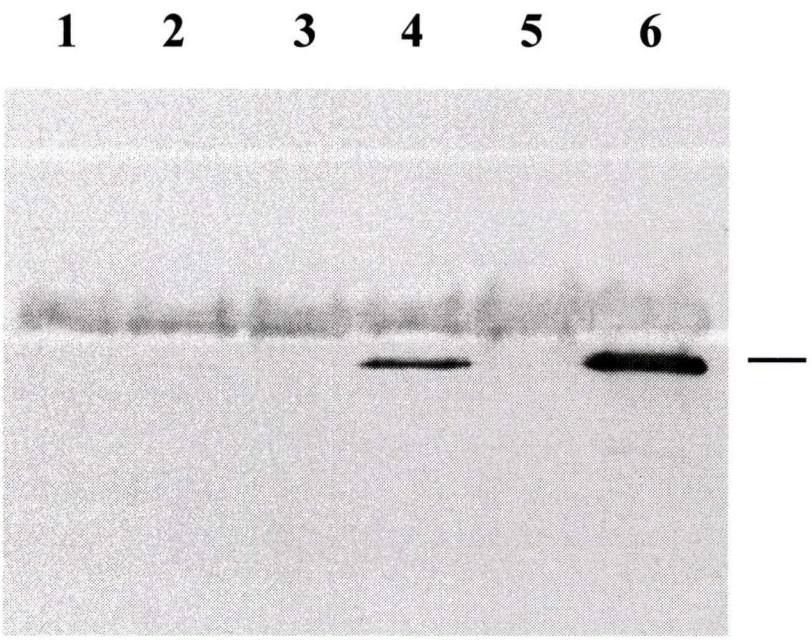


Figure 3. **The Δloop variant is not secreted into the culture supernatant.** Supernatant samples were collected from CB3:pSB1 (lanes 1, 3, and 5) and CB3:pNB5 cultures (lanes 2, 4, and 6) induced with 1 mM IPTG. The samples were then separated by SDS-PAGE and immunoblotted according to Materials and Methods. Lanes 1 and 2 were collected 2 hours following induction; lanes 3 and 4, 4 hours; and lanes 5 and 6, 20 hours. The bar indicates the location of purified proaerolysin.

The Δloop variant is insoluble

The fact that the Δloop variant was not secreted into the culture supernatant (Fig. 3) and the observation that CB3:pSB1 cells contain detectable quantities of Δloop variant even after only 4 hours induction with 1 mM IPTG (Fig. 2) lead to the question of where this variant was located within the cells. To begin answering this question CB3:pSB1 cells were subjected to osmotic shock, a method of cell fractionation that selectively releases periplasmic proteins (Willis *et al.* 1974). The results from this experiment, presented in Fig. 4, indicated that the majority of the Δloop protein fractionated with the shocked cells and not with the shock fluid (periplasmic fraction). This result suggests that the variant is located within the cytoplasm, is associated with either the inner or outer membranes of the cell, or is insoluble. Comparison of the migration of the Δloop variant on SDS-PAGE with that of the wild type protein confirmed that the loop variant is smaller that wt proaerolysin (Fig. 4). As the loop variant contains 31 fewer amino acids than wt (32 amino acids were replaced with 1 alanine bridge), one would expect this variant to migrate faster than wt. However, if the Δloop variant were present in the cytoplasm, where the N-terminal signal sequence would not have been removed, this variant would be only 9 amino acids shorter than wt found within the periplasm. Because there is such a difference in mobility between Δloop and wt found within the cells, it is likely that the loop variant has at least partially passed through the inner membrane resulting in removal of the leader peptide. Therefore, this result reduces the possibility of a cytoplasmic location for the Δloop variant.

Having demonstrated that the Δloop variant is unlikely to be located in the cytoplasm, I wanted to determine whether the variant was membrane-bound. To

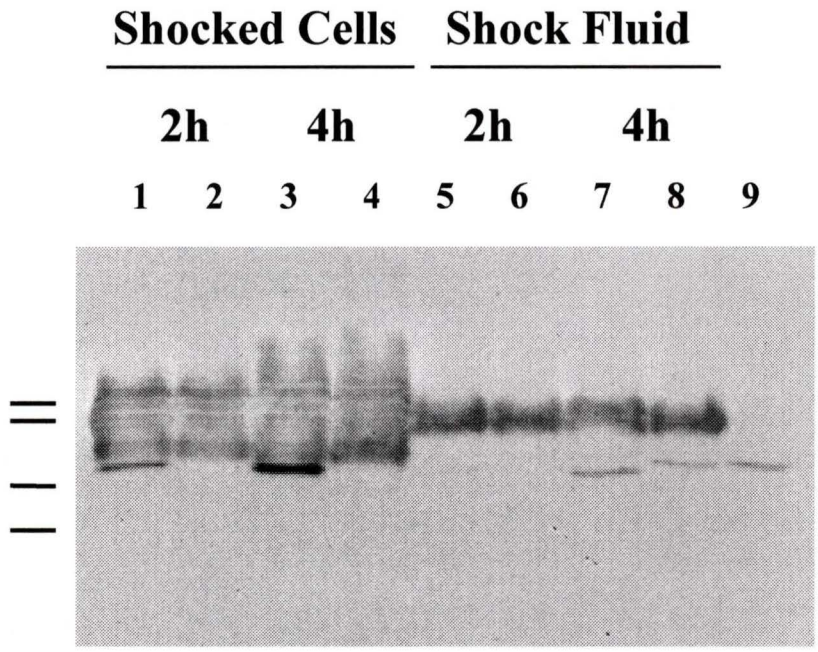


Figure 4. **The Δloop variant fractionates with shocked cells.** CB3:pSB1 (lanes 1, 3, 5, and 7) and CB3:pNB5 cells (lanes 2, 4, 6, and 8) were induced with 1 mM IPTG and then osmotically shocked 2 hours (lanes 1, 2, 5, and 6) and 4 hours (lanes 3, 4, 7, and 8) following induction. Lane 9 contains purified proaerolysin. Samples were separated by SDS-PAGE and immunoblotted according to the text. Bars mark the locations of the molecular mass markers (from top, 106, 81, 47.5, and 35.3 kDa). Following longer exposure times, proaerolysin can be detected in lane 6 (not shown).

accomplish this, CB3:pSB1 cells that had been induced with 1 mM IPTG for 4 hours were broken open by passage through a French pressure cell. The resulting cell lysate was layered on top of a sucrose gradient and the cellular contents were separated within the gradient by ultracentrifugation. This particular experiment was unsatisfactory; small amounts of the loop variant were found throughout the entire gradient (data not shown). In an effort to improve the experiment, the *aerA* gene containing the Δloop mutation was cloned into an overexpression system. This system made use of the fact that removal of a potential stem-loop structure found at the start of the *aerA* gene increases proaerolysin production (see below). It was thought that this system would express greater quantities of the loop variant, and that this would allow for clearer results from the sucrose density gradient. To this end, a new *aerA* construct was created lacking not only the loop found within Domain 3 but also the stem-loop structure found near the start of the *aerA* gene. As the results presented in Fig. 5 indicate, this expression system did result in greater protein production; cells expressing the new construct (CB3:pSB2 cells) produced far more of the Δloop variant than CB3:pSB1 cells. Therefore, the sucrose density gradient was repeated using lysate from CB3:pSB2 cells. The results shown in Fig. 6 reveal that the loop variant was not found in association with either the inner or outer membranes. Instead, the mutant protein was found at the bottom of the sucrose gradient indicating that it was insoluble.

Trypsin treatment of Δloop indicates the variant is incorrectly folded

As the cytoplasm, inner membrane, and outer membrane were all ruled out as possible locations of the Δloop variant, it seems likely that this protein is found within the

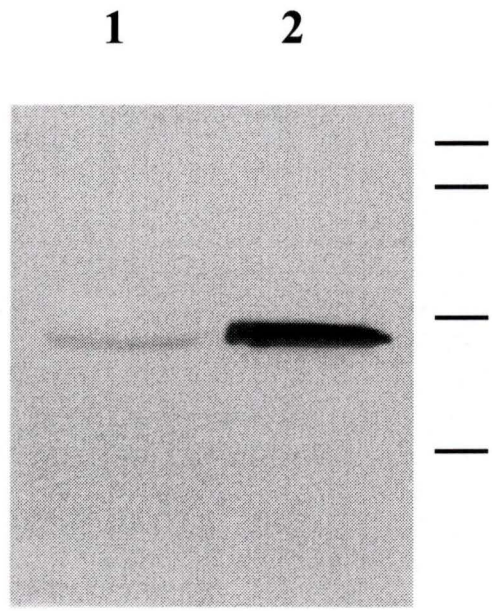


Figure 5. **Overexpression of the Δloop variant.** CB3:pSB1 (lane 1) and CB3:pSB2 cells (lane 2) were induced for 4 hours with 1 mM IPTG. Samples were separated by SDS-PAGE and immunoblotted. Bars indicate the locations of the molecular mass markers (from top, 100, 77, 50, and 34.3).

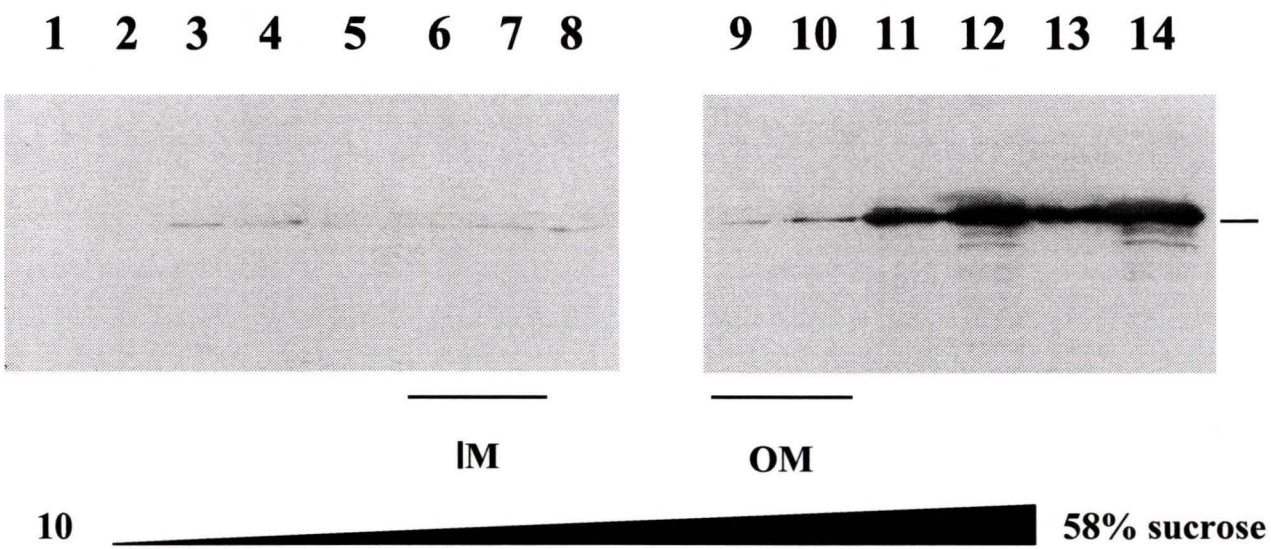


Figure 6. **Δloop migrates to the bottom of a sucrose density gradient.** CB3:pSB2 cell lysate was separated on a sucrose density gradient according to the text. Gradient fractions were then separated by SDS-PAGE and immunoblotted. Lane numbers correspond to fraction numbers where 1 represents the top of the gradient and 13 represents the bottom. Fractions containing inner membrane (IM) and outer membrane (OM) proteins are indicated. Lane 14 contains a sample of the cell lysate. The bar indicates the position of purified proaerolysin.

periplasm. However, as the protein is insoluble, it did not fractionate with the periplasm upon osmotic shock. Furthermore, as wt proaerolysin is soluble, the results obtained from this study suggest that removal of the loop alters the folding of the protein.

Additional evidence to support this idea was obtained upon trypsin treatment of the Δloop variant. Treatment of wt proaerolysin with trypsin results in the removal of a 43 amino acid peptide from the C-terminal end of the protein (Howard and Buckley, 1985a). The effects of this can be seen in Fig. 7. In contrast, treatment of lysate obtained from CB3:pSB2 cells with trypsin resulted in degradation of the Δloop variant (Fig. 7) indicating that it was not correctly folded. Similar degradation of Δloop was seen upon treatment with chymotrypsin and proteinase K (data not shown).

As protein secretion *via* the Type II system has previously been shown to be blocked when exoproteins are not correctly folded (Hirst and Holmgren, 1987: Kornacker and Pugsley, 1989), the inability of CB3:pSB1 and CB3:pSB2 cells to secrete the Δloop variant is likely do to the incorrect folding of the protein and not the absence of the loop *per se*. Therefore, we are unable to determine if this region plays any direct role in proaerolysin secretion. In addition, as treatment of the Δloop variant with trypsin resulted in degradation of the protein, we have been unable to determine the role this region plays in the oligomerization process.

Removal of the C-terminal end of proaerolysin

The second region of the proaerolysin molecule to be examined was the C-terminal or activation peptide (see Fig. 1). Removal of this peptide by proteolytic cleavage, for example by trypsin treatment (see above), results in activation of the

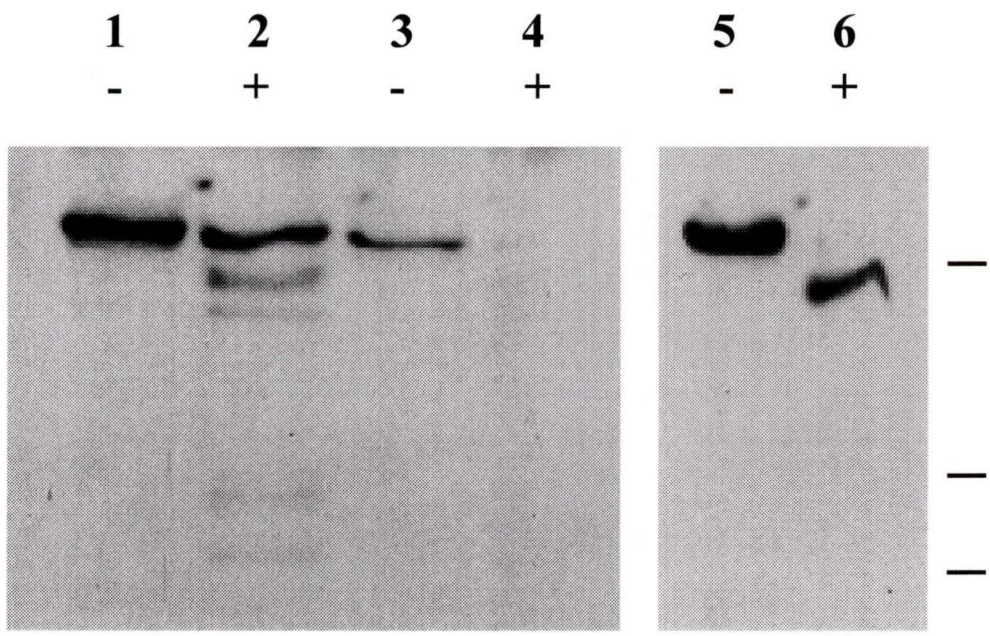


Figure 7. **Treatment of Δloop with trypsin.** Samples of shocked cells (lanes 1, and 2) and shock fluid (lanes 3, and 4) were treated with (+) or without (-) trypsin as indicated. Lanes 5 and 6 contain purified proaerolysin treated in the same manner. Samples were separated by SDS-PAGE and immunoblotted. Bars indicate the locations of the molecular mass markers (from top, 50, 34.3, and 28.8 kDa).

aerolysin toxin. There have been no published studies that examine the role of this peptide during proaerolysin secretion. However, one group has investigated the role played by the C-terminal peptide of *A. sobria* haemolysin, a toxin that displays approximately 73% sequence homology to aerolysin (Nomura *et al.*, 1999; Nomura *et al.*, 2000). The work carried out by Nomura and colleagues found that removal of 10 amino acids from the C-terminus of haemolysin blocked secretion of the protein. I was interested to see if similar mutations in aerolysin would affect its secretion. To accomplish this, two proaerolysin variants were constructed. The first, termed End440, contained two stop codons inserted following residue 440. Likewise, the mutation, End450 contained two stop codons inserted after residue 450.

The truncated proaerolysin variants are not secreted

As removal of the C-terminal peptide results in activation of aerolysin, it was possible that these truncated aerolysin variants would be active. If this were true, such mutations would be lethal. For this reason, the End440 and End450 mutations were originally created using an *aerA* gene containing another mutation, Y215C as a template. Previous studies in our laboratory have indicated that the Y215C variant is inactive, as it is unable to oligomerize (unpublished data). However, this mutation does not affect secretion of the protein. As before, *aerA* genes containing these mutations were cloned into the expression vector pMMB66HE. The resulting plasmids were named pSB4-1 (Y215C/End440) and pSB5-1 (Y215C/End450). As expected, expression of these plasmids in *A. salmonicida* CB3 resulted in truncated proteins (Fig. 8) indicating that insertion of the stop codons was successful. The results presented in Fig. 8 also reveal

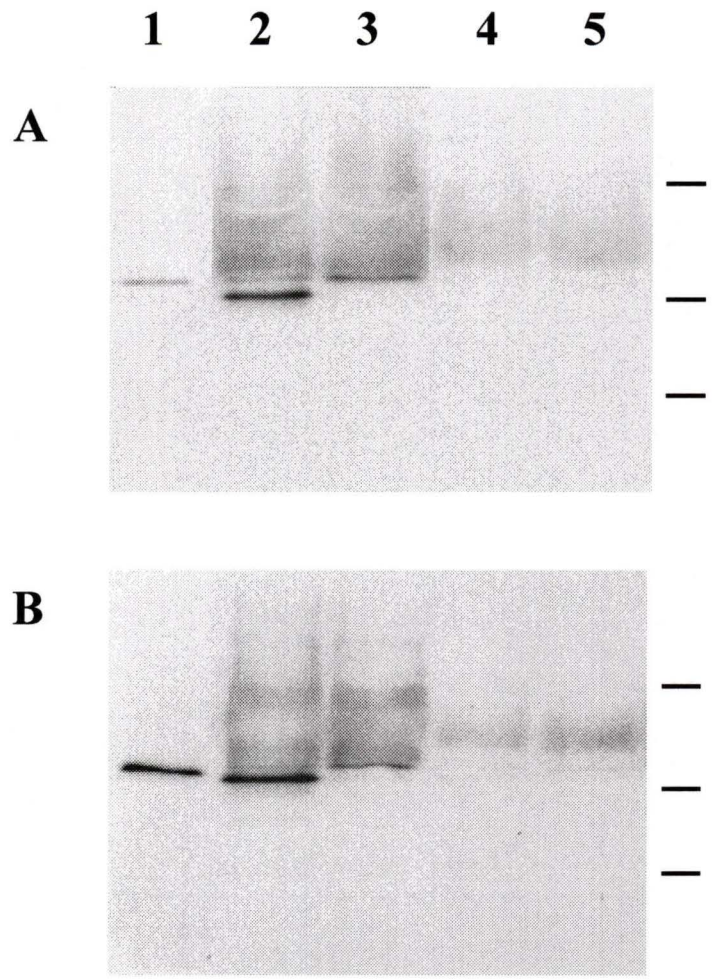


Figure 8. **Expression of End440/Y215C and End450/Y215C.** CB3:pSB4-1 cells (A) and CB3:pSB5-1 cells (B) were induced for 4 hours with 1 mM IPTG. Lane 1, purified proaerolysin: lane 2, induced cells; lane 3, uninduced cells; lane 4, induced culture supernatant; and lane 5, uninduced culture supernatant. Samples were separated by SDS-PAGE and immunoblotted according to the text. Bars indicate the locations of molecular mass markers (from top, 77.0, 50.0, and 34.3 kDa).

that cells expressing these mutations (CB3:pSB4-1 and CB3:pSB5-1) are unable to secrete the Y215C/End440 and Y215C/End450 variants. This result indicates that the loss of the C-terminal end of the activation peptide prevents proaerolysin secretion.

The truncated proaerolysin variants are inactive

Having demonstrated that truncation of the C-terminal peptide prevents secretion of the toxin, I wished to examine how such a mutation affects the activity of proaerolysin. With this aim in mind, the End440 and End450 mutations were cloned into wt *aerA*. Cells carrying these new variants (CB3:pSB4 and CB3:pSB5) were induced with 1 mM IPTG. The growth of these induced cells was then compared with that of uninduced cells. The results (shown in Fig. 9) indicated that cell growth was unaffected by either mutation, suggesting that removal of up to 32 amino acids from the C-terminal domain is not sufficient for activation of the toxin. [This experiment also confirmed the previous finding that truncating the C-terminal peptide prevents proaerolysin secretion (data not shown).]

A haemolytic titre assay was then performed to further study the effect of the End440 and End450 mutations on proaerolysin activity. In this experiment, aliquots of cell lysate obtained from both CB3:pSB4 and CB3:pSB5 cells were treated with trypsin and then incubated with red blood cells (see Materials and Methods). The results, seen in Table 2 revealed there was no difference in the haemolytic activity of either CB3:pSB4 or CB3:pSB5 cell lysate treated with trypsin or without. Furthermore, these cells displayed no more haemolytic activity than CB3 cells not carrying the *aerA* gene on a plasmid.

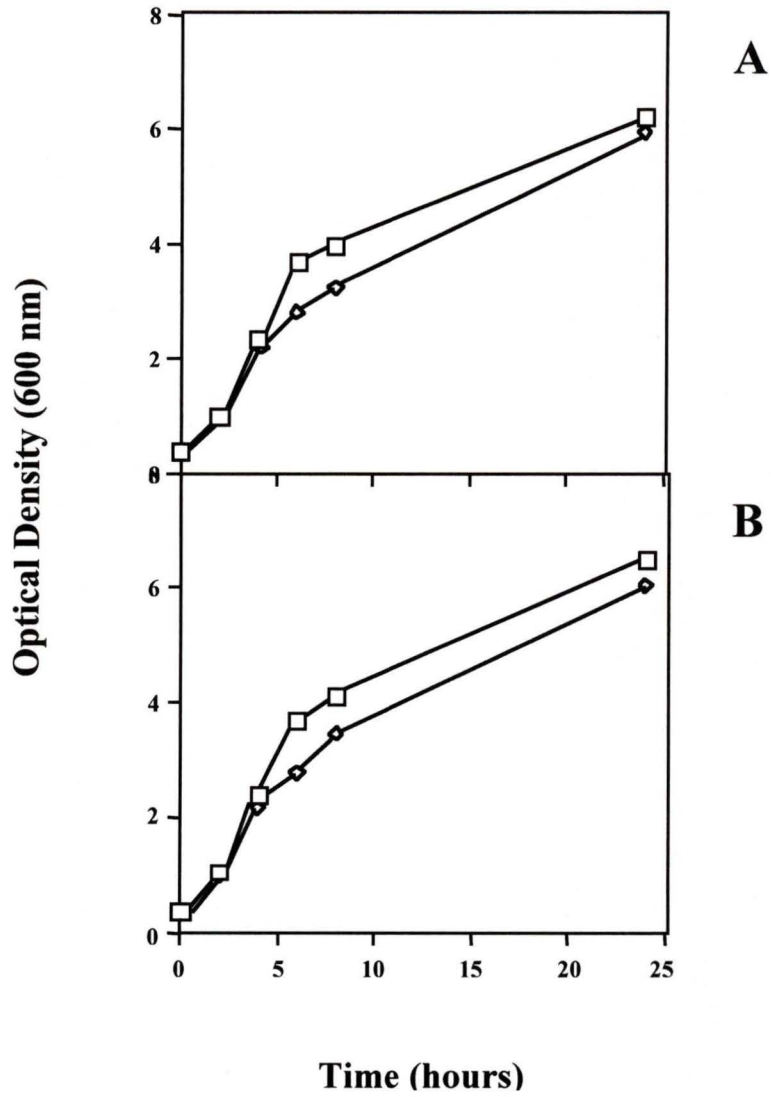


Figure 9. **Growth of CB3:pSB4 and CB3:pSB5 cells.** CB3:pSB4 (A) and CB3:pSB5 (B) cultures were grown in the presence (□) or absence (◊) of 1 mM IPTG. The optical density (600 nm) of the cultures was monitored for 24 hours following induction.

Table 2. Haemolytic titre of CB3:pSB4 and CB3:pSN5 cell lysate treated with or without trypsin.

Cell	**Variant**	**+/- Trypsin**	**Haemolytic Titre[1]**
CB3:pSB4	End 440	+	4
CB3:pSB4	End 440	-	4
CB3:pSB5	End 450	+	3.5
CB3:pSB5	End 450	-	4
CB3	N/A	+	4
CB3	N/A	-	4
N/A	Purified wt (8μg/ml)	+	9

[1] titre obtained following 20 hours incubation with 0.4% human erythrocytes

N/A - not applicable

These data suggest the End440 and End450 variants do not possess any haemolytic activity.

Finally, CB3:pSB4 and CB3:pSB5 cells that had been induced for 4 hours with 1 mM IPTG were osmotically shocked in the presence or absence of 40 μg/ml trypsin. The results of this experiment (see Fig. 10) indicate that both the End440 and End450 variants fractionate with the shocked cells. Furthermore, cells shocked in the absence of trypsin contain significantly greater quantities of each variant than cells that were shocked in the presence of the protease. This result suggests that trypsin is able to degrade the End440 and End450 variants indicating they are not correctly folded.

Activation of proaerolysin by proteinase K

While trypsin is able to cleave proaerolysin following residue 427, other proteases are also able to remove the C-terminal peptide resulting in activation of the toxin. For example, chymotrypsin cleaves proaerolysin after R-429 (Fig. 11; van der Goot *et al.*, 1992) and furin cuts the protein following residue R-432 (Fig. 11; Abrami *et al.*, 1998). Proteinase K is also able to cleave the toxin (Garland and Buckley, 1988) however the cut site of this protease had not previously been identified. To find this site, the weight of the aerolysin variant, A330C/T253C following treatment with proteinase K was determined using mass spectrometry. As this variant is unable to oligomerize (Rossjohn *et al.*, 1998), proteolytic removal of its C-terminal peptide would not produce oligomers that might interfere with the mass spectrometry reading. The results obtained from the mass spectrometry analysis indicated that the mass of the cleaved product was approximately 47581 Da. This best fits with processing of the protein following V-428 (Fig. 11);

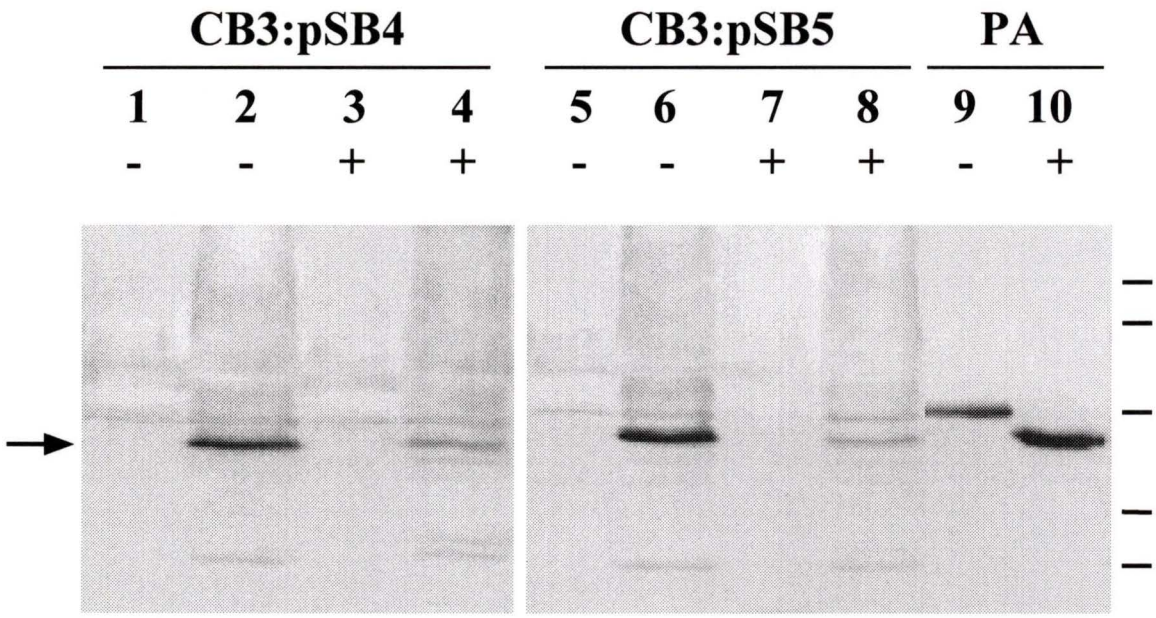


Figure 10. **Treatment of End440 and End450 with trypsin.** CB3:pSB4 (lanes 1-4) and CB3:pSB5 (lanes 5-8) cells were induced for 4 hours with 1 mM IPTG and then osmotically shocked in the presence (+) or absence (-) of 40 μg/ml trypsin as indicated. Lanes 1, 3, 5, and 7, shock fluid; lanes 2, 4, 6, and 8, shocked cells; lanes 9 and 10, purified proaerolysin (PA). Samples were separated by SDS-PAGE and immunoblotted according to the text. The arrow identifies the position of the End440 variant. Bars indicate the locations of the molecular mass markers (from top, 116, 80, 52.5, 34.9 and 29.9 kDa).

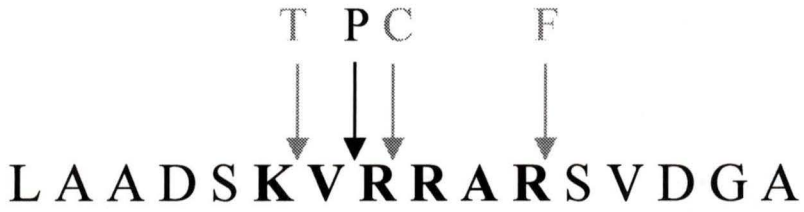


Figure 11. **Protease cut sites.** Proaerolysin residues 422 - 437 are shown. Cut sites for trypsin (T), chymotrypsin (C), and furin (F) are indicated. Proteinase K (P) nicks proaerolysin following V-428. The activation sequence is shown in bold.

cleavage of the A330C/T253C variant following this residue would be expected to generate a product of 47602 Da.

Removal of the activation sequence

The fact that proteinase K cuts proaerolysin following V-428 puts the cleavage site for this protease very close to the cleavage of site of trypsin, chymotrypsin and furin (Fig. 11). The region encompassing these cut sites is referred to as the activation sequence. A novel proaerolysin variant was constructed lacking the activation sequence (residues 427 - 432). Expression of the *aerA* gene containing this mutation (termed Act$^-$) in *A. salmonicida* CB3 (CB3:pSB6 cells) resulted in secretion of the variant (Fig. 12). Therefore, one can conclude that the activation site does not play a role in the secretion of the proaerolysin molecule.

As the Act$^-$ variant was secreted, I was able to purify the protein from culture supernatant. Like wt proaerolysin, the Act$^-$ variant responded well to purification using our published procedure (Buckley, 1990), a process that utilizes hydroxyapatite and ion-exchange chromotography (data not shown).

The Act$^-$ variant is processed by proteinase K

As expected, treatment of purified Act$^-$ with trypsin or chymotrypsin did not result in reduction of the mass of the protein, indicating it could not be nicked by these proteases (Fig. 13). In contrast, the results displayed in Fig. 13 indicate that proteinase K was able to process the proaerolysin variant. This result is perhaps not surprising if one examines the sequence left after removal of the activation site (see Fig. 11). Removal of

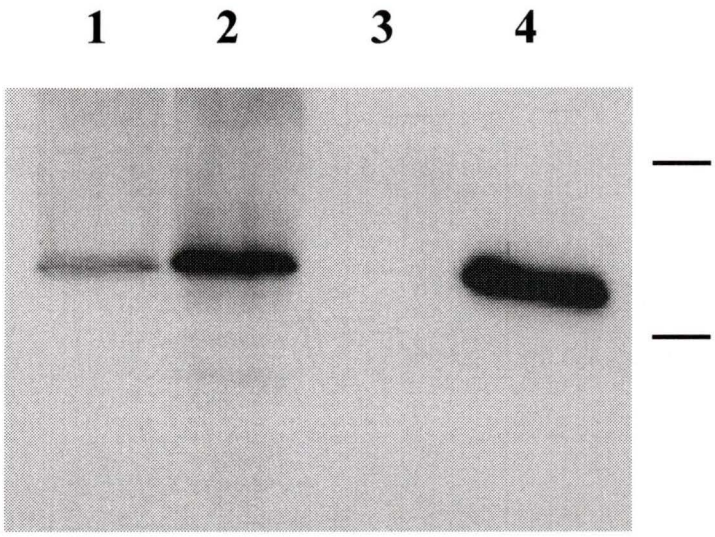


Figure 12. **Secretion of Act⁻ variant.** Cell samples (lanes 1, and 2) and culture supernatants (lanes 3, and 4) from CB3:pSB6 cells (uninduced, lanes 1, and 3; induced lanes 2, and 4) were collected 4 hours following induction with 1 mM IPTG. Samples were separated by SDS-PAGE and immunoblotted. Bars mark the locations of the molecular mass markers (77, and 50 kDa).

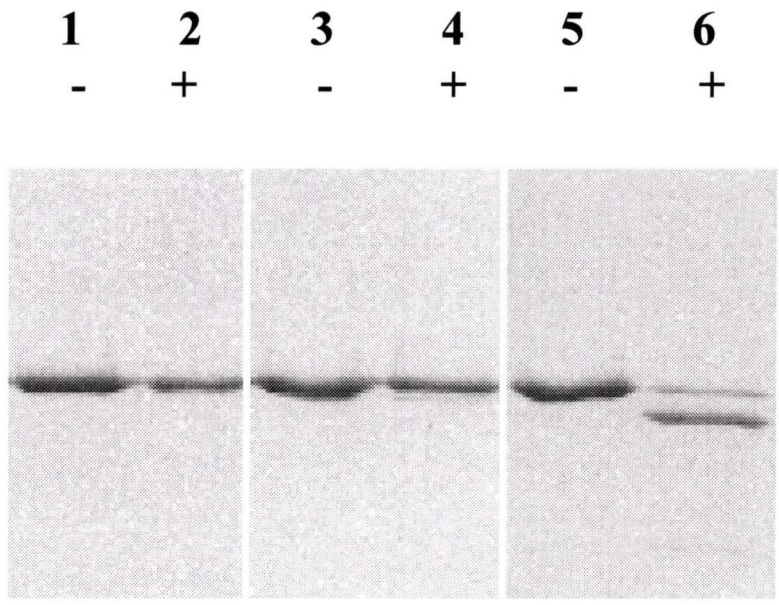


Figure 13. **Protease treatment of Act⁻.** Equal amounts of purified Act⁻ variant were treated with or without 2μg/ml trypsin (lanes 1, and 2), 100 μg/ml chymotrypsin (lanes 3, and 4), or 10 μg/ml proteinase K (lanes 5, and 6) as indicated. Proteins were separated by SDS-PAGE and stained with Coomassie blue.

the activation sequence, KVRRAR moved the valine residue found at position 434 forward six positions, so that it is in position 428; the same position that serves as the cut site for proteinase K in wt proaerolysin.

I next wanted to learn whether or not processing of the Act⁻ variant by proteinase K led to the production of active aerolysin. To this end, I carried out a spectrophotometric assay involving the addition of Act⁻ pretreated with proteinase K, to a stirred suspension of erythrocytes. If the erythrocytes within the sample lysed due to aerolysin activity, the absorbance at 600 nm of the suspension would be expected to decrease due to a decline in light scattering. The results of this experiment can be seen in Fig. 14. Clearly, treatment of Act⁻ with proteinase K resulted in a decrease in absorbance thereby indicating activation of the variant. In contrast, there was no decline in the absorbance of a sample treated with trypsin. This result is in agreement with the results presented in Fig. 13, which indicated that treatment of Act⁻ with this protease did not alter the size of the toxin.

As proteinase K was shown to activate the Act⁻ variant, I was eager to learn if the activated variant displayed the same degree of haemolytic activity as wild type aerolysin. To accomplish this, a haemolytic titre was performed using equal amounts of Act⁻ and wt proaerolysin activated with proteinase K. The results of this experiment are shown in Table 3. After one hour incubation with human erythrocytes, an 80 μg/ml sample of wt aerolysin generated a titre of 11.5 wells. In contrast, the same concentration of Act⁻ generated a titre of only 10.5 wells indicating the protein is either less active than wt or that proteinase K is not as efficient at nicking the variant than it is in processing the wt protoxin. Samples of wt and Act⁻ that had been incubated with 10 μg/ml proteinase K for

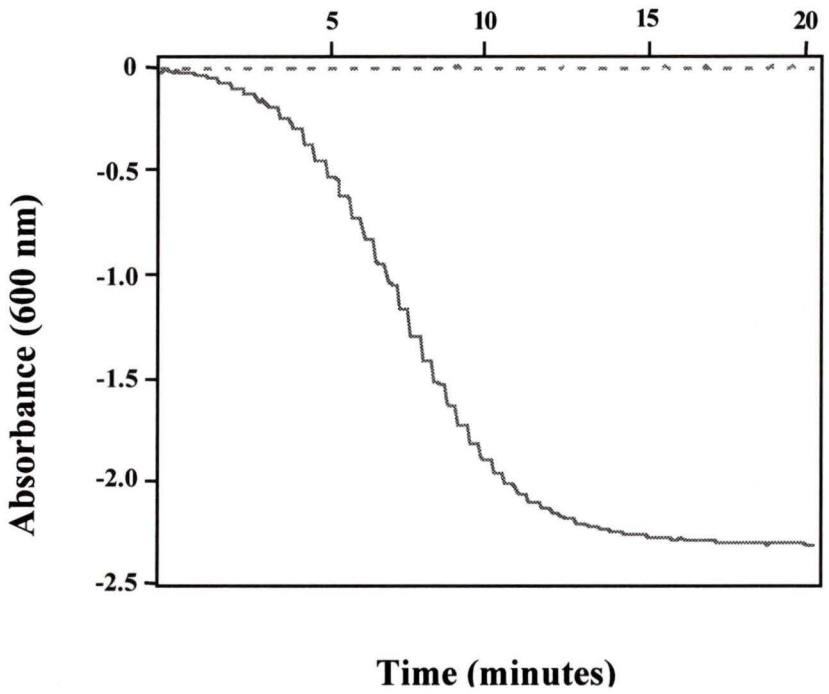


Figure 14. **Processing by proteinase K activates Act⁻ variant.** Act⁻ was pretreated with 10 μg/ml proteinase K (solid line) or 2 μg/ml trypsin (broken line) for 10 minutes at RT. Haemolytic activity against human erythrocytes was then measured at 37°C.

Table 3. Haemolytic titre of Act⁻ and wt aerolysin.

Sample	**Protease**	**Haemolytic Titre**[1]
Act⁻	Proteinase K	10.5
Act⁻	Trypsin	0
wt	Proteinase K	11.5
wt	Trypsin	11.5

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

10 minutes were then separated by SDS-PAGE and stained with Coomassie blue. The results of this procedure can be seen in Fig. 15. Clearly proteinase K was able to process all of the wt proaerolysin present, however it was not able to process an equivalent amount of Act$^-$ variant in the same time. This result indicates that proteinase K is not as efficient at processing the Act$^-$ as it is at processing the wt protoxin.

T-lymphomas are not sensitive to the Act$^-$ variant

Previous studies in our laboratory have shown that T-lymphomas are equally sensitive to aerolysin and proaerolysin, indicating these cells can activate the toxin (Nelson *et al.*, 1997). It is thought that furin, a proprotein convertase present on the cell surface, is primarily responsible for activation (Abrami *et al.*, 1998). I performed a viability assay comparing wt and the variant protoxin. The results, displayed in Fig. 16, indicate that incubation with this variant did not markedly affect the cells' viability. Indeed, even treatment with toxin concentrations in the 10^{-9} molar range did not result in significant cell death. In contrast, the same concentration of wt proaerolysin resulted in only 10% cell viability. Therefore, the removal of the KVRRAR activation sequence prevents activation of proaerolysin by a T-lymphoma's own proteases. Furthermore, this result indicates that there are no proteinase K-like proteins found on the surface of these cells.

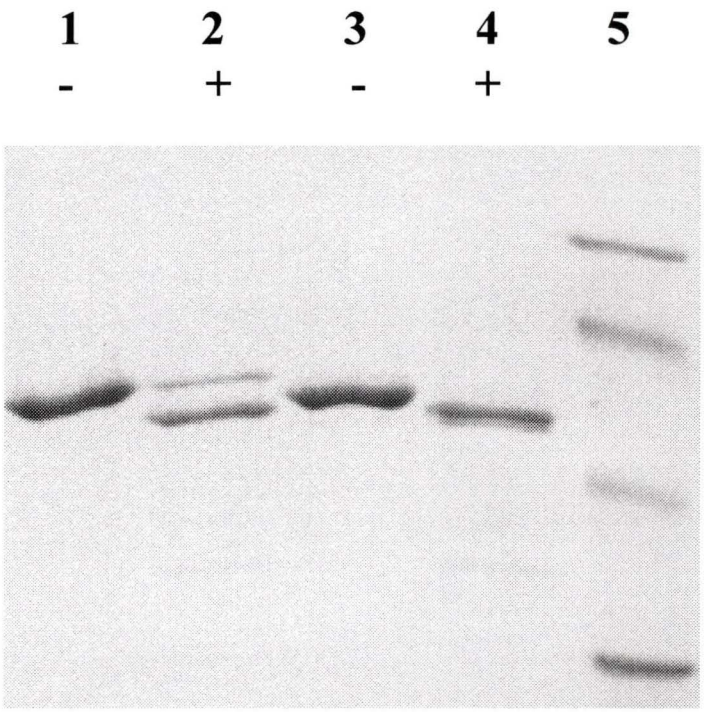


Figure 15. **Processing of Act⁻ by proteinase K.** Purified Act⁻ (lanes 1 and 2) and wt proaerolysin (lanes 3 and 4) were treated with (+) or without (-) 10 μg/ml proteinase K as indicated for 10 minutes at 25°C. Lane 5, molecular mass standards (from top 97.4, 66.2, 45.0, and 31.0 kDa). Samples were separated by SDS-PAGE and stained with Commassie blue.

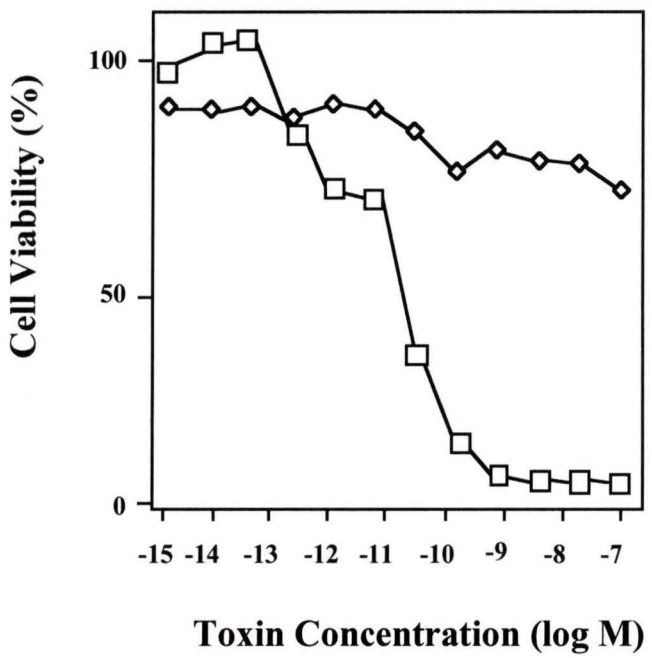


Figure 16. **T-lymphomas are less sensitive to Act⁻ than to wt proaerolysin.** EL4 cells (10^6 cells/ml) were treated with a range of Act⁻ (□) or wt proaerolysin (◊) concentrations as indicated. Cell viability was determined using an MTS/PMS viability assay as described in Materials and Methods.

OVEREXPRESSION OF PROAEROLYSIN

Removal of potential stem-loop structure increases proaerolysin expression

Sequence analysis of the 5′ end of the aerolysin gene has revealed the presence of two inverted repeats upstream of the start site (Howard and Buckley, 1986). The second of these repeats is part of a stretch of 27 basepairs (including the ribosome binding site and start codon) that has the ability to form a stem-loop structure (Fig 17). Such secondary structure has the potential to affect expression of the aerolysin gene either at the level of gene transcription or translation. An earlier publication from our laboratory has described the creation of a novel *aerA* variant in which one half of the potential stem loop was removed and replaced with a new sequence (Diep *et al.*, 1998; see Fig. 17). As a result, this construct, $aerA^{-loop}$ is unable to form the stem loop (Diep *et al.*, 1998). CB3:γ123 cells (cells expressing the $aerA^{-loop}$ construct) display a marked increase in proaerolysin production. This can be seen in Fig. 18, where proaerolysin production by cells expressing wt proaerolysin, CB3:pNB5 is compared to that of CB3:γ123. The data presented in this figure clearly indicate that removal of the potential stem-loop leads to an increase in the amount of proaerolysin secreted into the culture supernatant as well as an accumulation of the toxin within the cells.

Haemolytic titre was used to further compare the relative amount of proaerolysin present in the supernatant of CB3:pNB5 and CB3:γ123 cultures. The results of this experiment are presented in Fig. 19. Clearly, the amount of proaerolysin present in the culture supernatants of CB3:γ123 cells is higher than that found within CB3:pNB5 supernatants at every IPTG concentration tested.

A

A-U
U-A
A-U
A-U
C-**G**
U-**G**
G U-**G**
U-**G**
A-U
A-U
C-G
U C
G-U
U-**A**
A-**U**
C-**G**
G-C
5′-GAAU-AAAAAAUAAAA-3′

B

5′-CTGCAGAAGAAGGAGATATACATATGCAAAAAATAA-3′

Figure 17. **Potential stem-loop structure located at the beginning of the aerolysin mRNA.** A. Schematic representation of the 5′ end of the aerolysin mRNA. The ribosome binding site and AUG start codon (indicated in bold) are confined within the stem structure. B. The new DNA sequence used in engineering the γ123 construct. This sequence replaces the original sequence that had the potential to code for the stem structure.

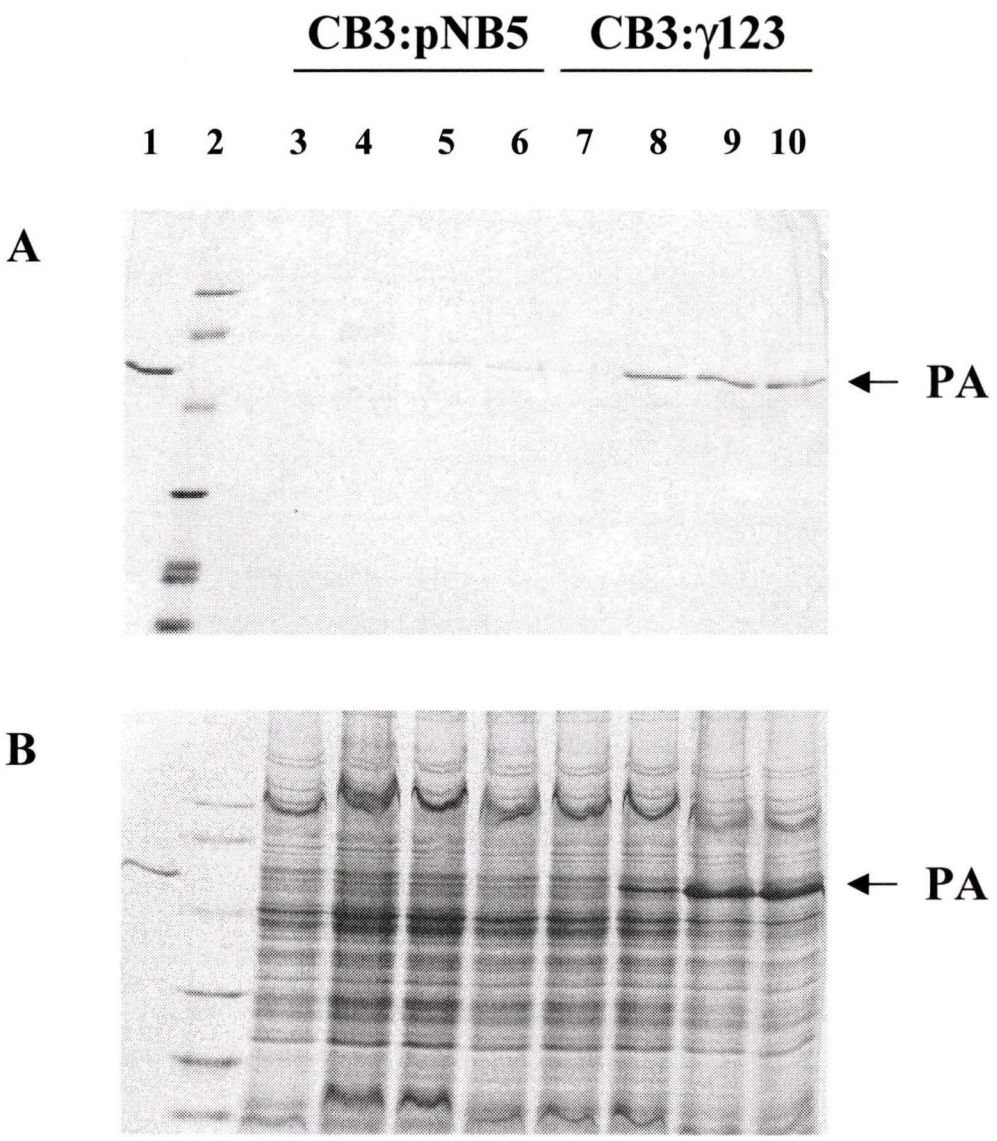


Figure 18. **Increased proaerolysin production by CB3:γ123.** CB3:pNB5 cells (lanes 3-6) and CB3:γ123 cells (lanes 7-10) were induced with increasing concentrations of IPTG (lanes 3 and 7; 0mM; lanes 4 and 8, 0.02mM; lanes 5 and 9, 0.1mM; lanes 6 and 10, 1.0mM). Culture supernatants (A) and whole cells (B) were collected 4 hours following induction and separated by SDS-PAGE. The proteins were then stained with Coomassie blue. The arrow indicates the position of proaerolysin (PA). Lane 1 contains purified proaerolysin and lane 2 contains molecular mass markers (from top to bottom: 97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa). Corresponding amounts of cells and supernatants were applied to each lane.

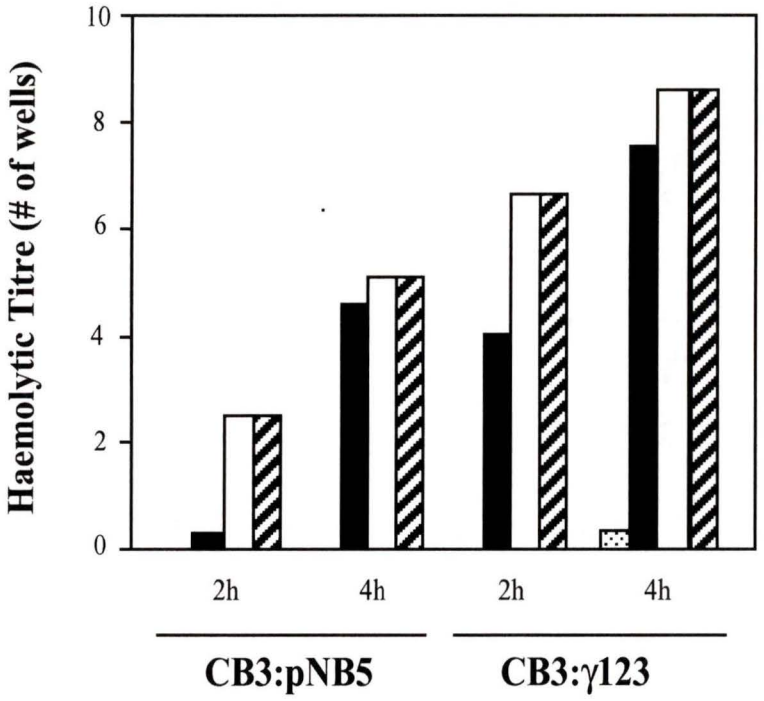


Figure 19. **Increased proaerolysin secretion by CB3:γ123.** The haemolytic titre of culture supernatants from CB3:pNB5 and CB3:γ123 cells following 2 and 4 hours induction with increasing concentrations of IPTG (dotted bars; 0mM; solid bars, 0.02mM; open bars, 0.1mM; hatched bars, 1.0mM) was determined as described in the text.

I was interested in quantitating the amount of proaerolysin found within CB3:γ123 cells. To accomplish this, cells that had been induced for 2 hours with IPTG were subjected to osmotic shock. The amount of proaerolysin within these cells was then determined using the haemolytic titre assay. The results of this experiment indicated that a 10 µl sample of shock fluid generated a haemolytic titre of 6.5 wells (not shown). Previous studies in our laboratory have established that 6 µg of proaerolysin generates a titre of 10 wells, and an increase in titre of one well corresponds to a doubling of proaerolysin concentration. Using this information, one can determine that the 10 µl sample of shocked cells contained approximately 0.56 µg of proaerolysin. As the shock fluid is five times concentrated in comparison to the bacterial culture (see Materials and Methods), one can determine the amount of proaerolysin present in the shock fluid from one ml of culture according to the equation:

$$\frac{0.5625\ \mu g\ \text{proaerolysin}}{10\ \mu l \times 5} \times \frac{1000\ \mu l}{ml} = \frac{11.25\ \mu g\ \text{proaerolysin}}{ml\ \text{culture.}}$$

The total number of cells in a ml of culture can then be determined using the relationship 1×10^9 cells / ml = OD_{600} 1.6. Therefore:

$$\frac{1.43\ OD_{600} \times 1 \times 10^9\ \text{cells}}{1.6\ OD_{600}} = 8.9375 \times 10^8\ \text{cells.}$$

With this information, the amount of proaerolysin found in the shock fluid of a single cell can be calculated as:

$$\frac{11.25 \times 10^{-6}\ g\ \text{proaerolysin}}{8.9375 \times 10^8\ \text{cells}} = \frac{12.59 \times 10^{-15}\ g\ \text{proaerolysin.}}{\text{cell}}$$

This amount of proaerolysin found within the shock fluid of CB3:γ123 cells, 12.6×10^{-15} g of proaerolysin per cell, is more than ten times the size of the intracellular pool found within CB3:pNB5 cells previously calculated as approximately, 1.2×10^{-15} g per cell

(Wong and Buckley, 1989; Letellier *et al.*, 1997). However, what is even more interesting is that while proaerolysin accumulates primarily within the shockable fraction of CB3:pNB5 cells, the pool of proaerolysin found within the shockable fraction of CB3:γ123 cells accounts for only one half of the total intracellular proaerolysin. The remainder of the toxin is not released by osmotic shock; instead it remains associated with the shocked cells (Fig. 20).

Secretion of the accumulated proaerolysin pool

Letellier and colleagues (1997) have reported that the relatively small periplasmic pool of proaerolysin found within CB3:pNB5 cells is rapidly secreted into the extracellular environment. Indeed, such cells treated with chloramphenicol to arrest further translation of the protein, are able to secrete 90% of this proaerolysin pool within the first 20 minutes following addition of the poison. I was interested in examining the cells' ability to secrete the much larger accumulated pool of proaerolysin found within CB3:γ123 cells. With this aim in mind, CB3:γ123 cells were induced for two hours with 0.1 mM IPTG in order for the intracellular pool to increase in size. After this time, the cells were moved into fresh media and protein synthesis was arrested by the addition of 120 μg/ml chloramphenicol. The subsequent secretion of the accumulated proaerolysin pool was then monitored.

The results presented in Fig. 21 indicate that, following the addition of chloramphenicol, there is an immediate decrease in the amount of proaerolysin found within the shockable fraction of the cells. As a corresponding increase in proaerolysin was found in the culture supernatant, one could conclude that the decrease of

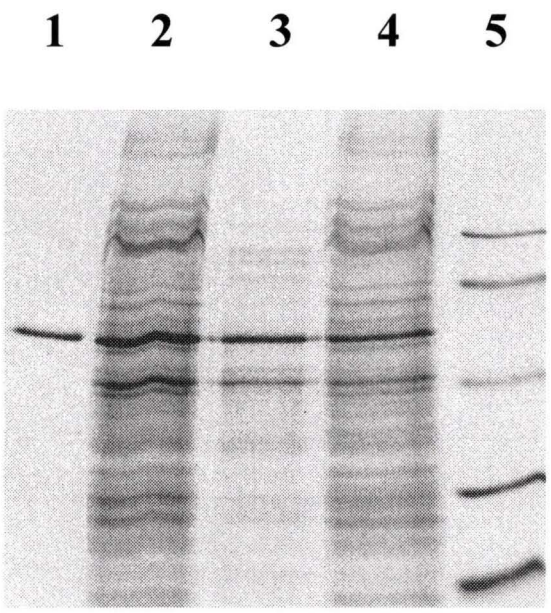
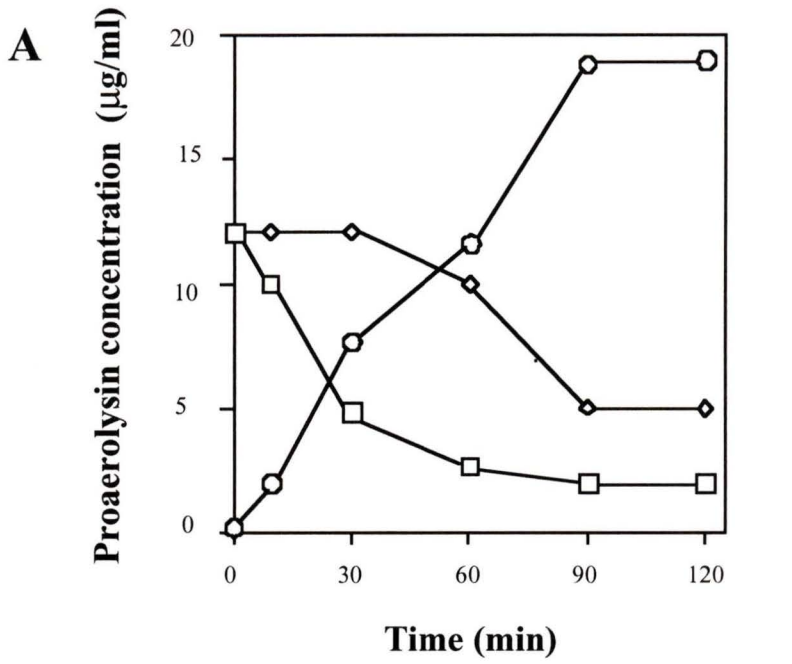


Figure 20. **Proaerolysin distribution upon osmotic shock.** CB3:γ123 cells were induced for 2 hours with 0.1 mM IPTG and then osmotically shocked as described in Materials and Methods. Lane 1, purified proaerolysin; lane 2, whole cells: lane 3, shock fluid; lane 4, shocked cells; lane 5, molecular mass standards (from top 97.4, 66.2, 45.0, 31.0, and 21.5 kDa). Samples were separated by SDS-PAGE and stained with Commassie blue.



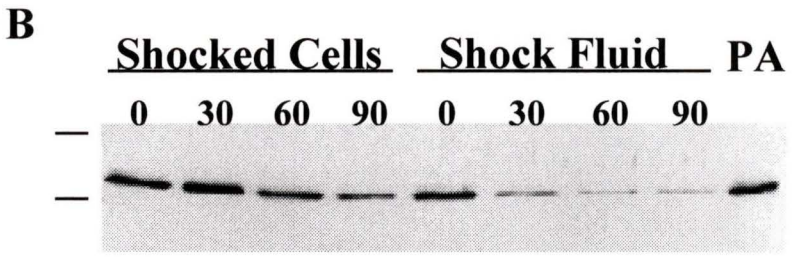


Figure 21. **Secretion of the accumulated proaerolysin pool.** CB3:γ123 cells were induced for 2 hours with 0.1 mM IPTG, then treated with chloramphenicol according to the procedure described in the text. A. The secretion of proaerolysin found within shocked cells (◊) and shock fluid (□) into the culture supernatant (○). Proaerolysin concentrations were determined by haemolytic titre assay as described in the text. B. Immunoblot of the shocked cells and shock fluid samples taken at indicated time intervals (minutes) following the addition of chloramphenicol. Purified proaerolysin (PA) is seen in the last lane and bars mark the locations of molecular mass markers (77 and 50 kDa). Proteins were separated by SDS-PAGE and immunoblotted.

proaerolysin within the shock fluid represents the second step in proaerolysin secretion, transfer across the outer membrane. Thus, even when proaerolysin is overexpressed to such high levels, secretion across the outer membrane of the cell remains active. However, the other proaerolysin pool, associated with the shocked cells, does not decrease immediately following the addition of chloramphenicol (Fig. 21). Instead, the amount of proaerolysin within this pool remains relatively constant during the first 30 minutes of the experiment and a significant decrease in this pool is not seen until 90 minutes following chloramphenicol addition. Furthermore, no additional secretion of proaerolysin into the culture supernatant is seen after this time, yet approximately 40% of the non-shockable pool of proaerolysin remains within the cells. This is compared to approximately 10% of the shockable pool that remains within the cells at this time. These results may indicate that the pool of proaerolysin associated with the shocked cells is not readily available for secretion.

Pulse-chase labeling followed by immunoprecipitation was used to further examine the secretion of the non-shockable pool of proaerolysin. Again, CB3:γ123 cells were induced for two hours with 0.1 mM IPTG in order for the intracellular pools to accumulate. After this time, the cells were moved into Met^- minimal media and labeled by the addition of ^{35}S-methionine. Two minutes following the pulse, the label was chased with cold methionine and the cells were immediately placed back into the rich LB media. The subsequent secretion of labeled proaerolysin was monitored.

The results of this initial experiment are presented in Fig. 22. Here, the amount of labeled proaerolysin seen in the culture supernatant gradually increases during the course of the experiment. However, the amount of label seen within the intact cells also

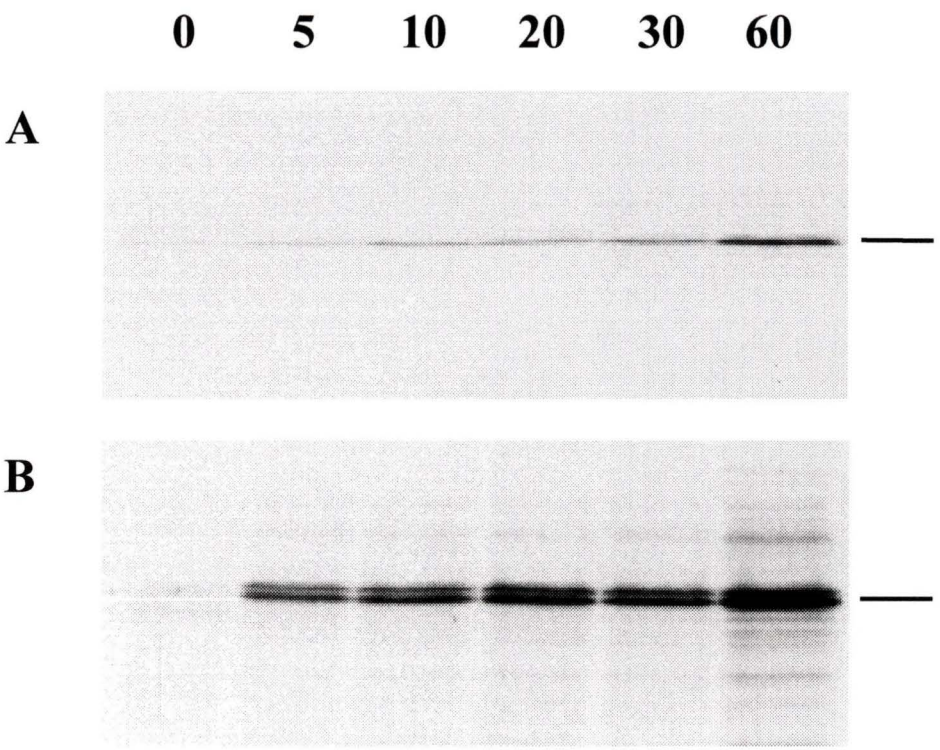


Figure 22. **Initial pulse-chase and immunoprecipitation of CB3:γ123.** CB3:γ123 cells were induced for 2 hours with 0.1 mM IPTG and then pulsed with ^{35}S-methionine. Aliquots of culture supernatants (A) and whole cells (B) were collected at indicated time intervals (minutes) and immunoprecipitated as described in the text. The bar marks the position of purified proaerolysin.

increases over time. This suggests that the chase with unlabelled methionine was unsuccessful; that following the chase, a substantial amount of radiolabeled methionine remains within the cells' amino acid pools.

In an effort to prevent incorporation of the labeled methionine into proaerolysin chains produced following the pulse, the experiment was repeated, however 120 μg/ml chloramphenicol was added to the culture media immediately following collection of the five-minute sample. The results obtained from this experiment indicate that this procedure was successful; while the amount of label seen within the cells increases during the first five minutes following resuspension in LB media, there is no further increase in the label after this time (Fig. 23A).

The autoradiograph presented in Fig. 23A also indicates that less than one half of the label found within the intact cells at the five minute mark is incorporated into the prepro-species of the toxin (proaerolysin that still possesses the N-terminal signal sequence). As the experiment continued, evidence of preproaerolysin processing can be seen. The sample collected 30 minutes following resuspension in LB media displays less preproaerolysin. This trend continues throughout the course of the experiment, with the sample collected at 120 minutes containing mostly proaerolysin. As expected, the same trend is seen within the shocked cells (Fig. 23B). One can therefore conclude that movement of proaerolysin across the inner membrane (as evidenced by processing of the leader peptide) occurs slowly.

The results obtained from this experiment also indicate that, five minutes following resuspension in LB media, the shocked cells and shock fluid (or shockable fraction) contain similar amounts of labeled proaerolysin (Fig. 23B and Fig. 23C). The

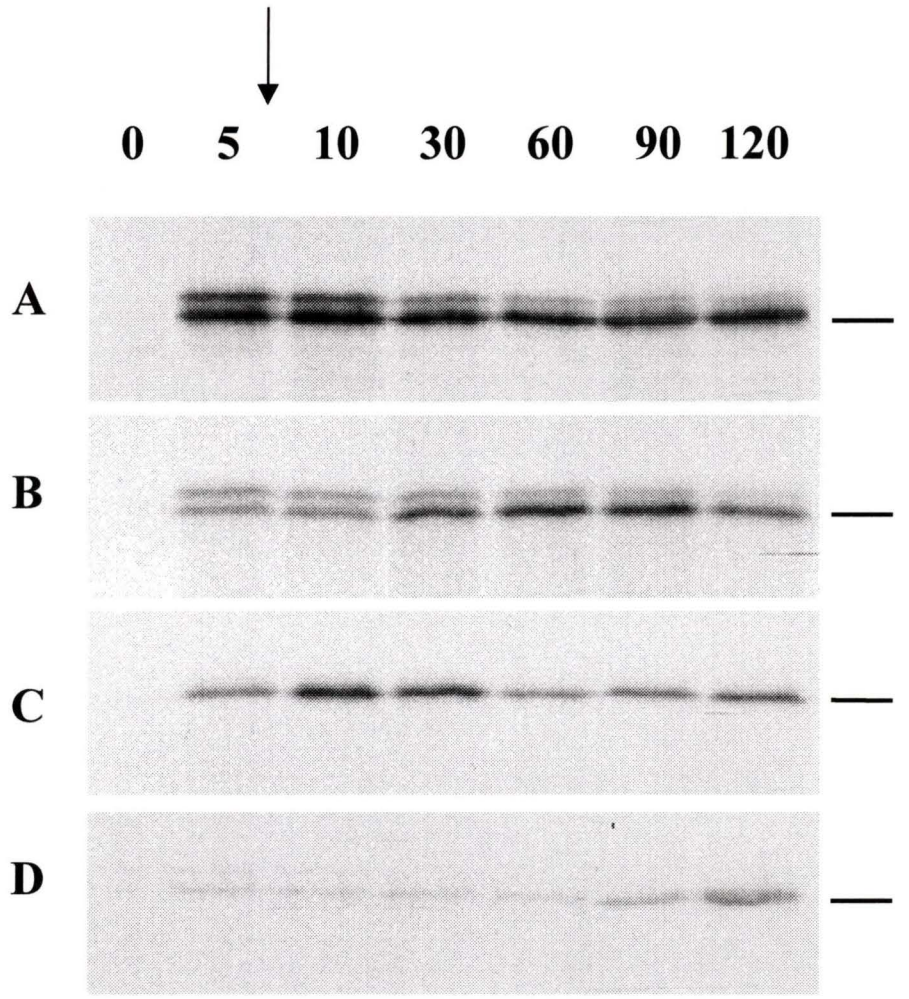


Figure 23. **Pulse-chase and immunoprecipitation of CB3:γ123 in the presence of chloramphenicol.** CB3:γ123 cells were induced for 2 hours with 0.1 mM IPTG and then pulsed with ^{35}S-methionine. Aliquots of whole cells (A), shocked cells (B), shock fluid (C) and culture supernatant (D) were collected at indicated time intervals (minutes) and immunoprecipitated as described in the text. The arrow indicates the addition of chloramphenicol to the culture flask. Bars mark the position of purified proaerolysin.

amount of labeled proaerolysin in the shockable fraction then increases during the next 25 minutes, indicating movement of labeled toxin into this pool (Fig. 23C). After this time, a decrease in the amount of label within the shockable fraction is seen. As this is accompanied by an increase in labeled proaerolysin in the culture supernatant (Fig. 23D), the decrease in proaerolysin seen in the shockable fraction likely represents secretion across the outer membrane. The decrease in labeled proaerolysin within the shockable fraction (seen in the first 60 minutes) is followed by a slight increase at 90 minutes and again at 120 minutes. These increases appear to correspond to a decrease in label seen within the shocked cells, suggesting that at this time the label is moving out of the pool associated with the shocked cells and into the shockable pool. Decreases in labeled proaerolysin associated with the shocked cells occur only within the last half-hour of the experiment; during the beginning of the experiment no significant decrease in this pool is seen. This result is in good agreement with the results obtained in the initial secretion experiment where movement out of the proaerolysin pools was quantitated (see above and Fig. 21) again suggesting that the pool of proaerolysin, associated with the shocked cells is not readily available for secretion.

The cell-associated proaerolysin pool is correctly folded

One possible explanation for why the pool of proaerolysin associated with the shocked cells was not being readily secreted is that the protein in this pool had not correctly folded. To examine this possibility, induced CB3:γ123 cells were subjected to osmotic shock in the presence of 40 μg/ml trypsin. This experiment yielded a 48 kDa form of aerolysin that comigrated on an SDS-PAGE gel with aerolysin produced when

wild-type proaerolysin is treated with this protease (Fig. 24). This result appears to exclude the possibility that CB3:γ123 cells are unable to secrete the accumulated pool due to incorrect folding of the protein.

The cell-associated pool does not contain proaerolysin dimers

Another experiment was performed to determine whether the proaerolysin present within the nonshockable fraction had formed dimers. In this experiment, osmotic shock was carried out in the presence of 25 mM DSP. The results of this experiment indicate that, after cross-linking, the majority of the proaerolysin found within the shockable fraction migrated to the same position on an SDS-PAGE gel as purified proaerolysin treated in the same manner (Fig. 25). As proaerolysin is a dimer in solution (van der Goot *et al.*, 1993), this result indicates that the proaerolysin found within the shockable fraction has formed dimers within the cell. This result is in good agreement with results published by Hardie *et al.* (1995), which reveal that proaerolysin dimerizes in the periplasm. However, as seen in Fig. 25, most of the proaerolysin found within the shocked cells was not recovered as dimers following cross-linking. Also of interest, were the presence of two additional bands found within the shocked cells both of which migrate slightly higher than the proaerolysin dimer. These bands are not detected in the shock fluid. Initially, I thought that these bands may represent a close association between proaerolysin and components of the secretion machinery. Preliminary experiments were carried out to see if this were true. The blot displayed in Fig. 24 was repeated and developed using antibodies against selected Exe proteins (namely ExeA, ExeB, ExeD, ExeF, ExeK, and ExeN). None of these antibodies detected the two bands

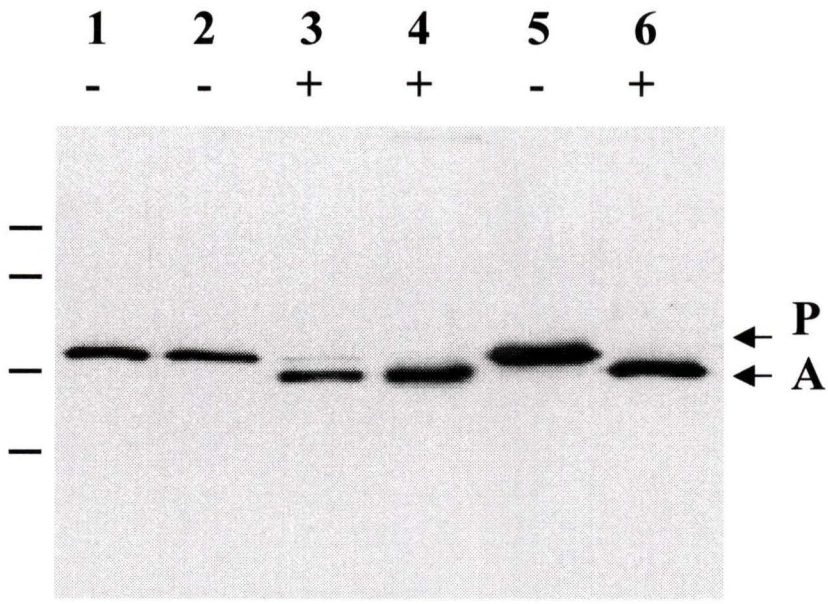


Figure 24. **Activation of the cell-associated pool of proaerolysin.** CB3:γ123 cells were induced for 2 hours with 0.1 mM IPTG and then osmotically shocked in the presence (+) or absence (-) of 40 μg/ml trypsin as indicated. Lanes 1 and 3, shock fluid; lanes 2 and 4, shocked cells; lanes 5 and 6, purified proaerolysin. Samples were separated by SDS-PAGE and immunoblotted. Arrows indicate the position of proaerolysin (P), and aerolysin (A) and bars the locations of the molecular mass markers (from top, 103, 77, 50, and 34.3 kDa).

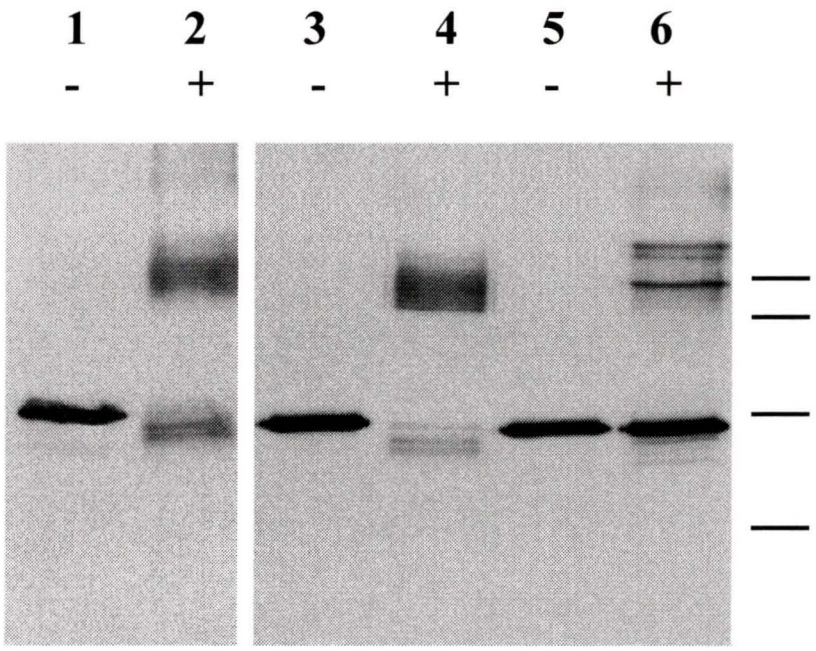


Figure 25. **Chemical cross-linking of CB3:γ123 cells.** CB3:γ123 cells were induced for 2 hours with 0.1 mM IPTG and then osmotically shocked in the presence (+) or absence (-) of 0.25 mM DSP as indicated. Lanes 1 and 2, purified proaerolysin; lanes 3 and 4, shock fluid; lanes 5 and 6, shocked cells. Samples were separated by SDS-PAGE and immunoblotted. Bars mark the locations of molecular mass markers (from top, 116.0, 80.0, 52.5, and 34.9 kDa).

of interest (data not shown). Subsequent studies have shown that the complexes represented by these bands are soluble (not shown). As a result, it is unlikely that they represent an interaction between proaerolysin and the membrane-bound Exe proteins. As yet, the identity of these bands remains unknown.

The location of the cell-associated pool

I wished to pinpoint the exact location of all proaerolysin found within CB3:γ123 cells. The fact that the bulk of the intracellular toxin is in the proaerolysin form, indicating that transport across the inner membrane has occurred, is in agreement with the observation that all of the protein is available to trypsin upon osmotic shock (Fig. 24). However, it was possible that trypsin treatment resulted in lysis of the cells thereby exposing a cytoplasmic pool of proaerolysin. In order to examine this possibility, an assay for isocitrate dehydrogenase (ICDH; a cytoplasmic enzyme) was performed on both the trypsin treated shocked cells and shock fluid. The results of this experiment revealed that ICDH was only present in the shocked cells; it was not present in the shockable fraction (not shown). This indicated that the integrity of the inner membrane remained intact during the osmotic shock procedure. Therefore, all proaerolysin that is processed by trypsin must be found outside the inner membrane. Taken together, these data are good evidence that proaerolysin accumulates outside the inner membrane. However, these data are seemingly inconsistent with the observation that a portion of the intercellular proaerolysin is not released from the cells upon osmotic shock. This is especially apparent as assaying for β-lactamase, a periplasmic marker, indicated that osmotic shock releases 100% of the total β-lactamase activity (not shown). This would

in turn indicate that the method of osmotic shock used here is efficient at releasing periplasmic proteins. The apparent discrepancy between these results is unclear.

The cell-associated pool does not fractionate with periplasmic proteins

Other methods of cell fractionation, which others have shown to selectively release periplasmic proteins, were also performed in an effort to release all of the cell-associated proaerolysin found within CB3:γ123 cells. However, none of the methods, including polymixin B treatment (Thorstenson *et al.*, 1997), chloroform treatment (Thorstenson *et al.*, 1997) and treatment with lysozyme-EDTA, were successful (data not shown).

Two methods of cell fractionation, Triton-X 100 extraction (Thorstenson *et al.*, 1997) and solubilization by French pressing did release the bulk of the cell-associated proaerolysin (not shown). However, both of these methods lysed the cells, as evidenced by the release of isocitrate dehydrogenase thereby providing us with little explanation as to the exact location of the accumulated pool.

The cell-associated pool of proaerolysin is not membrane-bound

I also utilized sucrose density-gradient ultracentrifugation to determine whether the nonshockable proaerolysin pool was membrane-bound. The results from these experiments indicated that after French-pressing, this pool was found within the soluble fraction of the cell; it was not bound to either the inner or outer membrane of the cell (data not shown).

Accumulation of proaerolysin causes periplasmic swelling

As I was unable to identify the location of the cell-associated proaerolysin using conventional fractionation methods, I employed the use of electron microscopy to directly visualize the cells. This method provided the opportunity to investigate the possibility that the overexpression of proaerolysin by CB3:γ123 had resulted in packaging of the toxin into membrane vesicles or inclusion bodies. While electron microscopic examination of CB3:γ123 cells (induced with 0.1 mM IPTG) did not reveal the presence of either of these structures, it did reveal that the periplasm of the cells was enlarged at one pole (Fig. 26B). In contrast, no enlargement of the periplasm could be seen in CB3:pNB5 cells that were induced in the same manner (Fig. 26A) suggesting that the periplasmic swelling in CB3:γ123 cells is the result of proaerolysin overexpression.

Electron microscopy of immunogold labeled thin sections from CB3:γ123 cells (induced with 0.1 mM IPTG) with polyclonal antisera raised against proaerolysin resulted in no labeling of the cytoplasm or of the inner and outer membranes (Fig. 26C). This result is in good agreement with previous results obtained from cell fractionation experiments (see above). Instead, all labeling of the CB3:γ123 cells was localized within the periplasmic space, particularly in the enlarged space at the pole of the cell (Fig. 26C). This result provided direct evidence to support a periplasmic location of the accumulated proaerolysin. However, it is still not clear why a portion of this proaerolysin is retained within the cells after osmotic shock.

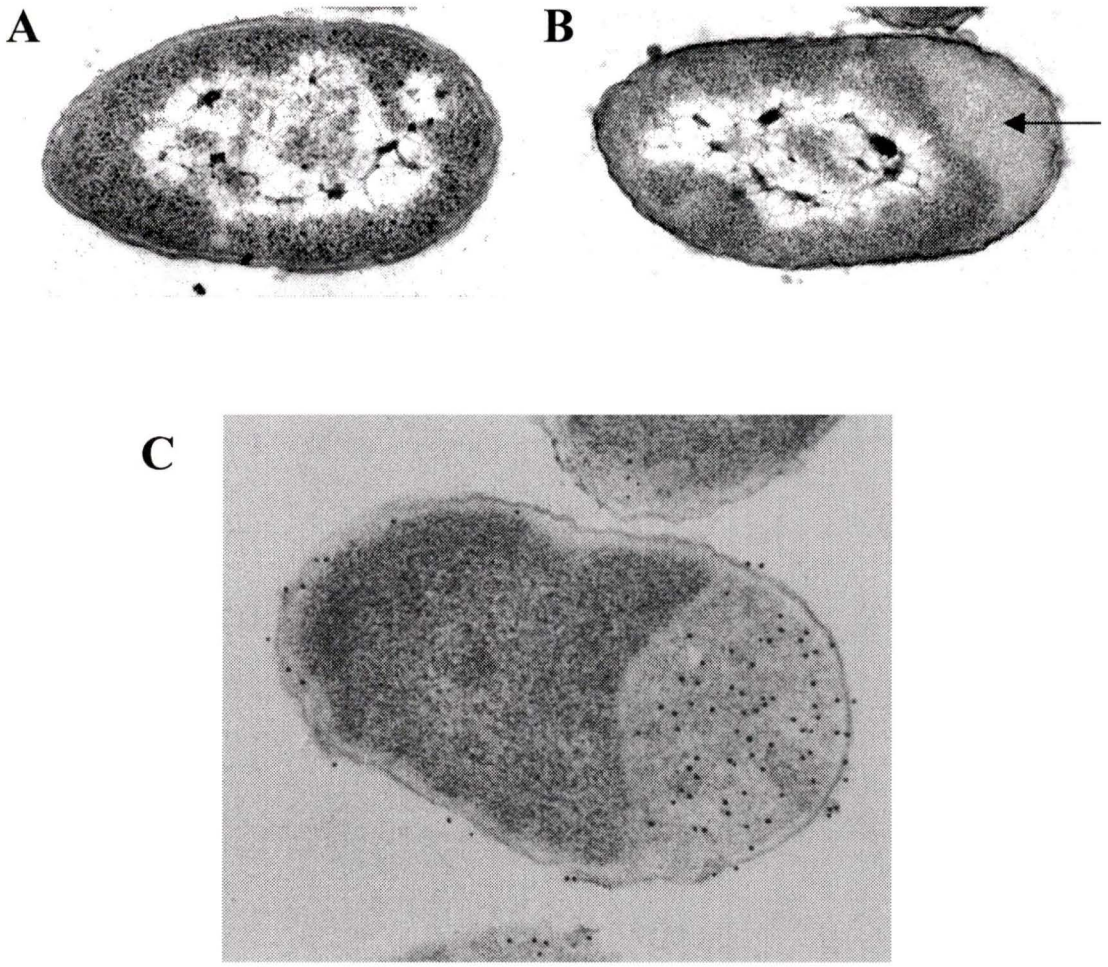


Figure 26. **Periplasmic accumulation of proaerolysin in CB3:γ123 cells.** CB3:pNB5 cells (A) and CB3:γ123 cells (B) were induced with 1 mM IPTG before examination by electron microscopy. The arrow indicates the enlarged periplasmic space seen within the CB3:γ123 cells. C. Immunogold labelling of thin sections of CB3:γ123 cells was performed using polyclonal antisera raised against proaerolysin. Preparation of cells was carried out as described in Materials and Methods.

DISCUSSION

Protein folding and its role in the secretion process

While proaerolysin and other proteins secreted by Type II systems are known to contain N-terminal signal sequences that direct their transport across the inner membrane, the secretion signal for translocation across the outer membrane remains unclear. Many groups have suggested that such a signal is not simply a linear sequence of amino acids but rather a three-dimensional signal that comes together upon correct folding of the protein. This hypothesis is supported by the fact that comparison of the sequences of Type II secretory proteins has failed to find any common linear sequence. In addition, many studies have found that these proteins take on tertiary structure within the periplasm. For example, intramoleclular disulphide bridges form within pullulanase and proaerolysin prior to their secretion from the periplasm (Pugsley, 1992; Hardie *et al.*, 1995). Cellulase produced by *E. chrysanthemi* also forms disulphide bridges within the periplasm, however in contrast to pullulanase and proaerolysin, the disulphide bridges within cellulase appear to be critical to the secretion of the protein (Borotoli-German *et al.*, 1994).

The results described within this thesis clearly demonstrate a link between protein folding and proaerolysin secretion. Three proaerolysin variants containing internal deletions (Δloop, End440, and End450) fold incorrectly as evidenced by their susceptibility to degradation by trypsin (Figs. 7 and 10). Furthermore, the results obtained from sucrose density gradients indicated that the Δloop variant, containing a deletion of the loop found within domain 3 of proaerolysin was insoluble (Fig. 6). The results shown

in Figs 3 and 8 indicate that none of these deletion variants were successfully secreted by *A. salmonicida.* However, one can not conclude whether the inability of the bacterium to secrete these variants was caused by the removal of a secretion signal or rather by the incorrect presentation of a secretion signal as a result of protein misfolding. For this reason, it is unlikely that further examination of deletion variants will prove useful in determining the secretion signal of proaerolysin.

The C-terminal peptide

The observation that variants containing deletions of the C-terminal activation peptide do not fold correctly is surprising considering that folding of the protein begins as the N-terminal end of the polypeptide chain is exported into the periplasm (see Introduction). How deletions at the C-terminal end of the protein (the last part of the protein to be synthesized) are able to exert such a dramatic effect on protein folding is unclear. However, the finding that these variants (End440 and End450) were not secreted is consistent with other studies of propeptides and protein secretion. Two such studies have analyzed the secretion of elastase, an endopeptidase produced by *P. aeruginosa* (McIver *et al.*, 1995; Braun *et al.*, 1996). Like aerolysin, elastase is secreted as a proprotein *via* a Type II secretion pathway. However, there are many differences between the elastase and aerolysin propeptides. For example, processing of the elastase propeptide occurs within the bacterial periplasm rather than in the extracellular environment (McIver *et al.*, 1991). Furthermore, after processing, this propeptide remains noncovalently associated with the mature domain, and the elastase-propeptide complex is then secreted by the cell (Kessler *et al.*, 1998). Despite these differences,

studies involving secretion of the *P. aeruginosa* elastase have shown that like the activation peptide of proaerolysin, the propeptide of elastase is important in the correct folding and secretion of the protein (Braun *et al.*, 1996). When a truncated variant of the enzyme (lacking the propeptide) was expressed in *P. aeruginosa*, its secretion was inhibited. Interestingly, coexpression of the propeptide with mature elastase resulted in the formation of an active enzyme that was actively secreted, suggesting that the propeptide acts as an intramolecular chaperone for elastase (McIver *et al.*, 1995; Braun *et al.*, 1996). It would be interesting to see if the activation peptide of aerolysin plays a similar role.

Further findings implicating a link between propeptides and protein secretion has been demonstrated by Nomura *et al.* (2000) who reported that the secretion of *A. sobria* haemolysin variants lacking amino acid residues from the C-terminal end was lower than that of wt haemolysin. However, this decrease in secretion was attributed to a decrease in stability of the haemolysin variants. Indeed, pulse chase analysis revealed substantial degradation of a variant lacking the 10 C-terminal amino acid residues within 15 minutes following its production (Nomura *et al.*, 2000). As yet, there is no evidence to suggest that deletion of the aerolysin propeptide decreases the stability of this protein.

In addition to their study of the role of the haemolysin propeptide in toxin secretion, Nomura and colleagues have investigated the role the propeptide plays in toxin activity (Nomura *et al.*, 1999). They have reported that the deletion of amino acid residues from the C-terminal end of the protein resulted in a decrease in haemolytic activity of the toxin. In contrast, the truncated proaerolysin variants created in this study displayed no haemolytic activity at all (Table 2). This apparent discrepancy likely lies

within the fact that Nomura and colleagues only removed up to 10 amino acids from the C-terminal end of haemolysin. In contrast, the studies described here involved the removal of a minimum of 20 amino acids. Furthermore, Nomura *et al.* reported that removal of an increasing number of amino acids from the C-terminal end of haemolysin, corresponded to a greater decrease in haemolytic activity of the toxin (Nomura *et al.*, 1999). It is possible that proaerolysin variants having fewer amino acids removed from the C-terminus may display some level of haemolytic activity. It is important to note that the Act$^-$ variant (lacking the activation sequence KVRRAR) was active despite the fact that removal of the activation sequence effectively shortened the C-terminal end of the protein by 6 amino acid residues.

The activation sequence

The only proaerolysin variant described here that was successfully secreted by *A. salmonicida* is the Act$^-$ variant (Fig. 13). The fact that this variant was secreted suggests this region of the protein plays no role in the secretion process. However, my experiments involving this variant emphasize how important proteolytic removal of the C-terminal peptide is to the toxin's activity. For example, removal of the KVRRAR sequence prevents proteolytic nicking of the toxin by chymotrypsin and trypsin (Fig. 14). As a result, trypsin-treated Act$^-$ displays no haemolytic activity (Fig. 15). More significantly, removal of the activation sequence prevents activation of proaerolysin by T-lymphomas (Fig. 17). As proteinase K is able to activate Act$^-$ (Fig. 15), the observation that T-lymphomas can not activate this variant suggests there are no proteinase K-like proteins present on the surface of these cells. This in turn lends support

to the claim that the sensitivity mammalian cells display to proaerolysin, is a result of toxin activation by furin, a proprotein convertase found on the cell's surface (Abrami *et al.*, 1998).

Act$^-$ has since been used as a template for the creation of another proaerolysin variant in which the activation sequence KVRRAR was replaced with a new sequence QNYPIV, the recognition sequence for HIV-1 protease (Nutt *et al.*, 1988). Like Act$^-$, the new variant termed Act^{HIV}, could not be activated by trypsin or chymotrypsin (Burr and Buckley, unpublished observations). Similarly, T-lymphomas are unable to activate the variant. Act^{HIV} is however activated by treatment with either proteinase K or purified HIV-1 protease (Burr and Buckley, unpublished observations). Future studies involving the Act^{HIV} variant will investigate the interaction between the variant and HIV-infected cells. We anticipate that these studies will contribute to our understanding of the HIV-1 protease and its activity and also to the possibility that aerolysin is internalized by target cells.

Overexpression of proaerolysin

CB3:γ123 cells possess two proaerolysin pools

The results presented in this thesis suggest that overexpression of proaerolysin by CB3:γ123 results in the formation of two distinct pools of toxin within the cell; both exterior to the cytoplasmic membrane. The first of these pools is released from the cells upon osmotic shock (Fig. 21) indicating it is located within the periplasm. Furthermore, the results displayed in Fig. 22 suggest that proaerolysin associated with this pool represents protoxin that is soon to be secreted by the cell. The size of the 'shockable'

pool of proaerolysin decreases immediately following chloramphenicol treatment. This is accompanied by an increase in proaerolysin found within the culture supernatant suggesting secretion of the protein into the external environment. This finding is consistent with previous results that demonstrate the periplasm is a part of the normal export route of proaerolysin (Howard and Buckley, 1985b; Wong *et al.*, 1989).

The second proaerolysin pool within CB3:γ123 is found associated with shocked cells (Fig. 21). In contrast to the 'shockable pool' of toxin, this pool does not seem to be readily available for secretion. As seen in Figs. 22 and 24, secretion of this pool appears to be delayed; significant movement of proaerolysin out of the 'nonshockable pool' does not begin until approximately 60 minutes following treatment with chloramphenicol. Furthermore, not all of this pool is secreted by the cell even as much as 2 hours following treatment with chloramphenicol. These findings suggest that CB3:γ123 cells are producing more proaerolysin than can be efficiently secreted across the outer membrane of the cell. It is conceivable that saturation of the Exe machinery is responsible for the initial accumulation of proaerolysin within the 'non-shockable pool'.

The results shown in Fig. 24 also indicate that movement of proaerolysin across the inner membrane (as evidenced by processing of preproaerolysin) occurs rather slowly when the periplasmic pool is large. Significant processing of the preproaerolysin present in CB3:γ123 cells does not occur until approximately 20 minutes following chloramphenicol treatment. This result suggests that overexpression of proaerolysin also results in blockage of the Sec system. Based on studies carried out in *E. coli*, the number of Sec translocases present in the cell is very high, approximately 500 (Matsuyama *et al.*, 1992; Pugsley, 1993). Furthermore, each translocase is estimated to export

approximately 80 polypeptides per minute (Pugsley, 1993). Thus, the total number of polypeptides translocated by the Sec system of a single cell is upward to 4×10^5 polypeptides per minute. This value is higher than the maximal rate of proaerolysin secretion into the culture supernatant, which was calculated to be 8×10^3 molecules of toxin per minute from the data presented in Fig. 22. Therefore, it is likely that overexpression of proaerolysin first results in saturation of the secretion machinery present in the outer membrane leading to toxin accumulation within the cell. This may then be followed by saturation of the Sec system located in the inner membrane.

The location of the 'non-shockable' proaerolysin pool

In an effort to further characterize the nature of proaerolysin that accumulates within the shocked cells, osmotic shock was carried out in the presence of trypsin (Fig. 25). The results of this experiment provided two important pieces of evidence. First, as the pool of proaerolysin associated with the shocked cells was not degraded by trypsin but rather correctly processed to form active aerolysin, one can conclude that this pool of toxin is correctly folded. This reduces the possibility that proaerolysin accumulates in this pool as a result of incorrect protein folding. [Similar conclusions have been drawn with regards to cell-associated proaerolysin found within *E. coli* and *Vibrio* species expressing the cloned *aerA* gene (Howard and Buckley, 1986; Wong *et al.* 1990).] Secondly, it indicates that this pool of proaerolysin is available to trypsin upon osmotic shock, that is, the protein has crossed the inner membrane. To demonstrate that this was not simply because the cells were lysed upon trypsin treatment thereby exposing a cytoplasmic pool, an assay for ICDH activity was performed. The results indicated that

this cytoplasmic enzyme remained cell-associated after shocking in the presence of trypsin and was not present in the shock fluid (not shown). Therefore, one can conclude that the inner membrane remained intact during the osmotic shock procedure and that all proaerolysin associated with the shocked cells must be located outside the inner membrane.

As electron micrographs of immunogold labeled CB3:γ123 cells indicated that the proaerolysin found within these cells is located in the periplasm (Fig. 28), the observation that there is a pool of proaerolysin that can not be shocked from the cells is certainly intriguing. It is conceivable that the proaerolysin found within the shocked cells has been shunted to a novel periplasmic compartment. This suggestion is consistent with early reports by Cerny and Teuber (1972) who predicted that the periplasm of Gram negative bacteria is not continuous but is instead compartmentalized. This prediction is supported by studies that have shown that the free movement of proteins within the periplasm is prevented by the cells' annuli (Foley *et al.*, 1989). The idea that proaerolysin may be found within specific periplasmic compartments raises several questions. For example, why is it that such a compartment is not sensitive to osmotic shock but can be released from the cell upon French pressing? What is the nature of such a periplasmic compartment? Certainly, if proaerolysin were being packaged into discrete compartments such as membrane vesicles, these structures would be visible by electron microscopy.

While membrane vesicles were not seen in electron micrographs of CB3:γ123 cells (Figs. 27 and 28), electron microscopic examination did reveal that the periplasm of the cells was enlarged. This phenomenon could even be seen in dividing cells, indicating

that periplasmic swelling is not merely an artifact of cell death (not shown). It is possible that enlargement or swelling of the periplasm disrupts critical contacts between the inner and outer membranes of the cell. Such disruption might prevent contact between ExeD and ExeS present in the outer membrane with the remainder of the Exe secretion apparatus found within the inner membrane. This may in turn prevent secretion of proaerolysin from any Exe secretion complexes located within the enlarged pole of the cell. Consistent with this hypothesis are the results obtained by Letellier *et al.* (1997) that indicate proaerolysin secretion is inhibited by hyperosmotic conditions (i.e. incubation of the cells in high sucrose or NaCl solutions). Plasmolysis of the cells as a result of these conditions may also disrupt critical contacts between the outer membrane and inner membrane components of the secretion apparatus (Letellier *et al.*, 1997) thereby preventing proaerolysin secretion.

Interestingly, similar enlargement of the periplasm has been seen in the *A. salmonicida* Tn5 mutant, A449-TM1, that is unable to transport the surface layer A-protein to the cell surface (Noonan and Trust, 1995). Immunogold labeling of this bacterium with polyclonal antisera raised against the A-protein revealed this protein is also localized within the enlarged periplasm. Still other examples of periplasmic swelling have been reported in *E. coli* by Wetzel *et al.* (1970) and Murgier *et al.* (1977).

As periplasmic enlargement was not seen in CB3:pNB5 cells expressing lower levels of proaerolysin (Fig. 26), it is reasonable to assume that the increase in proaerolysin production by CB3:γ123 cells is directly responsible for periplasmic swelling. By using the data presented in Fig. 22, the proaerolysin concentration within the periplasm has been estimated to be greater than 100 mg/ml. It is possible that this

increase in protein concentration within the periplasm disrupts the osmotic balance across the bacterial membranes. This may result in water being drawn into the periplasm from the extracellular environment or from the cytoplasm thereby causing the periplasm to expand.

Periplasmic swelling in CB3:γ123 cells was consistently seen at the cell poles rather then along the length of the cell (Fig. 27 and 28). While the reason for this is not clear, it is interesting to note that during mild osmotic upshock (for example when cells are placed in 10 to 20% sucrose) plasmolysis bays first form at the cell poles (Mulder and Woldringh, 1993; Woldringh, 1994). It is possible that the factor behind the increased sensitivity to plasmolysis displayed by cell poles also plays a role in the formation of enlarged polar regions within CB3:γ123 cells.

Dimerization of proaerolysin and its role in the protein's secretion

The results generated from chemical cross-linking of all cell-associated proaerolysin within CB3:γ123 cells have indicated that in contrast to the 'shockable pool' of proaerolysin, toxin found within the 'nonshockable pool' has not formed dimers (Fig. 26). It is possible that this is the key to why proaerolysin accumulates within this pool. Nomura and colleagues have suggested that dimerization is critical to the secretion of the *A. sobria* haemolysin (Nomura *et al.*, 2000). However, this conclusion was merely based on the observation that haemolysin dimers are present in *A. sobria* cells and culture supernatant. More convincing evidence for the role of dimerization in proaerolysin secretion has come from studies carried by Hardie *et al.* (1995). These authors observed that proaerolysin dimers held together by an interchain disulphide bridge were secreted as

efficiently as proaerolysin molecules that were unable to form this covalent bond. This finding, coupled with the fact that the large lobe of proaerolysin (composed of Domains 2-4) is monomeric and poorly secreted (Diep *et al.*, 1998) suggests that dimerization of proaerolysin may provide secondary structure that is important to the protein's secretion. The assembly of protein monomers into higher level structures is not uncommon among GSP proteins. For example, Hirst and Holmgren (1987) have shown that monomers of the cholera toxin B subunit must assemble into pentamers within the periplasm before they can be secreted across the outer membrane. If dimerization were indeed critical to proaerolysin secretion, it would provide an attractive explanation of why the pool of proaerolysin associated with the shocked cells is not readily secreted and instead accumulates within the cells.

Still, the observation that the pool of proaerolysin associated with the shocked cells has not formed dimers (Fig. 26) raises the question: why is this pool of proaerolysin monomeric? Protein folding within the periplasm is often assisted by folding catalysts, proteins that aid in specific rate-limiting steps of the folding process (Missiakas and Raina, 1997). For example, protein disulphide isomerases carry out thiol-disulphide exchanges. Other proteins, such as DsbA (see Introduction) catalyze the formation of disulphide bonds. These bonds in turn, contribute to the stabilization of the folded protein conformation (Creighton, 1986). While little is known about the dimerization process, it does seem as though the formation of disulphide bonds within proaerolysin monomers is not absolutely dependent on DsbA (Hardie *et al.*, 1995). Still, there may be other proteins that assist in the formation of the proaerolysin dimer. One such candidate is DsbC, identified in *E. coli* and *E. chrysanthemi* (Missiakes *et al.*, 1994; Shevchik *et al.*,

1994). Overexpression of proaerolysin seen in CB3:γ123 cells may be producing more proaerolysin molecules than the accessory protein(s) can efficiently assist. The production of increasing amounts of toxin may lead to a 'backlog' of protein waiting to be assembled into dimers.

Other effects of proaerolysin overexpression

While my research has shown that overexpression of proaerolysin by CB3:γ123 cells affects both protoxin secretion and the cell ultrastructure, the experiments appear to suggest that this overexpression may also affect amino acid pools in these cells. For example, the results obtained from the pulse-chase and immunoprecipitation experiments indicated there was no labeled proaerolysin immediately after a two minute pulse with radiolabeled methionine (Figs. 23 and 24). A similar experiment performed without immunoprecipitation of proaerolysin revealed there was in fact no labeling of any cellular proteins immediately following the pulse (not shown). This observation coupled with the findings presented in Fig. 23, which indicate that a chase with unlabelled methionine did not prevent subsequent incorporation of the labeled amino acid into proaerolysin, suggests that the amino acid pools within CB3:γ123 cells induced with IPTG do not equilibrate quickly. Such problems with labeling were not encountered when the experiment was repeated using CB3:pNB5 cells (not shown) suggesting that it is overexpression of proaerolysin that affects the amino acid pools of the bacterium.

A regulatory role for the stem-loop structure?

What is the purpose or significance of the stem-loop, found at the start of the *aerA* gene within the cell? It seems likely that this structure plays an important regulatory role in proaerolysin production. For example, the stem-loop may act at the level of gene transcription preventing premature interaction of the RNA polymerase with the *aerA* AUG start codon. Alternatively, the *aerA* gene may be constitutively transcribed by the bacterium at very low levels; unfolding of the stem loop in response to an environmental trigger may then result in translation of the mRNA transcripts. Such a trigger might be a change in temperature or pH, or might be provided upon interaction between the bacterium and a potential host. An example of such a system is the *E. coli* heat shock σ-factor, σ^{32}. σ^{32} is encoded for by the *rpoH* gene, which is constitutively expressed by the bacterium. However, because of extensive secondary structure within the 5′ end of the *rpoH* mRNA, the protein is not continually produced. Melting of this secondary structure occurs in response to high temperatures and results in rapid translation of the *rpoH* mRNA leading to increased levels of σ^{32} within the cell (Morita *et al.*, 1999a, and b).

Measuring the levels of *aerA* mRNA transcripts within *A. salmonicida* would likely provide insight into whether this potential secondary structure acts at the level of gene transcription or translation. For example, if CB3:γ123 and CB3:pNB5 cells were shown to contain equivalent levels of *aerA* mRNA, this would indicate that the stem-loop is acting at the level of translation. Alternatively, if CB3:γ123 cells were shown to contain significantly more *aerA* mRNA, one would conclude that the stem-loop is affecting gene transcription. Such studies may also be useful in determining the conditions required for expression of the chromosomally encoded *aerA* gene in *A.*

salmonicida. This would be particularly interesting as *A. salmonicida* does not express chromosomally encoded proaerolysin under laboratory culture conditions.

SUMMARY

In summary, the results presented within this thesis indicate a link between proaerolysin folding and the secretion process. Expression of proaerolysin variants containing internal deletions resulted in the production of misfolded proteins that could not be secreted by the cells.

The overexpression of proaerolysin by *A. salmonicida* was found to affect secretion of the toxin across both the inner and outer membranes. This overexpression also affected the ultrastruture of the cells resulting in proaerolysin accumulation within an enlarged periplasm.

FUTURE DIRECTION

While the results presented here provide convincing evidence that all proaerolysin found within CB3:γ123 cells is located in the periplasm, it is unclear why a portion of this proaerolysn is not released upon osmotic shock. It is unclear why the non-shockable pool of proaerolysin has not formed dimmers within the cell. Additional studies are needed to address these issues.

Future studies in this area might examine the relationship between this Type II secretion pathway and the biogenesis of Type IV pili. In particular, it would be interesting to learn whether the overexpression of proaerolysin adversely affects pili formation.

LITERATURE CITED

Abrami, L., Fivaz, M., Decroly, E., Seidah, N.G., Jean, F., Thomas, G., Leppla, S.H., Buckley, J.T., and van der Goot, F. (1998) The pore-forming toxin proaerolysin is activated by furin. *Journal of Biological Chemistry* **273:** 32656-32661.

Anderson, D.M., and Schneewind, O. (1997) A mRNA signal for the Type III secretion of Yop proteins by *Yersinia enterocolitica. Science* **278:** 1140-1143.

Arkowitz, R.A., and Wickner, W. (1994) SecD and SecF are required for the proton electrochemical gradient stimulation of preprotein translocation. *The EMBO Journal* **13:** 954-963.

Awram, P., and Smit, J. (1998) The *Caulobacter crescentus* paracrystalline S-layer protein is secreted by an ABC transporter (Type I) secretion apparatus. *Journal of Bacteriology* **180:** 3062-3069.

Ball, G., Chapon-Herve, V., Bleves, S., Michel, G., and Bally, M. (1999) Assembly of XcpR in the cytoplasmic membrane is required for extracellular protein secretion in *Pseudomonas aeruginosa. Journal of Bacteriology* **181:** 382-388.

Bardwell, J.C.A., McGovern, K., and Beckwith, J. (1991) Identification of a protein required for disulphide bond formation *in vivo*. *Cell* **67:** 581-589.

Berger, B.R., and Christie, P.J. (1994) Genetic complementation analysis of the *Agrobacterium tumefaciens* virB operon: virB2 through virB11 are essential virulence genes. *Journal of Bacteriology* **176:** 646-660.

Binet, R., and Wandersman, C. (1995) Protein secretion by hybrid bacterial ABC-transporters: specific functions of the membrane ATPase and the membrane fusion protein. *The EMBO Journal* **14:** 2298-2306.

Boman, H.G. (1991) Antibacterial peptides: key components needed in immunity. *Cell* **65:** 205-207.

Bolin, I., Portnoy, D.A., and Wolf-Watz, H. (1985) Expression of the temperature-inducible outer membrane proteins of *Yersiniae*. *Infection and Immunity* **48:** 234-240.

Bortoli-German, I., Haiech, J., Chippaux, M., and Barras, F. (1995) Informational suppression to investigate structural, functional and evolutionary aspects of the *Erwinia chrysanthemi* cellulase EGZ. *Journal of Molecular Biology* **246:** 82-94.

Braun, P., Tommassen, J., and Filloux, A. (1996) Role of the propeptide in folding and secretion of elastase of *Pseudomonas aeruginosa. Molecular Microbiology* **19:** 297-306.

Brumlik, M.J., van der Goot, F.G., Wong, K.R., and Buckley, J.T. (1997) The disulphide bond in the *Aeromonas hydrophila* lipase/acyltransferase stabilises the structure but is not required for secretion or activity. *Journal of Bacteriology* **179:** 3116-3121.

Buckley, J.T. (1990) Purification of cloned proaerolysin released by a low protease mutant of *Aeromonas salmonicida. Biochemistry and Cell Biology* **68:** 221-224.

Burns, D.L. (1995) Transport of pertussis toxin across bacterial and eucaryotic membranes. In *Membrane Transport Proteins*. Rothman, S. (ed). Greenwich, Conn: JAI Press, pp. 25-38.

Cerny, G., and Teuber, M. (1972) Comparitive polyacrylamide electrophoresis of periplasmic proteins released from Gram-negative bacteria by polymixin B. *Archiv Fur Mikrobiologie* **82:** 261-370.

Cheng, L.W., Anderson, D.M., and Schneewind, O. (1997) Two independent type III secretion mechanisms for YopE in *Yersinia entercolitica. Molecular Microbiology* **24:** 757-765.

Cohen, S.N., Chang, A.C.Y., and Hsu, L. (1972) Nonchromosomal antibiotic resistance in bacteria: Genetic transformation of *Escherichia coli* by R-factor DNA. *Proceedings of the National Academy of Sciences (USA)* **69:** 2110-2114.

Collazo, C.M., and Galan, J.E. (1997) The invasion-associated type-III protein secretion system in *Salmonella* - a review. *Gene* **192:** 51-59.

Cook, D.M., Farizo, K.M., and Burns, D.L. (1999) Identification and characterisation of PtlC, an essential component of the Pertussis toxin secretion system. *Infection and Immunity* **67:** 754-759.

Coote, J.G. (1992) Structural and functional relationships among the RTX toxin determinants of Gram-negative bacteria. *FEMS Microbiology Reviews* **88:** 137-162.

Cornelis, G.R. (1998) The *Yersinia* deadly kiss. *Journal of Bacteriology* **180:** 5495-5504.

Cornelis, G.R. (2000) Molecular and cell biology aspects of the plague. *Proceedings of the National Academy of Sciences (USA)* **97:** 8778-8783.

Covacci, A., and Rappuoli, R. (1993) Pertussis toxin export requires accessory genes located downstream from the pertussis toxin operon. *Molecular Microbiology* **8:** 429-434.

Creighton, T.E. (1986) Detection of folding intermediates using urea-gradient electrophoresis. In *Methods in Enzymology* Vol. 131. New York: Academic Press, pp.156-172.

Daefler, S., Guilvout, I., Hardie, K.R., Pugsley, A.P., and Russel, M. (1997) The C-terminal domain of the secretin PulD contains the binding site for its cognate chaperone, PulS, and confers PulS dependence on pIVfl function. *Molecular Microbiology* **24:** 465-475.

d'Enfert, C., Chapon, C., and Pugsley, A.P. (1987a) Export and secretion of the lipoprotein pullulanase by *Klebsiella pneumoniae*. *Molecular Microbiology* **1:** 107-116.

d'Enfert, C., Ryter, A., and Pugsley, A.P. (1987b) Cloning and expression in *Escherichia coli* of the *Klebsiella pneumoniae* genes for production, surface localisation and secretion of the lipoprotein pullulanase. *The EMBO Journal* **6:** 3531-3538.

d'Enfert, C., and Pugsley, A.P. (1989) *Klebsiella pneumoniae pulS* gene encodes an outer membrane lipoprotein required for pullulanase secretion. *Journal of Bacteriology* **171:** 3673-3679.

Diep, D.B., Lawrence, T.S., Ausio, J., Howard, S.P., and Buckley, J.T. (1998) Secretion and properties of the large and small lobes of the channel-forming toxin aerolysin. *Molecular Microbiology* **30:** 341-352.

Dinh, T., Paulsen, I.T., and Saier, M.H.J. (1994) A family of extracytoplasmic proteins that allow transport of large molecules across the outer membranes of Gram-negative bacteria. *Journal of Bacteriology* **176:** 3825-3831.

Driessen, A.J.M. (1992) Precursor protein translocation by the *Escherichia coli* translocase is directed by the proton motive force. *The EMBO Journal* **11:** 847-853.

Driessen, A.J.M., Fekkes, P., and van der Wolk, J.P.W. (1998) The Sec system. *Current Opinion in Microbiology* **1:** 216-222.

Duong, F., Lazdunski, A., Cami, B., and Murgier, M. (1992) Nucleotide sequence of a cluster of genes involved in synthesis and secretion of alkaline protease in *Pseudomonas aeruginosa*: relationships to other secretion pathways. *Gene* **121:** 47-54.

Duong, F., and Wickner, W. (1997) The SecDFyajC domain of preprotein translocase controls preprotein movement by regulating SecA membrane cycling. *The EMBO Journal* **16:** 4871-4879.

Economou, A., Pogliano, J.A., Beckwith, J., Oliver, D.B., and Wickner, W. (1995) SecA membrane cycling at SecYEG is driven by distinct ATP binding and hydrolysis events and is regulated by SecD and SecF. *Cell* **83:** 1171-1181.

Economou, A. (1999) Following the leader: bacterial protein export through the Sec pathway. *Trends in Microbiology* **7:** 315-320.

Farizo, K.M., Cafarella, T.G., and Burns, D.L. (1996) Evidence for a ninth gene, *ptlI*, in the locus encoding pertussis toxin secretion system of *Bordetella pertussis* and formation of a PtlI-PtlF complex. *Journal of Biological Chemistry* **271:** 31643-31649.

Farizo, K.M., Huang, T., and Burns, D.L. (2000) Importance of holotoxin assembly in Ptl-mediated secretion of pertussis toxin from *Bordetella pertussis*. *Infection and Immunity* **68:** 4049-4054.

Fekkes, P., van der Does, C., and Driessen, A.J.M. (1997) The molecular chaperone SecB is released from the carboxy-terminal of SecA during initiation of precursor protein translocation. *The EMBO Journal* **16:** 6105-6113.

Fekkes, P., de Wit, J.G., van der Wolk, J.P.W., Kimsey, H.H., Kumamoto, C.A. and Driessen, A.J.M. (1998) Preprotein transfer to the *Escherichia coli* translocase requires the co-operative binding of SecB and the signal sequence to SecA. *Molecular Microbiology* **29:** 1179-1190.

Felmlee, T., Pellet, S., Lee, E.Y., and Welch, R.A. (1985) *Escherichia coli* hemolysin is released extracellularly without cleavage of a signal peptide. *Journal of Bacteriology* **163:** 88-93.

Foley, M., Brass, J.M., Birmingham, J., Cook, W.R., Garland, P.B., Higgins, C.F., and Rothfield, L.I. (1989) Compartmentalization of the periplasm at cell division sites in *Escherichia coli* as shown by fluorescence photobleaching experiments. *Molecular Microbiology* **3:** 1329-1336.

Forsberg, A., Viitanen, A.M., Skurnik, M, and Wolf-Watz, H. (1991) The surface-located YopN protein is involved in calcium signal trasduction in *Yersinia pseudotuberculosis*. *Molecular Microbiology* **5:** 977-986.

Furste, J.P., Pansegrau, W., Frank, R., Blocker, H., Scholz, P., Bagdasarian, M., and Lanka, E. (1986) Molecular cloning of the plasmid RP4 primase region in a multi-host-range *tacP* expression vector. *Gene* **48:** 119-131.

Garland, W.J., and Buckley, J.T. (1988) The cytolytic toxin aerolysin must aggregate to disrupt erythrocytes, and aggregation is stimulated by human glycophorin. *Infection and Immunity* **56:** 1249-1253.

Genin, S., and Boucher, C.A. (1994) A superfamily of proteins involved in different secretion pathways in Gram-negative bacteria: Modular structure and specificity of the N-terminal domain. *Molecular and General Genetics* **243:** 112-118.

Gray, L., Machman, N., Nicaud, J.M., and Holland, I.B. (1986) The carboxy-terminal region of haemolysin 2001 is required for secretion of the toxin from *Escherichia coli. Molecular and General Genetics* **205:** 127-133.

Guilvout, I., Hardie, K.R., Sauvonnet, N., and Pugsley, A.P. (1999) Genetic dissection of the outer membrane secretin PulD: Are there distinct domains for multimerization and secretion specificity? *Journal of Bacteriology* **181:** 7212-7220.

Hakansson, S., Bergman, T., Vanooteghem, J., Cornelis, G., and Wolf-Watz, H. (1993) YopB and YopD constitute a novel class of *Yersinia* Yop proteins. *Infection and Immunity* **61:** 71-80.

Hakansson, S., Schesser, K., Persson, C., Galyov, E.E., Rosqvist, R., Homble, F, and Wolf-Watz, H. (1996) The YopB protein of *Yersinia pseudotuberculosis* is essential for the translocation of Yop effector proteins across the target cell plasma membrane and displays a contact-dependent membrane disrupting activity. *The EMBO Journal* **15:** 5812-5823.

Halter, R., Pohlner, and Meyer, T.F. (1984) IgA protease of *Neisseria gonorhoeae*: Isolation and characterisation of the gene and its extracellular product. *The EMBO Journal* **3:** 1595-1601.

Harayama, S., Tsuda, M., and Lino, T. (1980) High frequency mobilization of the chromosome of *Escherichia coli* by a mutant of plasmid RF4 temperature-sensitive for maintenance. *Molecular and General Genetics* **180:** 47-56.

Hardie, K.R., Schulze, A., Parker, M.W., and Buckley, J.T. (1995) *Vibrio* spp. secrete proaerolysin as a folded dimer without the need for disulphide bond formation. *Molecular Microbiology* **17:** 1035-1044.

Hardie, K.R., Lory, S., and Pugsley, A.P. (1996a) Insertion of an outer-membrane protein in *Escherichia coli* requires a chaperone-like protein. *The EMBO Journal* **15:** 978-988.

Hardie, K.R., Seydel, A., Guilvout, I., and Pugsley, AP. (1996b) The secretin-specific, chaperonlike protein of the general secretory pathway: separation of proteolytic protection and piloting functions. *Molecular Microbiology* **22:** 967-976.

Hartl, F.U., Lecker, S., Schiebel, E., Hendrick, J.P., and Wickner, W. (1990) The binding of SecB to SecA to SecY/E mediates preprotein targeting to the *E. coli* membrane. *Cell* **63:** 269-279.

Higgins, C.F. (1992) ABC Transporters - from microorganisms to man. *Annual Review of Cell Biology* **8:** 67-113.

Hirst, T.R., and Holmgren, J. (1987) Conformation of protein secreted across bacterial outer membranes: A study of enterotoxin translocation from *Vibrio cholera.* (1987) *Proceedings of the National Academy of Sciences (USA)* **84:** 7418-7422.

Howard, S.P., and Buckley, J.T. (1985a) Activation of the hole-forming toxin aerolysin by extracellular processing. *Journal of Bacteriology* **163:** 336-340.

Howard, S.P., and Buckley, J.T. (1985b) Protein export by a Gram-negative bacterium: Production of aerolysin by *Aeromonas hydrophila. Journal of Bacteriology* **161:** 1118-1124.

Howard, S.P., and Buckley, J.T. (1986) Molecular cloning and expression in *Escherichia coli* of the structural gene for the hemolytic toxin aerolysin from *Aeromonas hydrophila. Molecular and General Genetics* **204:** 289-295.

Howard, S.P., Critch, J., and Bedi, A. (1993) Isolation and analysis of eight *exe* genes and their involvement in extracellular protein secretion and outer membrane assembly in *Aeromonas hydrophila. Journal of Bacteriology* **175:** 6695-6703.

Howard, S.P., Meiklejohn, H.G., Shivak, D., and Jahagirdar, R. (1996) A TonB-like protein and a novel membrane protein containing an ATP-binding cassette function together in exotoxin secretion. *Molecular Microbiology* **22:** 595-604.

Hueck, C. (1998) Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiology and Molecular Biology Reviews* **62:** 379-433.

Inoue, H., Nojima, H., and Okayama, H. (1990) High efficiency transformation of *Escherichia coli* with plasmids. *Gene* **96:** 23-28.

Issartel, J.P., Koronakis, V., and Hughes, C. (1991) Activation of *Escherichia coli* prohaemolysin to the mature toxin by acyl carrier protein-dependent fatty-acylation. *Nature* **351:** 759-761.

Ito, K., Bassford, P.J., and Beckwith, J. (1981) Protein localisation in *E. coli*: Is there a common step in secretion of periplasmic and outer-membrane proteins? *Cell* **24:** 707-717.

Jahagirdar, R., and Howard, S.P. (1994) Isolation and characterisation of a second *exe* operon required for extracellular secretion in *Aeromonas hydrophila*. *Journal of Bacteriology* **176:** 6819-6826.

Jarvis, K., Giron, J.A., Jerse, A.E., McDonald, T.K., Donnenberg, M.S., and Kaper, J.B. (1995) Enteropathogenic *Escherichia coli* contains a putative type III secretion system necessary for the export of proteins involved in attaching and effacing lesion formation. *Proceedings of the National Academy of Sciences (USA)* **92:** 7996-8000.

Jiang, B., and Howard, S.P. (1992) The *Aeromonas hydrophila* ExeE gene, required both for protein secretion and normal outer membrane biogenesis, is a member of a general secretion pathway. *Molecular Microbiology* **6:** 1351-1361.

Johnson, F.D., and Burns, D.L. (1994) Dectection and subcellular localization of three Ptl proteins involved in the secretion of Pertussis toxin from *Bordetella pertussis*. *Journal of Bacteriology* **176:** 5350-5356.

Jones, R.N., Wilson, H.W., Novick, W.J., Barry, A.L., and Thornsberry, C. (1982) In vitro evaluation of CENTA, a new beta-lactamase-susceptible chromogenic cephalosporin reagent. *Journal of Clinical Microbiology* **15:** 954-958.

Jose, J., Jahnig, F., and Meyer, T.F. (1995) Common structural features of IgA1 protease-like outer membrane protein autotransporters. *Molecular Microbiology* **18:** 377-382.

Jose, J., Kramer, J., Klauser, T., Pholner, J., and Meyer, T.F. (1996) Absence of periplasmic DsbA oxidoreductase facilitates export of cysteine-containing passenger proteins to the *Escherichia coli* cell surface via the Iga_{β} autotransporter pathway. *Gene* **178:** 107-119.

Karlyshev, A.V., and MacIntyre, S. (1995) Cloning and study of the genetic organization of the *exe* gene cluster of *Aeromonas salmonicida*. *Gene* **158:** 77-82.

Kenny, B., Taylor, S., and Holland, I.B. (1992) Identification of individual amino acids required for secretion within the haemolysin (HlyA) C-terminal targeting region. *Molecular Microbiology* **6:** 1477-1489.

Kenny, B., Chervaux, C., and Holland, I.B. (1994) Evidence that residues -15 to -46 of the haemolysin secretion signal are involved in early steps in secretion, leading to recognition of the translocator. *Molecular Microbiology* **11:** 99-109.

Kessler, E., Safrin, M., Gustin, J.K., and Ohman, D.E. (1998) Elastase and the LasA protease of *Pseudomonas aeruginosa* are secreted by their propeptides. *Journal of Biological Chemistry* **273:** 30225-30231.

Klauser, T., Pohlner, J., and Meyer, T.F. (1992) Selective extracellular release of cholera toxin B subunit by *Escherichia coli*: dissection of *Neisseria* Iga_{β}-mediated outer membrane transport. *The EMBO Journal* **11:** 2327-2335.

Klauser, T., Kramer, J., Otzelberger, K., Pohlner, J., and Meyer, T.F. (1993) Characterization of the *Neisseria* Iga_{β}-core. The essential unit for outer membrane targeting and extracellular protein secretion. *Journal of Molecular Biology* **234:** 579-593.

Kornacker, M.M., and Pugsley, A.P. (1989) Molecular characterisation of *pulA* and its product, pullulanase, a secreted enzyme of *Klebsiella pneumoniae* UNF5023. *Molecular Microbiology* **4:** 73-85.

Koronakis, V., Koronakis, E., and Hughes, C. (1989) Isolation and analysis of the C-terminal signal directing export of *Escherichia coli* haemolysin protein across both bacterial membranes. *The EMBO Journal.* **8:** 595-605.

Koronakis, E., Hughes, C., Milisav, I., and Koronakis, V. (1995) Protein exporter function and *in vitro* ATPase activity are correlated in ABC-domain mutants of HlyB. *Molecular Microbiology* **16:** 87-96.

Koronakis, V., Li, J., Koronakis, E., and Stauffer, K. (1997) Structure of TolC, the outer membrane component of the bacterial type I efflux system, derived from two-dimensional crystals. *Molecular Microbiology* **23:** 617-626.

Koronakis, V., Sharff, A., Koronakis, E., Luis, B., and Hughes, C. (2000) Crystal structure of the bacterial membrane protein TolC central to multidrug efflux and protein export. *Nature* **405:** 914-919.

Koster, M., Bitter, W., de Cock, H., Allaoui, A., Cornelis, G.R., and Tommassen, J. (1997) The outer membrane component, YscC, of the Yop secretion machinery of *Yersinia enterocolitica* forms a ring-shaped multimeric complex. *Molecular Microbioloby* **26:** 789-797.

Kubori, T., Matsushima, Y., Nakamura, D., Urail, J., Lara-Tero, M., Sukhan, A., Galan, J.E., and Aizawa, S. (1998) Supramolecular structure of the *Salmonella typhimurium* Type III protein secretion system. *Science* **280:** 602-605.

Kumamoto, C.A., and Francetic, O. (1993) Highly selective binding of nascent polypeptides by an *Escherichia coli* chaperone *in vivo*. *Journal of Bacteriology* **175:** 2184-2188.

Letellier, L., Howard, S.P., and Buckley, J.T. (1997) Studies on the energetics of proaerolysin secretion across the outer membrane of *Aeromonas* species. *Journal of Biological Chemistry* **272:** 11109-11113.

Letoffe, S., Delepelaire, P., and Wandersman, C. (1990) Protease secretion by *Erwinia chrysanthemi:* the specific secretion functions are analogous to those of *Escherichia coli* α-haemolysin. *The EMBO Journal* **9:** 1375-1382.

Letoffe, S., Delepelaire, P., and Wandersman, C. (1991) Cloning and expression in *Escherichia coli* of the *Serratia marcescens* metalloprotease gene: secretion of the protease from *E. coli* in the presence of the *Erwinia chrysanthemi* protease secretion functions. *Journal of Bacteriology* **173:** 2160-2166.

Letoffe, S., Delepelaire, P., and Wandersman, C. (1996) Protein secretion in Gram-negative bacteria: assembly of the three components of ABC protein-mediated exporters is ordered and promoted by substrate binding. *The EMBO Journal* **15:** 5804-5811.

Lindeberg, M., Salmond, G.P.C., and Collmer, A. (1996) Complementtion of deletion mutations in a cloned functional cluster of *Erwinia chrysanthemi* out genes with *Erwinia carotovora* out homologs reveals OutC and OutD as candidate gatekeepers of species-specific secretion of proteins via the type II pathway. *Molecular Microbiology* **20:** 175-190.

Locht, C., and Keith, J. (1986) Pertussis toxin gene: nucleotide sequence and genetic organization. *Science* **232:** 1258-1264.

Mackman, N., and Holland, I.B. (1984) Secretion of a 107 k dalton polypeptide into the medium from a haemolytic *E. coli* K12 strain. *Molecular and General Genetics* **193:** 312-315.

Manting, E.H., and Driessen, A.J.M. (2000) *Escherichia coli* translocase: the unravelling of a molecular machine. *Molecular Microbiology* **37:** 226-238.

Manting, E.H., van der Does, C., Remigy, H., Engel, A., and Driessen, J.M. (2000) SecYEG assembles into a tetramer to form the active translocation channel. *The EMBO Journal* **19:** 852-861.

Matsuyama, S.I., Fujita, Y., Sagara, K., and Mizushima, S. (1992) Overproduction, purification and characterization of SecD and SecF, intergral membrane components of the protein translocation machinery of *Escherichia coli. Biochimica et Biophysica Acta* **1122:** 77-84.

Matsuyama, S.I., Fujita, Y., and Mizushima, S. (1993) SecD is involved in the release of translocated secretory proteins from the cytoplasmic membrane of *Eshcerichia coli. The EMBO Journal* **12:** 265-270.

McIver, K.S., Kessler, E., and Ohman, D.E. (1991) Substitution of active-site His-223 in *Pseudomonas aeruginosa* elastase and expression of the mutated *lasB* alleles in *Escherichia coli* show evidence for autoproteolytic processing of proelastase *Journal of Bacteriology* **173:** 7781-7789.

McIver, K.S., Kessler, E., Olson, J.C., and Ohman, D.E. (1995) The elastase propeptide functions as an intramolecular chaperone required for elastase activity and secretion in *Pseudomonas aeruginosa. Molecular Microbiology* **18:** 877-889.

Menard, R., Dehio, C., and Sansonetti, P.J. (1996) Bacterial entry into epithelial cells: the paradigm of *Shigella. Trends in Microbiology* **4:** 220-226.

Michel, G., Bleves, S., Ball, G., Lazdunski, A., and Filoux, A. (1998) Mutual stabilization of the XcpZ and XcpY components of the secretory apparatus in *Pseudomonas aeruginosa. Microbiology* **144:** 3379-3386.

Michiels, T., Wattiau, P., Brasseur, R., Ruysschaert, J., and Cornelis, G. (1990) Secretion of Yop proteins by *Yersinia. Infection and Immunity* **58:** 2840-2849.

Miller, J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 431.

Missiakes, D., Georgopoulos, C., and Raina, S. (1994) The *Escherichia coli dsbC* (*xprA*) gene encodes a periplasmic protein involved in disulphide bond formation. *The EMBO Journal* **13:** 2013-2020.

Missiakas, D., and Raina, S. (1997) Protein folding in the bacterial periplasm. *Journal of Bacteriology* **179:** 2465-2471.

Morita, M., Kanemori, M., Yanagi, H., and Yura, T. (1999) Heat-induced synthesis of σ^{32} in *Escherichia coli*: Structural and functional dissection of *rpoH* mRNA secondary structure. *Journal of Bacteriology* **181:** 401-410.

Morita, M.T., Tanaka, Y., Kodama, T.S., Kyogoku, Y., Yanagi, H., and Yura, T. (1999) Translational induction of heat shock transcription factor σ^{32}: evidence for a built-in RNA thermosensor. *Genes and Development* **13:** 655-665.

Mulder, E., and Woldringh, C.L. (1993) Plasmolysis bays in *Escherichia coli*: Are they related to development and positioning of division sites? *Journal of Bacteriology* **175:** 2241-2247.

Murgier, M., Pelissier, C., and Lazdunski, A. (1977) Aminopeptidase N from *Escherichia coli*: Unusual interactions with the cell surface. *European Journal of Biochemistry* **74:** 425-433.

Nelson, K.L., Raja, S.M., and Buckley, J.T. (1997) The glyocosylphosphatidylinositol-anchored surface glycoprotein Thy-1 is a receptor for the channel-forming toxin aerolysin. *Journal of Biological Chemistry* **272:** 12170-12174.

Neville, D.M. (1971) Molecular weight determination of protein dodecyl sulphate complexes by gel electrophoresis in a discontinuous buffer system. *Journal of Biological Chemistry* **246:** 6328-6334.

Neyt, C., and Cornelis, G.R. (1999) Insertion of a Yop translocation pore into the macrophage plasma membrane by *Yersinia enterocolitica*: requirement for translocators YopB and YopD, but not LcrG. *Molecular Microbiology* **33:** 971-981.

Nicaud, J.M., Mackman, N., Gray, L., and Holland, I.B. (1985) Characterization of HlyC and mechanism of activation and secretion of haemolysin from *Escherichia coli* 2001. *FEBS Letters* **187:** 339-344.

Nicosia, A., Perugini, M., Franzini, C., Casagli, M.C., Borri, M.G., Antoni, G., Almoni, M., Neri, P., Ratti, G., and Rappuoli, R. (1986) Cloning and sequencing of the pertussis toxin genes: operon structure and gene duplication. *Proceedings from the National Academy of Sciences (USA)* **83:** 4631-4635.

Nicosia, A., and Rappuoli, R. (1987) Promoter of the pertussis toxin operon and production of pertussis toxin. *Journal of Bacteriology* **169:** 2843-2846.

Nomura, T., Fujii, Y., and Okamoto, K. (1999) Secretion of hemolysin of *Aeromonas sobria* as protoxin and contribution of the protoxin to the proteolytic stability of the toxin. *Microbiology and Immunology* **43:** 29-38.

Nomura, T., Hamashima, H., and Okamoto, K. (2000) Carboxy terminal region of haemolysin *Aeromonas sobria* triggers dimerization. *Microbial Pathogenesis* **28:** 25-36.

Noonan, B., and Trust, T.J. (1995) Molecular analysis of an A-protein secretion mutant of *Aeromonas salmonicida* reveals a surface layer-specific protein secretion pathway. *Journal of Molecular Biology* **248:** 316-327.

Nouwen, N., Ranson, N., Saibil, H., Wolpensinger, B., Engel, A., Ghazi, A., and Pugsley, A.P. (1999) Secretin PulD: association with pilot PulS, structure, and ion-conducting channel formation. *Proceedings of the National Academy of Sciences (USA)* **96:** 8173-8177.

Nouwen, N., Stahlberg, H. Pugsley, A.P., and Engel, A. (2000) Domain structure of secretin PulD revealed by limited proteolysis and electron microscopy. *The EMBO Journal* **19:** 2229-2236.

Nutt, R.F., Brady, S.F., Darke, P.L., Ciccarone, T.M., Colton, C.D., Nutt, E.M., Rodkey, J.A., Bennett, C.D., Waxman, L.H., and Sigal, I.S. (1988) Chemical synthesis and enzymatic activity of a 99-residue peptide with a sequence proposed for the human immunodeficiency virus protease. *Proceedings of the National Academy of Sciences (USA)* **85:** 7129-7133.

O'Callaghan, D., Cazevieille, C., Allardet-Servent, A., Boschiroli, M.L., Bourg, G., Foulongne, V., Frutos, P., Kulakov, Y., and Ramuz, M. (1999) A homologue of the *Agrobacterium tumefaciens* VirB and *Bordetella pertussis* Ptl type IV secretion systems is essential for intracellular survival in *Brucella suis*. *Molecular Microbiology* **33:** 1210-1220.

Odenbreit, S., Puls, J., Sedlmaier, B., Gerland, E., Fischer, W., and Haas, R. (2000) Translocation of *Helicobacter pylori* CagA into gastric epithelial cells by type IV secretion. *Science* **287:** 1497-500.

Oliver, D. (1993) SecA protein: autoregulated ATPase catalyzing pre-protein insertion and translocation across the *Escherichia coli* inner membrane. *Molecular Microbiology* **7:** 159-165.

Osborn, M.J., Munson, R. (1974) Separation of the inner (cytoplasmic) and outer membranes of Gram-negative bacteria. In *Methods in Enzymology* Vol. 31. Fleischer, S., and Packer, L. New York: Academic Press, pp. 642-653.

O'Toole, P.W., Austin, J.W., and Trust, T.J. (1994) Identification and molecular characterization of a major ring-forming surface protein from the gastric pathogen *Helicobacter mustelae*. *Molecular Microbiology* **11:** 349-361.

Paetzel, M., Dalbey, R.E., and Strynadka, N.C. (1998) Crystal structure of a bacterial signal peptidase in complex with a beta-lactam inhibitor. *Nature* **396:** 186-190.

Park, S., Liu, G., Topping, T.B., Cover, W.J., and Randall, L.L. (1988) Modulation of folding pathways of exported proteins by the leader sequence. *Science* **239:** 1033-1035.

Parker, M.W., Buckley, J.T., Postma, J.P.M., Tucker, A.D., Leonard, K., Pattus, F., and Tsernoglou, D. (1994) Structure of the *Aeromonas* toxin proaerolysin in its water soluble and membrane-channel states. *Nature* **367:** 292-295.

Paulsen, I.T., Park, J.H., Choi, P.S., Sauer, M.H. (1997) A family of Gram-negative bacterial outer membrane factors that function in the export of proteins, carbohydrates, drugs and heavy metals from Gram-negative bacteria. *FEMS Microbiology Letters* **156:** 1-8.

Pepe, C.M., Eklund, M.W., and Storm, M.S. (1996) Cloning of an *Aeromonas hydrophila* type IV pilus biogenesis gene cluster: complementation of pilus assembly functions and characterisation of a type IV leader peptidase/N-methyltransferase required for extracellular protein secretion. *Molecular Microbiology* **19:** 857-869.

Persson, C., Nordfelth, R., Holmstrom, A., Hakansson, S., Rosqvist, R., anf Wolf-Watz. (1995) Cell-surface-bound *Yersinia* translocate the protein tyrosin phosphatase YopH by a polarized mechanism into the target cell. *Molecular Microbiology* **18:** 135-150.

Petosa, C., Collier, R.J., Klimpel, K.R., Leppla, S.H., and Liddington, R.C. (1997) Crystal structure of the anthrax toxin protective antigen. *Nature* **385:** 833-838.

Pizza, M.G., Covacci, A., Bugnoli, M., Manetti, R., and Rappuoli, R. (1990) The S1 subunit is important for pertussis toxin secretion. *Journal of Biological Chemistry* **265:** 17759-17763.

Plano, G.V., and Straley, S.C. (1995) Mutations in *yscC*, *yscD*, and *yscG* prevents high-level expression and secretion of V antigen and Yops in *Yersinia pestis*. *Journal of Bacteriology* **177:** 3843-3854.

Pohlner, J., Halter, R., Beyreuther, K., and Meyer, T.F. (1987) Gene structure and extracellular secretion of *Neisseria gonorrhoeae* IgA protease. *Nature* **325:** 458-462.

Possot, O., d'Enfert, C., Reyss, I., and Pugsley, A.P. (1992) Pullulanase secretion in *Escherichia coli* K12 requires a cytoplasmic protein and a putative polytopic cytoplasmic membrane protein. *Molecular Microbiology* **6:** 95-105.

Possot, O., and Pugsley, A.P. (1994) Molecular characterization of PulE, a protein required for pullulanase secretion. *Molecular Microbiology* **12:** 287-299.

Possot, O.M., Gerard-Vincent, M., and Pugsley, A.P. (1999) Membrane association and multimerization of secreton component PulC. *Journal of Bacteriology* **181:** 4004-4011.

Possot, O.M., Vignon, G., Bomchil, N., Ebel, F., and Pugsley, A.P. (2000) Multiple interactions between pullulanase secreton components involved in stabilization and cytoplasmic membrane association of PulE. *Journal of Bacteriology* **182:** 2142-2152.

Postle, K. (1993) TonB protein and energy transduction between membranes. *Journal of Bioenergetics and Biomembranes* **25**: 591-601.

Pugsley, A.P., d'Enfert, C., Reyss, I., and Kornacker, M.G. (1990) Genetics of extracellular protein secretion by Gram-negative bacteria. *Annual Review of Genetics* **24:** 67-90.

Pugsley, A.P., Kornacker, M.G., and Pouquet, I. (1991) The general protein-export pathway is directly required for extracellular pullulanase secretion in *Escherichia coli* K12. *Molecular Microbiology* **5:** 343-352.

Pugsley, A.P. (1992) Translocation of a folded protein across the outer membrane in *Escherichia coli. Proceedings of the National Academy of Sciences (USA)* **89:** 12058-12062.

Pugsley, A.P., and Dupuy, B. (1992) An enzyme with type IV prepilin peptidase activity is required to process components of the general extracellular protein secretion pathway of *Klebsiella oxytoca. Molecular Microbiology* **6:** 751-760.

Pugsley, A.P. (1993) The complete general secretory pathway in Gram-negative bacteria. *Microbiological reviews* **57:** 50-108.

Pugsley, A.P., Francetic, O., Possot, O.M., Sauvonnet, N., and Hardie, K.R. (1997) Recent progress and future directions in studies of the main terminal branch of the general secretory pathway in Gram-negative bacteria - a review. *Gene* **192:** 13-19.

Py, B., Loiseau, L., and Barras, F. (1999) Assembly of the type II secretion machinery of *Erwinia chrysanthemi*: Direct interaction and associated conformational change between

OutE, the putative ATP-binding component and the membrane protein OutL. *Journal of Molecular Biology* **289:** 659-670.

Randall, L.L., and Hardy, S.J.S. (1989) Unity in function in the absence of consensus in sequence: Role of leader peptides in export. *Science* **243:** 1156-1159.

Rosqvist, R. Forsberg, A., and Wolf-Watz, H. (1991) Intracellular targeting of the *Yersinia* YopE cytotoxin in mammalian cells induces actin microfilament disruption. *Infection and Immunity* **59:**4562-4569.

Rosqvist, R., Magnusson, K.E., and Wolf-Watz. (1994) Target cell contact triggers expression and polarized transfer of *Yersinia* YopE cytotoxin inot mammalian cells. *The EMBO Journal* **13:** 964-972.

Rossjohn, J., Raja, S.M., Nelson, K.L., Feil, S.C., van der Goot, F.G., Parker, M.W., and Buckley, J.T. (1998) Movement of a loop in Domain 3 of aerolysin is required for channel formation. *Biochemistry* **37:** 741-746.

Ruckdeschel, K., Roggenkamp, A., Schubert, S., and Heesemann, J. (1996) Differential contribution of *Yersinia entercolitica* virulence factors to evasion of microbial action of neutrophils. *Infection and Immunity* **64:** 724-733.

Russel, M. (1998) Macromolecular assembly and secretion across the bacterial cell envelope: Type II protein secretion systems. *Journal of Molecular Biology* **279**: 485-499.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning 1: A Laboratory Manual* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp. 6.3-6.7.

Sandkvist, M., Hough, L.P., Bagdasarian, M.M., and Bagdasarian, M. (1999). Direct Interaction of the EpsL and EpsM proteins of the general secretion apparatus in *Vibrio cholera. Journal of Bacteriology* **181:** 3129-3135.

Sauvonnet, N., Vignon, G., Pugsley, A.P., and Gounon, P. (2000) Pilus formation and protein secretion by the same machinery in *Escherichia coli. The EMBO Journal* **19:** 2221-2228.

Schiebel, E., Driessen, A.J.M., Hartl, F.U., and Wickner, W. (1991) $\Delta\mu H^+$ and ATP function at different steps of the catalytic cycle of preprotein translocase. *Cell* **64:** 927-939.

Schmitt, W., and Haas, R. (1994) Genetic analysis of the *Helicobacter pylori* vacuolating cytotoxin: structural similarities with the IgA protease type of exported protein. *Molecular Microbiology* **12:** 307-319.

Schoenhofen, I.C., Stratilo, C., and Howard, S.P. (1998) An ExeAB complex in the type II secretion pathway of *Aeromonas hydrophila*: effect of ATP-binding cassette mutations on complex formation and function. *Molecular Microbiology* **29:** 1237-1247.

Schulein, R., Gentschev, I., Mollenkopf, H.J., and Globel, W. (1992) A topological model for the haemolysin translocator protein HlyD. *Molecular and General Genetics* **234:** 155-163.

Scotti, P.A., Urbanus, M.L., Brunner, J., De Gier, J.L., van Heijne, G., van der Does, C., Driessen, A.J.M., Oudega, B., and Luirink, J. (2000) YidC, the *Escherichia coli* homologue of mitochondrial Oxa1p, is a component of the Sec translocase. *The EMBO Journal* **19:** 542-549

Shere, K.D., Sallustio, S., Manessis, A., D'Aversa, T.G., and Goldberg, M.B. (1997) Disruption of IcsP, the major *Shigella* protease that cleaves IcsA, accelerates actin-based motility. *Molecular Microbiology* **25:** 451-462.

Shevchik, V.E., Condemine, G., and Robert-Baudouy, J. (1994) Characterization of DsbC, a periplasmic protein of *Erwinia chrysanthemi* and *Escherichia coli* with disulphide isomerase activity. *The EMBO Journal* **13:** 2007-2012.

Smith, H., Bron, S., Van E.E., J., and Venema, G. (1987) Construction and use of signal sequence selection vectors in *Esherichia coli* and *Bacillus subtilis. Journal of Bacteriology* **169:** 3321-3328.

Song, L., Hobaugh, M.R., Shustak, C., Cheley, S., Bayley, H., and Gouaux, J.E. (1996) Structure of Staphylococcal α-hemolysin, a heptameric transmembrane pore. *Science* **274:** 1859-1866.

Sory, M.P., and Cornelis, G.R. (1994) Translocation of a hybrid YopE-adenylate cyclase from *Yersinia entercolitica* into HeLa cells. *Molecular Microbiology* **14:** 583-594.

Sory, M., Boland, A., Lambermont, I., and Cornelis, G.R. (1995) Identification of the YopE and YopH domains required for secretion and internalization into the cytosol of macrophages, using the *cyaA* gene fusion approach. *Proceedings of the National Academy of Sciences (USA)* **92:** 11998-12002.

Stanley, P., Koronakis, V., and Hughes, C. (1991) Mutational analysis supports a role for multiple structural features in the C-terminal secretion signal of *Escherichia coli* haemolysin. *Molecular Microbiology* **5:** 2391-2403.

Stein, M., Kenny, B., Stein, M., and Finlay, B.B. (1996) Characterization of EspC, a 100-kilodalton protein secreted by enteropathogenic *Escherichia coli* which is homologous to members of the immunoglobulin A protease-like family of secreted proteins. *Journal of Bacteriology* **178:** 6546-6554.

Strom, M.S., Nunn, D., and Lory, S. (1991) Multiple roles of the pilus biogenesis protein PilD: Involvement of excretion of enzymes from *Pseudomonas aeruginosa. Journal of Bacteriology* **173:** 1175-1180.

Tamano, K., Aizawa, S., Katayama, E., Nonaka, T., Imajoh-Ohmi, S., Kuwae, A., Nagai, S., and Sasakawa, C. (2000) Supramolecluar structure of the *Shigella* type III secretion machinery: the needle part is changeable in length and essential for delivery of effectors. *The EMBO Journal* **19:** 3876-3887.

Tardy, F., Homble, F., Neyt, C., Wattiez, R., Cornelis, G.R., Ruysschaert, J., and Cabiaux, V. (1999) *Yersinia enterocolitica* type III secretion-translocation system: channel formation by secreted Yops. *The EMBO Journal* **18:** 6793-6799.

Thanabalu, T., Koronakis, E., Hughes, C., and Koronakis, V. (1998) Substrate-induced assembly of a continguous channel for protein export from *E. coli*: reversible bridging of an inner-membrane translocase to an outer membrane exit pore. *The EMBO Journal* **17:** 6487-6496.

Thorstenson, Y.R., Zhang, Y., Olson, P.S., and Mascarenhas, D. (1997) Leaderless polypeptides efficiently extracted from whole cells by osmotic shock. *Journal of Bacteriology* **179:** 5333-5339.

Tommassen, J., Filloux, A., Bally, M., Murgier, M., and Lazdunski, A. (1992) Protein secretion in *Pseudomonas aeruginosa. FEMS Microbiology Reviews* **103:** 73-90.

Vallette, F., Mege, E., Reiss, A., and Adesnik, M. (1988) Construction of mutant and chimeric genes using the polymerase chain reaction. *Nucleic Acids Research.* **17:** 723-733.

van der Goot, F.G., Lakey, J., Pattus, F., Kay, C.M., Sorokine, O., Van Dorsselar, A., and Buckley, J.T. (1992) Spectroscopic study of the activation and oligomerization of the channel-forming toxin aerolysin: Identification of the site of proteolytic activation. *Biochemistry* **31:** 8566-8570.

van der Goot, F.G., Ausio, J., Wong, K.R., Pattus, F., and Buckley, J.T. (1993) Dimerization stabilizes the pore-forming toxin aerolysin in solution. *Journal of Biological Chemistry* **268:** 18272-18279.

Veiga, E., de Lorenzo, V., and Fernandez, L.A. (1999) Probing secretion and translocation of a β-autotransporter using a single-chain Fv as a cognate passenger domain. *Molecular Microbiology* **33:** 1232-1243.

Vogel, J.P., Andrews, H.L., Wong, S.K., and Isberg, R.T. (1998) Conjugative transfer by the virulence system of *Legionella pneumophila. Science* **279:** 873-876.

Weiss, A.A., Johnson, F.D., and Burns, D.L. (1993) Molecular characterisation of an operon required for pertussis toxin secretion. *Proceedings of the National Academy of Sciences (USA)* **90:** 2970-2974.

Wetzel, B.K., Spicer, S.S., Dvork, H.F., and Heppel, L.A. (1970) Cytochemical localisation of certain phosphatases in *Escherichia coli. Journal of Bacteriology* **104:** 529-542.

Willis, R.C., Morris, R.G., Cirakoglu, C., Schellenberg, G.D., Gerber, N.H., and Furlong, C.E. (1974) Preparations of periplasmic proteins from *Salmonella typhimurium* and *Escherichia coli. Archives of Biochemistry and Biophysics* **161:** 64-75.

Wilmsen, H.U., Leonard, K.R., Tichelaar, W., Buckley, J.T., and Pattus, F. (1992) The aerolysin membrane channel is formed by heptamerization of the monomer. *The EMBO Journal* **11:** 2457-2463.

Woestyn, S., Allaoui, A., Wattiau, P., and Cornelis, G.R. (1994) YscN, the putative energiser of the *Yersinia* Yop secretion machinery. *Journal of Bacteriology* **176:** 1561-1569.

Woldringh, C.L. (1994) Significance of plasmolysis spaces as markers for periseptal annuli and adhesion sites. *Molecular Microbiology* **14:** 597-607.

Wong, K.R., and Buckley, J.T. (1989) Proton motive force involved in protein transport across the outer membrane of *Aeromonas salmonicida. Science* **246:** 654-656.

Wong, K.R., Green, M.J., and Buckley, J.T. (1989) Extracellular secretion of cloned aerolysin and phospholipase by *Aeromonas salmonicida. Journal of Bacteriology* **171:** 2523-2527.

Wong, K.R., McLean, D.M., and Buckley, J.T. (1990) Cloned aerolysin of *Aeromonas hydrophila* is exported by a wild-type marine *Vibrio* strain but remains periplasmic in pleiotropic export mutants. *Journal of Bacteriology* **172:** 372-376.

Wong, K.R., and Buckley, J.T. (1991) Site-directed mutagenesis of a single tryptophan near the middle of the channel-forming toxin aerolysin inhibits its transfer across the outer

membrane of *Aeromonas salmonicida*. *Journal of Biological Chemistry* **266:** 14451-14456.

Yahr, T.L., Goranson, J., and Frank, D.W. (1996) Exoenzyme S of *Pseudomonas aeruginosa* is secreted by a type III pathway. *Molecular Microbiology* **22:** 991-103.

Yuk, M.H., Harvill, E.T., and Miller, J.F. (1998) The *BvgAS* virulence control system regulates type III secretion in *Bordetella bronchiseptica*. *Molecular Microbiology* **5:** 945-59.

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