Secretion of Proaerolysin Via the General Secretion Pathway

by

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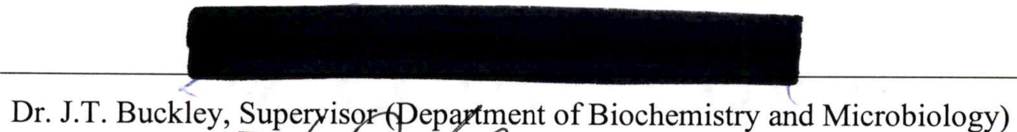
ABSTRACT

Proaerolysin is secreted by *Aeromonas salmonicida* and *Aeromonas hydrophila* via the General Secretion Pathway (GSP). Research presented in this thesis focused on the second step of the GSP, the Main Terminal Branch (MTB). Several approaches were taken to learn more about this process. Although a large amount was secreted into the extracellular medium, some proaerolysin accumulated inside the cell. The intracellular proaerolysin was localized both in the periplasm and in association with the inner membrane. The proaerolysin accumulated inside the cell could be secreted later. The maximal secretion rate of proaerolysin was calculated. A study of the effect of proaerolysin secretion on protease secretion showed that the appearance of protease in the culture supernatant was greatly reduced when proaerolysin secretion approached the maximum, suggesting that there was an upper limit to the GSP-MTB.

A study of secretion of domain II-IV of proaerolysin showed that domain II-IV could be secreted by itself. The secretion was poor compared to secretion of wild-type proaerolysin. When coexpressed with domain I of proaerolysin, domain II-IV did not show a great increase in secretion. However, parallel studies in our laboratory showed that secretion of both domain I and domain II-IV were increased when they were coexpressed in another way (Diep et al., in press). The *Clostridium septicum* α-toxin could not be secreted by *A. salmonicida*, despite the similarity in DNA sequence between α-toxin and aerolysin.

The energy requirements of the proaerolysin secretion were determined. Both ATP and the Proton Motive Force (PMF) were shown to be required for the GSP-MTB.



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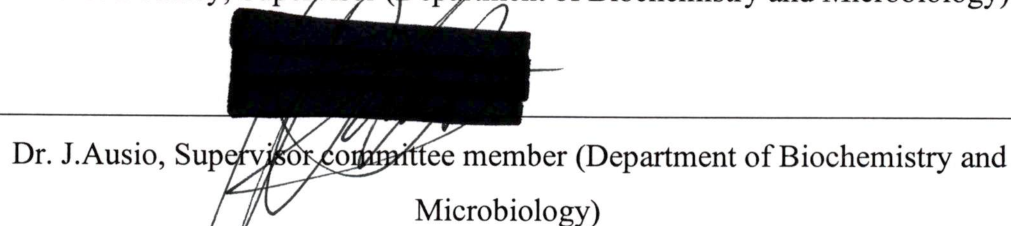

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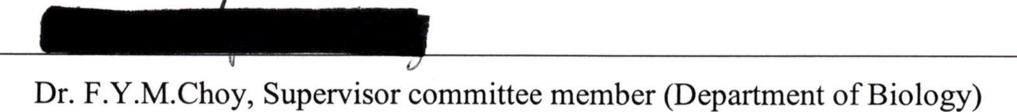

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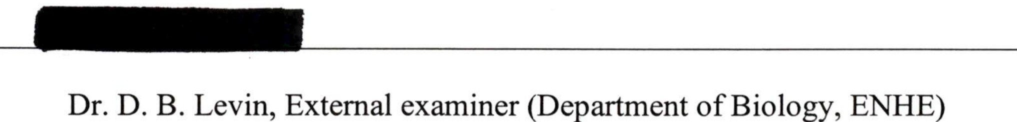

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ABBREVIATIONS

A	absorbance
aa	amino acid
ABC	ATP-binding cassette
Ala	alanine
ATP	adenosine triphosphate
bp	base pair
BSA	Bovine serum albumin
CCCP	carbonyl cyanide m-chlorophenyl hydrazone
CFTR	cystic fibrosis chloride channel
CPY	carboxypeptidase Y
CRA	Congo red agar
DBAP-B	dipeptidyl aminopeptidase B
dH_2O	deionized water
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EPEC	enteropathogenic *E. coli*
GCAT	glycerophospholipid:cholesterol acyl transferase
Gln	glutamine
Gly	glycine

GSP	General Secretion Pathway
GST	glutathione-S-transferase
hr	hour
IPTG	isopropyl-β-D-thiogalactopyranoside
ISO	inside out
kDa	kilodalton
LB	Luria-Bertani
Leu	leucine
LPS	lipopolysaccharide
mA	milliampere
MFP	membrane fusion protein
min	minute
MTB	main terminal branch
NBS	nucleotide binding site
nm	nanometer
OD	optical density
OMP	outer membrane protein
PADAC	{7-(thienyl-2-acetamido)-3[2-(4-N,N-dimethyl-aminophenylazo) pyridium methyl]-3cephem-4-carboxylic acid}
PAGE	polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBS-T	PBS-Tween 20

Phe	phenylalanine
PMF	proton motive force
PVDF	polyvinylidene difluoride
rpm	revolutions per minute
RSO	right side out
SDS	sodium dodecyl sulfate
Ser	serine
SN	supernatant
TM	transmembrane
Tween-20	polyoxyethylene-20-sorbitan monolaurate
w/v	weight/volume

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INTRODUCTION

Protein secretion in prokaryotic cells is a very complicated and interesting process, especially in Gram-negative bacteria, because two membranes are involved. Extensive research has provided evidence for three distinct secretion pathways: the type I, II, and III secretion systems. Detailed descriptions of these pathways will be provided in this section, followed by a brief introduction to Gram-positive bacterial secretion. The features of the genus *Aeromonas* will also be included in this introduction.

PART I. Bacterial secretion pathways

I. Secretion by Gram-negative bacteria

Type I System

The Type I secretion system is used by many Gram-negative bacteria to secrete proteins directly into the medium without a periplasmic intermediate. Secretion is mediated by a complex of three membrane proteins, one of which belongs to the ATP-binding cassette (ABC) superfamily of transport proteins.

1. General characteristics of the ABC protein exporters

The ABC protein exporters involve specific ATP-driven protein translocators of the ABC superfamily. ABC transporters are highly conserved and mediate transmembrane transport of a wide variety of substrates in both prokaryotes and eukaryotes (Higgins, 1992).

Over 50 ABC transporters are now known. The majority are from prokaryotic species but, increasingly, eukaryotic examples are being reported. Typically, ABC transporters utilise the energy of ATP hydrolysis to pump substrate across the membrane against a concentration gradient. Each ABC transporter is relatively specific for a given substrate. The variety of substrates handled by different transporters is enormous: ABC transporters specific for amino acids, sugars, inorganic ions, polysaccharides, peptides, and proteins have been characterised (Higgins, 1992 and references therein). Some ABC transporters are uptake (import) systems that accumulate substrate within the cell, while others export substrate from the cell. None has yet been identified that can pump in both directions.

ABC transporters require the function of multiple polypeptides/protein domains, organised in a characteristic fashion. The typical transporter consists of four membrane-associated domains. Two of these domains are highly hydrophobic and each consists of six membrane-spanning segments. These domains form the pathway through which the substrate crosses the membrane and they are believed to determine the substrate specificity of the transporter. The other two domains are peripherally located at the cytoplasmic face of the membrane, bind ATP, and couple ATP hydrolysis to the transport process. The sequences of these ATP-binding domains have been highly conserved throughout evolution.

The two transmembrane domains of ABC transporters are highly hydrophobic. Each is predicted from its sequence to consist of multiple α-helical segments that could span the membrane. The majority of transporters are predicted to have six membrane-spanning segments per domain (a total of twelve per transporter), with the N- and C-

termini on the cytoplasmic face of the membrane and three extracellular and two intracellular loops. This prediction has been confirmed first by identifying the six transmembrane segments of each domain of the oligopeptide permease of *Salmonella typhimurium*, using both biochemical and genetic data (Pearce et al, 1994).

The hydrophobic domains of the ABC protein translocators HlyB and PrtD have also been shown to span the membrane six times (Wang et al., 1991; Gentschev and Boebel 1992; Delepelaire and Wandersman 1991). These polypeptides seem likely to function as homodimers so each transport complex has the standard twelve membrane-spanning segments. For the mammalian multidrug resistance P-glycoprotein, studies with epitope-specific antibodies give direct support to the predicted topology (Yoshimura et al., 1989; Georges et al., 1990; Zhang and Ling 1991). The predicted topologies also place the glycosylation sites of P-glycoprotein and the cystic fibrosis chloride channel (CFTR) appropriately on the extracellular face of the membrane and the ATP-binding domains at the cytoplasmic face.

The ATP-binding domains of ABC transporters are their most characteristic feature. Each domain is about 200 amino acids long and the domains from different transporters share considerable sequence identity, varying between 30 and 50% depending on the transporters being compared (Higgins et al., 1986; Hyde et al., 1990). The sequence identity is generally greater between the two ATP-binding domains of a single transporter than between domains from different transporters: this may reflect functional constraints or may simply be a consequence of convergent evolution. The sequences conserved between the ATP-binding domains include two short motifs associated with many nucleotide-binding proteins (the Walker motifs; Walker et al.,

1982; Higgins et al., 1985,1986). However, it is important to emphasise that the sequence identity extends over the entire domain and is far more extensive than the two short Walker motifs. This clearly distinguishes the ABC domains from other nucleotide-binding proteins.

The ATP-binding domains are highly hydrophilic, include no potential membrane-spanning segments, and would not normally be expected to span the membrane. Indeed, the available data are consistent with a peripheral location, tightly associated with the cytoplasmic face of the membrane. The use of epitope-specific antibodies showed that the ATP-binding domains of P-glycoprotein are only accessible from the cytoplasmic side of the membrane (Georges et al., 1990; Yoshimura et al.,1989). Similarly, the ATP-binding components of the bacterial oligopeptide and histidine transporters, OppF and HisP respectively, are less accessible to proteolysis from the exterior of the cell than from the cytoplasm (Gallagher et al., 1989; Kerppola et al., 1991). The observation that the MalK component of the maltose transporter is cytoplasmic in the absence of the membrane-associated components also points to a peripheral association with the cytoplasmic face of the membrane (Shuman and Silhavy, 1981).

In several cases, the four domains of ABC transporters, whether single or multiple polypeptides, appear to be sufficient to mediate transmembrane translocation of solutes as demonstrated by in vitro ATP-dependent transport by the purified components reconstituted into proteoliposomes (Gottesman and Pastan, 1993; Gottesman et al., 1995). Nevertheless, certain ABC transporters have additional domains that serve regulatory or other peripheral functions. For example, the cystic fibrosis gene product, CFTR, has a

fifth domain, the R-domain, which has no equivalent in any other ABC transporter and serves a regulatory function (Cheng et al., 1991; Rich et al., 1991). The ATP-binding domain of the maltose transporter (MalK) has a C-terminal extension that has an enzymic function apparently independent of the transport process (Reidl et al., 1989).

Most of the bacterial Gram-negative ABC protein-mediated transport systems involve accessory proteins. For example, uptake systems require a soluble periplasmic substrate-binding protein (Boos and Lucht, 1996). Similarly, ABC protein-mediated exporters involved in protein secretion require in all cases two accessory envelope proteins. One is an inner membrane protein with a short N-terminal hydrophobic domain anchoring it in the inner membrane, a large hydrophilic domain presumably located in the periplasm, and a C-terminal domain with a possible β-sheet structure which could interact with the outer membrane. Because of their membrane topology, these proteins are classified as members of the membrane fusion protein family (MFP; Dinh et al., 1994). The second accessory component is an outer membrane protein (Wandersman and Delepelaire, 1990). The whole apparatus required for protein secretion, consisting of the ABC protein and the two accessory proteins, has been referred to as the ABC protein-mediated exporter.

The Gram-negative bacterial protein exporters are dedicated to the secretion of one or several closely related proteins belonging to the toxin, protease and lipase families. The genes encoding the three secretion proteins and the exoproteins are usually all linked, consistent with the specificity of the systems. For example, the four highly homologous metalloproteases of *Erwinia chrysanthemi*, PrtA, B, C and G, are encoded by genes clustered with the three genes encoding their ABC exporter, in the order of the

genes for PrtD, the ABC protein. PrtE, the MFP, and PrtF, the outer membrane component (Letoffe et al., 1990). The *Escherichia coli* α-hemolysin encoding gene, *hlyA*, is adjacent to the *hlyB* and *hlyD* genes which encode the ABC protein and the MFP, respectively (Mackman et al., 1986). Similarly, *has*A, the *Serratia marcescens* structural gene for an extracellular hemoprotein required for heme acquistion (Letoffe et al., 1994a,b), is linked to *hasD* and *has*E which encode the ABC protein and the MFP, respectively. In these two last cases, the genes encoding the outer membrane components are unlinked. For the hemolysin transporter, the outer membrane component TolC is also used by another *E. coli* ABC transporter, the colicin V transporter (Fath and Kolter, 1993).

TolC is a multifunctional protein known to be part of several membrane complexes including the hemolysin and colicin V exporters (Fath et al., 1991; Nikaido, 1994; Wandersman and Delepelaire, 1990), the AcrAE drug efflux (Nikaido, 1994), the colicin E1 import system (Morona et al., 1983), and LPS assembly (Schnaitman and Klena, 1993).

For the *S. marcescens* HasA transporter, the outer membrane component HasF shares a high degree of amino acid identity with *E. coli* TolC (Binet and Wandersman, 1996). These two proteins have 80% DNA identity and immunological cross reaction. HasF complements TolC for protein secretion and drug- and detergent- sensitivity phenotypes.

HasA can be secreted in *E. coli* by a hybrid secretion system (Letoffe et al., 1994a; Binet and Wandersman, 1996). The system consists of the two *S. marcescens* inner membrane-specific components, HasD and HasE, associated with an outer

membrane component such as either *E. coli* TolC protein, or the *E. chrysanthemi* protease transporter protein PrtF protein. However neither the genuine *E. chrysanthemi* protease transporter PrtD-PrtE-PrtF nor the hemolysin transporter HlyB-HlyD-TolC allows the secretion of HasA, indicating that the *E. chrysanthemi* transporter recognizes HasA and this recognization occurs at an early step of interaction between HasA and the inner membrane components. Similarly, *E. chrysanthemi* metalloproteases B and C can be secreted by a hybrid transporter, further demonstrating the complementation between outer membrane proteins for secretion (Letoffe et al., 1994b).

The exporter proteins are widespread among Gram-negative species, where they exhibit highly conserved sequence and function. When co-expressed in *E. coli*, exoproteins from the same family are efficiently secreted by heterologous exporters (Fath et al., 1991; Guzzo et al., 1991; Koronakis et al., 1992).

Unusually, *S. marcescens* secretes several unrelated proteins by means of ABC exporters. It secretes one metalloprotease (Nakahama et al., 1986), one lipase (Akatsura et al., 1995), and one heme-binding protein, HasA (Letoffe et al., 1994a). Recently, a gene cluster encoding a complete ABC exporter was identified in *S. marcescens*. It is not linked to any gene encoding a secreted protein. It consists of LipB, the ABC protein, LipC, the MFP and LipD, the outer membrane component. Expressed in *E. coli*, it promotes secretion of two unrelated enzymes by *S. marcescens*: the metalloprotease and the lipase. Several other proteins such as *Rhizobium leguminosarum* NodO, and *Pseudomonas fluorescens* lipases can be secreted by the *E. chrysanthemi* or the *P. aeruginosa* ABC transporters (Duong et al., 1994). This implies the presence of secretion signals on the exoproteins.

2. Nature of the secretion signal on the exoproteins

The proteins secreted by this pathway have C-terminal secretion signals located in the last 60 aa, which was first identified on a hemolysin using deletions and gene fusions (Mackman et al., 1986). Similarly, the highly homologous metalloproteases secreted by *E. Chyrsanthemi*, *S. marcescens* and *P. aeruginosa* were shown to have C-terminal secretion signals. Using one *E. Chyrsanthemi* protease, PrtG, it was shown that the smallest C-terminal sequence allowing efficient secretion contains the last 29 aa of PrtG and that low but significant secretion can be promoted by the last 15 aa of PrtG (Ghigo and Wandersman, 1994). Moreover, the extreme C-terminal motif, consisting of a negatively charged aa followed by several hydrophobic aa, must be exposed and is conserved among the homologous proteases.

Sequence comparison has revealed (Duong et al., 1994) that in addition to the proteases above, lipases and NodO display a very similar C-terminal motif. The importance of this motif is emphasised by the lack of overall sequence homology between these proteins and the protease family. NMR study of the C-terminal fragment of purified protease G has shown that it forms a stable α-helix located just upstream from the last 7 or 8 aa (Wolff et al., 1994).

The *E. Chrysanthemi* C-terminal protease secretion signal can promote the specific secretion of fused passenger polypeptides. However, the 4-aa C-terminal motif is not sufficient by itself to promote secretion. The study of fusion protein secretion has revealed the role of a domain located just upstream from the C-terminal signal on most of the exoproteins. Toxins, proteases and lipases secreted by ABC pathways all have such a domain consisting of a Gly-rich sequence (GGXGXD) that is repeated 4-36 times,

depending on the protein (Welch, 1991). Comparison of the secretion level of various heterologous polypeptides either fused directly to the signal or separated from it by the Gly-rich domain has shown that these repeats play a critical role in the secretion of some polypeptide passengers (Letoffe and Wandersman, 1992). An attractive hypothesis would be that the Gly-rich repeats act as internal chaperones, allowing better signal separation from the remainder of the protein.

3. Functions of the ABC protein

Since the ABC protein has a nucleotide-binding site, it is likely that this protein provides energy for the translocation. The cytoplasmic half of HlyB and the whole PrtD ABC proteins were overproduced and purified. They exhibit ATPase activity *in vitro* (Delepelaire, 1994). Biochemical evidence suggested a direct interaction between the secreted polypeptide and the ABC protein. The ATPase activity of purified PrtD protein was specifically inhibited by the addition of a C-terminal protease signal (Delepelaire, 1994).

The determination of the ABC protein function *in vivo* was facilitated by the identification of the *S. marcescens* HasA ABC transporter, which is highly homologous to the *E. chrysanthemi* protease transporter but dedicated to the secretion of HasA. Whereas proteases can use their own genuine transporter and the HasA transporter as well, HasA is secreted only by its specific transporter. Functional analysis of protease and HasA secretion through hybrid transporters obtained by combining components from each system demonstrated that the ABC protein is responsible for the substrate specificity (Binet and Wandersman, 1995).

4. Functions of the two helper proteins

Using HasA and GST-PrtC (a chimeric protein which has a glutathione-S-transferase moiety fused to a large C-terminal domain of protease C) as a protein substrate, Binet et al. (1997) developed a simple system based on substrate affinity chromatography to show an ordered association between the protein substrates and the three exporter components. The exoproteins PrtD, PrtE, PrtF, HasA and GST-PrtC were only found to bind to corresponding secretion proteins, indicating the substrate specificity of the secretion system. Similar experiments performed with incomplete, hybrid exporters constituted of only the ABC protein, or of the ABC component and the MFP showed that secretion protein interaction is ordered: the substrate recognizes the ABC protein, which interacts with the MFP which in turn binds the outer membrane component. It has also been shown that the substrate plays a critical role in the secretion protein association. HasA protein purified from extracellular medium, added to solubilized membrane proteins from cells containing the exporter but no substrate, was coretained on hemin agarose only with the ABC protein. In a similar experiment performed with solubilized membrane proteins from cells producing the secretion proteins together with one of the substrates, such as protease B or C-terminal peptide of protease B, the three secretion proteins all bound to the resin. The presence of both substrate and exporter is thus necessary for the formation of a complex prior to solubilization. Induction of multiprotein complex formation by the substrate is very likely initiated by substrate binding to the ABC protein, which may cause either a chemical or a conformational change of the ABC protein (Letoffe et al., 1996).

Type III system

It is now recognised that several animal and plant pathogens use a specialised protein secretion system termed either type III or host-cell contact-dependent for the secretion and/or delivery of virulence determinants (Salmond and Reeves, 1993; van Gijsegem et al., 1993). General features of this system include: (1) the secreted proteins do not have typical amino-terminal sequences characteristic of proteins exported in a Sec-dependent manner (for details of Sec system, see below); (2) the export machinery directs the translocation of the target proteins through two membranes without cleavage of their amino termini; and (3) an inducing extracellular signal, usually resulting from the interaction with host cells, is required for complete activation of the secretory apparatus. Some of the proteins that comprise these systems share homologies with polypeptides involved in the flagella assembly process in both Gram-positive and Gram-negative microorganisms (Dreyfus et al., 1993). Targets of these secretion systems have been identified in several bacterial pathogens. These include the Yop proteins of *Yersinia* (Straley et al., 1993); the Sip proteins of *Salmonella* (Kaniga et al., 1995a,b); the Ipa (Sansonetti, 1992),VirA (Uchiya et al., 1995), and IpgD (Allaoui et al., 1993) proteins of *Shigella*; the EaeB protein of enteropathogenic *E. coli* (EPEC; Donnenberg et al., 1992); and Pop (Arlart et al., 1994) proteins from several plant pathogens.

In *Yersinia*, the Yops and their secretion apparatus are encoded by a 70kb plasmid called pYV. The secretion apparatus itself is encoded by about 20 genes clustered in three operons called *virA* (*IcrA*), *virB* (*IcrB*) and *virC* (*IcrC*; Michiels et al., 1991; Plano and Straley, 1993; Bergman et al., 1994,1995b). The genetic information encoding the

components and targets of the type III secretory pathway in *Salmonella* is located in a contiguous region of the chromosome at centisome 63, which constitutes an example of a pathogenicity island (Mills et al., 1995). Other type III systems are also known to be encoded in either discrete chromosomal regions or in virulence associated plasmids. These observations, in conjunction with the consistent finding that the G + C content of these regions is significantly different from that of the chromosome of the host microorganism, suggest that these genetic determinants have their origins in a common ancestor (Galan et al., 1992; Ginocchio et al., 1992; Groisman and Ochman, 1993).

1. Components of the secretion apparatus

1). The inner membrane proteins:

Several proteins have been located in the inner membrane based on their secondary structures. In *Yersinia*, these include LcrD (Barve and Straley, 1990), YscR, and YscU (Allaoui et al., 1994). In *Salmonella*, the gene products have been especially well characterized. The inner membrane proteins include the InvA (Galan et al., 1992), SpaP, SpaR, and SpaS proteins (Collazo and Galan, 1996; Groisman and Ochman, 1993). InvA is a polytropic inner membrane protein with two domains: an N-terminal domain with at least eight putative transmembrane (TM) segments and a C-terminal region located in the cytoplasmic compartment (Galan et al., 1992). This topological organization has been confirmed using biochemical fractionation techniques and studies with the transposon *TaphoA* (Gonocchio, 1994). The structural features of InvA suggest that this protein may form a channel in the inner membrane through which the exported

polypeptides are translocated. However, thus far, there is no experimental evidence to substantiate this hypothesis.

The SpaP, SpaQ and SpaR proteins contain hydrophobic regions that are likely to span the membrane (Groisman and Ochman, 1993; Collazo and Galan, 1996). This observation is supported further by several studies in other systems that seem to indicate that some of the homologues of these proteins are located in the membrane. For example, protein fusion experiments using β-lactamase have indicated that the *Caulobacter cescentus* FliQ and FliR proteins, which are homologous to SpaQ and SpaR, are likely to be membrane associated (Zhuang and Shapiro, 1995). In fact, SpaQ is highly hydrophobic, containing at least two putative TM regions, which suggests that this protein could be almost completely embedded in the membrane. Studies in *Yersinia pestis* have indicated that the YscR protein, a homolog of SpaP, is an integral membrane protein with at least four TM spanning domains (Fields et al., 1994). Recent studies have shown that non-polar mutations in the *spa* genes abolished *Salmonella* entry and prevented protein secretion (Collazo and Balan, 1996). Therefore, the Spa proteins may be structural components of the translocase or may assist in the translocation process.

2). The outer membrane proteins

Several outer membrane proteins have been identified in the type III system. These include the YscC of *Yersinia* (Plano and Strdey et al., 1995), the SepC protein of pathogenic *E. coli* (Lee et al., 1997), the InvG protein of *Salmonella* (Kaniga et al., 1994), lipoproteins such as PrgH and PrgK proteins of *Salmonella* (Pegues et al., 1994), and VirG protein in *Yersinia* (Allaoui et al., 1995). YscC is an insoluble complex (Plano

and Straley, 1995). It belongs to the "secretins" family as protein pIV involved in the extrusion of the filamentous phages (Russel, 1994) and PulD involved in pullulanase secretion (see below). YscC presumably assembles in very stable multimers and forms a large channel in the outer membrane. In *Salmonella,* InvG also is a member of the PulD family of protein and it is the only component identified so far that share homology with components of type II protein secretion systems (Kaniga et al., 1993). However, this homology is restricted to a domain in the C-terminus, which suggests that this protein is organized in a modular fashion and that this conserved domain might be involved in localizing the protein to the outer membrane (Kaniga et al, 1993). InvG has been showed to play an essential role in bacterial uptake and in protein secretion (Kaniga et al., 1994). Based on studies on the pIV protein of filamentous phage, it is likely that InvG may be a multimeric protein that forms an outer membrane channel through which the secreted proteins enter the bacterial cell (Russel, 1995; Kazmierczak et al., 1994). The conservation of the InvG/PulD homologs in the type II, type III, and phage-assembly systems suggests that these proteins play a key role in the translocation of polypeptides across the outer membrane.

PrgH and PrgK contain structural motifs characteristic of the processing site of lipoproteins (Pegues et al., 1995). These lipoproteins are widely conserved since they have been found in all of the type III systems studied so far. PrgK shares sequence homology with the MixJ (Allaoui et al., 1992) and YscJ (Michiels et al.,1991) proteins of *Shigella* and *Yersinia,* respectively. PrgH is homologous to the *Shigella* MxiG protein (Allaoui et al., 1995).The genes encoding these proteins are part of the *prgHIJK* operon which has been proposed to be involved in protein secretion based on sequence

homologies to the *Yersinia* and *Shigella* systems (Pegues et al., 1995). However, non-polar mutations in each gene of this operon are required in order to clearly establish their involvement in both bacterial entry and protein export. Thus, at least two determinants encoded by the *prg* locus are likely to be outer membrane proteins that play an important role in bacterial internalization and protein secretion.

3). ATPase in type III system

Another very conserved member of the type III secretion apparatus is a protein that shares considerable homology with the β subunit of the F_0F_1 ATPases. Such a protein in *Salmonella* is InvC, which has been shown in biochemical studies to have ATPase activity (Eichelberg et al., 1994). A site directed mutant in the catalytic domain of InvC lacked ATPase activity and was unable to complement a non-invasive *S. typhimurium* strain carrying a null mutation in the *invC* gene. These observations suggest a role for InvC in energizing the translocation process.

4). Accessory chaperones

A group of proteins associate with the type III secretion systems might be involved in modification of the secreted targets, or in the stabilization of these targets prior to their secretion. For example, a distinct feature of the type III systems is the existence of cytoplasmic chaperones that assist in the translocation of the secreted targets by maintaining them in a conformation that is competent for export and/or by preventing their degradation (Wattiau et al., 1996). Usually, these chaperones are encoded next to or near the gene encoding their cognate protein. In addition, the chaperones identified so far

share certain structural features such as high charge, small size, and a potential to form α-helices.

i). SicA and InvI

SicA (Kaniga et al., 1995b) is a protein in *Salmonella* that shares sequence homology with the cytoplasmic chaperones IpgC (Baudry et al., 1988) and LcrH (SycD) (Bergman et al., 1991; Price et al., 1989) of *Shigella* and *Yersinia*, respectively. It is most likely involved in either guiding the secreted targets to the translocation machinery or in preventing their premature degradation. Mutations in *sicA* prevented the secretion of SipA, SipB, and SipC, but not InvJ, which indicates that the function of SicA is restricted to a subset of secreted proteins (Tucker et al., 1995). Another protein with putative chaperone function in *Salmonella* is InvI (Collazo et al., 1995). This protein, which is encoded immediately adjacent to *invJ* and *spaO*, exhibits some structural features observed in the type III chaperones such as small size, high charge and predicted potential to form α-helical structures. It is therefore possible that InvI may serve as a chaperone for the secreted proteins InvJ and SpaO. However, this hypothesis remains to be experimentally substantiated.

ii). IacP

IacP is a *Salmonella* protein that belongs to the family of acyl carrier proteins and therefore might be involved in the post-translational modification of exported proteins (Kaniga et al., 1995a). In fact, the modification of secreted proteins has been observed in other systems. In the type I system, for example, hemolysin requires an acyl carrier protein for its post-translational modification. This acylation is essential for the activation of this toxin and for its proper insertion into the plasma membrane of the mammalian cell

(Braun et al., 1993). It is possible that IacP modifies some important component of the invasion-associated secretion pathway that is targeted to the host-cell membrane. However, the target(s) that might be lipidated by IacP has not been identified thus far.

iii). SycE

SycE (YerA) was first identified by Firsberg and Wolf-Warz (1990), who characterized the region neighbouring *yopE* in *Y. pseudotuberculosis.* These authors found an open reading frame of 130 amino acids upstream of *yopE* and orientated in the opposite direction. A *Y. pseudotuberculosis* mutant unable to produce this protein displayed a dramatic decrease in the global level of YopE, one of a dozen proteins secreted by *Yersinia.* As a consequence, the protein was termed YerA. Studies devoted to the *Y.enterocolitica* counterpart of YerA demonstrated that this protein does not regulate transcription of the *yopE* gene but rather binds to YopE in order to assist its secretion (Wattiau and Cornelis, 1993). As this protein displays the characteristics of a chaperone, it was named "SycE". This designation avoids the term "regulatory" which can be misleading and it provides a generic name, Syc, that can be applied to functionally similar proteins of *Yersinia.*

SycE is an acidic (pI 4.55) cytosolic protein, the purified form of which is a homodimer. Intracellular amounts of YopE are the same in wild-type and $SycE^-$ *Yersinia,* indicating that YopE does not accumulate in the absence of its specific chaperone but is instead degraded by proteases. Using a simple blot experiment, it was observed that SycE binds with high affinity to YopE; total proteins including the putative target of SycE were separated on SDS gels, blotted on nitrocellulose membranes and incubated with a mixture containing the radiolabelled chaperone. Binding was then visualized by autoradiography.

This experiment was repeated with truncated derivatives of YopE, which showed that the SycE-binding site lies within the 98 N-terminal residues of YopE (Wattiau and Cornelis, 1993).

2. Exoproteins in type III system

Several proteins in the range of 23~85 kDa have been shown to use the type III secretion system to exit bacterial cells (Cornelis et al., 1997; Kaniga et al., 1995b; Pegies et al., 1995). These include the Yop proteins of *Yersinia* (Cornelis et al., 1997; Frithz-Lindsten et al., 1996; Galyov et al., 1993), and the InvJ (Gollazo et al., 1995), SpaO (Li et al., 1995; Bollazo and Galan, 1996), SptP (Kaniga et al., 1996) and Sip proteins in *Salmonella* (Kaniga et al., 1995a,b). These exoproteins can be divided into at least three functional categories. One group of these proteins plays an important role in protein secretion and a second group contains proteins that may be involved in the delivery of effector molecules into host cells. Effector molecules themselves are contained in the third group (Collazo and Galaz, 1996).

1) Exoprotein involved in secretion

Two proteins, InvJ and SpaO, are required for protein secretion through the type III system in *Salmonella* (Collazo and Galan, 1996). Introduction of mutations in their coding genes completely abolishes the secretion of all targets of the secretion apparatus including the Sip proteins (Collazo and Balan, 1996; see below). However, mutations in the sip genes do not prevent the secretion of InvJ, SpaO or other Sip proteins (Kaniga et al., 1995a). These observations suggest that a subset of exported targets is essential for

the translocation of another class of secreted proteins. InvJ and SpaO are encoded in the vicinity of the highly conserved *inv/spa* loci (Collazo and Galan, 1996; Collazo et al., 1995; Groisman and Ochman, 1993; Li et al., 1995). However, these proteins share little or no similarity to their cognate proteins in other microorgamisms, which suggests that they may be involved in functions that are specific for *Salmonella.*

Another protein that plays a role in the secretion process is SipD which is homologous to the *Shigella* IpaD protein (Kaniga et al., 1995a). Mutations in *sipD,* and to a less extent in *sipB,* lead to increased secretion of a subset of targets of the *inv/spa* translocon (Kaniga et al., 1995a, b). This is similar to the phenotype of mutations in the *Shigella ipaB* and *ipaD* genes (Menard et al., 1994; Parsot et al., 1995). How SipB and SipD modulate the secretion process is unknown. In *Shigella,* the IpaB and IpaD proteins are associated with the membrane and it has been speculated that they may block the secretion apparatus by forming a plug or lid (Menard et al., 1994). However, if the *Salmonella* homologues are involved in a similar function, the blockage of secretion can not apply to all targets since the secretion levels of InvJ are not affected in a *sipD* mutant (Kaniga et al., 1995b).

2) Exoproteins as delivery apparatus

YopD of *Yersinia* is the first element of the delivery apparatus that was identified (Rosqvist et al., 1994; Hartland et al., 1994; Sory and Corneils, 1994). It is encoded, together with two other Yops, LcrV and YopB, by the large *IcrGVHyopBD* operon. Recent analysis of non-polar *yopB* and *yopD* mutants showed that YopB is also individually required for translocation of the effectors across the eukaryotic cell plasma

membrane (Hakansson et al., 1996a; Boland et al., 1996). Further study on YopB showed that it causes hemolysis of erythrocytes and that this can be blocked by the addition of dextran but not raffinose (Hakansson et al., 1996b). These results suggest that YopB froms a pore through which the effector Yops are likely translocated. This pore formation has not yet been studied *in vitro*.

In *Salmonella*, it is known that SipB and SipC (Kaniga et al., 1995b) are required for the internalization of the bacteria into cultured epithelial cells. A specific function has not been assigned for these proteins.

3) Exoproteins as effector molecules

In *Yersina*, four Yop effector proteins have been identified: YopE, YopH, YpkA, and YopM. The 23kDa YopE causes disruption of the actin-microfilament structure of cultured HeLa cells. However, it does not disrupt actin filaments polymerized in vitro, even in the presence of NAD^+, suggesting that its action is indirect. The target of YopE is still unknown, but it is interesting to note that YopE shares homology with Exoenzyme S (ExoS) of *P. aeruginosa*, which was recently shown to be secreted by a contact secretion pathway (Yahr et al., 1996) and which elicits the same cytotoxicity as YopE when injected with a recombinant *Y. pseudotuberculosis* (Frithz-Lindsten et al.,1996). This may indicate that the two proteins have the same target(s). Since ExoS modifies small G-proteins involved in the regulation of the actin network (Goranson and Frank, 1996), it is possible that the effect of YopE is also mediated by some modification of small G-proteins.

YopH is a 51kDa, broad-spectrum protein tyrosine phosphatase related to eukaryotic PTPases. Though the catalytic domain is only about 20% identical to human PTP1B, the *Yersinia* PTPase contains all of the invariant residues present in eukaryotic PTPases and forming the phosphate-binding loop (P-loop), including the nucleophilic Cys-403, which forms a phosphocysteine intermediate during catalysis (Studkey et al., 1994). It acts on tyrosine-phosphorylated proteins of macrophages (Andersson et al., 1996), which contributes to the inhibition of bacterial uptake (Andersson et al., 1996) and oxidative burst (Bliska and Black, 1995), presumably by dephosphorylating key proteins involved in signal transduction.

YokA is an 81 kDa serine/threonine kinase that shows noticeable sequence similarity to eukaryotic counterparts (Galyov et al., 1993). It is targeted to the inner surface of the plasma membrane of the eukaryotic cell (Hakansson et al., 1996a). Given the kinase activity of YpkA and its spatial localization, it is reasonable to suggest that YpkA also interferes with some signal-transduction pathway of the eukaryotic cell.

YopM is an acidic 41 kDa protein that contains a succession of 12 repeated structures (Leung and Straley.,1989) related to the very common leucine-rich repeat (LRR) motifs (Kobe and Deisenhofer, 1994). It is suggested (Leung and Straket, 1989) that YopM could bind thrombin and interfere with platelet-mediated events of the inflammatory response. In agreement with this hypothesis, *in vitro* studies showed that YopM-containing culture supernatants of *Y. pestis* inhibit platelet aggregation, whereas culture supernatants of a *yopM* mutant do not (Leung et al., 1990).

Recently, a new target protein of the type III secretory system has been identified in *Salmonella*. This protein, which has been termed SptP, has a modular architecture and

consists of two domains (Kaniga et al., 1996). One domain, located at the N-terminus, shares sequence homology with ExoS of *P. aeruginosa* and the YopE protein of *Yersinia* spp. The other domain, located at the C-terminal end, is homologous to the catalytic domain of tyrosine phosphatases, such as that of the *Yersinia* YopH protein. Consistent with this latter homology, SptP has been shown to have tyrosine phosphatase activity in biochemical assays (Kaniga et al., 1996). This activity is abolished when an essential cysteine residue present in the catalytic site of the tyrosine phosphatase is substituted by a serine. A non-polar mutation in *sptP* does not prevent *Salmonella* from entering epithelial cells or macrophages.

4) Secretion signal of exoproteins

It was believed that the signal required to secrete a Yop is located in the N-terminal region of the protein, but it does not have the features of a classical signal peptide (see below) and it is not cleaved off during secretion. Sory et al.(1995)showed that this signal is contained within the first 15 and 17 residues of YopE and YopH, respectively. The effector Yops that are internalized must also be recognized by the translocation apparatus. To define the putative translocation signal on YopE and YopH, Sory et al. (1995) engineered a panoply of *yop-cya* hybrid genes by gradual deletions. Internalization into cultured macrophages only required the 50 N-terminal amino acid residues of YopE and 71 N-terminal residues of YopH. YopE and YopH are thus modular proteins composed of a secretion domain, a translocation domain and an effector domain (Sory et al., 1995). The same applies to YopM (Boland et al., 1996).

In a more recent study, Deborab et al. (1997) proposed that the secretion signal for type III system was encoded in messenger RNA rather than the peptide sequence. Taking two Yop proteins as subjects, the authors performed a series of genetic and biochemical experiments. Systematic mutagenesis of the secretion signal (first 15 residues) yielded mutants defective in Yop translation; however, no point mutants could be identified that specifically abolished secretion. Frameshift mutations that completely altered the peptide sequence of the signals also failed to prevent secretion. These results led to the proposal that the signals are encoded in the mRNA sequence. This novel proposal needs more confirmation.

3. Conservation of heterologous type III secretion systems

Several animal and plant pathogens have similar strategies for the export of virulence determinants using the type III system (van Gijsegem et al., 1993). The structural components of these systems are highly conserved at the protein sequence level. In addition, heterologous complementation among components from different species has been observed. A non-invasive *Salmonella spaP* mutant was complemented by the *Shigella spa24* gene (Groisman and Ochman, 1993). In addition, a more detailed study has shown that although the *Shigella mxiA* gene is able to complement a non-invasive *mxiA* mutant, the IcrD gene of *Yersinia* was unable to complement this mutant (Ginocchio and Galan, 1995). However, a chimeric protein consisting of the terminus of LcrD and the C-terminus of InvA was able to restore the ability of the *invA* mutant to enter epithelial cells. These results indicate that there are regions of specificity that are located in the C-termini of these proteins.

The type III secretory machinery is capable of exporting heterologous proteins. For example, the IpaB protein of *Shigella* is secreted in *Y. pseudotuberculosis* and the YopE protein of *Yersinia* is translocated by *S. typhimurium* via the Inv-secretory machinery (Rosqvist et al., 1995). Secretion requires the presence of the cognate chaperones for both proteins. Furthermore, in addition to heterologous secretion, functional complementation has been observed among secreted targets of *Salmonella* and *Shigella* (Hermant et al., 1995). A *Shigella ipaB* mutant strain was complemented by a plasmid carrying the *sipB* gene and its corresponding chaperone. These results suggest that SipB is probably exported by *Shigella* and that these two proteins may be functional homologues.

4. Regulation of the secretion process

The type III secretion process is regulated at both the transcriptional and post-transcriptional levels. Several factors such as growth phase (Lee and Falkow, 1990), oxygen tension (Ernst et al., 1990; Lee and Falkkow, 1990), and osmolarity (Galan and Curtiss, 1990) affect the ability of *Salmonella* to enter epithelial cells as well as the expression of the invasion genes. Although the molecular mechanisms of this type of regulation are not completely understood, changes in the degree of DNA supercoiling are likely to be involved (Galan and Curtiss, 1990).

At least two regulatory proteins associated with the invasion phenotype, InvF and HilA, have been identified in the centisome 63 region of the *Salmonella* chromosome. InvF is a member of the AraC family of transcriptional activators (Kaniga et al., 1994). Although this gene is clearly essential for bacterial entry, its regulatory target(s) has not

been identified. HilA is a member of the OmpR/ToxR family of transcriptional activators (Miras et al., 1995; Bajaj et al., 1995). Like InvF, this protein is required for bacterial internalization. Some of the regulatory targets of HilA are *orgA, sipC* and *invF* (Baja et al., 1995). These findings suggest a complex regulatory loop in which a protein conceivably involved in regulation (InvF) is under the control of another regulator (HilA). The secretion apparatus and the invasion phenotype are also regulated by more global regulatory networks such as those that control intracellular survival (PhoP/PhoQ) (Behlau and Miller, 1993; Pegues et al., 1995), or flagellar assembly (Eichelberg et al., 1995).

In addition to the transcriptional regulatory mechanisms, the function of the secretion apparatus is regulated by other factors that do not require gene expression or protein synthesis (Macbeth and Lee, 1993;Ginocchio et al., 1994; Zierler and Galan, 1995). This regulatory network is manifested by the rapid stimulation of the type III secretion system upon bacterial contact with host cells (Ginocchio et al., 1994; Zierler and Galan, 1995). Studies have shown that InvJ secretion is stimulated when *Salmonella* is exposed to cultured epithelial cells, and, to a lesser extent, upon bacterial contact with culture dishes coated with serum (Zierler and Galan, 1995). Indeed, the contact-induced InvJ secretion is dependent on an intact type III secretion system, since mutations in the *invC* and *invG* genes prevent the contact-stimulated secretion of this protein (Galan et al., 1996). These observations implicate an essential role for the secretion apparatus upon *Salmonella* exposure to mammalian cells. Another consequence of the interaction of *Salmonella* with host cells is the assembly of appendage-like organelles called invasomes on the bacterial surface (Ginocchio et al., 1994). These structures are transient and have a

very short half-life. The *inv* locus encoding the type III secretory system is required for the assembly of these structures since *invC* and *invG* mutants prevent their formation (Galan et al., 1996). Similar to *Salmonella, Shigella* form organized structures consisting of extended sheets when grown under certain inducing conditions (Parsot et al., 1995). Some of the Ipa proteins are components of these filamentous structures. It is possible that the extended sheets observed in *Shigella* may be related to the invasomes identified in *Salmonella.* The correlation between protein secretion and invasome assembly needs to be understood at the molecular level.

In *Yersinia,* the genes involved in Yop synthesis, secretion and delivery into host cell are organized as a single regulon under dual transcriptional control. The first level of regulation, which puts the genes on the alert when the temperature reaches 37 °C, results from the temperature-influenced interplay between a transcription activator, VirF (LcrF) and chromatin structure. The second regulation prevents full expression of *yop* genes as long as the secretion apparatus is closed. By analogy with the secreted anti-σ factor involved in regulation of flagellum synthesis (Hughes et al., 1993), the most likely hypothesis is that feedback inhibition is mediated by an inhibitor that is normally expelled via the Yop secretion apparatus. Genetic evidence shows that the secreted LcrQ protein of *Y. pseudotuberculosis* is involved in this negative control of *yop* gene expression but it remains to be shown if LcrQ is directly or indirectly involved in the control of *yop* genes (Petterssin et al., 1996). Hence, upregulation of *yop* expression and polarized translocation of effector Yops are triggered by the opening of the secretion apparatus in response to the signal generated upon interaction of the pathogen with its target cell (Lee et al, 1997).

Type II system: the General Secretion Pathway (GSP)

Most of the bacteria that have type II systems can secret multiple enzymes and/or toxins. The secretion of these proteins involves two steps. In the first step, precursors of the proteins are translocated across the cytoplasmic membrane (inner membrane of Gram-negative bacteria). This step is completed with the help of up to seven proteins, the Sec proteins, which form a prokaryotic translocation system. The second step of the GSP has several branches which lead the proteins to different locations. Most of proteins are secreted into the medium via the main terminal branch (MTB).

1. Sec system and transport across the inner membrane:

1). The signal sequence

Signal sequences play a central role in the targeting and translocation of nearly all secreted proteins, as well as many integral membrane proteins in both prokaryotes and eukaryotes. The sequence, first described by von Heijne (1985), is composed of three domains: the C domain, the H domain and the N domain. There is no strict homology among these domains of all known signal sequences. The most conservative site is the cleavage site since it must be recognized by signal peptidase. However, the three domains do display common distributions of residue type. Counting from the cleavage site, there are usually five to seven residues (including the "-1,-3 rule"residues) that comprise the C domain (von Heijne, 1983; Perlman and Halvorson, 1983). Although not generally charged, these residues are of higher polarity on average than those in the H domain immediately N-terminal to the C domain. The H domain is rich in Leu, Ala, Met, Val, Ile,

Phe, and Trp but may contain an occasional Pro, Gly, Ser or Thr residue. This hydrophobic core (H domain) is the true hallmark of signal sequences. Its length (10±3) distinguishes it from membrane-spanning sequences (24±2 residues long) and from hydrophobic segments of globular proteins (6-8 residues in length). Statistical results suggest that overall hydrophobicity is the major requirement in the H domain (von Heijne, 1985. The N domain is of highly variable length and composition, but always carries a net positive charge (on average +1.7). In eukaryotes, this charge is contributed by the N-terminus and charged residues; in prokaryotes, the N-terminus retains a formyl-Met, and the charge comes exclusively from basic residues.

The studies of signal sequences were first carried out in eukaryotes. In an early experiment, β-hydroxy-leucine was incorporated into the preprolactin signal sequence. This mutation led to a cytoplasmic protein which escaped binding to the SRP (Signal Recognization Particle; Hortin and Biome, 1980; Walter et al., 1981; Walter and Blobel, 1981). Substitution of this polar Leu analogue in a protein whose signal sequence has no or few Leu residues does not impair export. This result suggests that the hydrophobic core of eukaryotic signal sequences mediates their recognition by SRP.

In bacteria, early studies of both Emr et al. (1980) and Bedouelle et al. (1980) showed that the H domain is the most important region of the signal sequence. A series of mutants which contained H-regions composed of alanines and leucines was used to demonstrate that incremental changes in the hydrophobicity could affect protein export (Doud et al., 1993). At ratios of alanine to leucine of less than 3:7 in the H-region, the signal peptide functioned efficiently. As more alanines replace leucines in this region, the translocation efficiency decreased in a non-linear fashion. A polyleucine hydrophobic

region was also able to increase the efficiency of a PhoA mutant which had been made export-deficient by placing 6 serine residues at the N-terminus (Rusch and Kendall, 1994). Export efficiency was greater than that observed for wild type PhoA. The authors suggested that it may be possible to optimize the composition of the signal peptide for export of foreign proteins in *E. coli* by altering their hydrophobicity.

The role of the positively charged residues in the N-region has been less clearly defined. *In vivo* studies of mutants of the major *E. coli* lipoprotein in which positively charged amino acids were replaced with negatively charged residues showed a loss of export activity (Vlasuk et al., 1983). However, replacement of the basic residues with neutral amino acids did not result in the accumulation of lipoprotein precursor (Vlasuk et al., 1983). A similar change in the *E. coli* porin PhoE N-region also had no effect on its export (Bosch et al., 1989). These results contrast with *in vitro* studies which showed a loss of export activity for the *E. coli* porin protein OmpF when the N-region basic residues were replaced with either acidic or neutral amino acids (Sasaki et al., 1990). It may be that a component lacking in the *in vitro* system was able to compensate for the decreased positive charge.

Although the signal sequence theory has been generally accepted, the belief that signal sequences are all essentially the same is challenged by a growing body of evidence, largely gathered in yeast *Saccharomyces cerevisiae*, which demonstrates that there are distinct modes of targeting different proteins depending on their signal sequences. It was shown first that mutations of yeast cellular components have differential effects on translocated proteins, which provides an indication as to their pathway preference. Mutations that block the function of proteins important for SRP-

independent translocation compromise *in vitro* translocation of prepro-α-factor and preproCPY (carboxypeptidase Y) but not invertase (Deshaies and Schekman, 1989; Feldheim and Schekman, 1994). Furthermore, pathway specificity is determined by the signal sequence and not the mature protein, as maturation of chimeric secretory proteins with a signal sequence of CPY, but not that of invertase, was severely compromised in a mutant strain lacking a protein important for SRP-independent targeting (Feldheim and Schekman, 1994).

In another study (Hann and Walter, 1991), disruption of the gene encoding the yeast SRP54 subunit was found to cause a severe defect in the maturation of dipeptidyl aminopeptidase B (DBAP-B) without causing any apparent defect in the maturation of CPY. Other proteins, including Kar2p, prepro-α-factor, and invertase were affected to varying degrees.

Combined with other results (Ng et al., 1996), a qualitative ranking of signal sequences can be set up in terms of their dependence on the yeast SRP: DPAP-B> invertase, propre-α-factor> preproCPY. They also confirmed that the information specifying the pathway preference (SRP-dependent or SRP-independent) in yeast resides in the signal sequence (Ng et al., 1996). Therefore it was proposed that the hydrophobicity of signal sequences correlates with preference for translocation pathway. Signal sequences for proteins that follow the SRP-independent pathway were found to be relatively less hydrophobic than those from proteins targeted by SRP or those utilizing both pathways.

Similar results have not been found with bacterial signal sequences, although the proposal of mRNA signals for the type III system (see above) may lead to a

breakthrough. Therefore, the possible specificity of signal sequence remains to be further explored.

2). Sec translocation machinery

The translocation across the bacterial inner membrane (cytoplastic membrane) is completed by a complex called translocase. The translocase is a multi-subunit protein complex with the integral membrane subunits SecY, SecE, and SecG, and with SecA as the dissociable peripheral subunit. These subunits have been found in all prokaryotes studied thus far and also in eukaryotic organelles evolved from bacteria, such as the thylakoidal membrane of the plant chloroplast (Yuan et al., 1994). Preproteins can be bound as nascent chains at the ribosome by the cytosolic chaperone SecB. SecB promotes the interaction of the preprotein with SecA to form a ternary complex. SecA interacts with negatively charged phospholipids and with the integral subunits SecY, SecE, and SecG of the translocase. Other proteins like SecD and SecF are thought to function in the later stages of translocation, such as in correct folding of the protein.

i) SecA

SecA is the central component of this translocation pathway. This 102-kDa protein (Schmidt et al., 1988) is functional as a dimer (Akita et al., 1991; Driessen 1993). SecA performs at least three essential tasks: (i) it is the physical link between all of the components of the reaction: preprotein, SecB, SecYEG, lipids, SecA mRNA and ATP (Ulbrandt et al., 1992; Oliver, 1993; Arkowitz and Bassilana, 1994; Breukink et al., 1995; Fekkes et al., 1997); (ii) it is the translocase receptor for secreted substrates and their

chaperones (Wickner and Leonard, 1996); (iii) it generates the energy necessary for translocation by converting ATP free energy (Economous and Wickner, 1994).

SecA has two essential nucleotide binding sites (NBS; Matsuyama et al., 1990b; Klose et al., 1993; Mitchell and Oliver 1994): a high-affinity binding site (K_D=0.13 μM) confined to the amino-terminal domain of the protein (NBS-I), and a low-affinity binding site (K_D=340 μM) located approximately at two-thirds along the sequence of 901 amino acids (NBS-II; Mitchell and Oliver 1994; van der Wolk et al., 1993, 1995).

Mutations in NBS-I block the translocation activity of SecA, consistent with this sequence being part of the phosphate binding site (Van der Wolk et al., 1993; Mitchell and Oliver 1994). These mutations interfere with the release of SecA from the membrane (van der Wolk et al., 1993), suggesting a coupling between tranlocation and the temporal insertion/withdrawal of SecA into the membrane (Economou and Wickner 1994). Mutations in NBS-II block in translocation ATPase activity, i.e., in the stimulation of the ATPase activity by preproteins when SecA is bound at the SecYEG protein. Both NBS-I and NBS-II are indispensable for the translocation activity of SecA.

Sodium azide is an inhibitor of the Sec-dependent protein translocation pathway in *E. coli* (Oliver et al., 1990) and in *B. subtilis* (Nakane et al., 1995). Protein translocation by sodium azide is inhibited by blocking the translocation ATPase activity of the SecA protein. Azide-resistant mutants show an elevated translocation ATPase activity. Another effect of these *azi* mutations is that they, like the *prlD* mutations, enable the translocation of proteins with defective signal sequences (Huie and Silhavy 1995). The *prl* (for protein localization) class of mutants, isolated as suppressors of signal sequence mutations, all have been found to contain mutations in SecA (*prlD*)

(Fikes and Bassford 1989; Schatz et al., 1991; Huie and Silhavy 1995). It has been proposed that *prl* suppressors function not by restoring the recognition of altered signal sequences but rather by preventing the rejection of defective preproteins from the export pathway (Osborn and Sihavy 1993). For the *prlD* mutants, this could be achieved by a reduction of the ATPase activity, thereby prolonging the presentation of the preprotein to the translocase (Huie and Sihavy 1995). According to this hypothesis, SecA would have a proofeading activity.

Preproteins weakly associate with SecA in solution, but associate with high affinity with membrane-associated SecA. SecB promotes this interaction by binding SecA (see below) and prevents the premature release of the preprotein from SecA. Biochemical evidence demonstrates that SecA interacts with preprotein through recognition of the positive charge at the amino terminus of the signal peptide (Akita et al., 1989) and through binding of the mature domain of the preprotein (Cunningham and Wicker, 1989; Lill et al., 1990; Akita et al., 1991; Kumamoto, 1991). Cross-linking studies with a large variety of amino-terminal and carboxy-terminal SecA peptides suggest that the domain in SecA that interacts with the preprotein is located between amino acid residues 167 and 340 (Kimura et al., 1991). The ADP-bound form of SecA has a higher affinity for the preprotein than the ATP bound form (Schinkai et al., 1991). ATP hydrolysis dissociates the SecA/preprotein complex bound at the SecYEG protein (Schiebel et al., 1991).

Proteoliposomes reconstituted with a detergent-soluble fraction of urea-stripped inside-out (ISO) vesicles (i.e., vesicles containing urea-extraction-resistant SecA), have been shown to be capable of protein translocation, whereas SecA-depleted

proteoliposomes are inactive (Watanabe and Blobel, 1993). This shows that at least part of the active SecA population is in a membrane-integral form. Proteolysis studies with right side-out (RSO) membrane vesicles of *E. coli* suggest that SecA penetrates the cytoplasmic membrane in its membrane-bound form, exposing domains to the periplasm (Kim et al., 1994).

In ISO membrane vesicles, a 35-kDa membrane-interacting domain of SecA has been found that is relatively trypsin insensitive. Using monoclonal antibody mapping, this fragment has been shown to consist of amino acid residues in the region 1-310 (Tanneke and Driessen, 1996). This fragment may overlap with the 31 kDa membrane-inserting domain (Economou and Wickner, 1994) and the SecA peptide (1-239) that are found entirely associated with the membrane (Cabelli et al., 1991). A second, carboxy-terminal domain of SecA consisting of at least part of the amino acid sequence 850 up to 901 has been found to be embedded in the membrane or in the translocase and to be accessible from the periplasmic face of the membrane in RSO membrane vesicles. This implies that the carboxy-terminal region of SecA penetrates through the entire membrane.

ii) SecB

SecB is the only molecular chaperone found thus far that is dedicated to the Sec-dependent transport of proteins. It enhances the translocation of precursors of outer membrane proteins and some periplasmic proteins (Kumamoto and Francetic, 1993; Powers and Randall, 1995). SecB binds the preprotein as a nascent chain when it emerges from the ribosome (Kumamoto and Gannon, 1988) and prevents premature folding (Liu et al., 1989) and aggregation due to hydrophobic interactions (Lecker et al., 1990). SecB targets the preprotein to the SecA subunit of the translocase (Hartl et al., 1990). SecB is a

homotetrameric protein of 15-kDa subunits (Watanabe and Blobel, 1989) that forms a stoichiometric complex with the mature domain of the preprotein (Lecker et al., 1989; Hartl et al.,1990). It appears to be designed to interact with unfolded polypeptide segments at high rates, but with low specificity (Fekkes et al., 1996). The signal peptide is generally not involved in binding (Randall and Hardy, 1995), but is thought to delay stable folding of the preprotein (Park et al., 1988) and to enhance the affinity for the SecB/SecA interaction (Hartl et al., 1990; Breukink et al., 1995). SecB is only indispensable for protein translocation at high growth rates (Randall and Hardy, 1995) or when translocation is impaired by mutations causing a decrease in the translocation efficiency (Derman et al., 1993; Flower et al., 1994). This suggests that SecB is essential when preproteins queue for translocation. Both membrane-bound and soluble SecA bind the preprotein/SecB complex through direct recognition of SecB and of the signal sequence and the mature domain of the preprotein (Hartl et al., 1990; Hoffschulte et al., 1994). The carboxy-terminal 70 amino acids of SecA are essential for protein translocation, and removal of these residues abolishes the ability of SecA to cause aggregation of negatively charged phosphatidylglycerol vesicles (Breukink et al., 1995). This phenomenon is inhibited by SecB, suggesting the presence of a SecB binding site at the carboxyl terminus of SecA.

iii). SecY, SecE and SecG:

The integral membrane subunits SecY, SecE, and SecG of the translocase co-purify as a heterotrimeric complex when a cytoplasmic membrane detergent extract is fractionated. Furthermore, the complex can be immunoprecipitated with anti-SecY, anti-SecE, and anti-SecG antisera (Brundage et al., 1990,1992). Overproduced SecY is

extremely labile without the presence of overproduced SecE, suggesting that SecY and SecE physically interact (Matsuyama et al., 1990a). Recent studies have shown that SecY, SecE, and SecG assemble *in vivo* as a stabel heterotrimeric complex (Joly et al., 1994). SecY is a 49-kDa polypeptide with ten putative transmembrane helices (TMH) (Ito, 1992), SecE is a 14-kDa polypeptide that contains three TMHs in *E. coli* and in many other bacteria only one TMH (Schatz et al., 1989). The first two TMHs of *E. coli* SecE (residues 7-78) are dispensable for protein translocation (Schatz et al., 1991). Interestingly, SecY and SecE are homologous to the α- and γ-subunists of the heterotrimeric Sec61 complex involved in preprotein translocation into the yeast and mammalian endoplasmic reticulum (Gorlich and Rapoport, 1993; Jartmann et al., 1994). These proteins may thus have similar roles in the bacterial and mammalian systems.

SecG is a 11.4-kDa polypeptide with two putative TMHs and a large carboxy-terminal cytoplasmic domain (Nishiyama et al., 1993), which is essential for cell growth and protein translocation at low temperatures, but is dispensable under non-restrictive conditions (Nishiyama et al., 1994). SecG function can be compensated for by an increase in the level of acidic phospholipids, which are essential for the association of SecA with the membrane (Kontinen and Tokuda, 1995). Therefore, it is thought that SecG might facilitate the insertion of SecA at the translocase, especially at low temperatures when the membrane fluidity is low (Kontinen and Tokuda, 1995).

iv). SecD and SecF

Both SecD and SecF have been suggested by genetic studies to be involved in a late step of protein translocation (Gardel et al., 1990; Bieker-Brady and Silhavy, 1992). The direct participation of SecD in this step was demonstrated with spheroplasts by

means of an immunochemical technique (Matsuyama et al., 1993). It was suggested that SecD plays a role in the release of translocated proteins from the outer surface of the cytoplasmic membrane.

SecF exists in membranes at an about 10-fold lower level than other membrane Sec proteins (Mizushima et al., 1996), but its exact function is not clear (Mizushima et al., 1996).

3) Energy requirements for translocation

One of the features of the bacterial translocase is that it is driven by two different energy sources, ATP and the proton motive force (PMF; Driessen, 1992; Arkwitz and Baxxilana, 1994). *In vitro* studies have revealed that the two energetic components act at distinct stages of the translocation reaction, with ATP being required first (Schiebel et al., 1991) and used via SecA, the only ATPase of the system (Oliver, 1993). The PMF is also present during the early stages of translocation. However, its contribution can be seen more clearly after preproteins have translocated about halfway through the membrane. At that stage, the PMF can drive completion of translocation in the absence of ATP, but only if SecA is inactivated beforehand by antibodies (Schiebel et al., 1991). It is possible that several membrane components of the reaction are simultaneously affected by the PMF. It may affect translocase directly by optimizing the rate of ATP by 100-fold (Shiozuka et al., 1990) and by imposing directionality to the vectorial transfer (Driessen, 1993; Arkowitz and Bassilana, 1994). A direct involvement of SecY in PMF-driven translocation was suggested by an important recent observation: a single aminoacyl residue substitution in this protein renders the *in vivo* and *in vitro* translocation PMF

independent (Nouwen et al., 1996). While the ATP-driven reactions are essential for translocation, those of the PMF are not (Schiebel et al., 1991; Dressen, 1993), but the imposition of a PMF leads to a two- to 10-fold increase in translocation rates (Shiozuka et al., 1990; Schiebel et al., 1991; Driessen, 1993). The SecA/ATP and the PMF components of the reaction are somehow co-ordinately linked, as excess of SecA allows translocation of a precursor that is otherwise absolutely PMF dependent for its translocation (Yanada et al., 1989). Furthermore, PMF-driven translocation (after SecA inactivation) is slowed down when SecA is added back to the system in the absence of ATP (Schiebel et al., 1991).

4). Insertion and Translocation

Several models of preprotein insertion and translocation have been proposed (Pugsley, 1993; Tanneke and Driessen, 1996; Economou, 1998). Generally, it is supposed that this process involves four steps. In step one, the signal sequence of preprotein/SecB complex binds to the ADP-bound compact state of SecA. The tertiary complex traverses to or along the membrane until it encounters the integral membrane translocase subunits. Interaction of SecA with these subunits stimulates the protein for ADP/ATP exchange, and the binding of ATP to SecA causes insertion of the carboxyl terminus of SecA into the membrane and the release of bound SecB. Membrane insertion of SecA makes the signal peptide domain of the bound preprotein accessible to SecY and SecE, and proofreading takes place. In step two, the membrane-inserted signal sequence may enter the translocation channel laterally, as suggested for the translocase of mammalian endoplasmic reticulum (Martoglio et al., 1995). If SecY and SecE are able to recognize

and to bind the signal sequence, the translocase channel opens, and concomitantly allows access of the amino-terminal domain of SecA with the mature part of the preprotein. In step three, the ATPase activity of SecA is stimulated at the high-affinity nucleotide binding site, causing SecA to release the preprotein. Subsequent ATP hydrolysis at the low-affinity nucleotide binding site induces the compact conformation of ADP-bound state and causes withdrawal of SecA from the membrane. In step four, in the presence of the proton motive force, translocation will be completed with involvement of SecYEG and later SecD, SecF, but not SecA (Schiebel et al. 1991; Driessen, 1992). Alternatively, SecA may bind a mature part of the preprotein and re-enter the ATP-driven translocation until completion (Schiebel et al. 1991).

2. The Main Terminal Branch (MTB)

In Gram-negative bacteria, after preproteins are transported across the inner membrane, they enter into another cellular compartment, the periplasm. Resident or periplasmic proteins go no further. Other proteins insert into the outer membrane and remain as outer membrane proteins (OMPs). Still others take the second step of the GSP, termed as the Main Terminal Branch (MTB), and are secreted into the medium.

1) The periplasm and protein folding

The periplasm is the region between the inner and outer membranes of Gram-negative bacteria. Estimates of its size vary considerably (van Wielink and Duine, 1990). Combining data from electron microscopy and biochemical experiments, van Wielink

and Duine (1990) argued that the periplasm constitutes about 30% of the total cell volume of *E. coli*, which means that it is 50nm in width.

It has been argued that the periplasm has a gel-like structure. Hobot et al. (1984), based on electron microscopy data with special sample preparation procedures, have put forward a model that the periplasm is essentially filled with a peptidoglycan matrix with large pores. This matrix, as proposed, is more tightly cross-linked towards the outer membrane and has a high content of bound water. It is believed that this structure is responsible for the low diffusion coefficients of proteins in the periplasm (Brass et al., 1986). Another feature of the periplasm is the energetics of this compartment. It was argued that there was no ATP exist in the periplasm, because only periplasmic phosphatases and nucleotides can be found (Rosen, 1987). It is also improbable that ATP could be secreted into the periplasm since the inner membrane is thought to be impermeable to nucleotides (Rosen, 1987).

Protein folding in the periplasm was demonstrated first in *E. coli* (Bardwell et al., 1991). It was found that proteins would form disulphide bonds in the periplasm with involvement of a novel protein called DsbA. This enzyme has a high-energy disulphide bond, similar to disulphide isomerase (Noiva and Lennearz, 1992; Bulleid, 1993). The crystal structure of DsbA has been obtained (Martin et al., 1993) and the redox potential has been determined as –0.089 V at pH 7.0 (Wunderlich and Glockshuber, 1993). It is thus a significantly stronger oxidant than the cytoplasmic thio-redoxin and it more closely resembles the eukaryotic disulphide isomerases (Zapun et al., 1993).

DsbA has been shown to be required for the correct formation of disulphide bonds of some periplasmic proteins *in vivo* and *in vitro* (Bardwell et al., 1991, 1993; Kamitani

et al., 1992; Akiyama and Ito, 1993), and for the production of recombindant eukaryotic proteins in the periplasm of *E. coli* (Knappik et al., 1993). The fraction of the recombinant molecules which becomes correctly folded can be smaller than that of natural periplasmic proteins, and is sequence dependent (Knappik et al., 1993). Overproduction of DsbA did not help to increase the proportion of correctly folded proteins, indicating that other steps limit their folding in the periplasm (Knappik and Pluckthun, 1994).

A second protein involved in periplasmic disulphide bond formation is DsbB. DsbB is an integral membrane protein spanning the inner membrane. It is believed to be involved in the reoxidation of DsbA and thus may form part of a chain that links an electron transfer step to the formation of disulphide bonds in the periplasm (Bardwell et al., 1993).

Besides these folding catalysts, there are some molecular chaperones which are believed to be involved. These chaperones are thought to control protein-protein interactions, thereby preventing side-reactions and they do not actually catalyze protein folding. Several periplasmic chaperones have been found, including ClpB (Squires et al., 1991), which has been proposed as a general chaperone to bind to non-native proteins and lead them to a folded state (Woo et al., 1992).

2). The Outer membrane proteins (OMPs)

Bacterial outer membrane proteins utilize the Sec system to cross the inner membrane (Nikaido, 1992) but are not retained because they lack hydrophobic stretches of 20 or more amino acids (Nikaido and Saier, 1992).

It is now accepted that the outer membrane proteins have periplasmic intermediates before their insertion into the outer membrane (Nikaido et al., 1990). It was suggested that OmpF and porin proteins PhoE of *E. coli* can insert into the outer membrane via a soluble periplasmic intermediate in a spontaneous process determined by the conformation of the protein itself and independent of exogenous factors such as molecular chaperones (de Cook et al., 1990, 1992; Eisele et al., 1990; Sen and Nikaido, 1990). However, more recent studies showed that LPS is an important outer-membrane component required for efficient folding of PhoE before it can trimerize and insert into the assembly site in the outer membrane (de Cock and Tommassen, 1996).

3). The Main Terminal Branch (MTB)

The main terminal branch is the most widespread of the many branch pathways of the GSP. It was first characterized during the study of the secretion of *Klebseilla oxytoca* pullulanase (Pugsley, 1990). Since then, numerous bacteria species have been shown to use this pathway to secrete various proteins, including toxins and enzymes. Of these secretion systems, the secretion of pullulanase by *K. oxytoca* and *E. coli*, the secretion of proaerolysin by *Aeromonas* species and the secretion of Exotoxin A by *P. aeruginosa* are best characterized.

Secretion of the *K. oxytoca* pullulanase

Pullulanase is a 120 kDa lipoprotein that is secreted by *Klebsiella* grown in medium containing starch, maltose or intermediate-sized dextrins. The enzyme cleaves α-1,6 linkages in branched maltodextrin polymers, such as glycogen or amylopectin, to

release linear dextrins that can be transported and metabolized by the cells. Pullulanase contains a number of motifs that are conserved in other amylases and in particular in isoamylases, which also hydrolyses α-1,6 linkages (Kornacker and Pugsley 1989). These motifs define a large, putative catalytic domain in the central region of the pullulanase polypeptide. Although pullulanase uses the main terminal branch (MTB) of the GSP to cross the outer membrane, it is relatively unique in that it remains anchored to the cell surface by its fatty acylated N-terminal cysteine residue.

i). Genetics of the GSP-MTB

The first studies of the pullulanase structural gene (*pulA*) revealed the predicted presence of a lipoprotein-type signal peptide (Michealis et al. 1985). This finding was later confirmed and extended in experiments which demonstrated that the enzyme in its natural host is anchored to the cell surface by covalently attached lipids. In contrast, the enzyme remains facing the periplasm when *pulA* cloned on a 6.0 kb DNA fragment is expressed in *E. coli* (Pugsley et al. 1986). This encouraged a search for genes from *Klebsiella* that would permit *E. coli* to secret pullulanase. This search was guided by the fact that a gene facing in the opposite direction to *pulA* (initially call *malX*) was found to be coregulated with *pulA* and was incomplete in the DNA fragment that was initially cloned. It was reasoned that this gene, being expressed only when *pulA* was also expressed, would code for a protein that allowed pullulanase secretion. It turned out that this gene (now called *pulC*) is the first gene of an operon of 13 genes, all of which are needed for pullulanase secretion (d'Enfert et al., 1989; Possot et al., 1992; Pugsley and Reyss, 1990; Reyss and Pugsley, 1990). An independently-transcribed gene (*pulS*) located upstream from *pulA* is also required for secretion (d'Enfert and Pugsley, 1989).

Thus, 15 linked genes from the *K. oxytoca* genome are suffcient to promote pullulanase production and secretion in *E. coli*. The organization of these genes is more or less conserved, even in *E. coli* K-12, where they are not expressed under any known conditions (Francetic and Pugsley, 1996). The level of sequence identity between homologous components of these secretion pathways ranges from 20% to 80%, depending on the species. Indeed, cross-complementation assays show that some but not all components of these pathways are interchangeable (Lindeberg et al., 1996; Pugsley and Dupuy, 1992; Pugsley, 1996; Possot and Pugsley, 1997).

ii) The GSP-MTB machinery

All proteins secreted by the GSP-MTB have signal peptides which direct them to the first part of the GSP, the Sec pathway for translocation across the cytoplasmic membrane (see above). This was verified experimentally in the pullulanase secretion pathway in which the two secretion steps can be totally uncoupled (Pugsley et al., 1991a,b). Thus, despite the fact that many of the GSP-MTB components appear to be located in the cytoplasmic membrane, they are exclusively involved in protein transport across the outer membrane.

PulO

PulO is an intergral cytoplasmic membrane protein with both endopeptidase and N-methyl transferase activities. Experiments to demonstrate these activities were based on studies by Strom and Lory on the corresponding protein, PilD, from *P. aeruginosa*. (Strom et al.1993a, b; Strom and Lory 1992). PilD (also called XcpA), which shares over 40% sequence identity with PulO, cleaves a short peptide from the N-terminus of the

major type IV pilus subunit (pilin) of this bacterium and then N-methylates the new N-terminal amino acid (usually phenylalanine). The sequence around the cleavage site in pilin, and the first 26-30 amino acids of the protein, are very similar to the sequences of the corresponding regions of PulG, PulH, PulI and PulJ proteins, one of which, PulG, has been shown to be processed and N-methylated by PulO (Pugsley, 1993b). Studies in Lory's group showed that the *P. aeruginosa* homologues of all four proteins are processed (Nunn and Lory, 1992). The generic name prepilin peptidase has been given to this family of proteins. *E. coli* K-12 has at least two prepilin peptidase genes, neither of which is expressed under normal laboratory conditions (Francetic and Pugsley, 1996).

PulG

The similarity between PulG and pilin led to the proposal that PulG and its sister Pul proteins could form a rudimentary pilus-like structure (the pseudopilus) that spans the periplasm to act as a scaffold or motor to drive secretion (Pugsley, 1993b; Pugsley and Possot, 1993; Hobbs and Mattick 1993). However, this proposal still needs experimental support.

PulE and membrane partners

The PulE protein shares homology with the *P. aeruginosa* protein PilB, and PulF is similar to PilC (Possot et al., 1992). Inactivation of PilB or PilC blocks piliation (Nunn et al., 1990), leading to the idea that they could be involved in type IV pilus assembly. This idea has been extended to PulE and PulF, which are proposed to be involved in pseudopilus assembly. PulE and PilB have consensus ATP binding sites that are

absolutely essential to their function (Possot and Pugsley, 1994; Turner et al., 1993), as well as other conserved residues which, though not essential, are required for maximum efficiency of the pullulanase secretion pathway (Possot and Pugsley, 1994, 1997). A homologue of PulE from *V. cholerae* has been shown to have autokinase activity, although the relevance of this observation for its function is unclear (Sandkvist et al. 1995).

PulE is anchored to the cytoplasmic face of the cytoplasmic membrane. In the absence of all other pullulanase secretion factors, PulE forms aggregates that fail to associate with this membrane. Studies in *V. cholerae* show that association with the membrane is mediated by the homologue of PulL, an intergral cytoplasmic membrane protein with a long cytoplasmic tail (Sandkvist et al. 1995). Genetic studies suggest that the interaction between PulE and PulL is mediated by the N-terminal region of PulE (Possot and Pugsley, 1994).

PulD (secretin)

The PulD protein shares substantial sequence similarity with gpIV proteins encoded by filamentous bacteriophages such as f1 and M13. Studies by Russell's group have led to the idea that gpIV could form a multimeric channel in the outer membrane to permit extrusion of the bacteriophage (Russell, 1994b). Parallel studies in Pugsley's group led to the idea that PulD could form a similar channel. All of the PulD homologues, collectively referred to as secretins, have relatively well conserved C-terminal halves, but their N-terminal halves are only conserved within groups of proteins with related functions. All secretins appear to form large multimeric complexes which,

characteristically, are not dissociated by heating in SDS (Drake and Koomey, 1995; Hardie et al., 1996a; Linderoth et al., 1996). These complexes are presumed to represent the natural multimeric state of the channel which must be large enough to permit the movement of macromolecules, such as pullulanase or filamentous phages.

PulS

A remarkable feature of PulD is that it is not inserted into the outer membrane in the absence of PulS protein. Furthermore, PulD protein produced in the absence of PulS is clipped near its C-terminal end, although multimerization is apparently unaffected. These observations led to the idea that PulS could be a secretin-specific molecular chaperone (Hardie et al., 1996), an idea that has been substantiated by subsequent studies (Hardie et al., 1996b). It appears that PulS has two dissociable functions, to protect PulD from proteolysis and to guide it to the outer membrane. PulS is an outer membrane lipoprotein; outer membrane association is necessary for its piloting activity but not for its ability to protect PulD. Although PulD and PulS have been shown to interact, they do not appear to remain associated, cross-linking studies indicate that multimers formed by PulD do not contain any other Pul protein (Hardie et al., 1996b).

Other Pul proteins

The roles of the less well characterized Pul proteins remain unknown. One proposal put forward by several groups is that they contribute to a trans-envelope protein complex, the "secreton", possibly including the hypothetical pseudopilus, that both controls the opening of the hypothetical outer membrane channel and couples energy to

the movement of pullulanase through this membrane. This idea is supported by studies in our laboratory (Wong and Buckley, 1989), and confirmed in Pugsley's group, showing that secretion via the GSP-MTB depends on the proton motive force across the cytoplasmic membrane. Thus, like the TonB protein involved in energy coupling for siderphore import across the outer membrane (Skare et al., 1993), the secreton might couple the PMF to secretion by a process that remains to be determined.

iii). Secretion signal

It has been established that secreted proteins (Pugsley, 1992; van der Goot et al., 1993) adopt complex tertiary and even quaternary structures before they are secreted across the outer membrane. This means that the information which determines that these proteins are specifically recognized by the secretion machinery must be presented on the surface of the folded polypeptide. After screening over a thousand deletions, it was determined that the minimum region of pullulanase that is necessary for β-lactamase (the reporter protein) secretion comprises two segments, each of approximately 80 amino acids (out of a total of 1071 amino acids), one located immediately after the signal peptide and one near the end of the presumed catalytic domain of the polypeptide (Sauvonnet and Pugsley, 1996).

The recognition of these regions of pullulanase as potential secretion signals is the first step toward the identification of secretion proteins with which this exoprotein interacts. Two strategies can be envisaged: the identification of genetic suppressors that overcome the effects of secretion-inhibiting mutations affecting one or another of the

identified segments or affinity chromatography to identify interacting proteins (Pugsley et al., 1997a). Research using these strategies is being carried out.

iv). An MTB in *E. coli* K-12

It is widely accepted that the special *E. coli* strain K-12 does not naturally secrete extracellular proteins. When proteins are found in the culture supernatants of this species, it is invariably the result of either a more or less severe perturbation of membrane physiology (often caused by the 'secreted' protein itself) or of the presence of a functional secretion system encoded by genes cloned from a different Gram-negative bacterium. It was assumed that *E. coli* K-12 does not possess homologues of genes coding for known secretion factors such as those of the GSP-MTB. The first indication that this might not be the case came with the partial sequencing of an open reading frame (Andrews et al., 1989) that appeared to code for a protein with homology to prepilin peptidases such as the MTB PulO (Whitchurch and Mattick, 1994). The gene (initially called *hopD* and now renamed *gspO*) was subsequently shown to be intact, to be non-essential (Whitchurch and Mattick, 1994) and to encode a fully functional prepilin peptidase that could substitute for PulO in the PulA secretion pathway and that could promote processing of type-IV prepilin (Pugsley et al., 1997). Furthermore, Sojiljkovic et al. (1995) showed that genes located upstream from the *gspO* gene were homologous to the MTB genes F,G and H, and that the product of one of these genes, *gspG*, is cleaved by the GspO protein and that it can be substituted for the G protein in the pullulanase secretion pathway. Finally, this entire region of the *E. coli* chromosome was sequenced

by Blattner and his colleagues as part of their genome sequencing project. They found a complete set of intact genes coding for MTB components (Plunkeett, 1995).

Despite the discovery of MTB genes in the *E. coli* strain, no laboratory conditions thus far have been found cause their expression. This was demonstrated both by the failure to detect functional *gspG* or *gspO* products and by measuring the strength of the *gspC* promoter, using a *lacZ* reporter gene operon fusion integrated into the chromosome (Pugsley et al., 1997b).

Secretion of the *P. aeruginosa* proteins

The opportunistic human pathogen *Pseudomonas aeruginosa* secretes several pathogenicity-related proteins into the environment, including elastase, lipase, alkaline protease, exoenzyme S and exotoxin A (Liu, 1974; Nicas and Iglewski, 1986). Although all three types of secretion pathways are present in *P. aeruginosa* (Tommassen et al., 1992; Yahr et al., 1996), most of the known secreted proteins are transported via the type II pathway. In *P.aeruginosa,* 12 *xcp* genes have been identified that are essential for the GSP-MTB (Filloux et al., 1990; Bally et al., 1991; 1992; Akrim et al., 1993). Among the encoded 12 proteins, XcpT, XcpU, XcpV and XcpW are homologues of PilA, the major subunit of type IV pili. XcpR and XcpS are homologues of PilB and PilC, which are believed to be chaperones involved in the process (Yoshihiro et al., 1998). XcpQ is the only outer membrane protein and therefore is the main candidate to constitute the actual protein translocation channel (Akrim et al., 1993). XcpA is a prepilin peptidase which is required for the cleavage and methylation of XcpT-W and PilA (Lu et al., 1997).

i). XcpQ

XcpQ belongs to the large family of homologous proteins, the secretins (Genin and Boucher, 1994; Hardie et al., 1996a). Like PulD, XcpQ has been shown to form highly stable, multimeric complexes too large to enter the separating gel in standard SDS-PAGE (Newhall et al., 1998; Kazmierzak et al., 1994; Hardie et al., 1996b; Chen et al., 1996; Wilbert et al., 1998). XcpQ, like other secretins, is supposed to consist of two domains. The C-terminal region, corresponding to residues 326-605 of XcpQ, is conserved in all members of the superfamily and has been shown to be important for complex formation and to be located in the outer membrane (Russel and Kazmierczak, 1993; Chen et al., 1996). The N-terminal region, corresponding to residues 53-300, is thought to extend into the periplasm and to interact with other components of the export apparatus (Brissette and Russel, 1990; Genin and Boucher, 1994; Wilbert et al., 1998). A series of mutant analyses showed that the C-domain is sufficient for multimerization, while mutations in the N-domain do not prevent oligomerization but inhibit the function of the protein, probably by disturbing the interactions between XcpQ and other Xcp components (Wilbert et al., 1998).

XcpQ, like other outer membrane proteins, has been found to have 13 putative transmembrane β-strands (Gromiha et al., 1997). All of these β-strands are located in the C-domain. Mutations in these β-strands prevent normal oligomerization of the protein, while mutations in other areas of the C-domain are more permissive. These results suggest that the C-domain forms a β-barrel structure (Wilbert et al., 1998).

Electron microscopy showed that the XcpQ homomultimeric complex is a ring-shaped structure with an apparent large central cavity. The size of the cavity is 9.5 nm,

which is sufficient to allow for the transport of completely folded proteins (Wilbert et al., 1998). For example, one of the proteins secreted via this pathway, elastase, has a maximum diameter of 6.0 nm (Thayer et al., 1991).

As bacterial outer membranes act as permeability barriers for harmful compounds, such as antibiotics, and generally only allow the passage of small hydrophilic molecules through porin channels, the large apparent pores formed by XcpQ complexes must be gated. Although the complex may exist in two different conformations, recent results showed that XcpQ complexes have only one conformation (Wilbert et al., 1998). Alternatively, other Xcp proteins could form a plug that closes the channel. The pilin-like Xcp proteins, i.e. XcpT, U, W and X (Tommassen et al., 1992; Bleves et al., 1998), would be candidates to fulfil this function by forming some rudimentary pilus-like structure or compex that interacts with XcpQ.

ii). XcpR

One of the conserved components of the MTB-GSP machinery, the XcpR protein of *P. aeruginosa*, has been studied extensively in several laboratories. This protein is a member of a large family of proteins, which are distinguished by conserved motifs common to proteins involved in ATP binding and hydrolysis, including the highly conserved Walker A consensus sequence: GXXXXGKT/S (Walker et al., 1982). It has been well established for XcpR that this consensus sequence is essential to the functioning of these proteins in extracellular protein secretion. Additionally, it has been observed that mutations in the Walker A motif and other regions of XcpR XcpE and XcpF are transdominant, resulting in a block in extracellular protein secretion (Turner et

al., 1993; Possot and Pugsley, 1994; Sandkvist et al., 1995). This dominant negative phenotype suggests that XcpR and its homologues interact with other components of the secretory apparatus and that the mutant proteins interfere with such interactions.

XcpR has been found to form dimers *in vivo* (Turner et al., 1997) and this dimerization is directed by a relatively short domain near its extreme N-terminus. There is a close correlation between deletions in this domain and interference with protein secretion when expressed in wild-type *P. aeruginosa* (Turner et al., 1997).

Several functions have been attributed to XcpR (Bally et al,m 1992; Nunn and Lory, 1993; Turner et al., 1997). XcpR may participate in multiple protein-protein interactions, perhaps involving the formation of transient complexes consisting of other proteins of the secretion machinery and homodimers of XcpR. Furthermore, the dimerization of XcpR may be an essential step in the process of energy transduction to the secretory apparatus.

iii). XcpT, U, V and W

The four pilin-like proteins XcpT, U, V and W are homologous to PilA, the major subunit of type IV pili (Russel and Darzins,.1994; Alm and Matack, 1995, 1996; Koomey, 1995). In addition to sequence similarities, both of these proteins require the bifunctional enzyme XcpA (PilD) for posttranslational modifications (Nunn et al., 1991; Nunn and Lory, 1992; Lauer et al., 1993; Strom et al., 1993; Lory, 1994). The location of XcpT-U is almost exclusively in the inner membrane, as is the location of unassembled pilin monomers (Nunn and Lay, 1993). Cross-linking studies show that XcpT can form concentration-dependent dimeric complexes with XcpU, XcpV, XcpW and interestingly,

with PilA (Lu et al., 1997). The heterodimer with PilA is the major dimeric form of XcpY when *xcp* genes are expressed from single chromosomal loci in the wild-type bacteria. Furthermore, it was found that there was a significant delay in protein secretion in PilA-deficient *P. aeruginosa* strains. Secretion can be complemented by cloning PilA into the bacteria, indicating that the defect was due to the absence of pilin monomeric subunits (Lu et al., 1997). Therefore, it can be concluded that the pilin monomers directly participate in the GSP-MTB in *P. aeruginosa*, establishing a functional link between the pili synthesis and secretion pathway.

iv). Secretion signal

Studies of hybrid protein secretion in *P. aeruginosa* suggest that a specific 60 amino acid domain might contain the necessary targeting information for translocation of exotoxin A across the outer membrane via the GSP-MTB (Lu and Lory, 1996). This domain was shown to help in the secretion of different proteins into the culture medium and is rich in anti-parallel β-sheets (Lu and Lory, 1996). In the three dimension view, the targeting domain is on the surface of exotoxin A where it might be easily accessible to the components of Xcp machinery (Lu and Lory, 1996).

GSP-MTB secretion by other species

In *V. cholerae*, the GSP-MTB is required for the secretion of several proteins including chitinase, enterotoxin,and protease through the outer membrane (Overbye et al., 1993; Sandkvist et al., 1993). 12 components of the system have been identified: the Eps proteins (Sandkvist et al., 1993; Overbye et al., 1993; Sandkvist et al., 1997). Mutations

in the *eps* genes result in aberrent outer membrane protein profiles, indicating at least some components of the GSP-MTB are required not only for secretion of soluble proteins but also for proper outer protein assembly (Sankvist et al., 1997). Another feature of the Eps system is that thus far no homologue of prepilin peptidase has been found in *V. cholerae*. In *K. oxytoca* and *P. aeruginosa,* such proteins (PulO and PilD) are required to process some of the components (see above).

The plant pathogens *Erwinia chrysanthemi* and *Erwinia carotovora* secrete extracellular pectate lyases (Pels) via a GSP-MTB named the Out system. Intriguingly, these two bacteria cannot secrete the enzymes encoded by heterologously expressed genes from the other species, suggesting the presence of species-specific recognization factors in the Out systems of the two *Erwinia* species (He et al., 1991; Py et al., 1991). Complementation analysis showed all the mutations of out protein species could be complemented by homologues from the other species except for OutC and OutD (Lindeberg et al., 1996). Therefore it was suggested that OutC and OutD were gatekeepers of the Out system involved in recognition of Pels targeted for secretion and that their assembly in the two bacteria species might be different, explaining the failure of complementation (Lindeberg et al., 1996). A possible signal for this species-specific secretion was recently identified (Lindeberg et al., 1998). Differential secretion of hybrid proteins, which were constructed with parts of PelC and PelI, revealed that the primary targetting signal was contained within the external loop and was also dependent on specific residues for proper positioning (Lindeberg et al., 1998).

Some other bacteria like *Xanthomonas campestris* (Dums et al., 1991; Hu et al., 1992), *Pseudomonas solanacearum* (Kang et al., 1994) and *Serratia marcescens* (Sah and

Benedik, 1997) can also secrete various proteins via the GSP-MTB. The secretion of aerolysin and some other proteins in *Aeromonas* species, which is one of the best characterized process of GSP-MTB, will be discussed in details below.

II. Secretion by Gram-positive bacteria

Despite the differences in their cell wall structure, Gram-positive and Gram-negative bacteria seem to have some similarities in their export apparatus. For example, homologues of SecA (Gilbert et al., 1996), SecY (Istiguy et al., 1996; Hale et al., 1995), SecD and SecF (Morosoli et al., 1997) and SecE (Miyake et al., 1994) have been found in *Streptomycetes*. However, SecY from *Streptococcus lividans* does not complement the temperature dependent lethality of the *E. coli* SecY mutant (Ostiguy et al., 1996) and the complete SecA from *S. lividans* does not complement *E. coli* MM52, which carries a thermosensitive mutation in the *secA* gene. On the other hand, a hybrid polypeptide consisting of the N-terminal portion (first 242 amino acids) of *S. lividans* SecA and of C-terminal protion (657 amino acids) of the wild-type *E. coli* SecA was able to complement that mutant (Gilbert et al., 1996).

As in Gram-negative bacteria, export in Gram-positive bacteria requires signal sequences, which are interchangeble with those of Gram-negative bacteria (Simonen and Palva, 1993; Wang et al; 1993). The average signal sequence length for Gram-positive bacteria is 29-31 amino acids, in contrast to 24-25 amino acids for Gram-negative bacteria (Gilbert et al., 1995). The additional amino acids are located in the N-terminal portion of the signal sequence and consist, on average, of four positively charged residues

which are mostly arginines that are often closely linked and designated the "arginine cluster" (Gilbert et al., 1995).

PART II. The genus *Aeromonas*

1. General features

Aeromonads are widespread, facultatively anaerobic, Gram-negative baccili, usually found in aquatic environments. Their size range is 0.3-10 μm in diameter and 1.0-3.5 μm in length and they exist singly, in pairs or in short chains. The biochemical properties include the ability to ferment glucose and they are oxidase positive (Austin et al., 1996).

Among the numerous species of *Aeromonas*, the fish pathogens *A. hydrophila* and *A. salmonicida* have been used in our lab for the study of aerolysin. *A. salmonicida* is typically a non-motile, fermentative, Gram-negative rod which cannot grow at 37 °C. Besides catalase and oxidase, *A. salmonicida* can produce a brown water-soluble pigment in tryptone-containing medium. Recently, non-pigmented and oxidase-negative strains have been isolated (Wiklund, 1995; Wilson and Holliman, 1994). Another interesting trait of *A. salmonicida* is the ability to grow into different types of colonies such as rough, intermediate and smooth. Electron microscopy has demonstrated that the rough or smooth forms reflect the presence of absence of an A-layer (Udey, 1985). The A-layer is a 50 kDa outer membrane protein which confers virulence by providing the organism with a protective barrier against the host defense. Congo Red Agar (CRA) has been used to detect the A-layer and as an indicator of cell surface integrity (Ishiguro et al., 1985). *A.*

salmonicida has a large plasmid in the size range of 60 to 150 kb. It also has two additional low-molecular weight plasmids each about 5 kb, which have been used as epidemiological markers (Neilsen et al., 1993).

A. hydrophila is one of the motile species of *Aeromonas*. It is also an opportunistic human pathogen, which is responsible for about 60% of human disease caused by *Aeromonas* (Chaudaury et al., 1996). The S-layer outer membrane protein, the metalloproteases and the hemolysin, aerolysin are thought to be the main virulence factors (Gosling et al., 1996).

2. Hemolytic toxin aerolysin

The hemolytic toxin aerolysin is produced by a number of *Aeromonas* species (Hirono and Aoki, 1991; Husslein et al., 1988). Aerolysin is synthesized as a 54 kDa precursor called preproaerolysin, which has a typical 23 amino acid signal sequence for the GSP (Howard et al., 1987; van der Goot et al., 1992). The signal sequence is removed while export across the inner membrane, which occurs via the Sec system (Howard and Buckley, 1985a). The protoxin then is called proaerolysin. Proaerolysin is composed of 470 amino acids (Figure 1). The crystal structure of proaerolysin has been solved (Parker et al., 1994, see Figure 2). The protein consists of a small lobe and a large lobe joined by a short arm. Proaerolysin is secreted into the extracellular medium via the GSP-MTB by *A. salmonicida*, *A hydrophila* and *V.cholerae*, but not *E. coli* (Howard et al., 1985, 1986; Wong et al., 1990). Proaerolysin is proteolytically converted to active aerolysin in the extracellular midium (Howard and Buckley, 1985). Many proteases, including trypsin and chymotrypsin can activate proaerolysin by removing approximately 40 amino acids

at the C-terminal of the protoxin (Howard and Buckley, 1985; van der Goot et al., 1992). Before the second step in secretion, proaerolysin folds and forms a homodimer in the periplasm (Hardie et al., 1995). This dimerization does not require the formation of disulphide bonds because unoxidized protein can be secreted by cells grown in beta-mercaptoethanol and by a *dsbA* mutant of V. cholerae (Hardie et al., 1995). Various regions in proaerolysin have been tested for possible targeting signal. Thus far only one region was found where mutations affect secretion (Wong and Buckley, 1991). This is in domain III of the protein in an area enriched in serine and theonine residues (Wong et al., 1991). Both PMF and ATP energize proaerolysin secretion (Wong and Buckley, 1989; Letellier et al, 1997), although the mechanism is still unknown.

Active aerolysin is able to disrupt cells by inserting into their membranes and forming channels (Wilmsen et al., 1990; Chakaborty et al., 1990). Aerolysin must oligomerize before it can enter into membranes (Garland and Buckley, 1988; van der Goot., 1993). Recently, it has been shown that a loop in domain III must move away from the β-sheet that forms the main body of the protein before oligomerization can occur (Rossjoha et al., 1998).

Aerolysin is able to bind to the glycosylphosphatidylinositol (GPI) anchors of some membrane proteins such as Thy-1 (Diep et al., 1998). The GPI-anchored proteins can provide the means by which aerolysin can be concentrated on the cell surface and then move laterally to oligomerize (Nelson et al., 1997).

The experiments described in this thesis represent an attempt to determine how proaerolysin interacts with the GSP-MTB. A number of different approaches have been used. The efficiency of secretion was determined by calculating the maximum secretion rate. The effect of proaerolysin secretion on other protein was discussed. The nature of the secretion signal has been attempted to find. Finally, the energy requirements for secretion were investigated.

```
1    ATG CAA AAA ATA AAA CTA ACT GGC TTG TCA TTA ATC ATA TCC GGC CTG CTG ATG GCA CAG
1    M   Q   K   I   K   L   T   G   L   S   L   I   I   S   G   L   L   M   A   Q

61   GCG CAA GCT GCA GAG CCC GTC TAT CCA GAC CAG CTT CGC TTG TTT TCA TTG GGC CAA GGG
21   A   Q   A   A   E   P   V   Y   P   D   Q   L   R   L   F   S   L   G   Q   G

121  GTC TGT GGC GAC AAG TAT CGC CCC GTC AAT CGA GAA GAA GCC CAA AGC GTT AAA AGC AAT
41   V   C   G   D   K   Y   R   P   V   N   R   E   E   A   Q   S   V   K   S   N

181  ATT GTC GGC ATG ATG GGG CAA TGG CAA ATA AGC GGG CTG GCC AAC GGC TGG GTC ATT ATG
61   I   V   G   M   M   G   Q   W   Q   I   S   G   L   A   N   G   W   V   I   M

241  GGG CCG GGT TAT AAC GGT GAA ATA AAA CCA GGG ACA GCG TCC AAT ACC TGG TGT TAT CCG
81   G   P   G   Y   N   G   E   I   K   P   G   T   A   S   N   T   W   C   Y   P

301  ACC AAT CCT GTT ACC GGT GAA ATA CCG ACA CTG TCT GCC CTG GAT ATT CCA GAT GGT GAC
101  T   N   P   V   T   G   E   I   P   T   L   S   A   L   D   I   P   D   G   D

361  GAA GTC GAT GTG CAG TGG CGA CTG GTA CAT GAC AGT GCG AAT TTC ATC AAA CCA ACC AGC
121  E   V   D   V   Q   W   R   L   V   H   D   S   A   N   F   I   K   P   T   S

421  TAT CTG GCC CAT TAC CTC GGT TAT GCC TGG GTG GGC GGC AAT CAC AGC CAA TAT GTC GGC
141  Y   L   A   H   Y   L   G   Y   A   W   V   G   G   N   H   S   Q   Y   V   G

481  GAA GAC ATG GAT GTG ACC CGT GAT GGC GAC GGC TGG GTG ATC CGT GGC AAC AAT GAC GGC
161  E   D   M   D   V   T   R   D   G   D   G   W   V   I   R   G   N   N   D   G

541  GGC TGT GAC GGC TAT CGC TGT GGT GAC AAG ACG GCC ATC AAG GTC AGC AAC TTC GCC TAT
181  G   C   D   G   Y   R   C   G   D   K   T   A   I   K   V   S   N   F   A   Y

601  AAC CTG GAT CCC GAC AGC TTC AAG CAT GGC GAT GTC ACC CAG TCC GAC CGC CAG CTG GTC
201  N   L   D   P   D   S   F   K   H   G   D   V   T   Q   S   D   R   Q   L   V

661  AAG ACT GTG GTG GGC TGG GCG GTC AAC GAC AGC GAC ACC CCC CAA TCC GGC TAT GAC GTC
221  K   T   V   V   G   W   A   V   N   D   S   D   T   P   Q   S   G   Y   D   V

721  ACC CTG CGC TAC GAC ACA GCC ACC AAC TGG TCC AAG ACC AAC ACC TAT GGC CTG AGC GAG
241  T   L   R   Y   D   T   A   T   N   W   S   K   T   N   T   Y   G   L   S   E

781  AAG GTG ACC ACC AAG AAC AAG TTC AAG TGG CCA CTG GTG GGG GAA ACC CAA CTC TCC ATC
261  K   V   T   T   K   N   K   F   K   W   P   L   V   G   E   T   Q   L   S   I

841  GAG ATT GCT GCC AAT CAG TCC TGG GCG TCC CAG AAC GGG GGC TCG ACC ACC ACC TCC CTG
281  E   I   A   A   N   Q   S   W   A   S   Q   N   G   G   S   T   T   T   S   L

901  TCT CAG TCC GTG CGA CCG ACT GTG CCG GCC CGC TCC AAG ATC CCG GTG AAG ATA GAG CTC
301  S   Q   S   V   R   P   T   V   P   A   R   S   K   I   P   V   K   I   E   L

961  TAC AAG GCC GAC ATC TCC TAT CCC TAT GAG TTC AAG GCC GAT GTC AGC TAT GAC CTG ACC
321  Y   K   A   D   I   S   Y   P   Y   E   F   K   A   D   V   S   Y   D   L   T

1021 CTG AGC GGC TTC CTG CGC TGG GGC GGC AAC GCC TGG TAT ACC CAC CCG GAC AAC CGT CCG
341  L   S   G   F   L   R   W   G   G   N   A   W   Y   T   H   P   D   N   R   P
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1081 AAC TGG AAC CAC ACC TTC GTC ATA GGT CCG TAC AAG GAC AAG GCG AGC AGC ATT CGG TAC
 361 N   W   N   H   T   F   V   I   G   P   Y   K   D   K   A   S   S   I   R   Y

1141 CAG TGG GAC AAG CGT TAC ATC CCG GGT GAA GTG AAG TGG TGG GAC TGG AAC TGG ACC ATA
 381 Q   W   D   K   R   Y   I   P   G   E   V   K   W   W   D   W   N   W   T   I

1201 CAG CAG AAC GGT CTG TCT ACC ATG CAG AAC AAC CTG GCC AGA GTG CTG CGC CCG GTG CGG
 401 Q   Q   N   G   L   S   T   M   Q   N   N   L   A   R   V   L   R   P   V   R

1261 GCG GGG ATC ACC GGT GAT TTC AGT GCC GAG AGC CAG TTT GCC GGC AAC ATA GAG ATC GGT
 421 A   G   I   T   G   D   F   S   A   E   S   Q   F   A   G   N   I   E   I   G

1321 GCT CCC GTG CCG CTC GCG GCT GAC AGC AAG GTG CGT CGT GCT CGC AGT GTG GAC GGC GCT
 441 A   P   V   P   L   A   A   D   S   K   V   R   R   A   R   S   V   D   G   A

1381 GGT CAA GGC CTG AGG CTG GAG ATC CCG CTC GAT GCG CAA GAG CTC TCC GGG CTT GGC TTC
 461 G   Q   G   L   R   L   E   I   P   L   D   A   Q   E   L   S   G   L   G   F

1441 AAC AAC GTC AGC CTC AGC GTG ACC CCT/GCT GCC AAT CAA taacggcagcgcgttgtagtgatggaac
 481 N   N   V   S   L   S   V   T   P / A   A   N   Q

1508 cgggcctctgtggcccggtttttgtttgcactgttcgggcttggtaaaggcttgtgctttccatttccccacttatactg

1588 gcgccatcttgtcggagtgccaaccgtcgaacgacccgaggctgagaccgttaattcgggatccgtggaacctgatcccc

1668 gggaattc  1675
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Figure 1. Gene sequence of proaerolysin.

The first 23 amino acids are the signal sequence.

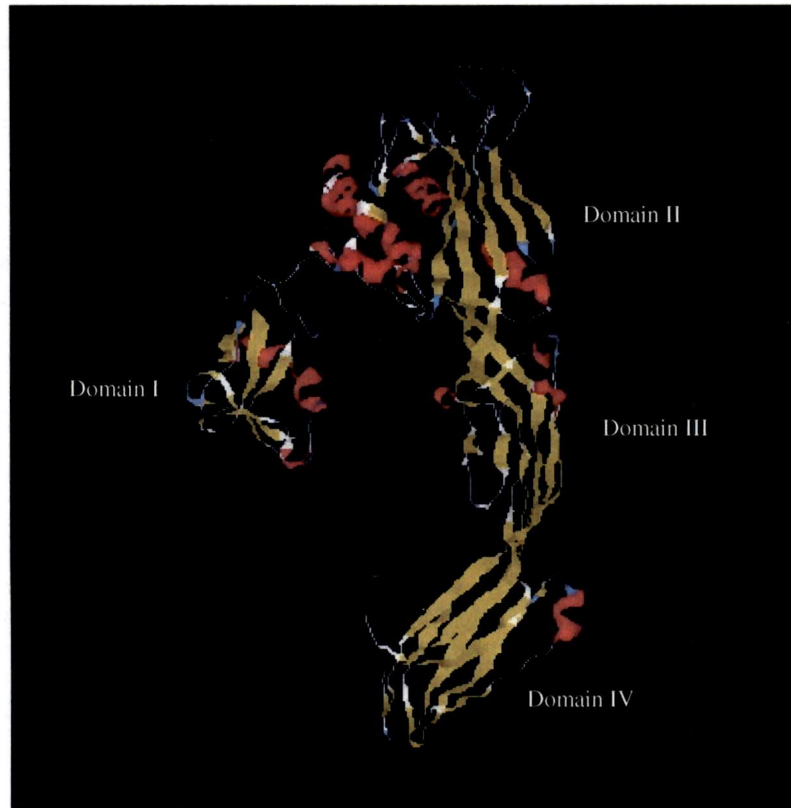


Figure 2. 3-D structure of proaerolysin (From Parker et al., 1994).

Proaerolysin is composed of two lobes: the small lobe (Domain I) is connected to the large lobe (Domains II-IV) by a short arm.

Materials and Methods

Media and reagents

Bovine serum albumin (BSA), carbonyl cyanide m-chlorophenyl hydrazone (CCCP), trypsin, trypsin inhibitor, ampicillin, kanamycin, tetracycline, rifamycin chloroamphenicol and hide powder azure were purchased from Sigma. Protein molecular weight standards were from Pharmacia. Nucleic acid molecular weight standards, restriction endonucleases, and isopropyl-β-D-thiogalactopyranoside (IPTG) were obtained from Boehringer Mannheim. {7-(thienyl-2-acetamido)–3[2-(4-N,N-dimethyl-aminophenylazo) pyridinium methyl]-3-cephem–4-carboxylic acid} (PADAC) was from Calbiochem, NaCl, $MgCl_2$, NaOH, $CaCl_2$, glucose, sucrose, ethylenediamine tetraacetic acid (EDTA) and glycerol were from BDH. Skim milk powder was from Oxiod. Sodium dodecyl sulfate (SDS) and polyoxyethylene -20- sorbitan monolaurate (Tween - 20) were from Fisher Scientific. Acrylamide and N,N'-methylene- bisacrylamide for gel electrophoresis were from BDH. All other reagents were supplied by Sigma unless otherwise specified.

Luria-Bertani (LB, supplied by Difco) media was prepared as described by Sambrook et al. (1989) except that the pH was adjusted to 7.5. One part 10× modified Davis buffer (Ashton et al., 1980) was added to nine parts LB to prepare LB-Davis media. Glucose was added 1:100 to media when required from a 20% sterile stock solution. Phosphate buffered saline (PBS) was 10 mM NaH_2PO_4 (pH7.4), 0.15 M NaCl. PBS-T was made by the addition of 0.5% Tween-20 to PBS. 4× sample buffer for SDS-PAGE sample (8% SDS, 20% β-mercaptoethanol, 40% glycerol, 108 mM H_2SO4, 220

mM Tris-HCl pH 6.1 with bromophenol blue) was added 1:3 to liquid samples, which were diluted in dH2O. 1× sample buffer, for re-suspending pellets or dried samples, was prepared by diluting 4× sample buffer 1:3 with dH2O.

Bacterial strains and plasmids

The bacterial strains and plasmids used in this study with their sources are listed in Table 1. The strain Rif-1 was isolated from wild-type strain AS440 (Wong et al, 1986). CB3 is a Rif-1 strain with an insertion of transposon Tn5 into the chromosomal DNA (Buckley, 1990). It is widely used in this study because it is deficient in protease secretion, so that secreted proaerolysin is not degraded in the culture supernatant. I2.66 is an *A. hydrophila* mutant strain with a Tn5 insertion in the *aerA* gene (Wong et al., 1992). Therefore it is deficient in proaerolysin expression. L1.97 is an *A. hydrophila* mutant strain with a mutation in one component of the secretion machinery, *exeE* (Jiang and Howard, 1991). This makes the strain unable to secrete any proteins through GSP. The *E. coli* strains which were used for cloning and preliminary study for secretion.

The wide-host-range vector pMMB66HE was used to move *aerA* into *A salmonicida*. The wide-host-range plasmid pMMB208 (Morales et al, 1981) was used to move constructs into *A. hydrophila*. Details of the plasmid γ123 will be discussed later.

Strain/plasmid	Description	Source
Aeromonas salmonicida		
AS440	Wild-type	ATCC
Rif-1	Rif^r strain of AS440	This laboratory
CB3	Rif-1:Tn5; defecient in secreted protease; Km^r and Rif^r	Buckley, 1990
Aeromonas hydrophila		
Ah65	Wild-type	This laboratory
L1.97	AH65 with mutation in exeE	Jiang and Howard, 1991
I2.66	AH65::Tn5; defecient in proaerolysin production	This laboratory
Escherichia coli		
DH52	Strains for high efficient	F.E Nano
HB101	cloning	E.E Ishiguro
JM109	Helper strains for	This laboratory
MM297	transconjugation	This laboratory
PMMB66HE	RSF1010 Δ(PstI-PvuII, 2.8kb) (lacI9 tacP rrnBblaNruI-AhaIII, 3.0kb) Ap^r	Furste et al.,1986
PMMB 208	IncQ, lacI9, cat (Dmr), Ptac, rmB with polylinker from M13mp19	Morales et al.,1981
γ123		This study

Table M-1: Bacterial strains and plasmids used in this study

Bacterial culture

All *E. coli* cultures were grown in a New Brunswick Scientific Gyrotary Water Bath Shaker (Model G-76) at 37°C and 250 rpm. All *Aeromonas* cultures were grown at 27°C and 250 rpm in a New Brunswick Scientific Controlled Environmental Incubator Shaker (Model G-25). Growth was followed by measurement of the optical density at 600 nm (OD_{600}) using a Cary 1 Bio UV-VIS spectrophotometer. Cultures were normally grown to an OD_{600} of 0.5 before adding IPTG to the desired concentration.

Cell fractionation

Culture supernatants were obtained by centrifuging cells in either a Beckman JA20 or JA14 rotor at approximately 9000 X for 10 minutes, or in a Fisher microcentrifuge for a period from 1 minute to 10 minutes at room temperature or at 4°C.

Shock fluids were obtained by the method of Willis et al. (1974). Pellets obtained by centrifuging 1 ml of cells were resuspended in 1 ml of shock solution (20% sucrose, 33mM Tris-HCl, 1 mM EDTA) and left at room temperature for 5 minutes before centrifuging for 2 minutes. The supernatants were removed and the pellets were resuspended in 200 µl ice-cold water. The suspensions were put on ice for 5 minutes before centrifuging for 3 minutes. The supernatants were collected as shock fluids. The pellets, which contained the shocked cells, were either resuspended in 1× sample buffer, or further fractionated.

Cytoplasmic fractions were prepared by a freezing and thawing method. Shocked cell pellets were resuspended in 200 µl ice-cold PBS and put into a -70°C refrigerator. After freezing for up to 15 minutes, they were taken out and quickly thawed in 37°C

water. This process was repeated 3 times before centrifuging for 5 minutes. The supernatants are referred as freeze-thaw SN. The pellets referred as insoluble components.

Sodium-Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS polyacrylamide gels were prepared as described by Neville (1971) using the BIO-RAD mini-protein slab gel casting system. A 12% separating gels and a 3% stacking gel were used unless otherwise stated. Samples were prepared in sample buffer and boiled for three minutes before being loaded onto the gel. Gels were run at 16 mA (maximum 160 V) constant current until the dye front reached the bottom of the gel. Proteins were either stained by one of the methods given below, or transferred to nitrocellulose, as described.

SDS-PAGE gel staining

1). Coomassie staining

Gels were fixed and stained in 0.03% Coomassie blue, 25% isopropanol, 10% acetic acid for 15 minutes or until bands were visible. They were gradually de-stained by transferring to solutions containing progressively lower Coomasie blue concentrations. In preparation for drying, gels were de-stained fully by washing for 1 to 2 hours in 10% acetic acid, then soaked in water for 10 minutes before being transferred to Watman No.3 paper and dried under vacuum at 80°C.

2). Silver staining

Gels were fixed in 50% methanol, 10% acetic for a minimum of 30 minutes, then rehydrated in 10% methanol, 10% acetic acid with heating for 1 minute in a microwave, followed by a 5 minutes wash in water. The water was drained and 33.3 μM dithiothreitol was poured over the gels, and this was heated for 1 minute in a microwave. The gels were rinsed with water and incubated in 0.1% (w/v) $AgNO_3$, for 15 minutes. Excess $AgNO_3$ was washed away thoroughly with distilled H_2O before the gels were developed in 3% Na_2CO_3 (w/v), 0.037% formaldehyde (v/v). Once the protein bands were clearly visible, development was stopped with 5% acetic acid.

Western Blotting

Proteins were transferred from polyacrylamide gels to nitrocellulose (BIO-RAD) or polyvinylidene difluoride (PVDF) membranes (Applied Biosystems Problott membrane) using a BIO-RAD mini-cell transfer apparatus (wet cell) for 30 minutes at 90 V as described by Towbin et al. (1979).

Visualization of proteins immobilized on membrane

1). Alkaline phosphatase

Membranes were blocked with 5% (w/v) skim milk in PBS containing 0.05% Tween 20 for 1 hour at room temperature, or overnight at 4°C. All subsequent incubations were performed at room temperature on a lab shaker. The blocking solution was removed and 30 ml of PBS-T was poured over the membrane. An anti-proaerolysin polyclonal or monoclonal antibody, produced in this laboratory, was usually used as the

primary antibody in the next step. A 1:4000 dilution of the primary antibody was added to the solution and the blot was incubated for one hour. The primary antibody was removed and the membrane was washed 5 × 5 minutes with PBS-T. The membrane was then covered with PBS-T containing a 1:4000 dilution of either goat anti-rabbit (Caltag; for polyclonals) or goat anti-mouse IgG (for monoclonals) antibody linked to alkaline phophatase. Blots were incubated for 1 hour before being washed 3 × 5 minutes with PBS-T. Another 3 × 5 washes with a buffer containing 100 mM NaCl, 50mM $MgCl_2$, 100mM Tris-HCl, pH 8 were used before the blots were stained as described by Sambrook et al. (1989).

2). Enhanced chemiluminescence (ECL)

To increase sensitivity, some blots were developed using the Amersham ECL system. Western blotting was performed using the alkaline phosphatase method as described above, with the following exceptions: Caltag second antibody was omitted, and in its place a 1:4,000 solution of Amersham goat anti-mouse IgG horseradish peroxidase conjugate was used. After 1 hour of incubation with this antibody conjugate, the blots were washed eight times in PBS-Tween (three times for 5 minutes, twice for 15 minutes then a further three times for 5 minutes), before they were treated with ECL reagent and exposed to film according to the manufacturer's instruction.

DNA purification and plasmid construction

Restriction digests of plasmids were performed at room temperature for 1 hour with restriction enzymes from Boehringer Mannheim. Digestions were stopped by

addition of 40% sucrose, 60mM EDTA, 0.25% bromophenol blue. Ligations were performed by mixing 100-200 ng of vector DNA with at least an equimolar amount of insert DNA, 2 units of T4 DNA ligase (BRL) and 1 × ligation buffer supplied by the company. Ligations were incubated at 37°C for 1 –2 hours before being used to transform competent cells (see below).

Mini plasmid preparations were obtained either by the alkali lysis method described by Sambrook et al. (1989), or using a QIAEX kit and the protocol supplied by the manufacturers. Mini preparations supplied enough DNA for most cloning experiments, however sometimes large preparations were performed using Wizard Megapreps kits. *E. coli* or *Aeromonas* strains containing the desired plasmid were used for plasmid preparations. All DNA preparations, restriction digests and ligations were checked by agarose gel electrophoresis using 0.8-1.5% agarose gels in 40 mM Tris-acetate, 4 mM EDTA. Submerged miniature electrophoresis tanks (Biorad) were used, normally at a constant voltage between 80-100 V.

Plasmid transformation and transconjugation

Plasmids and ligation mixtures were transformed into *E. coli* strain JM109 using the $CaCl_2$ method described by Sambrook et al. (1989) with the following changes: 50 mM $CaCl_2$ was used; cell pellets were re-suspended in 50 ml of $CaCl_2$ per 100 ml culture after the first centrifugation and 8 ml per 100 ml culture after the second centrifugation; LB medium and TSA plates were used instead of SOC medium and SOB agar containing $MgSO_4$ respectively. Plasmids were mobilized into *Aeromonas* species using a modification of the filter mating technique of Haryama et al. (1980). Both donor and

recipient cultures were grown in LB medium with shaking at 250 rpm and the optimum temperature for each species. The *E. coli* helper strain MM297 (Figurski and Helinski, 1979) was grown under the same conditions. All cultures were grown to an OD_{600} of 0.5 before being transferred in the ratio of 2:1:1 (recipient: helper: donor) onto sterile 0.45 μm millipore filters placed on a vacuum filter flask. Filters were incubated on TSA plates containing no antibiotics at 30°C or 37°C for at least 3 hours. The filters were then immersed in 5 ml of LB medium and vortexed to remove cells. Dilutions of the mating mixtures were plated onto antibiotic-selective HBA plates to select for transconjugates.

Preparation of human blood cells for titre

One volume whole blood (from the Royal Jubilee Hospital blood bank) was diluted with three volumes PBS. The suspension was centrifuged for 5 minutes at 2,000 × and the supernatant was removed using a tap aspirator. White blood cells and platelets were also aspirated. The blood was resuspended in the same volume of PBS, centrifuged and aspirated as before. This cycle was repeated until the supernatant was clear.

Hemolytic titre

Hemolytic titres were used to measure the activity of aerolysin. 50 μl samples were normally used. 10 μl of 20 μg/ml trypsin were used to activate the protein. The final volume was made up to 200 μl with PBS. After incubating for 10 minutes at room temperature, the samples were serially diluted 1 in 2 with PBS in 96-well microtitre plates. An equal volume of washed human erythrocytes was added to a final concentration of 0.4% packed cells and the plates were incubated at 37°C for 1 hour.

Hemolytic activity is expressed as the inverse of the largest dilution with which complete lysis occurred.

To more accurately measure the amount of aerolysin, the hemolytic titres were sometimes performed in microtitre plates with V-shaped bottoms. After the plates were incubated at 37°C for 1 hour, they were centrifuged for 10 minutes at 4,000 rpm. 100 μl aliquots of the supernatants were removed from the wells containing partially hemolyzed cells. Each aliquot was diluted with 900 μl of PBS and the absorbance at 413 nm was read. The amount of aerolysin in each original sample was determined from a standard curve prepared in the same microtitre plate with known amounts of aerolysin.

Enzyme assays

Protease activities were determined as described by Young and Broadbert (1982). A solution of 17 mg/ml hide powder azure (Sigma) in 20 mM Tris-HCL, pH7.4 was homogenized to get a homogeneous suspension. 50 μl supernatant samples and 950 μl Hide powder mixture were pre-warmed to 37°C before mixing. After incubating at 37°C for 15 minutes, they were centrifuged for 5 minutes and the OD_{595} of the supernatants was read.

β-lactamase was assayed using the chromogenic substrate PADAC (Calbiochem). Samples of 1-20 μl were added to cuvettes containing 900 μl of 20 mM Tris-HCl, pH 8.0, The ΔOD_{572} was then measured in a Cary 1 Bio UV-VIS spectrophotometer for 10 minutes. The slopes of the linear parts of the recorded reaction curves were used to indicate the enzyme activity.

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used to quantify the amounts of certain proteins. The 96-microwell ELISA plates were used for this study. Samples containing proaerolysin or other proteins of interest were added to the first column of wells and serially diluted 1 in 2 to the other 11 wells. The plates were then incubated at 37°C overnight to remove water and coat the wells. The coated plates were then washed with PBS+ 0.05% Tween20 once and blocked with 3% BSA in PBS and incubated at 37°C for 30 minutes with shaking. After the blocking, the plates were washed 3 more times with PBS-T. 1:4,000 first antibodies (polyclonal anti-proaerolysin antibodies) and second antibodies (goat-anti-rabbit alkaline phosphatase) were sequentially added to the washed plates, with a 3 washes in between, After the second antibody, p-nitrophenylphosphate tablets were dissolved in diethanolamine buffer to 1 mg/ml to prepare the substrate. 200 μl substrate were added to each well and the plates were incubated in the dark for 20 minutes at room temperature. The absorbance at 405 nm was read and the amounts of protein were determined by comparing to standards.

Electron microscopy

A. salmonicida and *A. hydrophila* were grown overnight in LB to OD_{600} about 3.0 with appropriate antibodies. The overnight cultures were diluted 1 in 10 before staining. Copper grids (200 mesh) were coated with 0.3% polyvinylformaldehyde resin (Formvar) dissolved in chloroform. Drops of culture were added to the coated grids and subsequently stained by floating the grids on a drop of 0.5% phosphotungstic acid (pH

7.1) for 30 seconds. Specimens were then viewed using a HITACHI H-7000 TEM at 75 KV and photographed with enclosed camera.

RESULTS

Aeromonas species secrete a wide variety of proteins including proaerolysin, lipases, proteases, amylases, and nucleases (reviewed by Pemberton et. al., 1997). In addition to studies on proaerolysin secretion, detailed studies of secretion of the lipase GCAT have been undertaken by Buckley and co-workers (Wong et al., 1989; Hilton et al., 1990, 1991; Robertson et al., 1994; Brumlik et al., 1997). My studies focused on the secretion of proaerolysin and a serine protease by the Gram-negative bacterium, *Aeromonas salmonicida*.

Electron microscopy studies revealed that *A. salmonicida* is a short rod, although the outer structure surrounding the cell makes it appear coccoid (Figure 3). The bacterium is about 2.0 µm in length and 1.0 µm in diameter. No flagella were observed. This confirmed previous findings (Graham et al., 1991).

Secretion of proaerolysin by *A. salmonicida*

A series of preliminary experiments was conducted to confirm the optimum conditions for secretion of proaerolysin, which have been described previously (Howard et al., 1986; Wong et al., 1989, 1991, 1993; Buckley, 1991, 1992; Hardie et al., 1995). The mutant strain *A. salmonicida* CB3 (Buckley, 1990) containing the plasmid pKW5 with the *aerA* gene (Wong et. al. 1989) was used for these studies. The advantage of this strain is that it does not secrete proteases, so the proaerolysin molecules are not activated. Because proaerolysin remains stable during secretion it can be easily detected. Optimum

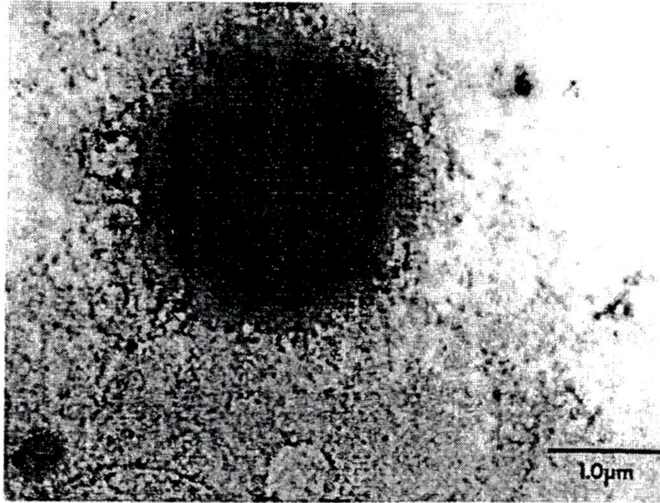


Figure 3. Electron micrograph of *A. salmonicida.*

conditions (hereafter, standard culture conditions) for secretion of proaerolysin included growing *A. salmonicida* CB3 cells in LB medium, pH 7.4, containing 1× Davis buffer and 0.2% glucose. Secretion of proaerolysin was induced by the addition of 1mM IPTG when the OD_{600} of the culture reached 0.5 (Figure 4). Cultures grown in the absence of IPTG served as controls. Preliminary experiments showed that the cells were not healthy when they were induced with IPTG concentrations higher than 1 mM. Samples were taken 3 hours after the addition of IPTG and centrifuged for 2 minutes. Cell pellets and culture supernatants were resuspended in 1× and 4× sample buffer, respectively, and then subjected to SDS-PAGE gel electrophoresis. Proaerolysin was detected in both the cell pellet and supernatant of induced samples, but not in uninduced samples (Figure 5). This result confirmed that proaerolysin is secreted by *A. salmonicida* CB3 (Howard et al., 1986; Wong et al., 1989, 1991, 1993; Buckley, 1991, 1992; Hardie et al., 1995).

In contrast to *A. salmonicida*, *E. coli* did not secrete proaerolysin under any of the conditions tested, confirming the findings of Howard et al. (1985). Proaerolysin was detected in the *E. coli* culture supernatant after a certain period of incubation; however, its appearance was accompanied by a decrease in cell mass and an increase in activity of the periplasmic marker β-lactamase in the supernatant, indicating that the presence of proaerolysin in the culture supernatant was likely the result of cell lysis rather than secretion. Although *E. coli* K-12 cells possess the complete set of genes for type II secretion (Pugsley, 1996), no laboratory conditions have thus far been found in which they are expressed (Pugsley, 1996). In other words, in the absence of functional secretion machinery, proaerolysin accumulated in the cell, eventually causing cell death.

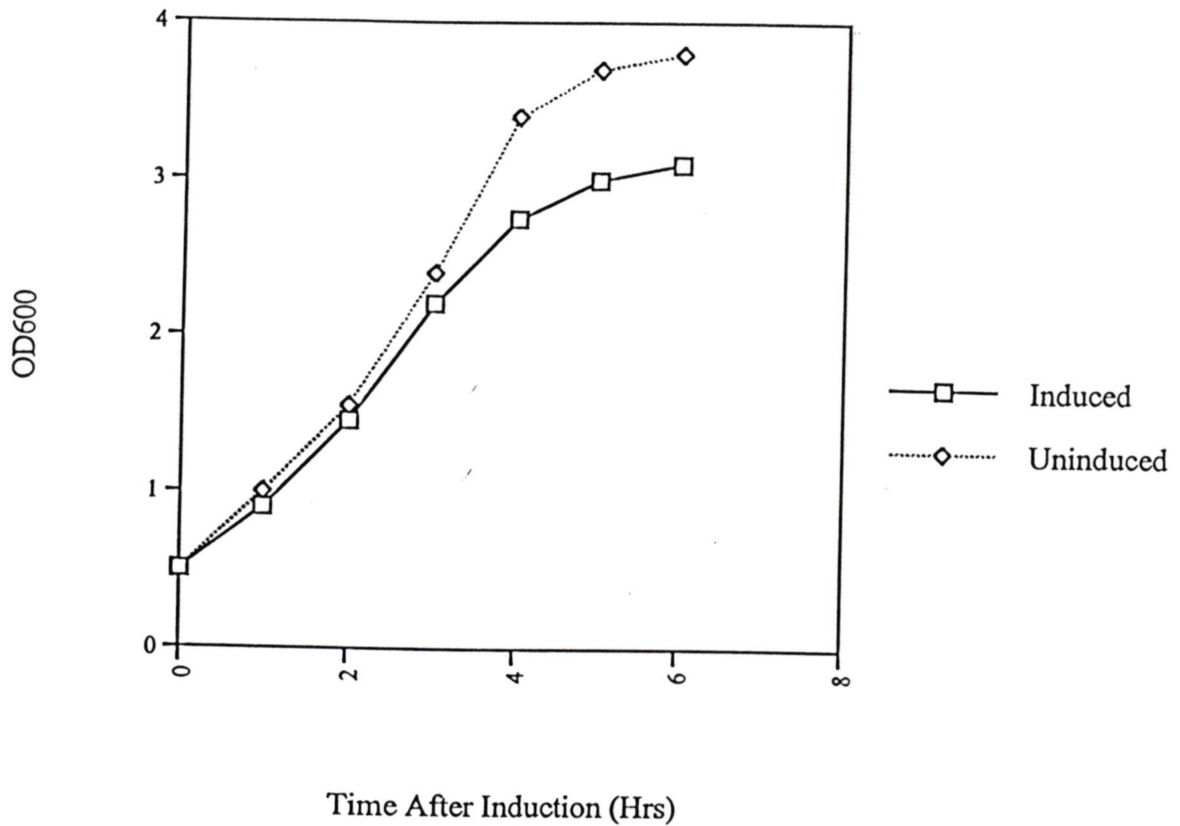


Figure 4. Growth curve of *A. salmonicida* CB3.

An overnight culture was sub-cultured 1 in 100 and induced at OD_{600}=0.5. Samples were taken periodically after induction and measured at OD_{600}. Experiments were repeated three times and the averages are shown in the figure. Variations between the results were minimal.

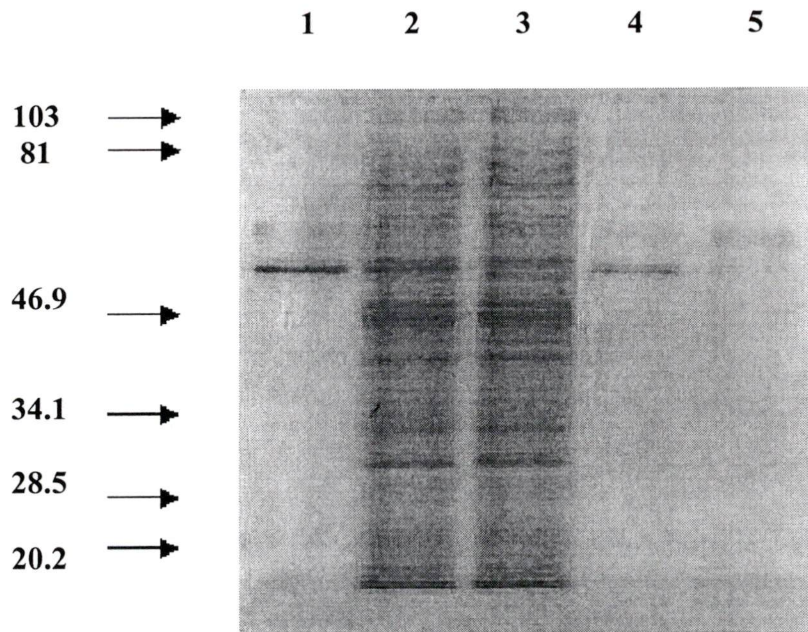


Figure 5. Release of proaerolysin by *A. salmonicida* CB3.

Samples taken 3 hours after induction were loaded on gels. Lane 1: purified proaerolysin standard; lane 2: induced cells; lane 3: uninduced cells; lane 4: induced supernatant (SN); lane 5: uninduced SN.

Location of proaerolysin inside bacterial cell

The preliminary series of experiments confirmed that *A. salmonicida* CB3 cells secrete proaerolysin; however a significant amount of the protoxin was found associated with the cells, as shown in lane 2 of Figure 5. Because the accumulation of proaerolysin in the cytoplasm could be lethal, it was of interest to determine the intracellular location of this proaerolysin. Therefore, *A. salmonicida* CB3 cells were grown under standard culture conditions and samples were removed at various times during the growth cycle. Samples were centrifuged and the culture supernatants and cell pellets were collected. The cells were then osmotically shocked and the shocked cells were pelleted. The inner membrane was then broken by repeatedly (three times) freezing and thawing the osmotically shocked cells. The broken cells were then centrifuged and the resulting supernatants and pellets (hereafter called freeze-thaw supernatants and pellets, respectively) were collected. Because the freezing and thawing cycles could release some membrane-associated proteins (d'Enfert et al, 1987), the freeze-thaw supernatants contained cytoplasmic as well as some inner-membrane-associated proteins. Proaerolysin was found localized both in the periplasm and freeze-thaw supernatants (Figure 6). Thus, although 70% of total hemolytic activity was found in the supernatant, about 30% of total hemolytic activity was associated with two intracellular fractions (shock and freeze-thaw supernatant, Figure 7).

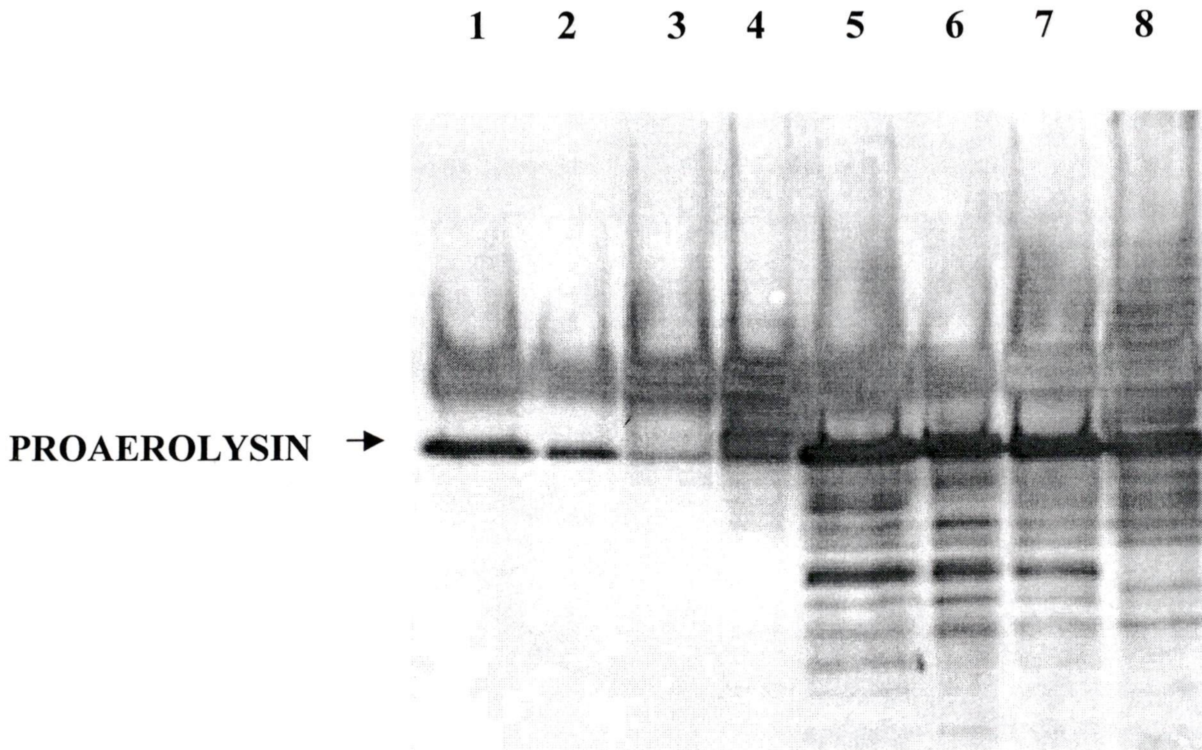


Figure 6. Location of proaerolysin in CB3.

Proaerolysin was detected by polyclonal antibody on this Western blot. Lanes 1-4: 1 hour samples; lanes 5-8: 4 hours samples; lanes 1,5: SN; lanes 2,6: shock; lanes 3,7: freeze-thaw (F-T) SN; lanes 4,8: F-T pellets The bands below the proaerolysin bands are breakdown products.

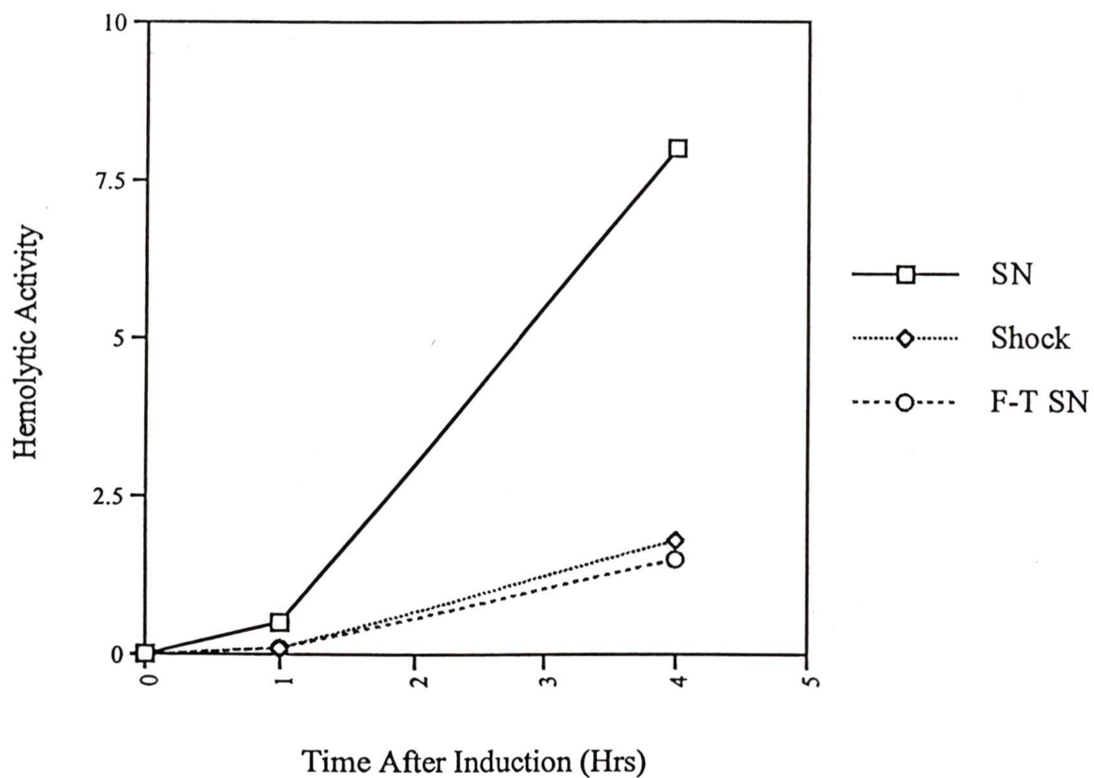


Figure 7. Hemolytic activity in different cellular compartments in CB3.

The shock fraction contained the periplasmic components and the freeze-thaw (F-T) SN contained cytoplasmic components and some membrane proteins.

The finding that some proaerolysin was detected in the freeze-thaw supernatants suggests that the accumulation of proaerolysin is associated with the inner membrane, because the inner-membrane translocation is cotranslational (Howard and Buckley, 1985). Therefore no proaerolysin exists in the cytoplasm. Another explanation is that the preparation of the periplasmic fraction was incomplete and some periplasmic proteins remained in the cytoplasmic fraction. This possibility was examined in the following experiments.

Location of β-lactamase

An experiment was designed to determine the completeness of the osmotic shocking of *A. salmonicida* CB3 cells using β-lactamase as a periplasmic marker (cf. Broome-Smith et al., 1990). Cellular fractions were prepared and it was confirmed that proaerolysin was present both in the shock fraction and in the freeze-thaw supernatant. Assay of β-lactamase indicated that >95% of the enzyme's activity was located in the shock fraction. The culture supernatant and freeze-thaw supernatant contained negligible β-lactamase activity (Figure 8). These data indicate that (1) the cells were intact because no β-lactamase activity was in the supernatant, and (2) the osmotic shocking was complete because there was no β-lactamase activity in the freeze-thaw supernatant. However, the presence of cytoplasmic β-lactamase activity could be a false negative, if β-lactamase

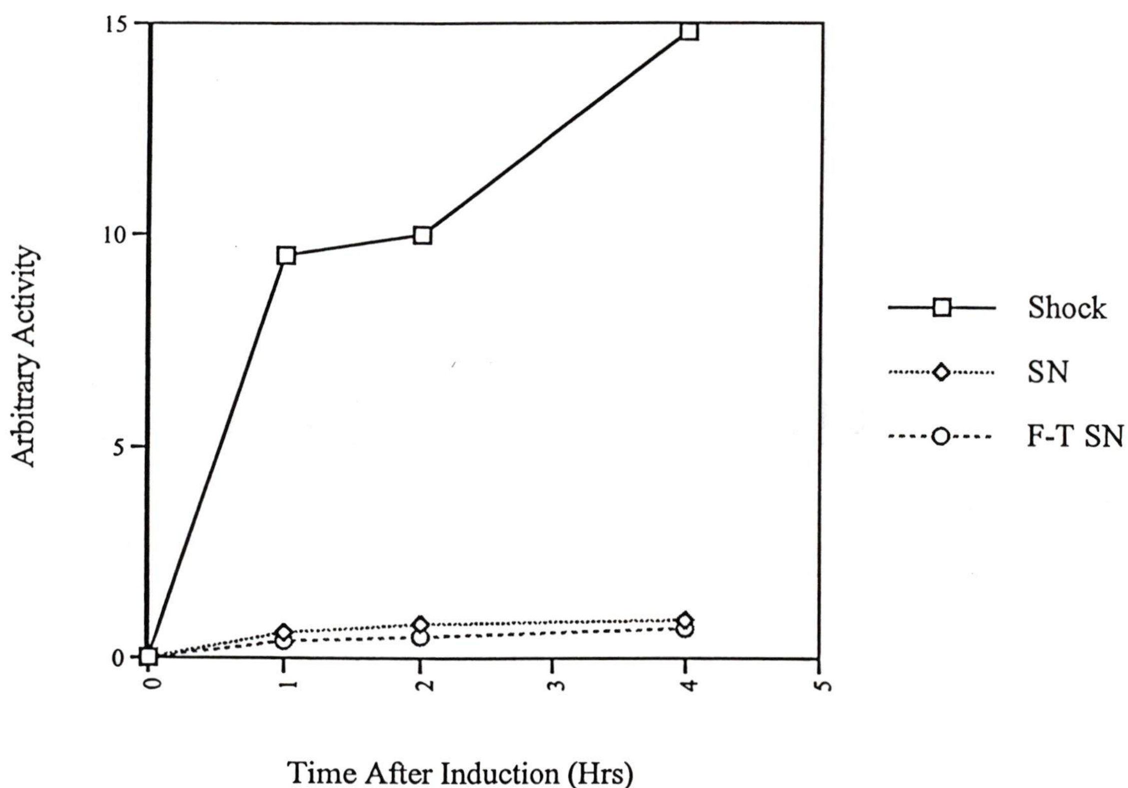


Figure 8. Location of β-lactamase in CB3.

Sample preparation was described in the text. Most of the enzyme activity could be localized in the shock fractions at all time points. The β-lactamase activity in culture SN and freeze-thaw SN remained constantly low. Experiments were repeated five times and similar results were obtained.

was degraded during the harsh freezing and thawing process. To test this possibility, *A. salmonicida* CB3 cells were grown under standard culture conditions. One set of samples was subjected to osmotic shock followed by freezing and thawing, whereas another set of samples was not osmotically shocked but was subjected directly to freezing and thawing. Direct freezing and thawing of the cells was expected to break the inner and outer membranes simultaneously, and so the resulting fraction (direct-freeze-thaw fraction) should contain periplasmic, cytoplasmic and some membrane-associated components. If the freezing and thawing method did not degrade β-lactamase, the amount of β-lactamase activity in the direct-freeze-thaw fraction should equal the sum of the β-lactamase activities in the shock fraction and the freeze-thaw supernatant fraction. On the basis of this criterion, the data presented in Figure 9 indicate that β-lactamase was not degraded by the freezing and thawing treatment. This series of β-lactamase experiments thus provide evidence that the osmotic shock treatment released all of the periplasmic components. Therefore, it seems reasonable to conclude that the accumulation of proaerolysin in the two intracellular compartments was not an artifact.

Release of accumulated proaerolysin from the intracellular pool

It was of interest to determine whether the proaerolysin accumulated in the cell could be secreted later. This would imply that it temporarily accumulated in the cell when the secretion machinery was saturated. On the other hand, if the accumulated proaerolysin could not be secreted, it might indicate that they lack a secretion signal because without a functional targeting signal, secretory proteins could not interact well with the secretion

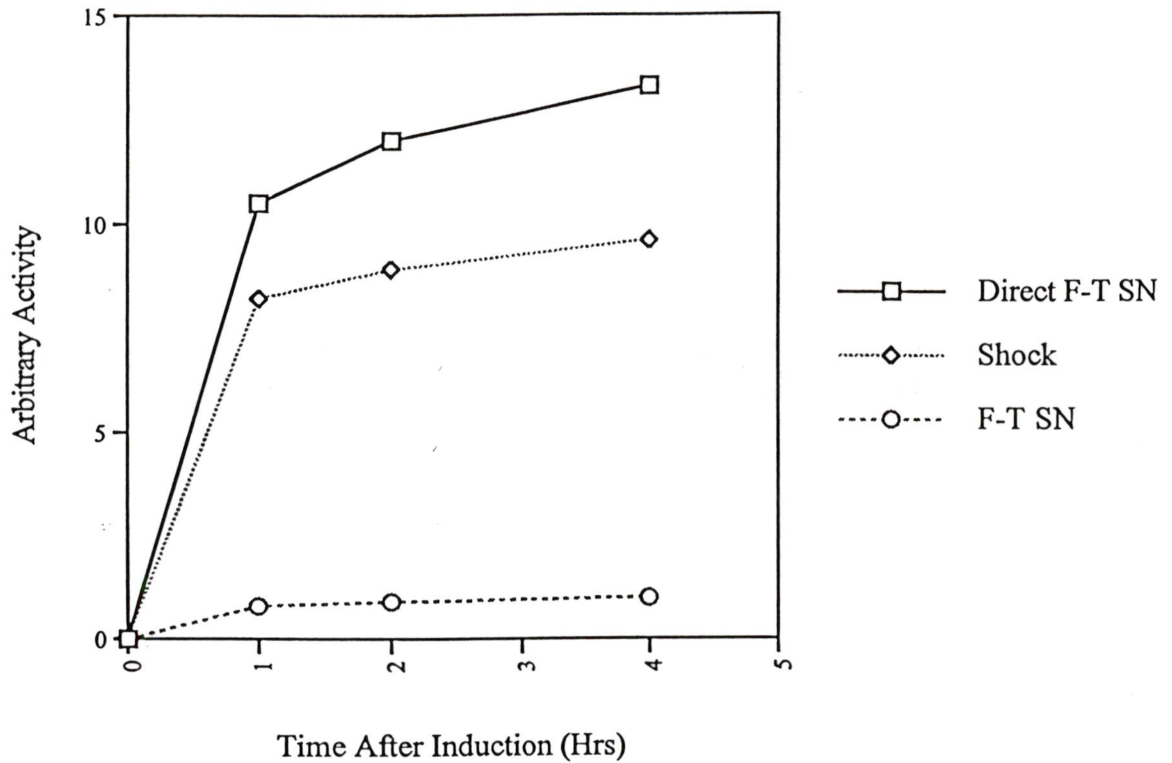


Figure 9. Effect of freeze-thaw method on β-lactamase.

Enzyme assays were carried out under similar conditions to those in Figure 8, except a direct-freeze-thaw sample was collected at each time point. The direct-freeze-thaw supernatant has a β-lactamase activity approximately equal to the sum of that in shock and freeze-thaw supernatant fractions.

apparatus and no translocation could occur. *Aeromonas salmonicida* CB3 cells were grown under standard culture conditions to the early stationary phase at which time a sample was taken and subcellular fractions prepared. Another sample was taken and centrifuged to remove the supernatant. The cell pellet was resuspended in fresh medium containing 100 μg/ml chloroamphenicol to stop protein synthesis. All of the fractions were then assayed for hemolytic activity over a one-hour period. Hemolytic activity in the two intracellular fractions decreased with time, whereas there was an increase in activity in the culture supernatant, indicating that the accumulated proaerolysin could be secreted (Figure 10, 11). The results also imply that the secretion process has an upper limit that is reached when either the machinery or some essential chaperone(s) is completely occupied, such that the system cannot increase its capacity any more.

Comparison of expression of *aerA* in new construct γ123 and construct pKW5

One way of testing whether the secretion system has an upper limit is to enhance the expression of the *aerA* gene. To this end, a study was undertaken to compare the expression of *aerA* in a new construct, γ123, with that in construct pKW5. The γ123 construct was prepared by Dzung Diep (Diep et al., in press). Analysis of the DNA sequence of *A. hydrophila* upstream of *aerA* (Howard and Buckley, 1985) revealed two regions containing inverted repeats (Dzung Diep, personal communication). One of these repeats, which is next to the signal sequence, resembles the DNA sequence for an mRNA intrinsic terminator. This mRNA sequence has a hairpin structure with a short G-C region in the stem and a single

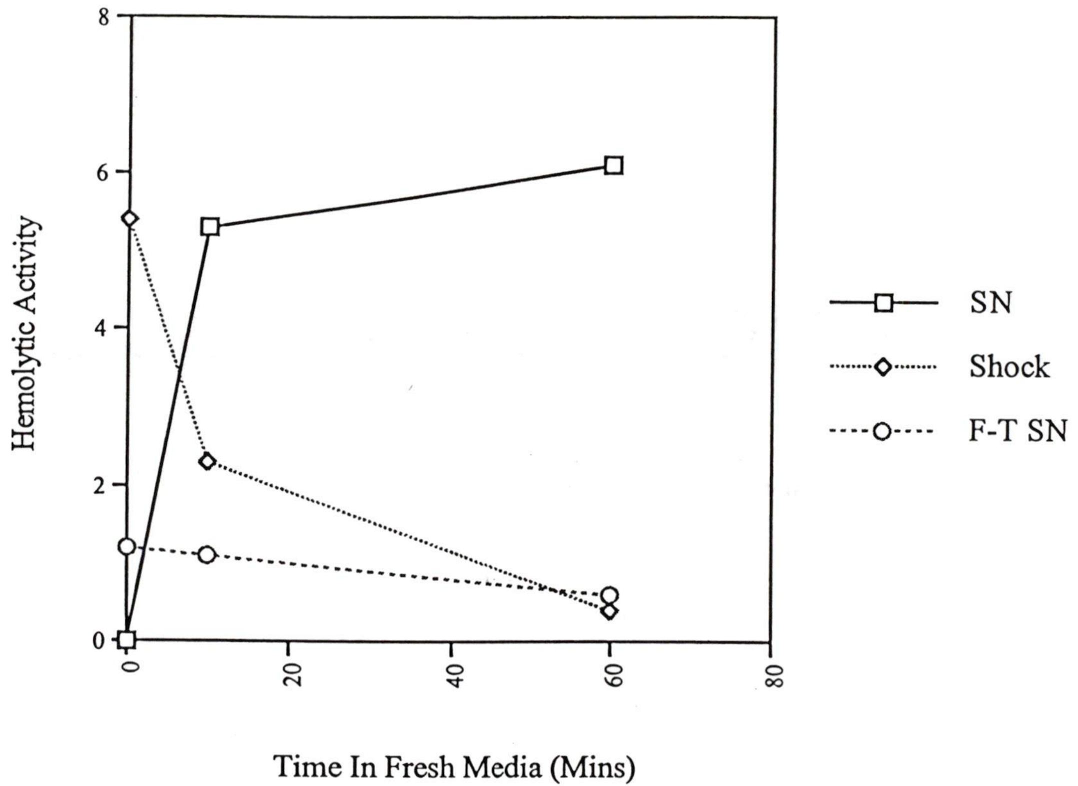


Figure 10. Release of accumulated proaerolysin.

A. salmonicida CB3 cells were grown and samples were prepared as described in the text. Hemolytic activities in different fractions were tested. Experiments were repeated three times and the averages are shown in this figure. Variations between the results were minimal.

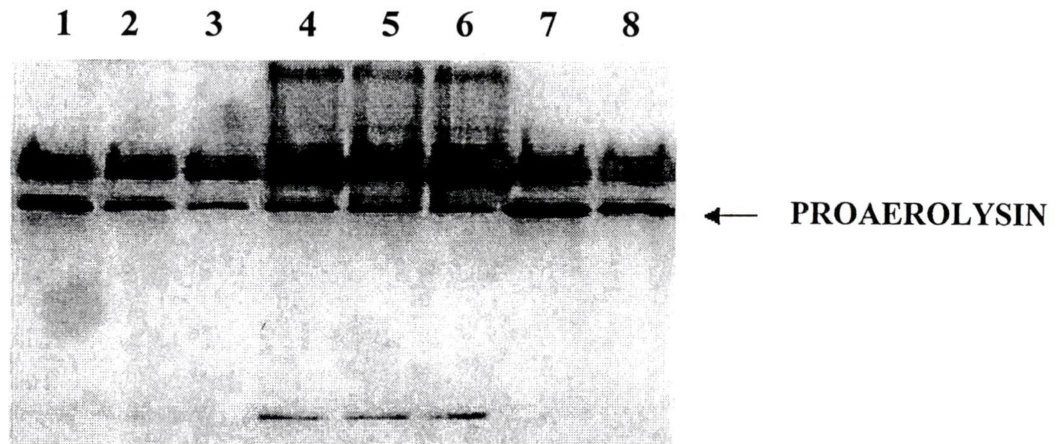


Figure 11. Western blot of the same samples collected in Figure 8.

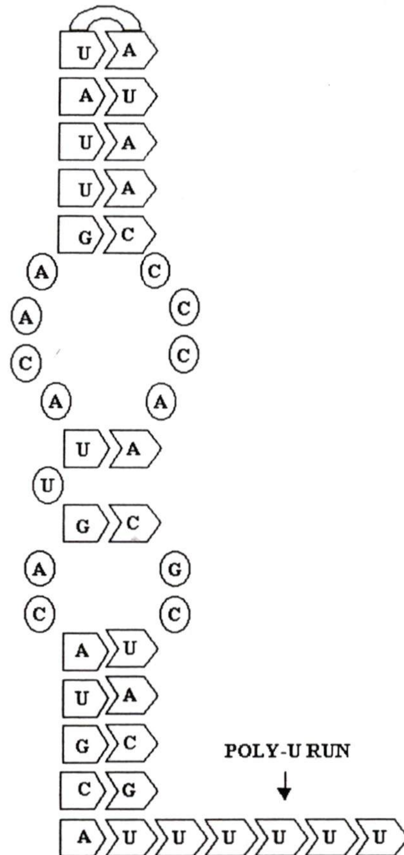
Proaerolysin was detected by polyclonal antibody. Lanes 1-3: shock samples at 0', 10', and 60', respectively; lanes 4-6: freeze-thaw SN samples at 0', 10', and 60', respectively; lanes7, 8: SN at 60' and 10', respectively. The thick bands above the proaerolysin band are backgrounds because of nonspecific binding.

stranded poly-U run (Figure 12). This structural feature has been shown to be a terminator for prokaryotic transcription (Levin et al., 1993). Dzung Diep removed this repeating region with the expectation that it would lead to higher transcription and expression of proaerolysin than the pKW5 construct. The new construct γ123 was then introduced into *E. coli* JM109 and *A. salmonicida* CB3 by Dzung Diep. Expression of *aerA* in the new construct was compared with expression of *aerA* in pKW5. *A. salmonicida* containing the γ123 construct produced far more proaerolysin than bacteria containing the *aerA* gene in pKW5 (Figure 13). The specific activity of the aerolysin produced was similar in both constructs, indicating that the deletion did not affect the proaerolysin gene product.

Secretion of overexpressed proaerolysin

The overexpressing γ-123 construct enabled the design of an experiment to determine the effects of production of large amounts of proaerolysin on secretion. *Aeromonas salmonicida* CB3 cells containing the γ-123 construct were grown under standard culture conditions, except that four concentrations of IPTG were tested. Samples were removed throughout the induction period and cell samples and culture supernatants were analyzed. The amount of proaerolysin accumulated inside the cells increased with induction time, whereas the amount of proaerolysin secreted into the supernatant did not increase significantly except for the first two hours of induction (Data not shown). More precise quantification of proaerolysin production and secretion was achieved by analyzing hemolytic activity (Table 2). After 4 hours of

(A).



(B). 5'------TGCATGTCAATGTTCAATATATTGGGGTTGCTATGCAAAAA-----GCA (actual proaerolysin gene) -------3'

Figure 12. Assumed mRNA structure and corresponding DNA sequence of one of the upstream repeats in proaerolysin gene. (A) mRNA structure; (B) DNA sequence

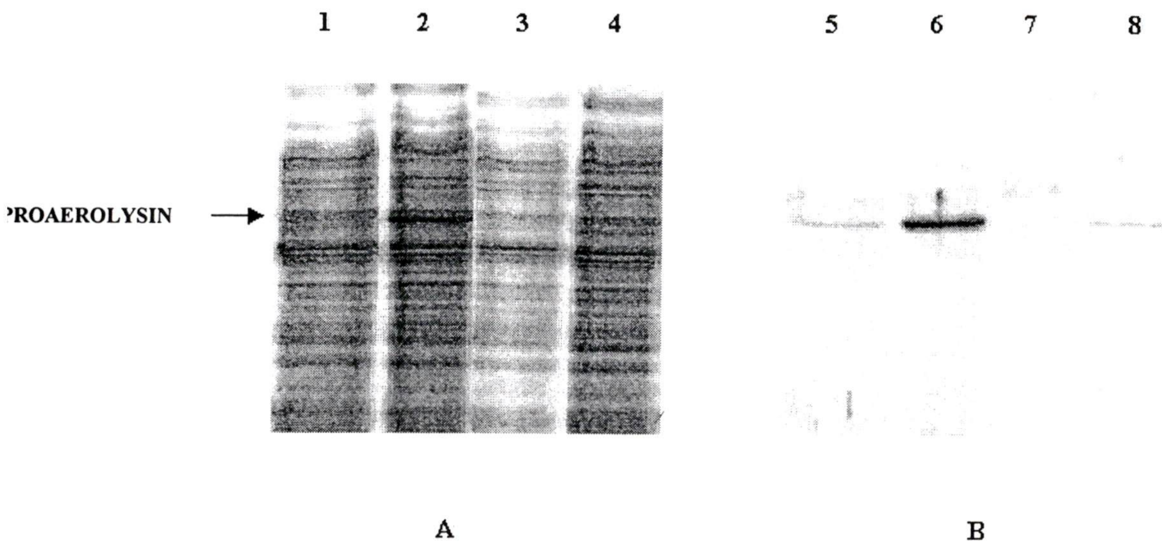


Figure 13. Comparison of the expression of the two *aerA* constructs.

A. salmonicida CB3 was grown under the standard conditions and induced with 0.05 mM IPTG. Samples were collected after 1 hr and 4 hrs induction. (A): Cells; (B): SN. Lanes 1,5: 1 hr NWT (with γ123); lanes 2,6: 4 hrs NWT; lanes 3,7: 1hr OWT (with pKW5); lanes 4,8: 4 hrs OWT.

(A).

TIME	IPTG 0.02 mM	0.05 mM	0.1 mM	0.2 mM
1 hour	5	6	6.75	6.75
2 hours	7	8.25	8.5	8.5
3 hours	8	9.25	9.25	9.25
4 hours	9	10.25	10.25	10.25

(B).

TIME	IPTG 0.02 mM	0.05 mM	0.1 mM	0.2 mM
1 hour	0.75	3	5	5.5
2 hours	2	4.75	7	7.25
3 hours	4	7	7.25	8.25
4 hours	5	8	8.25	9

Table 2.Titre of new wild-type (γ123) aerolysin with different concentrations of IPTG induction. (A) SN; (B) Shock.

50 μl SN or shock samples were used in the titre of 0.4% human blood. Wells in which the blood was completely lysed were counted as the hemolytic activity. Experiments were done in duplicates and the averages are shown in the table.

induction, the secretion system seemed to be saturated because the accumulation of proaerolysin in the periplasm increased with increasing concentration of IPTG, whereas the amount of proaerolysin secreted in the supernatant was independent of IPTG concentration. This result is compatible with the suggestion that the efficiency of the secretion machinery and the availability of putative chaperones set the limit on secretion. Because extracellular proteins have to be correctly folded in the periplasm before they can be secreted (Pugsley, 1992; van der Goot et al., 1993), the folding process could suppress the secretion rate if it is the rate-limiting step in the sequence.

Calculation of maximal secretion rate

Because the secretion process appeared to saturate when a certain amount of proaerolysin accumulated inside the cell, it was possible to calculate the maximum secretion rate. The number of cells was calculated based on the optical density of the culture. Previous work in the laboratory established that, under normal growth condition, there are 1×10^9 CB3 cells per milliliter of culture when the OD_{600} reaches 1.6. The amount of aerolysin was calculated based on its hemolytic activity. It has been established in this laboratory that, under standard titre conditions, 10 μg of fully activated aerolysin will have a ten-well hemolysis after 1 hour. Based on these data, a standard table was constructed relating titres to amount of aerolysin (Table 3).

The maximum secretion rate was calculated as:

$$V = \frac{\Delta P}{C \times T}$$

where ΔP denotes the increase in the amount of aerolysin in the supernatant, C is the mean cell number during a certain period, and T is the growth time. Thus, calculation of

Partially lysed wells	Lysed wells					
	0~5	6	7	8	9	10
0	...	0.625	1.25	2.5	5	10
1/4	...	0.743	1.486	2.973	5.945	11.89
1/2	...	0.884	1.768	3.535	7.07	14.14
3/4	...	1.051	2.103	4.205	8.41	16.82

Table 3. Standard table for quantifying aerolysin using titre.

The titre was performed under standard conditions as described in Materials and Methods. Aerolysin amount is indicated as μg.

the secretion rate of CB3 cells induced with 0.05 mM IPTG between 3 and 4 hours after induction involves the following steps. In this example, the amount of aerolysin increased from 5.945 μg (titre 9.25) to 11.89 μg (titre 10.25, see data in Table 2). The mean cell number is $(2.07+2.78)/2 \times 10^{9}/1.6 = 1.52 \times 10^{9}$ cells / ml and the time period is 1 hour. According to the formula, the secretion rate is:

$$\frac{(11.89\text{-}5.945) \times 20}{1.52\times10^{9}\ \text{cell/ml} \times 1\ \text{hr}} = 78.22 \times 10^{-9}\ \mu\text{g/cell hr}$$

Because the mass of one aerolysin molecule can be calculated to be $47{,}500/6.023\times10^{23} = 7.886 \times 10^{-14}$ μg, the secretion rate can be expressed as $78.22\times10^{9} / 7.886\times10^{-14} = 9.92 \times 10^{5}$ mol/cell.hr or 1.65×10^{4} molecules/ cell. min. The same method was used to calculate the secretion rates obtained with different IPTG concentrations and induction periods (Table 4). The data indicated that the maximum secretion rate was about 1.65×10^{4} molecules per cell per minute, which was obtained at an IPTG concentration of 0.05 mM with an induction period of 1 hour. The maximum secretion rate of 1.65×10^{4} molecules/ cell. min., compared with previous findings that secretion of β-lactamase by the Gram-positive bacteria *Lactobacillus* was found to be 8,000 molecules per cell per minute (Savijoki et al., 1997), confirms that the GSP is a very efficient secretion system.

Effect of proaerolysin secretion on protease secretion

One way to demonstrate that there is an upper limit for the GSP-MTB is to estimate the maximum secretion rate of proaerolysin. Another way is to determine whether the

Induction period	IPTG			
	0.02 mM	0.05 mM	0.1 mM	0.2 mM
0-1hr	0.30	0.60	0.88	0.88
1-2 hr	0.44	0.89	0.89	0.89
2-3 hr	0.50	0.98	0.97	0.95
3-4 hr	0.66	1.65	1.62	1.62

Table 4. Average secretion rate of new wild-type (γ123) proaerolysin in CB3 with different concentrations of IPTG induction and in different time periods.

The unit of the secretion rate is $\times 10^4$ molecules/cell.min. Experiments were repeated three times and similar results were obtained.

secretion of one protein will affect the secretion of other proteins. It is known that *A. salmonicida* can secrete several enzymes including proaerolysin, a metalloprotease, an α-amylase and a lipase GCAT (Pemberton et al., 1997). To determine whether the secretion of proaerolysin affects the secretion of another of these proteins, the metalloprotease was selected for study. Because *A. salmonicida* CB3 is a protease-deficient mutant strain, it was not suitable for this study. Therefore, the plasmid γ-123 was purified from *A. salmonicida* CB3 and introduced into *A. salmonicida* strain Rif-1. No significant difference was seen in the growth and secretion of proaerolysin when Rif-1 and CB3 were compared (data not shown). To determine the effect of proaerolysin secretion on protease secretion, *A. salmonicida* Rif-1 was grown without and in the presence of several concentrations of IPTG and samples were taken at various times during the induction period. Analysis of the samples indicated that as the production of proaerolysin increased to a maximum, the amount of protease in the culture supernatants decreased. The data presented in Figure 14, 15, 16 show that the uninduced Rif-1 culture had much higher protease activity in the culture supernatant than the Rif-1 culture induced with 0.06 mM IPTG. Among the cultures, the Rif-1 culture induced with 0.06 mM IPTG had the maximum proaerolysin secretion rate: induction with higher concentrations of IPTG (up to 0.2 mM) did not further increase the amount of proaerolysin in the supernatant. Therefore, the reduced secretion of protease could be attributed to saturation of the secretion system when maximal proaerolysin secretion was achieved. These data, combined with the estimation of the maximum secretion rate, confirmed that there was an upper limit for this secretion system.

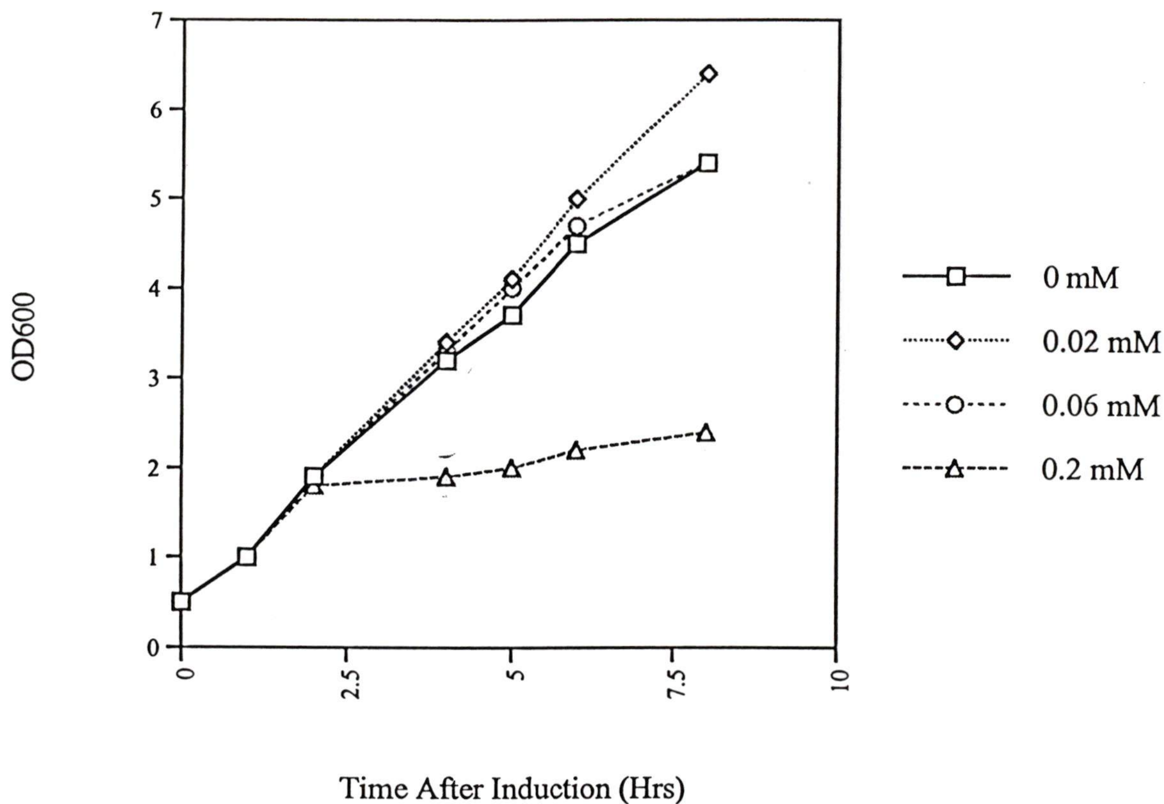


Figure 14. Growth of Rif-1 induced with different concentrations of IPTG in LB without glucose.

Experiments were performed three times and the averages are shown in this figure. Variations between the results were minimal.

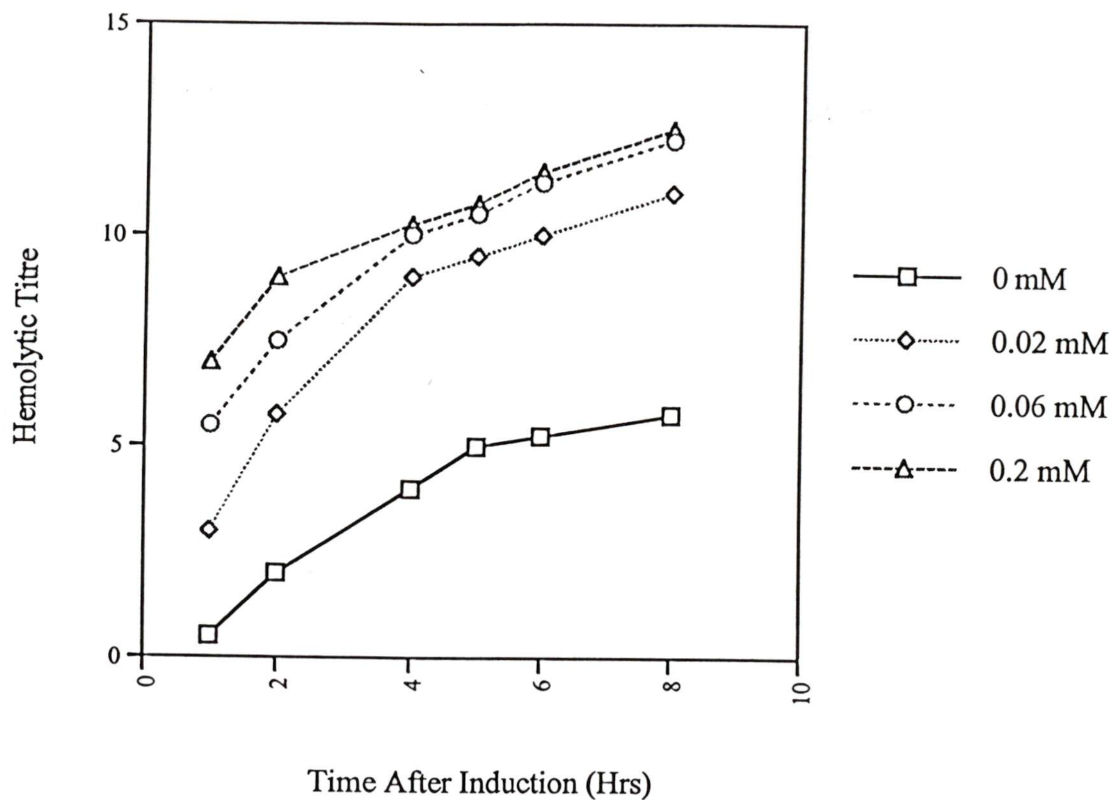


Figure 15. Hemolytic titer of Rif-1 culture supernatants with different concentrations of IPTG induction.

Experiments were performed three times and the averages are shown in this figure. Variations between the results were minimal.

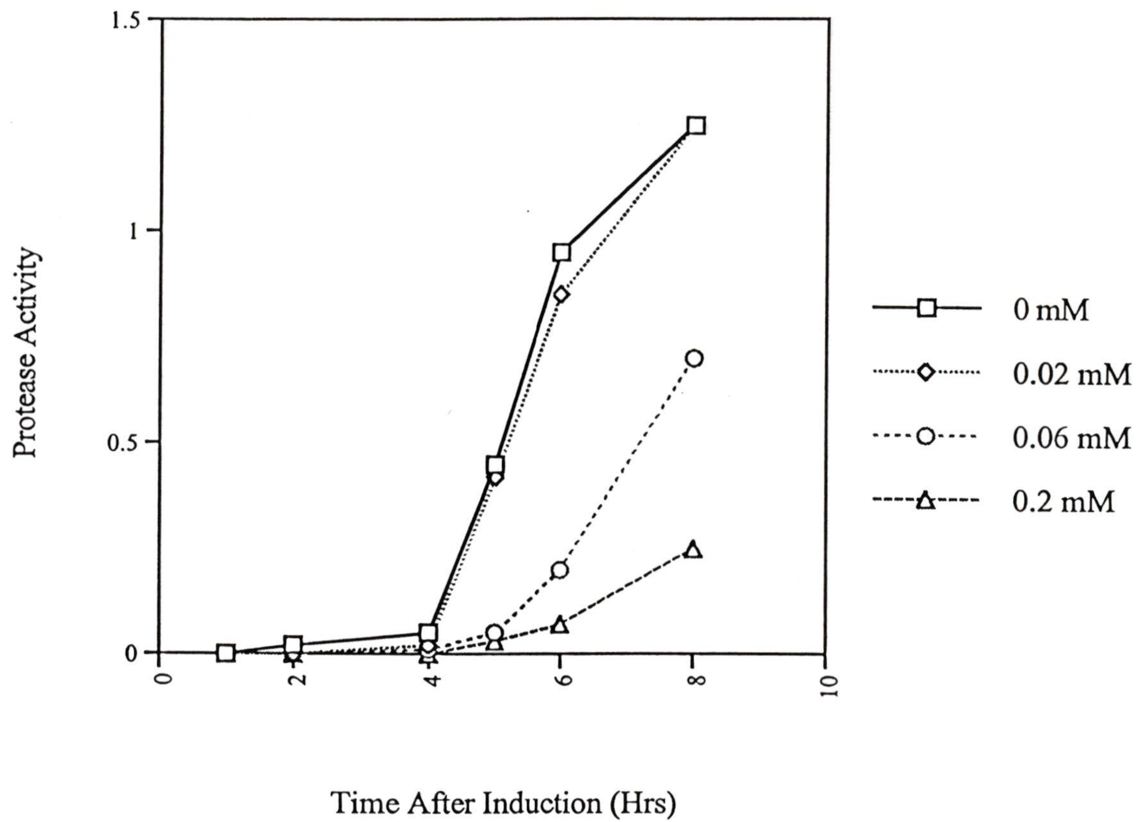


Figure 16. Protease activity of Rif-1 induced with different concentrations of IPTG.

Experiments were performed three times and the averages are shown in this figure. Variations between the results were minimal.

Secretion of non-hemolytic mutant proaerolysin H132N

It was possible that the intracellular accumulation of proaerolysin was a result of a toxic effect of the protoxin on the GSP. If this is true, a mutant proaerolysin lacking hemolytic activity should be secreted completely out of the cell. To test this possibility, the mutant H132N was chosen. Previous work in this laboratory has established that this mutant protein oligomerizes poorly and as a result it has greatly reduced hemolytic activity. The plasmid containing the H132N gene was purified by Dzung Diep and the upstream DNA sequence was removed to enhance productivity. The new H132N gene was then introduced into *A. salmonicida* CB3. Growth and proaerolysin secretion were compared at two IPTG concentrations (0.02mM and 1mM, Figure 17, 18). It was found that the bacteria with the mutant proaerolysin grew better than the bacteria with the wild-type proaerolysin; however, when the two proaerolysin secretions were compared, no difference in the amount of proaerolysin secreted was observed. This result, therefore, supported the assumption of secretion competition by excluding the possibility that accumulated toxicity impeded the secretion process. However, the study did not rule out the possibility that the mutant might be toxic to prokaryotic cells, even though it is not toxic to eukaryotic cells. For example, the non-hemolytic mutant Y221G showed greatly reduced activity to eukaryotic cells but caused death of bacterial cells (data not shown).

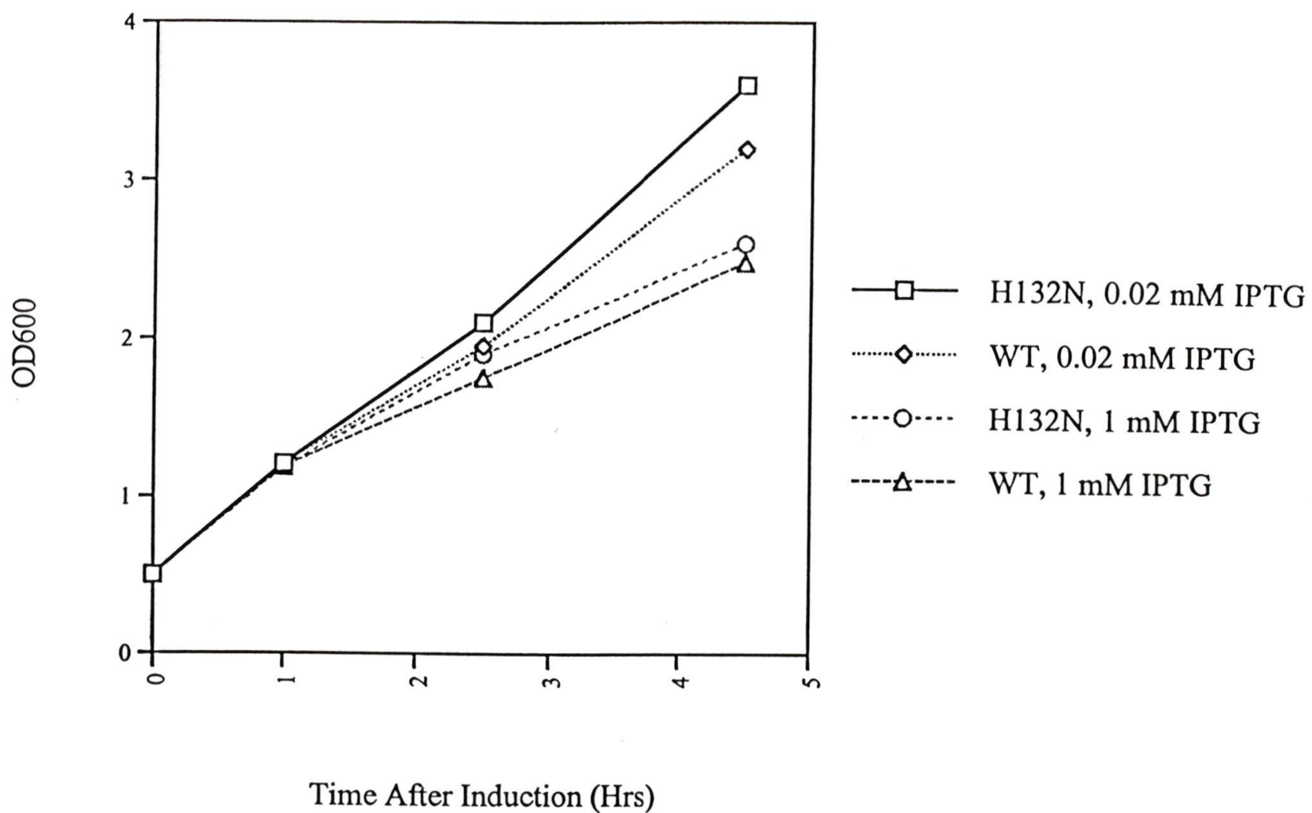


Figure 17. Growth of *A. salmonicida* CB3 expressing H132N or wild-type proaerolysin.

Cells were induced with 0.02 mM or 1 mM IPTG.

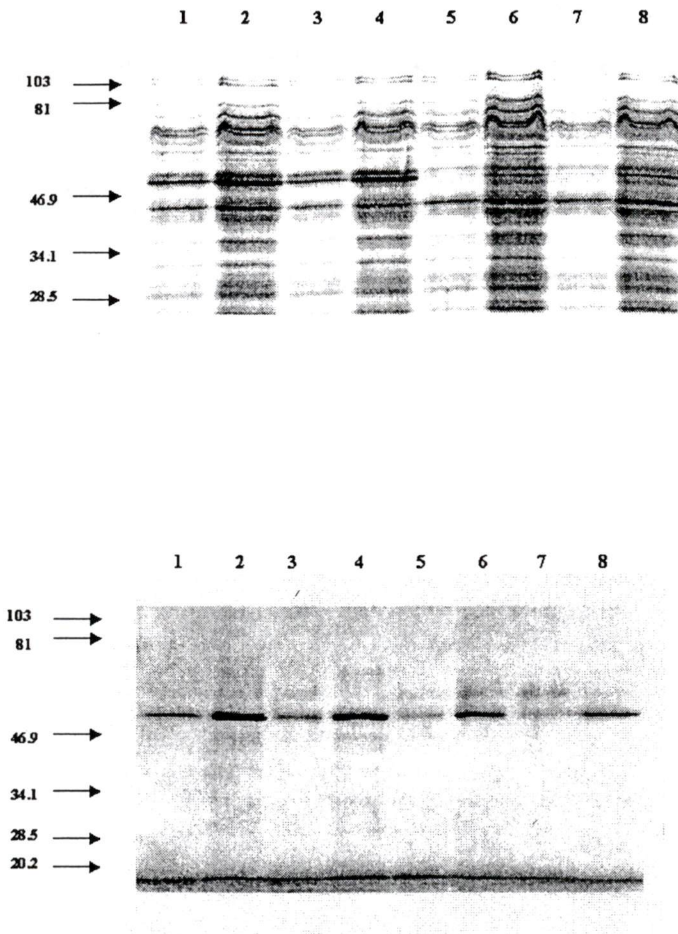


Figure 18. Comparison of wild-type and H132N proaerolysin secretion by CB3.

Cells (A) and supernatants (B) were compared. Lanes 1-4: 1 mM IPTG induced samples; lanes 5-8: 0.02 mM IPTG induced samples; lanes 1,5: 1 hr sample of H312N; lanes 2,6: 4 hrs sample of H132N; lanes 3,7: 1 hr sample of wild-type; lanes 4,8: 4 hrs sample of wild-type.

Secretion of domain II-IV of proaerolysin

Proaerolysin has a typical N-terminal signal sequence for translocation across the inner membrane via the Sec system (Howard and Buckley, 1985), however, the signal for targeting to the outside of the cell is unknown. Although various regions in other proteins have been found that are critical for secretion by way of the GSP-MTB (Sauvonnet and Pugsley, 1996; Lu and Lory, 1996), no such amino acid sequence has been found in proaerolysin. Various mutation analyses in this laboratory have provided evidence that such an amino acid sequence might not exist (Wong et al., 1991). Because secretory proteins must acquire their tertiary structure before they cross the outer membrane (Pugsley, 1992; Hardie et al., 1995), an alternative possibility is that the signal for the GSP-MTB is a secretion-competent tertiary structure of the protein. As shown in Figure 2, proaerolysin is composed of two parts: domain II-IV which is the main body and domain I which is linked to the main body by an arm structure. It is conceivable that if a tertiary structural signal exists, it is located in domain II-IV because domain II-IV represents more of the whole protein than domain I. To test this possibility, the gene sequence corresponding to domain I and the arm was removed from the *aerA* gene by Dzung Diep (Diep et al., in press). The truncated gene product was then introduced into *A. salmonicida* CB3 and proaerolysin secretion examined. After a four-hour induction, samples were removed and cell samples and culture supernatants prepared and subjected to SDS-PAGE. The gels were transferred to membranes by immunoblotting and the blots were developed by the ECL method. As shown in Figure 19, although most protein

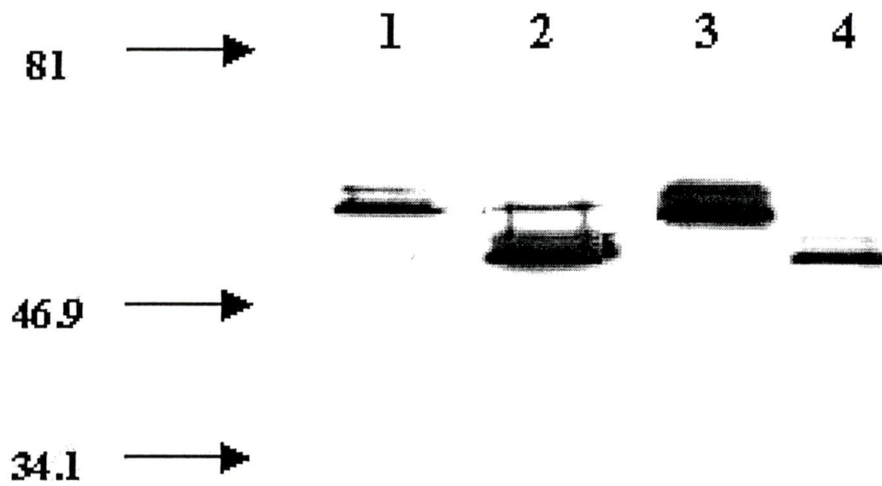


Figure 19. Secretion of domain II-IV of proaerolysin.

Experiments are described in the text. Proaerolysin and domain II-IV were detected by polyclonal antibody. Lane 1: WT cell; lane 2: domain II-IV cell; lane 3: WT SN; lane 4: domain II-IV SN.

remained inside the cell, some domain II-IV was detected in the culture supernatant. To rule out the possibility of nonspecific leakage, β-lactamase was assayed in the cell and supernatant fractions. Because more than 90% of the β-lactamase activity was found in the cell (data not shown), it was concluded that the membrane was intact and that domain II-IV could be secreted by itself. However, the amount of domain II-IV secreted was minor compared to the amount of wild-type proaerolysin (Figure 19).

Proteolytic degradation of domain II-IV of proaerolysin

Because proaerolysin must be in its tertiary configuration before it crosses the outer membrane, the accumulation of large amounts of domain II-IV inside the cell could be the result of an incorrect conformation. Correctly folded proaerolysin is processed at the C-terminus by proteases and is thus converted to aerolysin, which is resistant to further protease breakdown. If domain II-IV is correctly folded, the exposed proteolytic sites on domain II-IV of proaerolysin should be the same as in wild-type proaerolysin and so the trypsin-catalyzed degradation patterns should be similar. Therefore, to investigate the conformation of domain II-IV of proaerolysin, a series of trypsin assays were performed. *A. salmonicida* CB3 cells producing only domain II-IV of proaerolysin and cells producing wild-type proaerolysin were grown under standard culture conditions and samples were taken after 4 hours of induction and the cells fractionated by means of the osmotic shock and freeze-thaw methods. Trypsin was added to one sample of each cellular fraction to a final concentration of 0.4 μg/ml. The proteolytic reaction was allowed to proceed for 10 minutes at room temperature and then 10 μl of 1mg/ml of

trypsin inhibitor was added to terminate each reaction. Samples were subjected to SDS-PAGE and blotted for ECL. The degradation patterns of domain II-IV of proaerolysin for all of the cellular fractions are shown in Figure 20. Trypsin processed domain II-IV, resulting in a band of low molecular weight on the gel. The osmotically shocked sample without trypsin also had the low molecular weight band on the SDS-PAGE gel because of the presence of protease activity in the periplasm, whereas this band was absent from the supernatant sample because no protease was secreted by the *A. salmonicida* CB3 cells. The SDS-PAGE gels of the freeze-thaw pellet did not show the low molecular weight band because trypsin was unable to hydrolyze the insoluble and membrane-associated proteins comprising this cellular fraction. Similar degradation patterns were obtained with wild-type proaerolysin (data not shown), indicating that domain II-IV of proaerolysin could be correctly folded in the periplasm.

Secretion of domain II-IV of proaerolysin by an *A. hydrophila* mutant strain

One approach to determine whether domain II-IV of proaerolysin is secreted by the GSP-MTB is to measure its release by *A. hydrophila* mutant strain L1.97. This strain has a mutation in a component of the GSP-MTB, *exeE* (Jiang and Howard, 1991) and is therefore unable to secrete any protein by way of the GSP-MTB. The plasmid containing the domain II-IV gene was transferred into *A. hydrophila* L1.97 by Dzung Diep. As a control, Dzung Diep also transferred the same plasmid into another mutant strain of *A. hydrophila*, I2.66, which is deficient in autologous proaerolysin production (Howard and

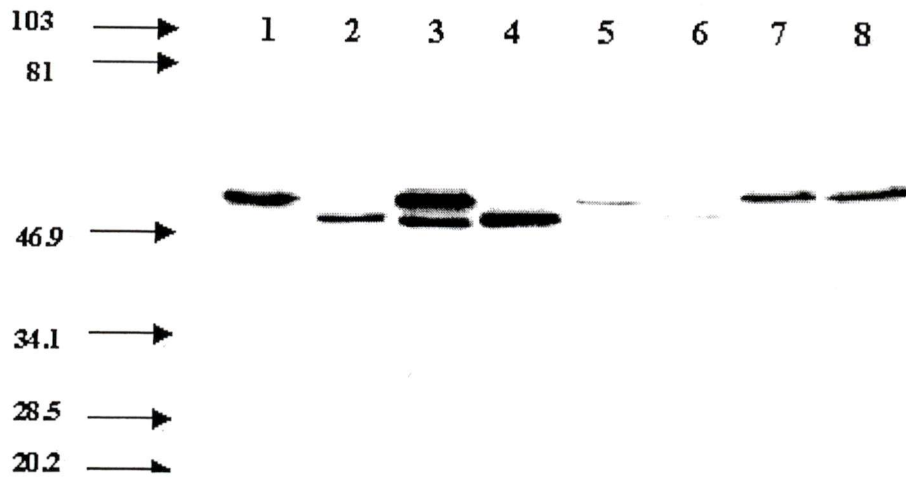


Figure 20. Trypsin degradation pattern of domain II-IV of proaerolysin.

Lanes 1,3,5,7: no trypsin; lanes 2,4,6,8: with 0.4μg/ml trypsin; lanes 1,2: supernatant; lanes 3,4: shock; lanes 5,6: freeze-thaw SN; lanes 7,8: freeze-thaw pellet.

Buckley, 1985). The rationale for choosing I2.66 as a control was to eliminate the possibility of any interference from background proaerolysin (i.e. no proaerolysin would compete with domain II-IV for the secretion apparatus). *Aeromonas hydrophila* L1.99 and I2.66 cells were grown under standard culture conditions and samples were taken during the induction period. Cell and supernatant samples were subjected to SDS PAGE and blotted for ECL. In I2.66, domain II-IV of proaerolysin appeared in the supernatant soon after induction, whereas, in L1.97, little trace of domain II-IV was detected in the supernatant even after 5 hours of induction (Figure 21). This result provided strong evidence that domain II-IV of proaerolysin is noramlly secreted by way of the GSP-MTB, although the secretion is less complete than for wild-type proaerolysin.

Estimation of secretion of domain II-IV of proaerolysin by ELISA

Secretion of wild-type proaerolysin was estimated by assaying its hemolytic activity. Unfortunately, this method could not be used for estimating the secretion of domain II-IV of proaerolysin because domain II-IV has little hemolytic activity. Instead, secretion of domain II-IV of proaerolysin was estimated by an enzyme-linked immunosorbant assay (ELISA). The ELISA is based on spectrophotometric measurement of the chromatic reaction between an additional reagent and an enzyme linked to the specific protein antibody. To exclude interference by background proaerolysin, the plasmid containing domain II-IV of proaerolysin was transferred into *A. hydrophila aerA*$^-$ mutant strain I2.66. Cells were grown under standard culture conditions and samples were taken after a four-hour induction period. Culture supernatants and cellular fractions were obtained and 100 μl of each sample was blotted on an ELISA plate. The plates were developed with

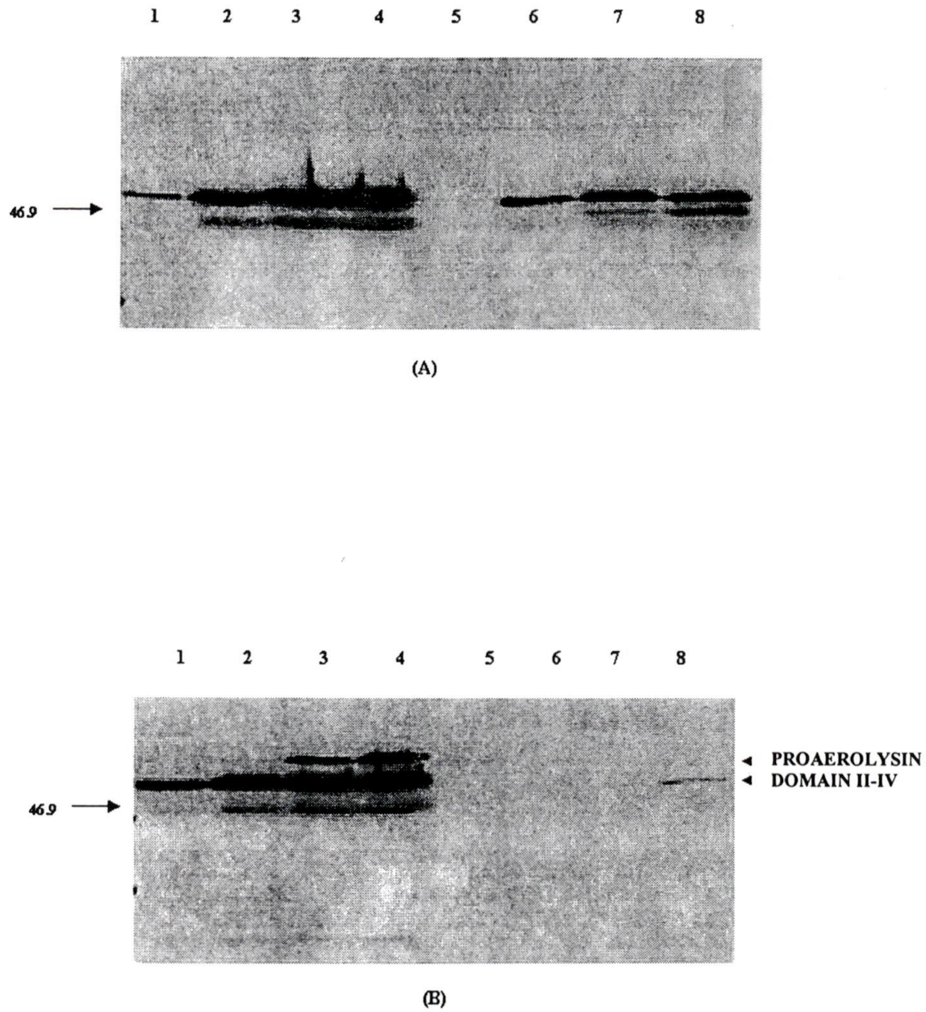


Figure 21. Secretion of domain II-IV of proaerolysin by *A. hydrophila.*

(A) I2.66 (B) L1.97. Lanes 1-4: cell; lanes 5-8: SN; lanes 1,5: 1hr after induction; lanes 2,6: 2 hrs; lanes 3,7: 3.5 hrs; lanes 4,8: 5 hrs.

alkaline phosphatase reagents and optical density at 450 nm measured. As a control, wild-type proaerolysin was also tested. All tests were duplicated. As shown in Table 5, although more than 70% of wild-type proaerolysin could be secreted, only about 25% of total domain II-IV could be secreted. This finding confirmed that domain II-IV of proaerolysin could be secreted but that the amount secreted was small compared with wild-type proaerolysin.

Secretion of co-expressed domain I and domain II-IV of proaerolysin

The finding that domain II-IV of proaerolysin could be secreted by the GSP-MTB prompted a study of the role of domain I in this process. The relatively poor secretion of domain II-IV compared to that of wild-type proaerolysin suggested that domain I might facilitate secretion, but that it was not an essential component. For example, domain I might be required for better folding of the protein in the periplasm to present a more competent secretion signal. Alternatively, domain I might be required to interact with some components of the *Exe* machinery to increase the efficiency of secretion. To investigate the role of domain I, the gene for domain I and the arm was constructed by Dzung Diep in the same plasmid containing the domain II-IV gene. The two reading frames were separated by termination signals. The plasmid was introduced into *A. salmonicida* CB3. The production and secretion of co-expressed domain I and domain II-IV of proaerolysin were tested under standard culture conditions. When domain II-IV was co-expressed with domain I, domain II-IV had a much higher production than when expressed by itself, and even higher than wild-type proaerolysin without removal of the

	SN	Shock	Freeze-thaw SN	Percent of Secretion
WT	1.380	0.232	0.212	75.6
D24	0.282	0.590	0.276	24.7

Table 5. Estimation of the secretion of domain II-IV of proaerolysin.

Readings at 450nm are listed and average rates were calculated.

upstream repeat. The production of such large amounts of proaerolysin resulted in cell death (Figure 22). To avoid this and permit estimation of proaerolysin secretion by the co-expressed domain I and domain II-IV, the experiment was repeated with a lower (0.05mM) IPTG concentration. As shown in Figure 23, the secretion of proaerolysin by domain II-IV was not significantly increased when it was co-expressed with domain I. Domain I could not be detected in the culture supernatant, probably because it was quickly broken down. These data indicate that domain I did not facilitate the secretion of domain II-IV, even though it increased the production of domain II-IV. However, in an experiment with a system in which the arm of domain I was linked with domain II-IV, co-expression of domain I and domain II-IV enhanced secretion of both domain I and domain II-IV (Diep et al., in press).

Release of *Clostridium septicum* α-toxin by *A. salmonicida*

The Gram-positive bacterium *Clostridium septicum* produces and secretes a toxin called α-toxin that is homologous to aerolysin, with 27% identity and 72% similarity (Ballard et all., 1995). The sequence of α-toxin corresponds to the C-terminal 350 amino acids of proaerolysin, which represent domain II-IV of proaerolysin (Ballard et al., 1995). Based on the finding that domain II-IV of proaerolysin can be secreted by itself, an experiment was conducted to determine whether the *C. septicum* α-toxin could be expressed and secreted in *A. salmonicida*. The plasmid containing the α-toxin gene was purified and introduced into *A. salmonicida* CB3 by Dzung Diep. The bacteria expressing the

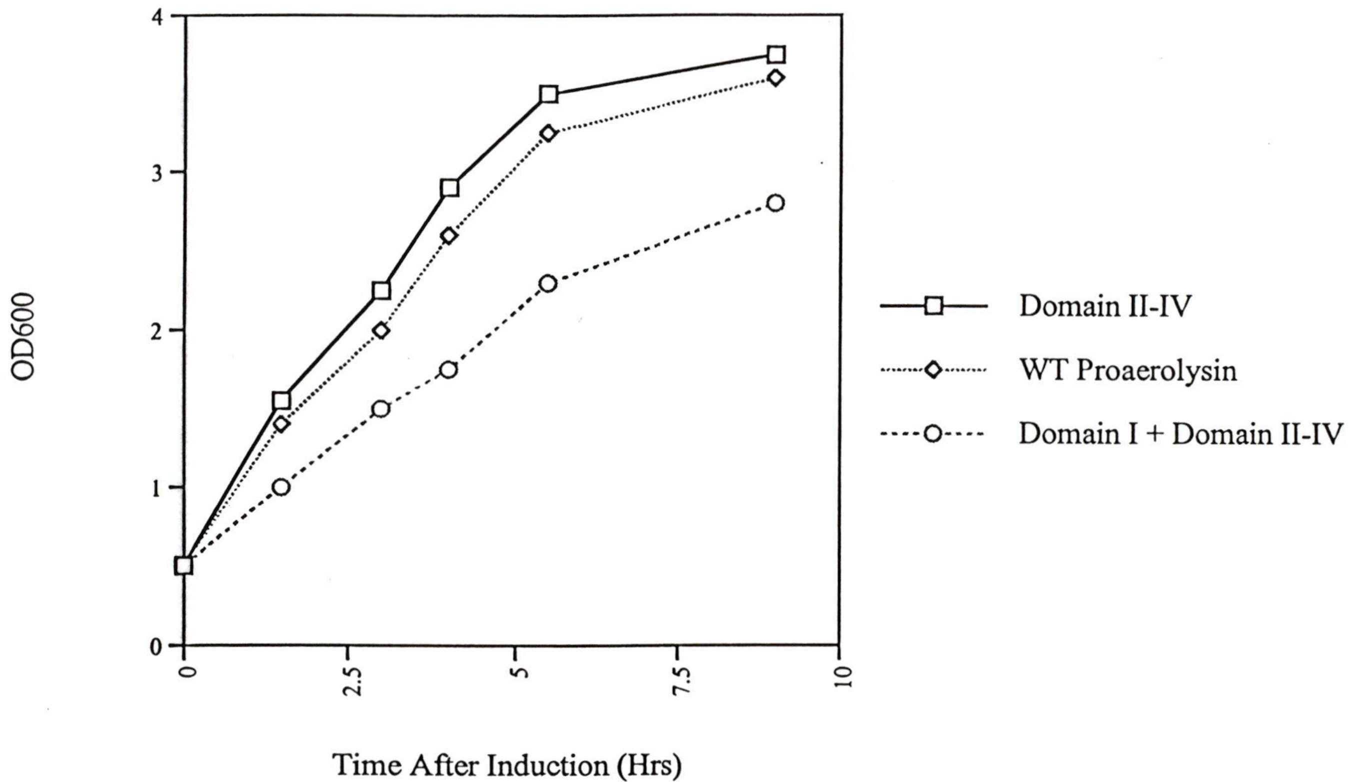


Figure 22. Growth of *A. salmonicida* CB3 with co-expressed domain I and domain II-IV of proaerolysin in comparison to growth with wild-type or domain II-IV of proaerolysin.

The cells were induced with 1 mM IPTG. Experiments were repeated three times and the averages are shown in the figure.

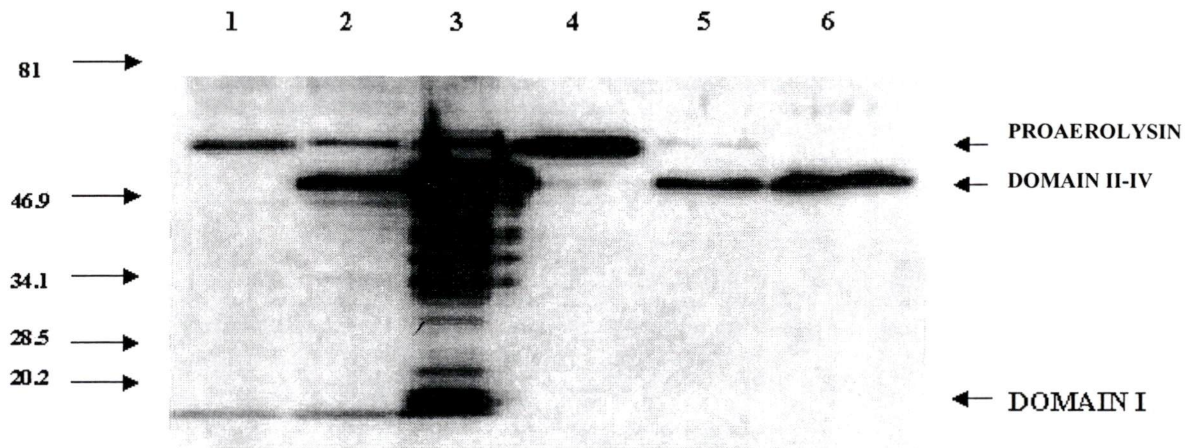


Figure 23. Secretion of co-expressed domain II-IV and domain I of proaerolysin in comparison to secretion of wild-type proaerolysin or domain II-IV .

CB3 cells were induced with 0.05 mM IPTG. Samples were taken after 4 hours of induction. Lanes 1-3: cell; lanes 4-6: SN; lanes 1,4: WT; lanes 2,5: domain II-IV; lanes 3,6: co-expressed domain II-IV and domain I. The several bands below the domain II-IV band in lane 3 are breakdown products.

heterologous toxin did not grow well under the standard culture conditions, even though production of α-toxin was lower than that of proaerolysin. When the secretion of α-toxin by CB3 was investigated, only traces of α-toxin could be detected in the culture supernatant after 4 hours of induction (Figure 24). Some toxin could be detected using anti-α-toxin polyclonal antibody after 10 hours of induction; however, the accompanying decrease in cell mass and increase in β-lactamase activity in the supernatant (data not shown) suggested that when a certain amount of α-toxin accumulated in the cells it leaked out and resulted in cell lysis. These results demonstrated that although α-toxin may have a similar structure to proaerolysin, it could not be secreted from *A. salmonicida* cells by way of the GSP-MTB.

Effect of arsenate on proaerolysin secretion

One component of the GSP-MTB machinery and several homologues have been found to have ATP-binding sites. Such components include PulE in *K. oxytoca* (Possot and Pugsley, 1994); XcpR in *P. aeruginosa* (Turner et al., 1993), and EpsE in *V. cholerae* (Sandkvist et al., 1995). In *A. salmonicida*, a novel inner membrane protein ExeA was recently found to have an ATP-binding site, suggesting its interaction with ATP (Howard et al., 1995). To confirm the use of ATP as an energy source for the GSP-MTB, the effect of arsenate on proaerolysin secretion was investigated. Arsenate can prevent cells using ATP as an energy source by non-specifically depleting the nucleotide triphosphate pool in the cell (Johnson and Taylor, 1993). *A. salmonicida* CB3 containing plasmid γ123 was grown under standard culture conditions and induced with 0.02 mM IPTG. After 4 hours

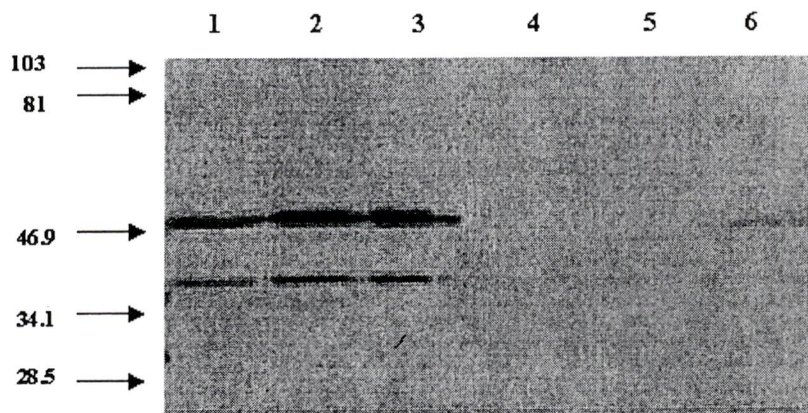


Figure 24. Expression and release of *C. septicum* α-toxin in *A. salmonicida*.

CB3 cells were grown as described previously, samples were taken at different time points and run on SDS-PAGE gel. The gel was later blotted for ECL. Lanes 1-3: cell; lanes 4-6: SN; lanes 1,4: 1 hr after induction; lanes 2,5: 2 hrs after induction; lanes 3,6: 4 hrs after induction.

of induction, samples were collected and centrifuged. The cell pellets were then resuspended in fresh medium with or without 10 mM arsenate. Samples were taken immediately after resuspension and at different times thereafter. The amount of proaerolysin in the supernatant was determined by hemolytic titre using the V-shaped titre plates as described in Materials and Methods. The cells incubated in the absence of arsenate secreted more proaerolysin than the cells incubated in the presence of arsenate, indicating that ATP was required by the GSP-MTB (Figure 25). However, the finding that arsenate could not completely block the secretion even when the concentration of arsenate was increased to lethal dose, suggested that other energy sources might be involved in the secretion process.

Effect of CCCP on proaerolysin secretion

Besides ATP, another energy source of the GSP-MTB is proton motive force (PMF). The electrochemical potential of the PMF can be used to produce ATP: a well-known example is the production of ATP in bacterial bioenergetics. However, the PMF can work as an energy source directly: Wong and Buckley (1989) obtained evidence that PMF is required for secretion. To confirm the requirement of PMF as an energy source for the GSP-MTB, the effect of carbonyl cyanide m-chlorophenyl hydrazone (CCCP) on proaerolysin secretion was investigated. The compound CCCP is a protonphore that can inhibit use of PMF by bacterial cells. Therefore, if PMF is required as an energy source by the GSP-MTB, the secretion of proaerolysin should be significantly reduced when bacterial cells are grown in a medium containing CCCP. This is exactly what happened.

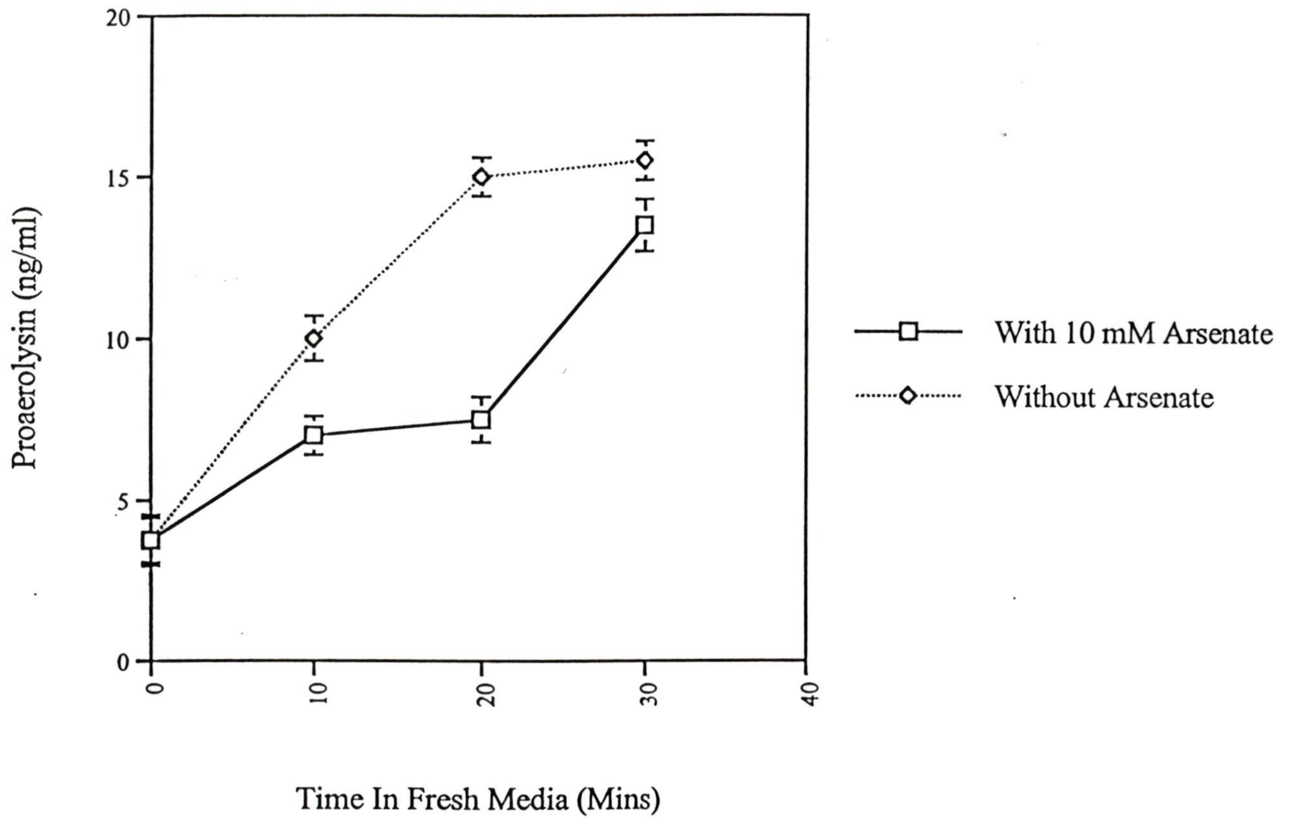


Figure 25. Effect of arsenate on secretion of proaerolysin.

Experiments were performed five times and the results are the means (± SEM) of the five experiments.

When *A. salmonicida* CB3 cells were grown under standard culture conditions and an experiment similar to the arsenate experiment was performed except that the 10 mM arsenate was replaced by 60 μM CCCP, it was found that the presence of CCCP almost completely blocked proaerolysin secretion (Figure 26). One interpretation of the results of the arsenate and CCCP experiments is that PMF is a more important energy source than ATP. An alternative explanation is that the inhibitory effect of CCCP on PMF is greater than that of arsenate on ATP; however, no such observations have been obtained.

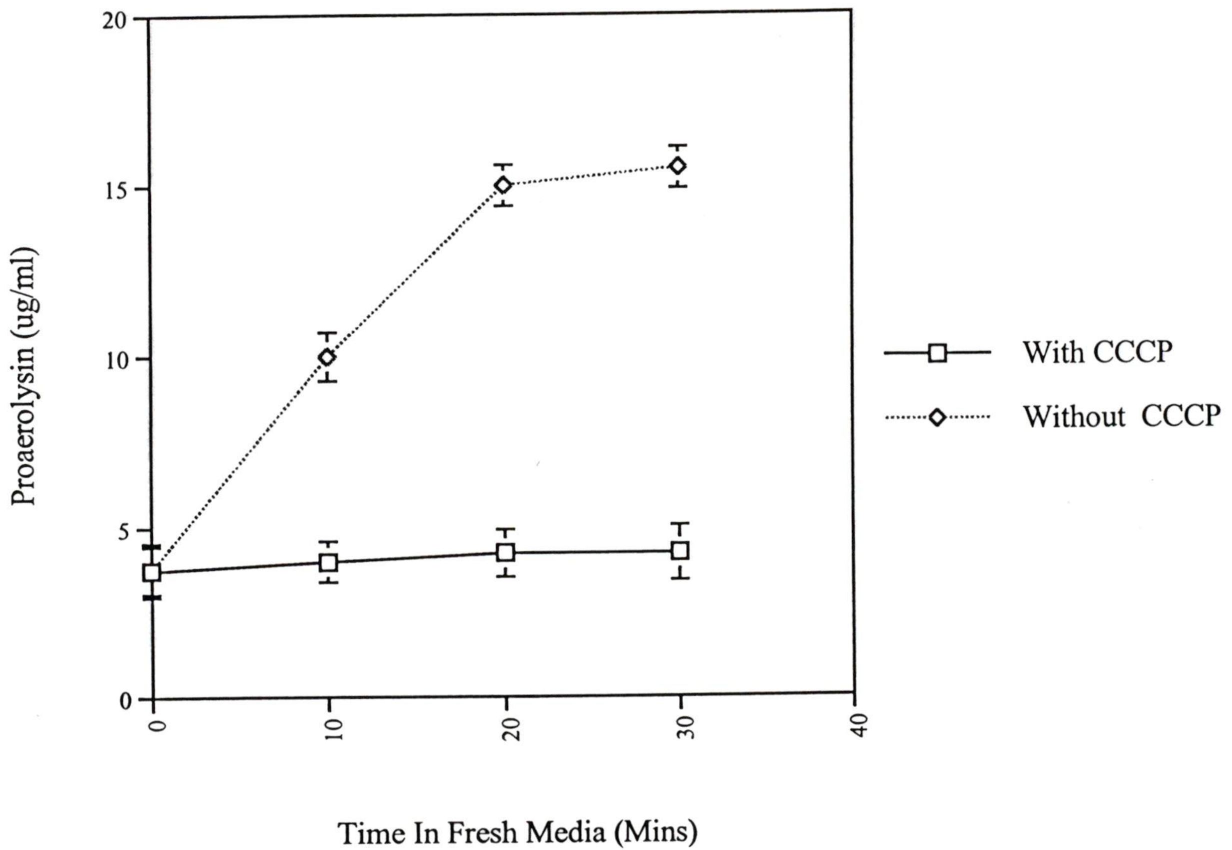


Figure 26. Effect of CCCP on secretion of proaerolysin.

Experiments were performed five times and the results are the means (± SEM) of the five experiments.

DISCUSSION

1. Secretion pathway of proaerolysin

Proaerolysin is synthesized as a 54kDa precursor with a typical N-terminal sequence (Howard et al., 1987; van der Goot et al., 1992). The first step in secretion is by means of the Sec system. During the cotranslational inner-membrane translocation, the signal peptide is removed (Howard and Buckley, 1985). The resulting proaerolysin must fold and dimerize before it crosses the outer membrane (Hardie et al., 1995). Whether this folding and dimerization is spontaneous or whether it requires the help of some other proteins are not clear, although it has been suggested that the disulphide bond formation within and between the proaerolysin monomers is independent of DsbA (Hardie et al., 1995). The observation made here that a certain amount of proaerolysin was detected in the freeze-thaw supernatant suggests that some proaerolysin was associated with the outside of the inner-membrane (Figure 6), because the fact that there is no signal peptide excludes the possibility of cytoplasm located proaerolysin. It is believed that the signal peptidase removes the signal peptide at the outside surface of the inner membrane (Dalbey, 1991). More direct evidence that proaerolysin is associated with the inner-membrane is not yet available. One future experiment is to isolate the inner membrane fraction from the freeze-thaw pellet and to determine whether proaerolysin can be detected in this membrane fraction. Nevertheless, the above observation may suggest that the folding or dimerization of proaerolysin requires the involvement of some inner-membrane proteins. Indeed, 11 of the 14 proteins that constitute the GSP-MTB are

integral inner-membrane proteins (Pugsley, et al., 1993). It is reasonable to speculate that at least some of these proteins may form a multicomponent complex, which helps the correct folding and oligomerization of secretory proteins. If this is true, the next barrier the folded proaerolysin faces is the peptidoglycan. Calculations of the effective pore size of peptidoglycan give an average pore radius of 2 nm (Demchick and Koch, 1996). The thickness of the proaerolysin monomer, however, is more than 4 nm (Parker et al., 1994). This may slow the movement of proaerolysin through the peptidoglycan and delay the second membrane translocation. If this is the case, it could partially explain the accumulation of proaerolysin in the periplasm (Figure 6). The ultimate secretion of the accumulated proaerolysin (Figure 10, 11) and the normal trypsin breakdown pattern (Figure 20) both indicate that this proaerolysin was correctly folded, suggesting that the speed limit step is after the folding of the protein. The next step is the translocation across the outer membrane. The two outer membrane GSP-MTB components, the D and S proteins (Pugsley, 1993), may form a temporary channel, which enables proaerolysin to cross the outer membrane. In summary, a four-step model of proaerolysin secretion can be proposed (Figure 27). 1) Preproaerolysin cotranslationally crosses the inner membrane. The signal peptide is removed before the protein reaches the periplasm. 2) Proaerolysin folds and dimerizes with the help of an inner-membrane multiprotein complex. 3) Proaerolysin dimer squeezes through the peptidoglycan layer with help from some chaperones and approaches the outer membrane. 4) Proaerolysin crosses the outer membrane through a channel formed by the D and S proteins.

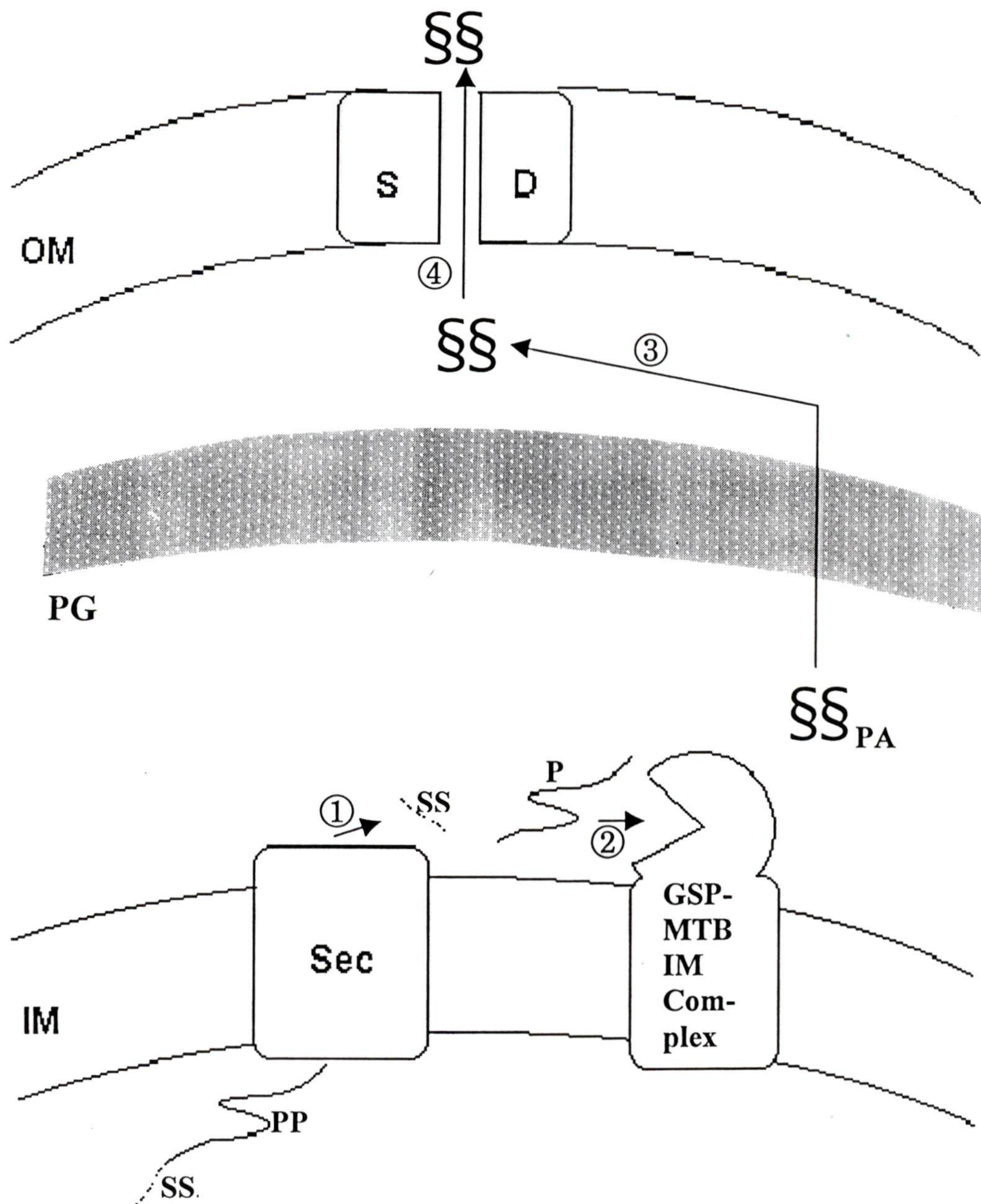


Figure 1. Model for proaerolysin secretion.

Secretion is proposed to consist of four steps as described in the text. PPA: preproaerolysin; PA: proaerolysin; SS: signal sequence; IM: inner membrane; PG: peptidoglycan; OM: outer membrane; Sec: Sec system; S, D: GSP S and D proteins.

2. Secretion efficiency of the GSP-MTB

The GSP-MTB is considered to be a very important secretion pathway (Pugsley, 1993), because it can secrete a great variety of proteins. However, no information about secretion rates of the GSP has appeared in the literature to date. The study in this thesis is the first attempt to quantify the efficiency of the GSP-MTB. The secretion rate of proaerolysin by the Gram-negative bacterium *Aeromonas* reached values up to about 16,500 molecules per cell per minute (Table 4). This rate is more than two times higher than the rate of β-lactamase secretion by the Gram-positive bacterium *Lactobacillus*, which was estimated at about 8,000 molecules per cell per minute (Savijoki et al., 1997). Secretion of proaerolysin from a Gram-negative bacterial cell involves two membrane translocations and two distinct systems, whereas β-lactamase from a Gram-positive bacterial cell needs only to cross one membrane. The higher secretion rate of proaerolysin therefore indicates that the GSP-MTB is more efficient than the Gram-positive bacterial secretion system. Alternatively, since β-lactamase is not antologous to *Lactobacillus*, the slow secretion rate may be due to that the heterologous protein can not interact well with the Gram-positive secretion apparatus. Therefore the actual secretion rate of Gram-positive bacteria may be much higher. Based on this assumption, one conclusion can be drawn that the translocation across the inner membrane is the speed limit step of the GSP.

3. Secretion substrates for the GSP-MTB

Aeromonas species secrete many enzymes by way of the GSP-MTB (Pemberton et al., 1997), including proaerolysin, protease, lipase, and amylase. A comparative study of

proaerolysin and protease secretion in *A. salmonicida* demonstrated that protease secretion occurs at a much later stage of the cell cycle. Proaerolysin secretion approached a maximum about 4 hours after the start of induction, whereas no significant protease secretion occurs before an 8-10-hour of IPTG induction (Figure 17). One explanation is that the GSP-MTB can secrete different substrate proteins at different growth times. However, the possibility that the GSP-MTB has sub-branches that share parts of the secretion machinery cannot be excluded. The secretion of *Serratia marcescens* nuclease may use a sub-branch, since its energy free feature is quite different from other GSP-MTB secretion pathways (Suh and Benedik, 1997). A second finding of the comparative study of proaerolysin and protease secretion in *A. salmonicida* was that the secretion of large amount of proaerolysin reduced the appearance of protease in the culture supernatant. Failure to observe this inhibitory effect in previous studies (Wong, unpublished data), can be explained on the basis that the over-expression of proaerolysin was insufficient to saturate the efficient GSP-MTB. In the present study, the removal of the upstream repeat greatly enhanced the expression and secretion of proaerolysin (Figure 13, Table 2), thereby making it possible to load the secretion apparatus fully. Although the reduced appearance of protease in the supernatant may be due to other reasons such as lowered activity, one best explanation is that the protease secretion was affected by the proaerolysin secretion. Some evidence was obtained that indicated that proaerolysin secretion dominated the GSP-MTB of the *Aeromonas* species examined. When the secretion machinery was fully loaded, the secretion of proaerolysin remained constant whereas the secretion of other protein like protease decreased.

4. Secretion signal for the GSP-MTB

Proaerolysin is composed of two lobes connected by a short arm (Parker et al., 1994; Figure 2). In this study, it was demonstrated that the large lobe (domain II-IV) could be secreted by itself (Figure 19), although secretion was much poorer than that of wild-type proaerolysin. The breakdown products of domain II-IV in the periplasm did not appear in the culture supernatant (Figure 20), indicating that the presence of domain II-IV in the supernatant was not a result of unspecific leakage. It was expected that domain II-IV could not be secreted without a functional secretion machinery. Therefore the failure of the *A. hydrophila ExeE* mutant to secrete domain II-IV (Figure 21) provided additional evidence that domain II-IV was secreted via the GSP-MTB.

Although I found that co-expressed domain I and domain II-IV of proaerolysin (the short arm was included in the small lobe) did not have a higher secretion than wild-type proaerolysin (Figure 23), parallel studies in this laboratory showed that when co-expressed by including the short arm in the large lobe, the secretion of both domain I and domain II-IV was increased (Diep et al., in press). The co-expression experiments provide some evidence about the secretion signal. The targeting signal for the GSP-MTB has been under extensive research by several groups. Two types of signals have been proposed. Some groups proposed that a linear stretch of amino acids within the correctly folded secretory protein might be the targeting signal (Pugsley et al., 1997). Some experimental data supported this idea. Lu and Lory (1996) identified a 60 amino acid segment of exotoxin A that enabled the secretion of β-lactamase in *P. aeruginosa*, indicating the signal is located in that region. Similar results have been obtained for

exotoxin A secretion (McVay and Hamood, 1995) and pullulanase secretion (Sauvonnet and Pugsley, 1996). However, studies in our laboratory do not support this idea. *A. salmonicida* has been found to be able to secrete several different proteins including proaerolysin, a metalloprotease, and *E. coli* alkaline phosphatase (Wong et al., 1991; Wong and Buckley, 1993). These proteins have no common sequences in their primary structures, leading to the proposal that the signal is a tertiary configuration (Parker et al., 1996). The co-expression experiments support the contention that the signal is not a primary structure, because if the signal is a linear amino acids sequence, the results from the two co-expression experiments should be similar. The explanation that best fits all of the observation is that the secretion signal depends on the correct and complete folding of the protein. If true, then co-expression by including the short arm in the large lobe must somehow enhance this folding process thereby leading to increased secretion. Perhaps the correct folding of the proaerolysin molecule presents a small domain on the protein surface that functions as a signal that is recognized by the secretion machinery. The finding that the α –toxin of *C. septicum* can not be secreted by *A. salmonicida* via the GSP-MTB (Figure 24), even though it has a similar amino acid sequence to domain II-IV of proaerolysin, may indicate that the folding of α-toxin does not result in a signal on the protein surface that is recognizable by the GSP-MTB of *A. salmonicida*.

5. Energy requirements of the GSP-MTB

The GSP-MTB has been shown to be an energy-driven process, first by Wong and Buckley (1989), and later confirmed by Letellier et al. (1997) and Pugsley's group (Pugsley et al., 1997). Suh and Benedik (1997) found that electrochemical proton

gradient and ATP hydrolysis were not required for secretion of nuclease across the outer membrane of *S. marcescens*. They suggested that such an energy-free translocation process, which operates as a second step of GSP, is located in a different branch than the MTB. The observations that both arsenate (Figure 25) and CCCP (Figure 26) inhibited the secretion of proaerolysin by the GSP-MTB suggest that both ATP and PMF are energy sources for this pathway. However, it is not known how the GSP-MTB utilizes these energies. Although various protein components of the GSP-MTB have been found to have ATP binding sites (Possot and Pugsley, 1994; Turner et al., 1993; Sandkvist et al., 1995; Howard et al., 1996), none of these proteins was able to hydrolyse ATP and provide energy for the GSP-MTB *in vitro*. Studies by Tommassen et al.(1992) showed that one of the components, XcpR, could provide energy for assembly of the secretion apparatus, but not for the actual secretion process. The observation that arsenate could not completely block proaerolysin secretion (Figure 25) suggests that ATP might not be essential as an energy source. The profile of the arsenate inhibitory effect was S-shaped (Figure 25), suggesting that ATP may be required for recycling of some of the essential components of the GSP-MTB. SecB of the Sec system has been shown to be a recycling component (Georgopoulos, 1992), although no energy is needed in this process (Driessen et al., 1995). If this suggestion is correct, arsenate will not strongly prevent secretion while the recyclable components are still available (see the initial slope of Figure 25), but the inhibitory effect will be stronger when all of the recyclable components are occupied (see the intermediate plateau of Figure 25). The slow yet gradual recycling of these components at a later stage would cause the arsenate to become less effective again (see the second slope in Figure 25). In such a scenario, ExeE and its homologues such as PulE

would be candidates for one of the recycling components. Other proteins such as ExeA or TonB in *A. salmonocida* may also function in this role.

Another energy source that is used by the GSP-MTB is the PMF. Although the existence of porin structures in the outer membrane appear to exclude the existence of such a cross-membrane electrochemical potential, there is experimental evidence to support the requirement for the PMF in the GSP-MTB. For example, it has been found that the secretion of various proteins is decreased in media with low pH (Wong and Buckley, 1989; Suh and Benedik, 1997). An inhibitory effect of CCCP on secretion by way of the GSP-MTB was demonstrated in the present study (Figure 26) and previous study in this lab (Wong and buckley, 1989). It has also been demonstrated by other groups (e.g., Possot et al., 1997). One explanation of the apparent dilemma created by postulating the co-existence of porin structures and PMF is that leakage of protons through the porin structure is localized in specific regions (near the porin) of the periplasm, whereas in general the cell can still maintain an intracellular proton pool. Although Bayer's bridges have been found to be artifacts (Kellenberger, 1990), some functional compartments in the periplasm may exist. The observation that proaerolysin is somehow protected in the periplasm from proteases lends support to this idea. Another explanation is that although some protons may leak out through the porin structure, they are recovered from the extracellular medium by an unknown mechanism. This postulated recovery process may function as an exchange system for moving certain important ions in or out of the cell. Thus far no such processes have been identified in prokaryotic cells, although they are prevalent in eukaryotic cells. Alternatively, this can be explained that the GSP-MTB machinery can use the PMF generated from the inner membrane to

translocate proaerolysin across the outer membrane. The TonB-like inner membrane protein, ExeB, may have this function (Howard et al., 1996; Letellier et al., 1997).

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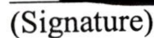

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