Secretion of the *Aeromonas hydrophila* Toxin Aerolysin Across the Outer Membrane of Gram-negative Bacteria

by

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We accept this thesis as conforming
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ABSTRACT

Aeromonas hydrophila secretes the hemolytic toxin aerolysin using the general secretion pathway (GSP). A number of different approaches were taken to learn more about this process. The aerolysin structural gene was cloned into *Escherichia coli*, *Aeromonas salmonicida*, and a marine *Vibrio* spp. It was expressed in all three bacteria, but only *A. salmonicida* and the *Vibrio spp.* were able to secrete it. The precursor form of the toxin, proaerolysin, could be detected in the periplasm in both these bacteria. In addition, the protoxin accumulated in the periplasm of *E. coli* and pleiotropic secretion mutants of the *Vibrio* spp. These observations support earlier proposals that proteins secreted via the GSP transiently enter the periplasm before crossing the outer membrane.

Site-directed mutagenesis was used to change Trp227 in proaerolysin to a Leu, Gly, or Phe. Secretion of all three mutant proteins by *A. salmonicida* was reduced, and the Leu227 and Gly227 forms became trapped in the outer membrane. Trapped Leu227 was sensitive to trypsin while purified Leu227 was as resistant as wild type, perhaps a sign of unfolding during secretion. These results suggest that the Trp at position 227 is important in secretion.

Fusions made between aerolysin and the *E. coli* periplasmic protein alkaline phosphatase (PhoA) were degraded to PhoA alone in the periplasms of both *E. coli* and *A. salmonicida*. The resulting PhoA was subsequently shown to be secreted by the *Aeromonas* spp. GSP when the pH of the medium was above 7.5, likely as a dimer. This would support earlier reports that GSP proteins can be secreted across the outer membrane in highly folded conformations.

Proaerolysin was prevented from leaving the periplasm of *A. salmonicida* by treating the cells with CCCP. The pool of protoxin was secreted when the cells were transferred into fresh media lacking the uncoupler. A similar effect was obtained by

reducing the pH of the medium. These results demonstrated for the first time that a proton motive force is required for the translocation of a GSP protein across the outer membrane.

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Abbreviation	
A	absorbance
Ala	alanine
Asn	asparagine
ATP	adenosine triphosphate
bp	base pairs
BSA	bovine serum albumin
CAT	chloramphenicol acyltransferase
CCCP	carbonylcyanide *m*-chlorophenylhydrazon
Cys	cysteine
EDTA	ethylenediamineacetic acid
EGZ	*Erwinia carotovora* cellulase
ER	endoplasmic reticulum
GCAT	glycerophospholipid:cholesterol acyl transferase
GDH	glutamate dehydrogenase
Gln	glutamine
Gly	glycine
GS+	Gene Screen Plus
GSP	general secretion pathway
GST	glutathione S-transferase
GTP	guanosine triphosphate
h	hour
HBA	human blood agar
HEPES	4-(2-hydroxyethyl)piperazine-N'-ethane sulfonic acid
HlyA	α-hemolysin

IgG	immunoglobulin G
IPTG	isopropyl-β-D-thiogalactopyranoside
kDa	kilodalton
LamB	λ-receptor protein
LacZ	β-galactosidase
LB	Luria-Bertani
Leu	leucine
LktA	leukotoxin A
LPS	lipopolysaccharide
MBP	maltose binding protein
min	minute
NADH	nicotinamide adenine dinucleotide
NEM	N-ethylmaleimide
nm	nanometer
OD	optical density
PADAC	7-(thienyl -2-acetamido)-3-[2-(4-N,N-dimethylamino-phenylazo)pyridinium methyl]-3-cephem-4 carboxylic acid
PAGE	polyacrylamide gel electrophoresis
PCM	prochymosin
PE	phosphatidylethanolamine
PEG	polyethylene glycol
PG	phosphatidylglycerol
Phe	phenylalanine
PhoA	alkaline phosphatase
Pipes	1,4-piperazinediethanesulfonic acid

PMF	proton motive force
RF	replicative form
RNA	ribonucleic acid
rpm	revolutions per minute
S	Svedberg unit
SAC	*Staphylococcal aureus* cells
SDS	sodium dodecyl sulphate
Ser	serine
SLS	sodium lauryl sarcosinate
SRP	signal recognition particle
SRP54	54 kilodalton SRP peptide
SSRα	signal sequence receptor
Thr	threonine
TRAM	translocating chain-associating protein
Tris	Tris(hydroxymethyl)aminomethane
Trp	tryptophan
Tween 20	polyoxyethylenesorbitan monolaurate
Tyr	tyrosine
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
XP	5-bromo-4-chloro-3-indolyl-β-D-phosphate

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INTRODUCTION

While most proteins are found inside the cell, a number are released from the cell to function extracellularly. These include antibodies, hormones, and structural proteins like keratin which are released by eukaryotic cells. Bacteria secrete proteins to help obtain nutrients, avoid host defense systems and attach to host cells. Translocation machinery has been found which identifies proteins that are to be secreted and that selectively moves them across cellular membranes. Secreted proteins of eukaryotic cells first enter the endoplasmic reticulum (ER). There, transport vesicles are formed which are successively directed to the Golgi apparatus and the plasma membrane in a series of endo- and exocytotic steps. Prokaryotic cells have no ER or Golgi apparatus, and export proteins directly across the cytoplasmic membrane. In the case of Gram-negative bacteria, the outer membrane must also be crossed for release into the extracellular milieu. This thesis deals with the process of protein secretion across the outer membrane.

Export of proteins across the prokaryote cytoplasmic and eukaryote endoplasmic reticulum membranes

The initial steps in eukaryotic and prokaryotic secretion are similar. Signals found within the amino acid sequence of secreted proteins direct them to the export machinery, which is located in the cytoplasmic membrane of prokaryotes and the ER membrane of eukaryotes. While the ER membrane is the site for phospholipid biosynthesis and the modification of secreted proteins (Bishop and Bell, 1985), the bacterial inner membrane also contains all the enzymes required for energy transduction and oxidative phosphorylation in the cell (Cronan et al., 1987). The lipid composition of the two membranes also differs considerably. The ER membrane is mainly composed of phosphatidylcholine (PC), as well as smaller amounts of other phospholipids and cholesterol (Jain and Wagner, 1980). In contrast, phosphatidylethanolamine is the major

lipid which makes up the bacterial inner membrane. No PC or cholesterol are found in this bilayer, but phosphatidylglycerol (PG) and diphosphatidylglycerol are two lipids typically found in bacterial cytoplasmic membranes (Burnell et al., 1980). In spite of the differences in lipid composition of the two membranes, both utilize similar mechanisms for transporting proteins. The similarities and differences between these two translocation processes are outlined below.

1. Export in eukaryotes

A) The signal sequence

George Palade and his co-workers first determined that eukaryotic secreted proteins are translated on ribosomes that are attached to the ER membrane (reviewed in Palade, 1975). They postulated that there must be a signal which directs secreted proteins to the ER membrane. Support for this came from the work of Milstein et al. (1972) who discovered that the IgG1 molecule which was translated in cell-free *in vitro* systems was larger in size than secreted IgG1. Peptide analysis of the larger molecule showed that the N-terminus had an extra 15-20 amino acids (Schechter et al., 1974). The rest of the molecule was indistinguishable from secreted IgG, suggesting that the *in vitro* product was a precursor. The authors proposed that this extra region at the N-terminus could be a secretion signal. In another study, polysomes detached from rough microsomes containing partially synthesized IgG1 chains were isolated and the synthesis of the nascent chains completed *in vitro* (Blobel and Dobberstein, 1975). The resulting products were a mixture of the larger precursor and the secreted form of IgG1. This indicated that the putative signal was removed from the nascent polypeptide chain before translation had been completed. This led to the following 'signal hypothesis' (Blobel and Dobberstein, 1975). Translation of the N-terminal region of secreted proteins would expose a secretion signal sequence on the surface of the messenger RNA-ribosome polysome complex. This

sequence could then direct the polysome to the ER membrane, perhaps to a location containing a channel. As the polypeptide continued to grow, it would travel through the ribosome, across the channel and into the lumen of the ER. The signal would be proteolytically cleaved during translocation, and when the protein had been completely transferred, the polysome would dissociate from the ER membrane. Over time, this scheme has been shown to be correct, and many of the components of the system have been identified.

The N-terminal signal sequences of many proteins have now been identified and compared (von Heijne, 1990). Although they vary in size from 15-30 amino acids and share little or no homology, they all contain regions with similar characteristics. In eukaryotic proteins, their N-termini normally contain 1-2 basic amino acids, followed by a stretch of hydrophobic amino acids of varying length. Only small amino acids (Ala or Ser) are found at the -1 position (on the N-terminal side of the cleavage site), while the -3 position always contains a non-polar residue other than proline (von Heijne, 1983). Bacterial signal sequences have similar characteristics and indeed have been found to be interchangeable with eukaryotic signal sequences (Talmadge et al., 1980). Due to the ease with which bacterial DNA can be manipulated, more studies with prokaryote signal sequences have been carried out (See section on prokaryotic signal sequences below).

B) The signal recognition particle

As the signal sequence is exposed to the cytoplasm during translation, it is recognized by an 11S complex composed of 6 polypeptides (molecular masses of 9, 14, 19, 54, 68 and 72 kDa; Walter and Blobel, 1980), and a 7S RNA molecule (Walter and Blobel, 1982). This complex is known as the signal recognition particle (SRP). Walter and Blobel (1981) showed that SRP binds specifically to the signal sequence and subsequently arrests translation of the nascent polypeptide chain. They suggested that this

would give the polysome time to move to the secretion components in the ER membrane so that the growing peptide could be exported co-translationally.

Walter and Blobel (1983) also found that the SRP complex could be dissociated into its components with ethylenediaminetetraacetic acid (EDTA). The 54 kDa (SRP54) and 19 kDa proteins were isolated as monomers while the 9/14 kDa and 68/72 kDa proteins were heterodimers (Walter and Blobel, 1983). Siegel and Walter (1988) showed that the 7S RNA could be reconstituted into an active SRP complex when mixed with the six peptides in the presence of Mg^{2+}. They also showed that the RNA was required as the backbone of the complex and that the peptides associated with the complex in a specific order. Exploiting the ability to reconstitute fully active SRP *in vitro*, they modified each of the proteins separately with N-ethylmaleimide (NEM) alkylation before placing them back in the complex. They found that when SRP54 was modified, the SRP could no longer recognize and bind to the signal sequence of the nascent polypeptide chain. Further support for a role for SRP54 in signal recognition came from the work of Kurzchalia et al. (1986) who were able to cross-link the signal sequence of a nascent peptide chain to SRP54. If SRP54 were proteolytically cleaved into two halves using V8 protease, the C-terminal fragment was found to bind to a signal sequence when the SRP was reconstituted (Zopf et al., 1990). Sequencing of SRP54 revealed that this fragment contains a methionine-rich domain which was proposed to be responsible for recognizing the signal sequence and binding to the 7S RNA (Bernstein et al., 1989). The N-terminal fragment was found to contain a putative GTP-binding site, suggesting that GTP may play a role in signal sequence recognition.

Alkylation of the 9 kDa polypeptide did not prevent the formation of the 9/14 kDa heterodimer, but blocked the translational arrest normally observed (Siegel and Walter, 1988). However, SRP-complexes reconstituted with the modified 9/14 kDa heterodimer were able to bind to the signal sequence, allowed translation and were functional in

microsome translocation assays. Translational arrest does not then appear to be necessary for export across the ER membrane *in vitro*, but it is not known if the same is true *in vivo*. Modification of the 72 kDa protein did not inhibit the formation of the 68/72 heterodimer. However, SRP-complexes formed with this modified protein were not directed to microsomal membranes. This has been shown to be due to their inability to interact with the signal recognition particle receptor on the endoplasmic reticulum (See section (c) below). Finally, modification of the 19 kDa protein resulted in fully functional SRP complexes. It had been proposed earlier that the 19 kDa peptide was required for SRP54 to properly attach to the 7S RNA (Walter and Blobel, 1983). When the complete SRP complex was treated with NEM, the alkylation sites of the various proteins were protected from modification, and a fully functional SRP complex was maintained (Siegel and Walter, 1988).

C) The SRP and the endoplasmic reticulum membrane

The polysome complex is directed to a specific receptor on the ER membrane by the SRP 68/72 heterodimer. This SRP receptor, originally known as "docking protein" (Meyer et al., 1982), was shown to be associated with the membrane by treatment of microsomes with elastase. A 60 kDa fragment of a 72 kDa ER membrane protein was released, preventing removal of the translational block caused by SRP. Addition of the 60 kDa fragment released the translational block. Gilmore et al. (1982) disputed this finding, claiming that the portion of the 72 kDa protein bound to the elastase-treated microsomes was also necessary to remove translational arrest, and they confirmed that the 60 kDa preparations used by Meyer et al. (1982) did in fact contain fragments of microsomal membranes. Cloning and sequencing of the gene for the proposed SRP receptor (SRα) led to a predicted molecular mass of 69.7 kDa (Lauffer et al., 1985). The protein consists of a large 52 kDa cytoplasmic domain, with one or two membrane-spanning domains located at

the N-terminus. The SRP receptor was later found to contain a second subunit. When the SRα protein was purified on affinity columns using monoclonals to SRα, a 30 kDa protein (SRβ) was copurified (Tajima et al., 1986). Furthermore, when SRβ was purified using monoclonal antibodies against SRβ, SRα was also found in the eluted fractions. While SRα is known to interact directly with the SRP, a function for SRPβ has not yet been found.

GTP binding sites similar to the one identified in SRP54 have been found in both SRα and SRβ. Connolly and Gilmore (1989) have shown that release of SRP from the signal sequence requires GTP after the SRP-complex has bound to the SRP receptor. In the absence of GTP, SRP has been found associated with microsomal membranes in *in vitro* assays. Dissociation appears to be dependent on binding of the SRP complex to SRα, but it is not clear which of the three GTP-binding sites is necessary for this step. The initial step of binding to SRα has been shown to require functional GTP binding sites in both SRα (Rapiejko and Gilmore, 1992) and SRP54 (Zopf et al., 1993). The GTP hydrolysis activity of both of these GTP binding sites increases by 40-fold when SRP54 is bound to SRα, raising the possibility that there is a cooperative effect between the two (Connolly and Gilmore, 1993).

D) A protein translocation channel

The steps following dissociation of SRP from the signal sequence remain unclear. There have been two theories about the fate of the signal sequence once the translational arrest has been lifted. It either enters the lipid bilayer itself or it is directed to a channel which crosses the ER membrane. Gierasch (1988) performed studies which indicated that the signal sequence could change its conformation from β-sheet under aqueous conditions, to α-helix under non-polar conditions. Since membrane spanning domains typically adopt α-helical conformations, this suggested that the signal might be able to enter the ER

membrane spontaneously by changing its conformation at the aqueous/lipid interface (de Vrije et al., 1990). The other possibility, that the nascent peptide chain was directed to a pore in the ER membrane that is large enough for proteins to pass through, has been supported by recent evidence. Simon and Blobel (1991) identified potential protein channels in the ER membrane by fusing rough microsomes of pancreatic ER to planar lipid bilayers. Initially, a small number of channels with conductances of 60-120 picosiemens (pS) were found. These would be large enough to allow proteins to pass through. However, the number of putative pores, relative to the number of ribosomes observed on the rough ER, was calculated to be extremely small. The experiment was repeated in the presence of puromycin in an attempt to remove any nascent peptide chains which might be blocking the channels. Under these conditions, many more channels large enough to conduct proteins were observed.

Further evidence for the existence of protein-conducting channels was obtained using fluorescent probes which were incorporated into the preprolactin signal peptide (Crowley et al., 1993). Since the fluorescent lifetime and emission maximum of the probes used differed in aqueous and non-polar environments, the authors were able to determine the nature of the environment through which the signal sequence travelled during its translocation across the ER membrane. It was found that the signal sequence was always in an aqueous environment, further supporting the existence of a protein channel, and arguing against the theory that the signal sequence directly enters the membrane. Further, Simon and Blobel (1991) found that the protein-conducting channels could be detected in artificial bilayers of widely different phospholipid compositions. Insertion of the signal sequence into a bilayer would be expected to depend on the properties of the bilayer, as Batenburg et al. (1988) suggested that the positively charged signal sequence should preferentially interact with negatively charged phospholipids.

Simon and Blobel (1991) also found that the channels could be closed when the salt concentration was high enough to dissociate the ribosomes from the lipid bilayer. This suggested that ribosome binding to the ER membrane was required for opening the channels, while dissociation from the membrane closed the channels. This implied that translocation must occur co-translationally, possibly with the ribosome powering the translocation process by coupling it with the elongation of the polypeptide. Observations that small proteins (less than 100 amino acids long) could be translocated across microsomes post-translationally *in vitro* in the presence of ATP and independent of the SRP did not support this conclusion (Schlenstedt and Zimmerman, 1987; Schlenstedt et al., 1990). However, it now appears that such small proteins can be transported across the ER membrane with the help of membrane proteins known as ABC (ATP-binding cassette) transporters without the need for a signal sequence (Click et al., 1992). Recent cross-linking studies suggest that sec61p and TRAM (see below) may play a role in this export pathway as well as in signal sequence mediated export (Klappa et al., 1994).

To prevent the indiscriminate flow of ions from the cytoplasm to the ER it was predicted that ribosomes must form a tight seal around the channel. To test this, experiments were done by Crowley et al. (1993) to see if ribosomes prevented iodide ions from entering the channels from the cytoplasmic face of the ER membrane. They reasoned that iodide ions would quench the fluorescent probes incorporated into the signal sequences if they could gain access to the channels during translocation of the signal peptides. No quenching of the probes was observed, demonstrating that the ribosomes did form a seal with the channel which excluded the iodide ions.

While the complete composition of the protein-conducting pores has not been established, a number of likely components have been identified. Incorporation of photoreactive amino acid analogues into the signal sequences of nascent peptide chains was used to identify proteins which were close enough to the signal to cross-link to and which

may interact with the signal after its release by SRP (Krieg et al., 1989; Wiedmann et al., 1987). Two glycoproteins were identified in this manner. The first, which was cross-linked with longer nascent peptide chains, was termed signal sequence receptor (SSRα) and has now been shown to be part of a heterotetramer (Gorlich et al., 1990; Rapoport 1992). The majority of shorter peptide chains cross-linked with a 35 kDa glycoprotein, termed translocating chain-associating membrane protein (TRAM; Gorlich et al., 1992a). The potential importance of these glycoproteins was demonstrated by Nicchitta and Blobel (1990) who found that rough microsome vesicles, reconstituted from microsomal extracts depleted of glycoproteins by being passed through a concanavalin-Sepharose column, could not translocate *in vitro*-synthesized peptides. Gorlich and Rapoport (1993) further dissected the requirements for protein export by reconstituting transport-active proteoliposomes from pure phospholipids and purified ER membrane proteins. They found that the only components needed for translocation activity were SRα, SRβ and a novel complex composed of a non-glycosylated protein (Sec61p; Gorlich et al., 1992b) and two smaller polypeptides, Sec61-β and Sec61-γ (Hartmann et al., 1994). While TRAM was required for transport of some proteins, for others it only stimulated translocation. The SSR complex was not required for transport of any of the proteins tested. However, in other studies, monoclonal antibodies made against SSRα have been shown to inhibit protein translocation (Hartmann et al., 1989), suggesting that SSRα plays some role in the export of proteins, although it may not be absolutely required. These results have led to the theory that SEC61p and the two smaller polypeptides make up the protein-conducting channels, and that TRAM is also associated in some manner (Gilmore, 1993).

As the peptide enters the lumenal side of the channel, its signal peptide is cleaved by signal peptidase. Signal peptidase is not required in proteoliposomes to reconstitute the translocation process, so it does not appear to be necessary for export (Gorlich et al., 1992b). It remains to be seen if the signal sequence is passed onto a specific receptor in the

translocation channel once the SRP is released. It is also not clear how the signal peptide is directed to the signal peptidase as it enters into the ER lumen.

2. Export in Gram-negative bacteria

A) The signal sequence

The export of proteins across the bacterial cytoplasmic membrane is similar to export of proteins across the ER membrane. A signal sequence is required to direct the protein to the export machinery. The bacterial signal sequence is composed of the same three domains as eukaryote signal sequences: i) an N-region containing at least one basic amino acid residue, ii) a central hydrophobic region (H-region) and iii) a C-region containing a cleavage site which follows the same -1, -3 rule as eukaryotic signals (see above and von Heijne, 1984). Prokaryotic proteins typically have an extra basic amino acid residue in the N-region. This may be because the free amino group in the eukaryote initiator methionine residue could act as an extra positive charge. This methionine is formylated, and thus uncharged, in prokaryotes (von Heijne, 1984).

The relative ease of producing and screening mutants in prokaryotes has led to a large number of studies of the essential components of the signal sequence. In early experiments, a fusion protein was made between the maltose binding protein (*malE*) signal sequence and β-galactosidase (*lacZ*) of *Escherichia coli* (Bassford and Beckwith, 1979). This protein was unable to cross the *E. coli* inner membrane due to the inability of the β-galactosidase to adopt a translocation-competent conformation. Consequently, the fusion protein became trapped in the membrane, resulting in an inactive β-galactosidase. However, spontaneous mutants were isolated which showed LacZ activity, indicating that the fusion protein was located in the cytoplasm (Bassford and Beckwith, 1979). It was found that in each of these mutants the signal sequence had been modified and that a charged amino acid had replaced a hydrophobic or uncharged residue in the H-region

(Bedouelle et al., 1930). Thus the mutations in this region prevented the fusion protein from entering the export pathway. The mutant *malE* signal sequences were subsequently recloned into wild type *malE*, resulting in the accumulation of MalE precursors in the cytoplasm (Bedouelle et al., 1980). Similarly, mutants of the λ receptor protein (LamB) which could not be exported also contained substitutions of uncharged amino acids with charged residues in the H-region (Emr et al., 1980). Mutants with complete deletions in the LamB H-region were also found. In the studies of both Emr et al. (1980) and Bedouelle et al. (1980), no mutations were observed in the N- or C-regions, suggesting that the H-region is the most important region of the signal sequence.

Results similar to those found with the MalE-LacZ fusion proteins were obtained using alkaline phosphatase (*phoA*)-*lacZ* fusions, with substitution of leucine residues by glutamine or arginine resulting in cytoplasmic accumulation of the fusion protein (Michaelis et al., 1983). Replacing 10 amino acids within the H-region of the *E. coli* PhoA signal with 9 leucine residues resulted in a fully functional signal sequence (Kendall et al., 1986) but placing a serine residue in the middle of the polyleucine H-region diminished the export efficiency of the signal. Full translocation activity was also observed when the PhoA H-region was replaced with a polyisoleucine segment of 10-15 residues (Kendall and Kaiser, 1988). Replacement with polyvaline segments resulted in decreased export efficiency, while polyalanine replacement resulted in an almost nonfunctional signal peptide (Chou and Kendall, 1990). It was pointed out that polyleucine and polyalanine peptides would normally assume α-helical conformations while polyvaline and polyisoleucine peptides should assume β-sheet conformations. Thus the replacement results suggested that overall hydrophobicity, and not secondary structure, was the most important determinant of a signal sequence. This is not supported by spectroscopic studies of native H-regions, which have suggested that they do form stable α-helical structures, and that the secondary structure of this region is critical in its functioning in the translocation process (Bruch et al.,

1989). It remains to be seen how large a role secondary structure plays in signal sequence function.

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 replaced 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 and intriguingly, the PhoA construct became insensitive to disruption of the proton motive force (see below). 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 the 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. There are also results which implicate the positive charges in interactions with one of the Sec proteins (see the section on SecA below).

Regions outside of the signal sequence, close to the signal peptidase cleavage site, have also been implicated in protein export. Placement of 6 lysines within the first 30 residues of mature signal peptidase led to a block in its translocation while their placement elsewhere in the mature protein had no effect (Andersson and von Heijne, 1990). It was also observed that export of the cytoplasmic protein chicken muscle triosephosphate isomerase was possible in *E. coli* when it was fused to the signal sequence and the first 14 amino acids of β-lactamase (Summers and Knowles, 1989). Fusion of the signal sequence alone did not result in an export-competent protein.

B) SecB

Prokaryote signal sequences appear to keep nascent peptides in unfolded conformations. Chaperones have been shown to bind to unfolded proteins, and either prevent the folding or direct them to the translocation machinery located in the inner membrane. The cytoplasmic protein SecB appears to be such a chaperone. Mutations to *secB* result in a block in the export of a group of outer membrane and periplasmic proteins, although other proteins are exported normally. Thus MBP (maltose binding protein), OmpF and LamB are not translocated in *secB*$^-$ mutants, but alkaline phosphatase and ribose-binding protein are (Kumamoto and Beckwith, 1985).

The *secB* gene was shown to encode a 17 kDa polypeptide which interacts with other proteins as a tetramer (Collier et al., 1988). Studies following the denaturation of MBP with guanidinium hydrochloride and its subsequent refolding in the presence of SecB using tryptophan fluorescence revealed that the presence of a signal sequence increased the relaxation time of the refolding step (Park et al., 1988). The MBP signal sequence was not found to bind SecB itself, but it was able to keep the rest of the protein in a conformation that exposed other sites which could bind SecB (Randall et al., 1990). In agreement with this were studies using fusions of MBP to PhoA. While the signal sequence of MBP alone

was unable to confer SecB-dependent export on PhoA, if the N-terminal third of the mature protein were also fused to PhoA, the fusion protein became dependent on SecB for export (Gannon et al., 1989). This suggested that a region of the mature protein binds to SecB. Others have made similar observations. MacIntyre et al. (1991) have found that tail fibre protein of phage T4, which is normally not exported, can compete for SecB binding when fused to the OmpA leader sequence. A region of mature LamB that maps to amino acids 320-380 was shown to be able to interfere with export of other SecB dependent proteins (Altman et al., 1990a). The interference by this region could be increased by fusing it to a signal sequence. The signal sequence likely prevents the interfering peptide from folding, allowing the SecB binding sites to be exposed for a longer period of time and allowing a greater number of SecB tetramers to bind (Altman et al., 1990b). Co-immunoprecipitation of SecB with either prePhoE or mature PhoE using α-SecB monoclonal antibodies has also been observed (de Cock et al., 1992). When portions of the mature region of PhoE are deleted, co-immunoprecipitation still occurs, although at reduced efficiency. These results demonstrated that SecB recognizes and binds to the mature region of the targeted protein and in the case of PhoE, at more than one site. Recent results which show that a leaderless MBP and PhoA protein could be exported in *secY* mutants (see section on SecY and SecE below) of *E. coli*, albeit at 30% of the wild-type efficiency, suggest that the signal sequence is not essential in the prokaryotic export process (Derman et al., 1993). However, it was found that SecB was absolutely required for export of both these leaderless proteins, even though PhoA normally does not require SecB for export. The authors suggested that the absence of the signal sequence changed the timing of PhoA folding so that SecB became necessary to keep it in an export-competent conformation.

The SecB tetramer has a number of negatively charged areas on its surface which are thought to be able to bind positively charged peptides. Once these sites have been occupied, a conformational change has been observed which exposes hydrophobic sites

(Randall, 1992). These sites may interact with other regions on the target protein or they may interact with another component of the export machinery.

The results discussed here have led to the theory that SecB will bind to any protein in a non-native conformation, and that binding is dependent on how quickly a protein will fold into its mature conformation (Hardy and Randall, 1991). The presence of a signal sequence appears to slow the rate of refolding, allowing SecB to bind to sites within the mature region of the protein. While it was initially thought that SecB binding keeps a protein in an export-competent conformation, recent evidence suggests that this is not the case with prePhoE (de Cock and Tommassen, 1992). These studies, which were done *in vitro*, showed that the functional half-life of transport competent PhoE was 14 min with or without SecB. It is not clear if these results are significant *in vivo*. It has also been reported that SecB prevents the aggregation of proOmpA in its translocation-competent conformation (Lecker et al., 1990). Intermediate conformations may expose hydrophobic regions normally hidden in the mature form of the protein, which could lead to protein aggregation if they were not shielded from each other. Finally, SecB has been shown to interact with another component of the export machinery, SecA (Hartl et al., 1990), indicating that it can direct exported proteins to the next step in the export process.

C) Other potential chaperones

Other chaperone proteins have been shown to keep preproteins in an unfolded conformation in a similar way to SecB. These include the heat shock proteins DnaJ and DnaK (Hendrick et al., 1993), GroEL (Lecker et al., 1989), and trigger factor (Crooke et al., 1988; Kusters et al., 1989). GroEL is the main protein that can be cross-linked to pre-β-lactamase as it comes off the ribosome (Bochkareva et al., 1988) but there has been no evidence that it is necessary for its export. Neither DnaK nor GroEL alone is able to rescue the export defect of SecB$^-$ cells, but initiation of the heat shock response does restore partial

translocation activity (Altman et al., 1991). The translocation of PhoA, a SecB independent protein, is inhibited in *dnaJ*$^-$/*dnaK*$^-$ double mutants. Finally, overproduction of DnaJ and DnaK was able to restore partial export activity of *secB* mutants (Wild et al., 1992). Thus, it looks like heat shock chaperone proteins can substitute for SecB under certain conditions.

Since the signal sequences of eukaryotes and prokaryotes are interchangeable, it has been argued that there must be an SRP-like homologue in prokaryotes which interacts directly with the signal peptide. The sequence of an *E. coli* gene (*ffh*) shows a high degree of homology with SRP54, and a 4.5S RNA encoded by the *ffs* gene shares a conserved domain with the 7S RNA of SRP (Poritz et al., 1990). The *ffh* gene product (Ffh) has been shown to interact with eukaryotic 7S RNA as well as with *E. coli* 4.5 S RNA (Phillips and Silhavy, 1992) and an Ffh/4.5S RNA complex could be cross-linked with signal sequences which had photoreactive groups incorporated in them (Luirink et al., 1992). As well, mutations to either *ffh* or *ffs* resulted in decreased export of β-lactamase in *E. coli* (Luirink et al., 1992). On the basis of these results it would appear that an essential SRP-like complex also exists in *E. coli* which is required for protein export. However, the *in vitro* translocation of proOmpA has been reconstituted using purified proteins, and neither Ffh nor the 4.5S RNA was required for efficient transport (Brundage et al., 1990). While this would appear to rule out a role for an SRP-like complex in bacterial export, some groups still maintain that Ffh and the 4.5S RNA must play a role in translocation. Recently, Miller et al. (1994) found that an Ffh/4.5S RNA complex can bind to an *E. coli* protein, FtsY, which is related to SRα. They found that this interaction was dependent on GTP, activating a GTPase activity similar to that observed in the interaction between SRP and SRα (Connolly and Gilmore, 1989). It has been suggested that the bacterial SRP complex could bind to the signal sequence before SecB binds to a precursor protein, but this remains to be proven (Luirink and Dobberstein, 1994).

D) Insertion of the signal peptide into membranes

As with eukaryotes, bacterial signal peptides have been proposed to enter bilayers by themselves or to be directed to pores in the inner membrane to initiate the translocation process. Early evidence that signal peptides were able to insert into phospholipid monolayers led to the idea that insertion was required for export (Briggs et al., 1986). Addition of PhoE signal peptides to bilayer membranes caused changes in the ^{31}P-NMR spectra of dioleoylphosphatidylglycerol and dioleoylphosphatidylethanolamine, producing patterns resembling reversed hexagonal structures (H_{II}; Killian et al., 1990). Electron microscopy of freeze-fractured samples supported this idea, as many concave structures were observed on the bilayer surface. This suggested that leader sequences could cause localized perturbations of lipid bilayers, resulting in movement of the protein across the membrane. Since the signal peptide appeared to change conformation to an α-helix once it had inserted into the membrane (Wang et al., 1993b) it was also proposed that a conformational shift dragged the mature protein through the membrane. This was termed the "unlooping model" (de Vrije et al., 1990). However, the discovery of large channels able to conduct proteins across the inner membrane casts doubt on the relevance of these earlier ideas (see below; Simon and Blobel, 1992). In addition, it has now been calculated that the minimum number of signal peptides required to observe insertion of signal peptides into the bilayers used by Briggs et al. (1986) would be equivalent to 60 000 signal peptides per *E. coli* cell (Simon and Blobel, 1992). In contrast, Simon and Blobel (1992) found that the equivalent of 120 peptides per *E. coli* cell is required to open their putative inner membrane channels (see section on SecY and SecE below). This seems more reasonable for a cell which has to adapt to changing environments and export outer membrane and periplasmic proteins very quickly. Waiting for an accumulation of 60 000 molecules before initiating export seems improbable.

Earlier observations that negatively-charged phospholipids were required for the insertion of signal peptides into lipid bilayers (Batenburg et al., 1988) led to studies identifying a need for the negatively charged phospholipid PG in the translocation of proteins across the *E. coli* inner membrane (de Vrije et al., 1988). It was originally proposed that this might be due to an interaction between the basic amino acids in the N-region of the signal peptide and negatively charged phospholipids in the bilayer. However, it now appears that it may be SecA (one of the components of the export machinery; see below) that requires PG. It is still possible that insertion of the signal peptide into the membrane has a role in directing preproteins across the inner membrane in a PG-dependent manner. A possible example is a protein which does not require SecA for translocation but does require acidic phospholipids for export (Kusters et al., 1994). However, since this is a viral protein, it is likely that this is a specialized case. It is also possible that insertion of the signal peptide into a region of a bilayer adjacent to a translocation channel is required for entry into the channel. However, no evidence for this has been found.

E) The Sec translocation machinery

A number of proteins in the inner membrane make up the translocation machinery. These include the integral membrane proteins SecE, SecY, SecD and SecF, as well as the peripheral membrane protein SecA. The *sec* genes are located at different locations on the *E. coli* chromosome, with only *secD* and *secF* located together in an operon (Schatz and Beckwith, 1990). Early experiments identified suppressor mutations which restored translocation activity to LamB molecules containing altered signal sequences (Emr et al., 1981). These mutations were mapped to three different loci, one of which was termed *prlA*. Similar experiments located two other sites affecting protein export, which were termed *prlD* (Bankaitis and Bassford, 1985) and *prlG* (Stader et al., 1989). All of these loci have now been shown to contain at least one of the *sec* genes.

i) SecA

The *prlD* mutations were mapped to different sites within the *secA* gene (Fikes and Bassford, 1989). Mutations to *secA* had been shown to cause the accumulation of PhoA, LamB, OmpF and a *malE-lacZ* fusion protein in the *E. coli* cytoplasm (Oliver and Beckwith, 1981). A 92 kDa protein which fractionates as a peripheral inner membrane protein is encoded by *secA* (Oliver and Beckwith, 1982). It was observed that translocation of proOmpA across *E. coli* inner membrane vesicles could be inhibited by treatment of the vesicles with urea. Cytoplasmic extracts from an *E. coli* strain which overproduces SecA could restore the translocation activity, as could purified SecA (Cunningham et al., 1989). ProOmpA was shown to bind to the membrane vesicles by itself, but translocation would only proceed if SecA had bound to the membrane first.

SecA has been shown to contain an ATPase activity which is required for protein export (Lill et al., 1989). Using a photoreactive analogue of ATP it has been shown that SecA is selectively released from inner membrane vesicles upon UV-irradiation. The cross-linked SecA is unable to bind back to membranes to restore the translocation activity suggesting that release of the nucleotide plays an important role in SecA function. Breukink et al. (1992) found that binding to ATP also causes a conformational change in SecA which results in the insertion of SecA into the bilayer. SecA dissociates from the bilayer upon hydrolysis of the ATP and is able to repeat this cycle with the release of ADP (Breukink et al., 1992). This demonstrated that the ATPase activity is linked to the translocation activity of SecA. These studies, as well as site-directed mutagenesis studies of SecA, have identified both a high and low affinity ATP-binding domain (Mitchell and Oliver, 1993).

Cunningham and Wickner (1989) found that signal peptides can compete with proOmpA for binding sites on SecA, leading to speculation that SecA interacts with the

signal sequence. This inhibition by signal peptide competition has been found to occur early in the translocation process, and is not seen at later stages of export. Cross-linking studies revealed that OmpF which contained an uncleavable leader peptide would cross-link with SecA whereas mature OmpF would not (Akita et al., 1990). The interaction was enhanced as the number of basic residues in the leader sequence was increased, suggesting that the positively charged residues in the signal sequence were required for recognition and interaction with SecA. Cross-linking of different regions of SecA with proOmpF also identified an area close to, but distinct from the ATP binding site, which bound to proOmpF (Kimura et al., 1991). While signal peptides alone were unable to stabilize a membrane bound SecA complex, Lill et al. (1990) demonstrated that addition of mature OmpA along with the signal peptides did. This suggested that regions within the mature region of secreted proteins could stabilize the interaction between SecA and the membrane.

The ATPase activity of SecA has also been found to be stimulated by the acidic phospholipids PG and cardiolipin and by the integral membrane protein SecY (Lill et al., 1990). Liposomes which lacked PG were unable to bind with SecA, but fusion with liposomes containing PG restored the translocation competency (Hendrick and Wickner, 1991). Reduced efficiency of translocation due to decreased amounts of negatively charged phospholipids in the membrane could be reversed by the addition of elevated amounts of cytosolic SecA (Kusters et al., 1992). Insertion of SecA into the inner membrane has been shown to be the step which requires acidic phospholipids (Ulbrandt et al., 1992). This appears to be the cause of the effect noted earlier, which had led to the conclusion that signal peptides required acidic phospholipids for their insertion into the membrane as the first step of protein export (see above; de Vrije et al., 1988). Interaction with PG or cardiolipin, as well as with signal peptides, causes a change in the conformation of SecA, as it becomes more sensitive to proteolytic digestion in their presence but not in the presence of phosphatidylethanloamine (Shinkai et al., 1991).

It has been postulated that after dissociation, SecA may reassociate with the presecretory preprotein further along its C-terminus upon binding of another ATP molecule (Breukink et al., 1992). This cycle of events could repeat until the protein has completely moved across the inner membrane. Arkowitz and Wickner (1994) have shown that saturating concentrations of ATP in *in vitro* translocation assays allow for the complete SecA dependent translocation of proOmpA across membrane vesicles. Inactivation of SecA after the proOmpA protein has partially translocated across the membrane leads to reversal of the translocation process. Addition of SecA and ATP halted reversal but it was not inhibited by the addition of excess signal peptides (Schiebel et al., 1991). This indicated that SecA is able to promote export at later stages of the translocation process by binding to sites other than the signal sequence.

ii) SecY and SecE

As mentioned above, an interaction of SecA with SecY regulates its ATPase activity (Lill et al., 1990). The *secY* gene was mapped to the original *prlA* export mutation (Emr et al., 1980). SecY is a 30 kDa integral membrane protein which has been shown to contain 10 transmembrane segments, with 6 cytoplasmically exposed and 5 periplasmically exposed regions (Akiyama and Ito, 1987). Both the N- and C-termini of the protein were predicted to be exposed on the cytoplasmic side of the membrane. Antibodies made to SecY were used to disrupt the translocation of LamB and MBP. Neither of the preproteins was able to bind to the membrane when plasma membrane vesicles were first treated with α-SecY antibodies (Watanabe and Blobel, 1989). Antibodies specific to both the N- and C-termini of SecY were found to disrupt *in vitro* translocation of OmpF across proteoliposomes, confirming that both termini are exposed to the cytoplasm (Tokuda et al., 1990). SecY was found to interact with a second integral membrane protein, SecE (Bieker and Silhavy, 1990). SecE is a 13.6 kDa protein which crosses the membrane 3 times

(Schatz et al., 1989). The overproduction of SecE using a plasmid-encoded promoter results in the overproduction of SecY, suggesting the two are closely regulated together (Matsuyama et al., 1990).

Genetic studies using mutants of *secE* indicate that SecE interacts with SecY in the membrane to form a stable translocator complex (Bieker and Silhavy, 1990). Both SecY and SecE were found to be required for reconstitution of SecA ATPase-dependent translocation *in vitro* (Brundage et al., 1990). These two components were shown to form a complex in the inner membrane with a third component called band 1 (Brundage et al., 1992). All three of these proteins were co-precipitated from translocation-active membranes using α-SecY antibodies. These antibodies could also prevent the binding of SecA to inner membrane vesicles, suggesting that a SecY/SecE complex acts as a receptor for SecA (Hartl et al., 1990). Further evidence for this interaction came from the observation that addition of SecA protects the cytoplasmic domains of SecY from proteases (Hartl et al., 1990). SecA alone provides 60% protection against trypsin cleavage of SecY while SecA together with a SecB-proOmpA complex and ATP provides complete protection. Recent reports indicate that SecY is unstable and is rapidly degraded if uncomplexed with SecE (Taura, et al., 1993). This does not agree with the earlier observation that SecE and SecY remain unassociated in the inner membrane until SecE is bound by a SecA-preprotein complex (Bieker-Brady and Silhavy, 1992).

Protein-conducting channels which are similar in size to the ones observed in the rough ER membrane have now been found in the inner membrane of *E. coli* (Simon and Blobel, 1992). This indicates that a universal mechanism of protein translocation through membranes likely exists. It had been suggested earlier that SecY might play a role in forming a channel (Watanabe and Blobel, 1989). It has been found that SecY and SecE share homology with Sec61p (Gorlich et al., 1992b) and Sec61-γ (Hartmann et al., 1994) respectively, two of the putative channel forming proteins in the ER membrane. Using a

photoreactable cross-linking reagent incorporated into the mature regions of proOmpA, it was shown that proOmpA could cross-link to SecY and SecA but not to SecE, band 1 or to any phospholipids (Joly and Wickner, 1993). This suggested that proOmpA was shielded from the phospholipid bilayer by SecY, further evidence that SecY does take part in forming protein-conducting channels. One difference from the ER system that has been found is that binding of signal peptides, and not ribosomes, can open the *E. coli* channels (Simon and Blobel, 1992).

While a protein conducting channel can explain protein export, there must also be an explanation as to how inner membrane proteins are directed into the bilayer. If the observation by Bieker-Brady and Silhavy (1992) that SecY and SecE remain unassociated until SecA inserts into the membrane is true, then the translocation channel may be able to dissociate to allow the inner membrane proteins to enter the bilayer (Simon and Blobel, 1991). Simon and Blobel have suggested that the hydrophobic "stop transfer" signal found in inner membrane proteins may trigger this dissociation (See section on "Export of integral membrane proteins" below). Alternatively, the channels may contain gaps through which the proteins can be directed (Simon and Blobel, 1991).

iii) SecD and SecF

A fourth locus which affects export was located by using fusion proteins made between the signal sequence of PhoA and LacZ (Gardel et al., 1987). As was found in the earlier experiments of Bassford and Beckwith (1979) and Michaelis et al. (1983), these fusions became trapped in the inner membrane, resulting in an inactive LacZ moiety, unless a spontaneous mutation affected the signal sequence or one of the *sec* genes. Gardel et al. (1987) were able to identify two new genes, *secF* and *secD*, in a region of the *E. coli* chromosome separate from the other *sec* genes. These two genes were associated together as part of an operon (Gardel et al., 1990). Sequence analysis suggested that they encode

integral membrane proteins with large regions at the C-termini located in the periplasm (Gardel et al., 1990). The inability to produce *secD* or *secF* mutants by looking for suppressors of signal sequence defects in the earlier experiments (Emr et al., 1980) suggests that the proteins may act at a stage in the export process after the signal peptide has been cleaved off. This may also explain why large portions of the proteins are located in the periplasm. Addition of SecD or SecF did not affect the translocation activity of proteoliposomes composed of SecY, SecE and phospholipids, further suggesting that neither plays a role in the initial steps of translocation (Matsuyama et al., 1992). However, transposon mutagenesis of the *secD* and *secF* genes did prevent post-translational modification of *E. coli* prolipoprotein, resulting in accumulation in the inner membrane (Sugai and Wu, 1992). In addition,Pogliano and Beckwith (1994b) have shown that overexpression of SecD and SecF stimulated translocation *in vivo* and improved translocation of proteins with mutant signal sequences. These results support the view that both SecD and SecF play some role in export.

Matsuyama et al. (1993) demonstrated that anti-secD antibodies are able to inhibit export of OmpA and MBP across spheroplasts. Since a trypsin-sensitive form of MBP was exposed on the surface of these spheroplasts, it was proposed that SecD played a role in protein release from the export machinery, possibly by assisting in folding the exported proteins into their mature conformations (Matsuyama et al., 1993). This does not support the earlier findings of Brundage et al. (1990) who had found that neither SecD nor SecF was required for reconstituting OmpA export activity *in vitro*. Arkowitz and Wickner (1994) believe that this discrepancy can be explained by an effect that the proton motive force (PMF) has on protein export. They found that the *in vitro* export of preMBP and proOmpA did not require SecD or SecF if saturating concentrations of ATP were used. However, if subsaturating concentrations were used, proOmpA export required SecD, SecF and a PMF while preMBP export required a PMF and was stimulated 5-fold in the

presence of SecD and SecF. They suggested then that SecD and SecF interact with the PMF to stimulate export when concentrations of ATP are low (See section on energy requirements for export below).

The number of SecD molecules per *E. coli* cell has been estimated to be between 50 (Pogliano and Beckwith, 1994a) and 500 (Matsuyama et al., 1992). The latter corresponds to the estimates for SecY and SecE, suggesting that SecD might form a stoichiometric complex with SecY and SecE. It has been estimated that only 50 molecules of SecF exists per cell (Matsuyama et al., 1992; Pogliano and Beckwith, 1994a).

3. Export in Gram-positive bacteria

There do not appear to be major differences between the export of proteins by Gram-negative and by Gram-positive bacteria. Export by the latter also requires signal sequences which are similar to, and even interchangeable with, those for Gram-negative bacteria (Simonen and Palva, 1993; Wang et al., 1993a). Many components of the *sec* system have homologues in Gram-positive species. For example, the product of the *Bacillus subtilis divA* gene is homologous to *E. coli* SecA (Sadaie et al., 1991). Although it has not yet been shown that DivA plays a role in protein export, the N-terminal half of the DivA protein contains an ATP binding region similar to that found in SecA. In addition, the protein coded by a gene within the *B. subtilis spc* operon is homologous to SecY (Suh et al., 1990). In fact, the SecY homologue is able to complement an export defect in an *E. coli secY* mutant (Nakamura et al, 1990). An RNA molecule similar to the eukaryotic 7S SRP RNA and *E. coli* 4.5S RNA has also been found in *B. subtilis* (Struck et al., 1988). While its role in export has yet to be identified, deletion of the RNA gene resulted in defects in the expression of α-amylase and β-lactamase, which could be complimented by either the 7S SRP RNA or the 4.5S *E. coli* RNA (Nakamura et al., 1992).

One difference from *E. coli* is that *B. subtilis* requires a 33 kDa protein, encoded by the *prsA* gene, for export of a number of proteins (Kontinen et al., 1991). This protein shares no homology with any of the known *sec* gene products, but it appears to be homologous with the PrtM protein of *Lactococcus lactis*. Since PrtM is located on the cell wall side of the *L. lactis* cell membrane, it has been proposed that PrsA may have a similar location in *B. subtilis* and may play a role late in export (Simonen and Palva, 1993). There also seems to be a role for metal ions in the secretion of some *B. subtilis* proteins. Both Fe^{3+} and Ca^{2+} have been shown to increase the efficiency of export of the protein levansucrase (Chambert et al., 1990; Petit-Glatron et al., 1993). It appears that the metal ions catalyze the folding of the enzyme as it crosses the cell membrane. In the general export scheme suggested by Simon et al. (1992) it is proposed that this folding in part helps to energize the export process.

4. Export of integral membrane proteins

Most integral membrane proteins, in both eukaryotes and prokaryotes, are synthesized with typical signal peptides. As well, bacterial inner membrane proteins and ER proteins have been found to have transmembrane spanning α-helical regions (Singer, 1990; Nikaido and Saier, 1992). Such proteins utilize the *sec* machinery to cross into the inner membrane. However, it is not clear how they are released from the translocation channels. A stretch of 20 or more hydrophobic amino acids appears to be the signal required to release them into the ER (Verner and Schatz, 1988) or inner membrane (Singer, 1990). Simon and Blobel (1991) have suggested that the components of the translocation channels may dissociate when the hydrophobic region enters the channel. Alternatively, they suggested that the hydrophobic region may direct the proteins to gaps in the channels which they can slip through.

Bacterial outer membrane proteins are predominantly composed of anti-parallel-β-pleated sheets which can assemble into a β-barrel conformation in the outer membrane (Nikaido, 1992). They utilize the *sec* machinery 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). Most outer membrane proteins contain a Phe residue at the C-terminus which appears to be required for their insertion into the outer membrane (Struyve et al., 1991). OmpA has a Phe residue in the middle of the protein which is located at the end of the last membrane spanning region of the protein (Klose et al., 1988b) and is in a region that is homologous to the C-termini of other outer membrane proteins (Struyve et al., 1991). Klose et al. (1988a) found that deletion of this region results in the periplasmic accumulation of the deletion mutants. This suggests that the Phe residue and the last membrane spanning region are required for the correct localization of outer membrane proteins.

Mutations to the N-terminus of mature OmpA also result in the accumulation of the protein in the periplasm, suggesting that it is normally released there before incorporation into the outer membrane (Klose et al., 1988). Outer membrane proteins need not be directed to specific components in the periplasm for their insertion into the outer membrane as numerous studies have shown that they can spontaneously enter into lipid bilayers in an unfolded conformation (Eisele and Rosenbusch, 1990; Dornmair et al., 1990). On the other hand, there have been reports that lipopolysaccharide (LPS) is required to direct these porins into the outer membrane (Sen and Nikaido, 1991). The x-ray crystal structures of the *E. coli* OmpF, PhoE (Cowan et al., 1992) and *Rhodobacter capsulatus* (Weiss et al., 1991) outer membrane porins indicate that a number of charged acidic residues may be at the outer surface of the bilayer and it has been proposed that these regions could interact with the negatively charged groups on LPS through divalent cation bridges (Nikaido,

1994). It is not yet known if these proteins must fold into their final conformations before entering the membrane or if folding plays a role in the insertion process.

5. The energy requirements for protein export

As described earlier, export of proteins across the ER membrane requires the hydrolysis of GTP. This appears to be necessary for the binding and release of the SRP complex to the SRα and SRβ receptors. The driving force behind the movement of protein through the putative protein conducting channels has yet to be determined. It has been suggested that protein unfolding through the channel may power the translocation by way of "biased random thermal motion" (Simon et al., 1992). Factors such as chaperonins binding to translocating peptides, disulphide bond formation, coiling induced by pH or ionic gradients and glycosylations may play a role in the translocation process, but no evidence yet exists that any of these are involved.

Two energy sources play a role in the translocation process. Both ATP and the PMF across the inner membrane are used at different stages of protein export. Originally it was thought that the PMF alone was required for translocating proteins across the inner membrane (Bakker and Randall, 1984). Chen and Tai (1985) were later able to reconstitute export of OmpA and PhoA in *E. coli* inner membrane vesicles in the absence of a PMF. Translocation activity only required ATP. However, as first suggested by Geller et al. (1986) it now appears that both are important in the export of proteins.

A) The role of ATP in protein export

ATP is required in the early stages of translocation across the inner membrane (Lill et al., 1989). ATP binding to SecA is followed by insertion of the signal sequence and early portions of the protein through the secretion complex. Hydrolysis of the ATP leads to the dissociation of SecA from the SecY/SecE membrane complex followed by the release

of ADP (Schiebel et al., 1991). Chen and Tai (1985) as well as Geller and Green (1989) demonstrated that the subsequent steps in translocation could proceed using only ATP. In both cases, no PMF was required in reconstituting export activity *in vitro*. It was noted though that a PMF could enhance the translocation activity by up to five-fold (Geller et al., 1986). This enhancement could be diminished by increasing the levels of SecA in the reconstituted export system (Yamada et al., 1989). This suggests that SecA and ATP are utilized in the later stages of translocation and supports the theory described earlier (see section on SecA) that SecA sequentially binds to regions down the exported protein as the N-terminal regions cross the inner membrane (Breukink et al., 1992).

B) The role of the proton motive force in protein export

The role that the PMF plays in protein export is less clearly defined than for ATP. Initially it was believed that the charge difference across the inner membrane set up by the PMF could be used to move proteins by electrophoresis. However, model secretory proteins that lack any charged residues in their mature domain are exported like other proteins, discounting this possibility (Kato et al., 1992). Although the PMF does not appear to affect the initial insertion of exported proteins into the translocation complex, it may have a role in the dissociation of SecA from the complex. Shiozuka et al. (1990) found that the rate of translocation of an OmpF fusion protein *in vitro* was inhibited by the addition of ADP, but only in the absence of a PMF. The presence of a PMF lowered the level of ATP required for export. They speculated that the PMF helps to remove ADP from the translocation complex, causing SecA to dissociate and allowing the protein to proceed through the complex.

Following the initial steps of translocation, the PMF does not appear to be required, but it can increase the efficiency of the export process. In the absence of a PMF, translocation intermediates can be identified (Tani et al., 1989). These intermediates enter

the translocation complex in an ATP-dependent manner, then move slowly across the inner membrane in the absence of a PMF. They can be identified due to their increased susceptibility to proteases (Tani et al., 1989). Re-establishing a PMF allows the intermediates to move quickly across the membrane. Both the membrane potential ($\Delta\psi$) and the pH gradient (ΔpH) are able to increase the translocation rate (Driessen and Wickner, 1991). Since SecD and SecF stimulate the rate of translocation in the presence of a PMF (Arkowitz and Wickner, 1994) and are found on the periplasmic side of the inner membrane, it is possible that they play an important role in the PMF-associated mechanism of the later stages of protein export.

It has also been noted that if proOmpA contains an internal disulphide bond it will only be exported in the presence of a PMF. Under reducing conditions, ATP alone is sufficient to translocate the precursor (Tani et al., 1990). This would seem to indicate that it is the bulkiness of the molecule which determines the need for a PMF. This led to speculation that the PMF may regulate the size of the protein conducting channel, allowing larger molecules to be exported across the inner membrane (Geller, 1991; Pugsley, 1993a). The presence of a proline residue located near the signal sequence cleavage site of proOmpF lowers the PMF required for export (Lu et al., 1991). It was suggested that the proline increases the flexibility of the protein, diminishing the size of the channel required to allow the protein across the membrane.

The PMF has also been shown to prevent intermediates from moving backwards into the cytoplasm after the first ATP-dependent step (Schiebel et al., 1991). This backwards movement could also be prevented by the addition of ATP alone, but the greatest effect was observed in the presence of a PMF. Movement of proOmpA into proteoliposomes containing a PMF (inside acid and positively charged) could be supported by both ΔpH or $\Delta\psi$, and the direction could be reversed by reversing the polarity of the PMF (inside alkaline and negatively charged; Driessen, 1992). These results suggest that

the PMF is responsible for the direction of export across the inner membrane. However, none of these results are conclusive evidence that either ATP or the PMF is directly responsible for the translocation event. While ATP may help to initiate the process, and the PMF enhances the later stages by preventing any reverse translocation, the folding of the precursor to the mature protein may still power the movement of the protein through a channel, as has been suggested for ER proteins (Pugsley, 1993a; see above). However, it is possible that the translocation process is responsible for the unfolding of preprotein domains (Arkowitz et al., 1993). If this is the case, then other mechanisms will have to be suggested. The idea described earlier (see SecA section above; Schiebel et al., 1991), that SecA can bind sequentially to different regions of preproteins and push them through the export machinery by ATP hydrolysis, could fit this model. Since the PMF-associated enhancement of translocation seems to be mediated by SecD and SecF (Arkowitz and Wickner, 1994), it may be that SecD and SecF utilize the PMF to regulate the size of the opening in the translocation channel.

Translocation across the outer membrane of Gram-negative bacteria

While the mechanism of export across the ER membrane is similar to the export of proteins across the bacterial inner membrane, no such similarity appears to exist between the secretion of proteins across the eukaryotic cell membrane and the bacterial outer membrane. Eukaryotic proteins must cross through the Golgi apparatus before passing through the cell membrane. This is accomplished by successive blebbings of the ER and Golgi organelles, creating transport vesicles which contain the secreted protein. Fusion of the vesicles to the next organelle in line eventually delivers the proteins to the cell membrane, where their contents are released into the extracellular milieu by endocytosis. Much work on this process has shown that secreted eukaryotic proteins apparently have no secretion signals inherent in their primary or tertiary structures. Rather, secreted proteins

are allowed to pass through the different organelles because they lack a signal which would cause them to be retained. This has been described as the bulk flow default pathway (See Rothman and Orci, 1992 and Rothman, 1987 for reviews on this topic). While the ER retention signal has been well characterized (Munro and Pelham, 1987), only recently have transmembrane domains been identified as potential Golgi retention signals (Masibay et al., 1993; Nothwehr et al., 1993).

To be secreted by Gram-negative bacteria, proteins must cross the outer membrane after export across the inner membrane. The outer membrane is an asymmetric lipid bilayer composed of phospholipids in the inner leaf and LPS in the outer leaf (Nikaido and Vaara, 1985). The LPS is anchored to the outer membrane by the lipid A moiety, composed of a glucosamine disaccharide linked to 6 to 7 saturated fatty acid chains. Extending away from the outer membrane are polysaccharide chains linked to the lipid A. Due to the presence of negatively charged sugar residues, including KDO (2-keto-3-deoxyoctonic acid), these chains give the cell a hydrophilic surface which prevents hydrophobic compounds from entering. Small hydrophilic molecules are able to enter the cell through specific porin channels in the membrane while larger molecules are excluded. Thus the outer membrane acts as a protective layer as well as a selective permeability barrier (Hancock, 1991).

To date, there has been no evidence that proteins are transported from the inner to outer membrane in transport vesicles. Early electron microscopy studies of prokaryotic cells revealed areas ("Bayer bridges") in which the inner and outer membranes made contact with each other (Bayer, 1979). It was proposed at the time that these may be areas at which secreted proteins could cross both membranes in a single step. It has been possible to isolate membrane fractions on sucrose density gradients which are more dense than inner membrane fractions but less dense than outer membrane fractions (Ishidate et al., 1986). It was thought that these fractions demonstrated the existence of Bayer bridges. However, other studies suggest that Bayer bridges may be artifacts resulting from the

preparation of cells for the electron microscope and that they may not exist *in vivo* (Hobot et al., 1984; Kellenberger, 1990).

While there are some proteins which appear to cross both membranes in a single step (see section on *E. coli* α-hemolysin below) most secreted proteins appear to pass into a compartment between the two membranes before crossing the outer membrane. This compartment is called the periplasmic space. The width of this space has been a topic of controversy, with different techniques used in preparing samples for electron microscopy resulting in different values. However, the accepted range appears to be within 10-25 nm (Graham and Beveridge, 1991). The periplasm contains a number of proteins used for binding molecules as well as the peptide cross-linked carbohydrate polymer peptidoglycan. This polymer layer is linked to the outer membrane by lipoproteins and is responsible for maintaining the shape of the cells (Oliver, 1987). Peptidoglycan polymers which are not cross-linked are also found in the periplasmic space. It was proposed that these unlinked polymers could increase the viscosity of the periplasm, creating a gel which could maintain a regular distance between the inner and outer membranes (Hobot et al., 1984). Such a gel should be viscous, resulting in the slow diffusion of proteins within the periplasm. This was demonstrated by Brass et al. (1986) who used fluorescently labelled MBP to measure the rate of diffusion in the periplasm. By irreversibly photobleaching a small area of *E. coli* cells containing the labelled MBP with a laser, they were able to measure the diffusion of MBP back into the area by measuring the fluorescence. They found that the rate of diffusion was 1000-fold slower than that expected for diffusion in a non-viscous aqueous environment.

A number of different proteins have now been found to be actively secreted by a variety of Gram-negative bacteria (for reviews see Hirst and Welch, 1985; Pugsley, 1993a). However, there is no single secretion pathway common to all Gram-negative bacteria. Different Gram-negative organisms use a number of different pathways for

secreting their extracellular proteins. In the first of these pathways, it appears that all of the machinery required for secretion is contained within a single gene product. A signal sequence directs the secreted protein into the periplasm via the *sec* system while the C-terminal domain of the protein helps to transport it across the outer membrane. The IgA protease of *Neisseria gonorrhoeae* is the best characterized protein which utilizes this pathway. Another family of proteins has no typical signal sequence and appears to cross both the inner and outer membranes in a single step with the help of a pair of integral membrane proteins. The α-hemolysin of *E. coli* has been the most extensively studied protein from this group. The majority of the remaining secreted proteins use the general secretion pathway (GSP). In this pathway, proteins use the *sec* system for export into the periplasm, but then depend on a series of 13-15 proteins in the inner and outer membranes to facilitate their translocation across the outer membrane. The *Klebsiella oxytoca* GSP was the first for which the complete secretion machinery was identified. Finally, the secretion of pertussis toxin has recently been found to utilize a novel pathway.

1. The secretion of *Neisseria gonorrhoeae* IgA protease

The IgA protease of *N. gonorrhoeae* was the first protein identified which apparently contains all that is needed for its own secretion. It is synthesized as a 169 kDa protein, while the mature active form located in the extracellular milieu has a molecular mass of 106 kDa (Pohlner et al., 1987). Sequencing the *iga* gene confirmed that a 169 kDa protein should be expressed. Four different domains were identified in this larger species: i) A signal sequence containing N-terminus, ii) the protease, (IgA_P) located next to the signal sequence and which corresponds to the 106 kDa species observed outside the cell, iii) a region at the C-terminus containing a high degree of β-sheet structure (the β-domain), and iv) a linker region (the α-domain) between the protease and C-terminal domains.

The protease contains a typical N-terminal 27 amino acid signal sequence which directs the precursor across the inner membrane using the *sec* system. Once the signal has been cleaved off, a 1505 precursor can be found in the periplasm (Pohlner et al., 1987). Deletion of the C-terminal portions of this protein results in accumulation of the mutant proteins in the periplasm. This was evidence that the β-domain is necessary for secretion across the outer membrane (Pohlner et al., 1987). When *iga* was cloned into *E. coli* , the protease was successfully secreted. This suggested that the *iga* gene product contains everything that is required for IgA protease secretion. It appears that the β-domain integrates into the outer membrane from the periplasm, followed by exposure of IgA_P and the α-domain on the surface, and finally separation from the β-domain by autoproteolysis (Klauser et al., 1992). The autoproteolytic event occurs after IgA_P has folded into its mature conformation, producing an active protease. Finally, autoproteolytic separation of IgA_P and the α-domain occurs in the extracellular milieu to produce the mature protease. Consensus IgA protease cleavage sites are found at both ends of the α-domain (Klauser et al., 1993b).

The region of the β-domain required for outer membrane insertion has been localized to the 274 amino acids at the C-terminus. This core region is predicted to form a β-barrel conformation like those found in many outer membrane proteins (Klauser et al., 1993a). Like outer membrane proteins, the β-domain core of IgA protease has a hydrophobic consensus sequence at its C-terminus which ends with a Phe residue (Struyve et al., 1991). This led Klauser et al. (1993b) to propose a model in which the β-domain inserts into the outer membrane, making a channel through which the IgA_P can pass. Support for this model has been obtained by using hybrid proteins in which the β-domain is linked to the B subunit of cholera toxin (CtxB). Expression in *Salmonella typhimurium* results in exposure of the hybrid protein on the cell surface (Klauser et al., 1990). The B subunit can be released from the β-domain, and into the culture supernatant by the addition

of trypsin or purified IgA protease. Expression of the hybrid proteins in *E. coli* results in release of the B subunit into the supernatant without the need for added proteases (Klauser et al., 1992). This has been shown to be due to proteolytic cleavage by the *E. coli* outer membrane protease OmpT.

The IgA protease system only transports unfolded proteins through the outer membrane. Thus translocation of the CtxB moiety of the fusion product described above only occurs in the presence of a reducing agent such as β-mercaptoethanol (Klauser et al., 1990, 1992) or in *E. coli* cells lacking the DsbA protein (required for disulphide bond formation in the periplasm; Klauser et al., 1993b). Secretion of the IgA protease itself is unaffected by DsbA and does not require β-mercaptoethanol, but this protein only contains 2 cysteines which are in close proximity to each other and which may not form a disulphide bridge (Pohlner et al., 1987). If a loop does form, it may be small enough to allow the protein to pass through the pore (Klauser et al., 1993b).

Other proteins have been identified that use an identical secretion mechanism. The IgA protease of *Haemophilus influenzae* (Poulsen et al., 1989), a cytotoxin of *Helicobacter pylori* (Klauser et al., 1993b) and the *Serratia marcescens* serine protease (Miyazaki et al., 1989) all contain C-terminal domains capable of inserting into the outer membrane and making potential channels for secretion.

Several other proteins are secreted by systems that share some properties with the IgA protease system. The hemolysin of *S. marcescens* is synthesized as a 165 kDa precursor (ShlA*; Poole et al., 1988). Secretion of ShlA* and activation to the mature ShlA form have been shown to require a 62 kDa outer membrane protein, ShlB (Schiebel et al., 1989). Both ShlB and ShlA* are synthesized with typical signal sequences which presumably allow their export across the inner membrane. It was found that ShlA* and ShlA had the same mobilities on SDS polyacrylamide gels, but ShlA* was sensitive to trypsin digestion while ShlA was resistant (Schiebel and Braun, 1989). It is not yet known

what the change is that differentiates ShlA from ShlA*, but it has been suggested that ShlB causes a conformational change or a chemical modification in ShlA. It is known that ShlB does not proteolytically cleave ShlA at the N-terminus, as the N-terminal sequence of both ShlA and ShlA* are identical (Schiebel et al., 1989). It is also not clear whether the change in ShlA* is required for the secretion of the hemolysin across the outer membrane.

The N-terminal 238 amino acids of ShlA* are required for secretion, as deletion mutagenesis of this region prevented ShlA* translocation across the outer membrane (Schonherr et al., 1993). As well, site-directed mutagenesis of two Asn residues in this region prevented the secretion of ShlA* (Schonherr et al., 1993). There may be a signal in this region which allows ShlA* to interact with ShlB. It has been suggested that ShlB could play a role similar to that of the β-domain of the IgA protease. Since secondary structure analysis predicts a high degree of amphipathic β-pleated sheet structure, ShlB may form a secretion channel in the outer membrane similar to the one made by the IgA protease β-domain (Schiebel et al., 1989). The hemolysin from *Proteus mirabilis* (HpmA; Uphoff and Welch, 1990) and the *B. pertussis* Fim/Fha hemagglutinin and fimbriae (Willems et al., 1994) also utilize a single outer membrane protein homologous to ShlB for their secretion. In fact, ShlB was able to activate the *P. mirabilis* HpmA hemolysin *in vitro* (Ondraczek et al., 1992). However, no studies have yet been done to see if ShlB can promote secretion of these heterologous proteins.

2. The secretion of *Escherichia coli* α-hemolysin

A number of strains of *Escherichia coli* secrete an α-hemolysin (HlyA). It has been shown that non-hemolytic *E. coli* strains are avirulent for rats, suggesting that HlyA is an important virulence factor. In agreement with this idea are results obtained by Welch et al. (1981) who found that *E. coli* could be transformed into a virulent strain by introducing the *hlyA* gene (and the genes required for HlyA transport) from a virulent *E. coli* isolated from

a human subject. Insertion of a transposon into the *hlyA* plasmid returned the strain to its avirulent phenotype.

It was originally found that all the determinants necessary for HlyA synthesis and secretion are located on a single transmissible plasmid (Noegel et al., 1979). Using transposon mutagenesis and restriction mapping, three different regions were identified which affected HlyA production and secretion (Noegel et al., 1981). The largest region, called cistron A, and a smaller upstream region called cistron C, are both required for hemolytic activity (now called *hlyA* and *hlyC* respectively). HlyC has been shown to assist in the acylation of HlyA, causing the activation of the inactive proHlyA *in vitro* and *in vivo* (Hardie et al., 1991; Issartel et al., 1991). The third region, cistron B, is required for the secretion of HlyA into the culture supernatant. Cistron B was found to contain two genes, originally called $hlyB_a$ and $hlyB_b$ (Wagner et. al., 1983; now termed *hlyB* and *hlyD* respectively). A fourth component, TolC, which is not part of the Hly operon, is also involved in hemolysin secretion. The role of each of the gene products is discussed below.

A) The HlyA protein

The *hlyA* gene isolated from a plasmid source was originally found to produce a protein estimated to be 107 kDa in size (Goebel and Hedgpeth, 1982). Early evidence suggested that this protein was proteolytically cleaved in the periplasm to an active 58 kDa product which crossed the outer membrane in *E. coli* minicells. It was later found that the 107 kDa species was the secreted form of HlyA (Mackman and Holland, 1984a; 1984b; Gonzalez-Carrero et al., 1985). A chromosomally encoded HlyA was sequenced and the predicted mass of the protein was determined to be 110 kDa (Felmlee et al., 1985a). The *hlyA* gene isolated from the plasmid also encodes a protein of 110 kDa, with 97.7% homology with the chromosomal HlyA (Hess et al., 1986).

Examination of the determined HlyA amino acid sequence reveals that it does not contain a typical signal sequence. As well, HlyA is released from the cell with its N-terminus unprocessed (Felmlee et al., 1985a). This shows that HlyA does not use the *sec* pathway to cross the inner membrane. Surprisingly then, fusions of the HlyA N-terminus to PhoA resulted in an active PhoA fusion protein. However, these fusions did not require HlyB or HlyD for this to occur. This indicated that a portion of PhoA had crossed the inner membrane but it did not use the normal hemolysin secretion machinery (Erb et al., 1987). Thus a region of the HlyA N-terminus was proposed to be able to enter into the lipid bilayer without assistance from HlyB or HlyD (Erb et al., 1987). However, only the C-terminus has been shown to be required for secretion of HlyA (see below).

Once it was clear that no identifiable signal sequences were located in the N-terminal regions of HlyA, other regions of the protein were examined for possible signals. The first evidence for a secretion signal was found by fusing the C-terminal 23 kDa region of HlyA in frame with the *tac* promoter. The resulting C-terminal peptide was secreted into the extracellular milieu, but only if HlyB and HlyD were also present (Nicaud et al., 1986). This identified the final 218 amino acids as containing the secretion signal. This region was shown to be necessary for secretion by studying OmpF-HlyA fusion proteins. These constructs lacked an OmpF signal sequence, but contained the β-galactosidase promoter and its first 8 amino acids, as well as varying portions of the HlyA C-terminus (Mackman et al., 1987). The presence of the 23 kDa C-terminal region enabled secretion of the chimeric protein into the culture supernatant, and none of the protein was detected intracellularly. Both the chimeric protein and the 23 kDa fragment alone could only be detected in the cell if HlyB and HlyD were not present. When the C-terminal 25 amino acids of PhoA were replaced with the final 60 amino acids of the HlyA C-terminal sequence, PhoA was also secreted into the culture supernatant in the presence of HlyB and HlyD (Gentschev et. al, 1990). Even though these fusions contained the native PhoA

signal sequence, no PhoA activity was observed without HlyB and HlyD. This suggested that the C-terminus of PhoA also contained information required for export by the *sec* system, possibly a recognition site for a chaperone protein.

Two other hemolysins, from *Proteus vulgaris* and *Morganella morganii*, are closely related to HlyA and both can be secreted by the *E. coli hlyB* and *hlyD* genes (Koronakis et al., 1987). Comparison of the C-terminal sequences of all three proteins revealed a high degree of divergence in primary structure. However, there appear to be 3 conserved features at the C-terminus of each: a hydrophobic sequence rich in hydroxylated residues (the 27 amino acids at the C-terminus; region III); a cluster of charged residues (29-34 amino acids from the C-terminus; region II); and a potential amphiphilic α-helix (34-52 amino acids from the C-terminus; region I). Deletion mutagenesis in any of these regions of *E. coli hlyA* results in complete elimination of Hly A secretion (Koronakis et al., 1989). In each case, HlyA is located intracellularly, but not in the periplasm. In another study using the previously described PhoA-HlyA fusions, deletions were made in regions I, II and III of HlyA (Hess et al., 1990). While deletion of region I caused a dramatic decrease in secretion of the fusion protein, replacement of this region by a non-amphiphilic, nine amino acid peptide containing two prolines resulted in almost complete restoration of secretion. This could mean that the amphiphilic nature of region I may not be important, and this region may only act to separate the C-terminal signal sequence from the rest of the protein, exposing it to the HlyB/HlyD secretion complex. Deletion of regions II and III also resulted in the loss of secretion ability. Fusion of either region II or III to PhoA resulted in low levels of secretion, indicating that these two regions are able to interact with the secretion machinery. However, the translocation activity of the individual regions appeared to be weak and it was proposed that there might be a cooperative interaction between the two.

A series of other HlyA fusions was made to N-terminal regions of the *E. coli* cytoplasmic proteins chloramphenicol acetyltransferase (CAT) and LacZ, as well as to the eukaryotic protein prochymosin (PCM; Kenny et al., 1991). Fusions containing either the N-terminal half of PCM or the full length PCM molecule were secreted in an HlyB and HlyD-dependent manner. However, it was noted that the full length PCM fusion was secreted at a lower efficiency. In addition, fusion of PCM to longer HlyA C-terminal sequences resulted in up to a 5-fold increase in secretion efficiency. Equivalent results were observed for the HlyA-CAT fusions. HlyA-LacZ fusions were only secreted when expressed at low levels. These results suggest that if cytoplasmic proteins are fused to the C-terminus of HlyA, secretion may be inhibited if enough of the cytoplasmic protein remains to allow folding into non-secretory conformations. It was also noted that the secretion signal may not be recognized efficiently if it is not separated from the passenger protein, as placement of linker regions between the C-terminus of HlyA and the PCM resulted in increased secretion efficiency. The passenger protein may interfere with presentation of the signal sequence to the HlyB/HlyD complex if it is too close to the signal (Kenny et al., 1991).

The C-terminus signal was further dissected by making several mutants using site-directed mutagenesis. In one study, it was concluded that the three regions identified earlier are the most important determinants in secretion of HlyA (Stanley et al., 1991). The final 48 amino acids were shown to be critical, but the proposed amphipathic helix (amino acids 973-990) was found to be unnecessary, as mutations which disrupted the helix did not prevent secretion. Two charged residues, E979 and K982, were found to be necessary for secretion, and it was proposed that the two might form a salt bridge which help to stabilize a critical helical structure. Replacement of any of the six hydroxylated amino acids at the very C-terminal region results in a decreased secretion efficiency (Stanley et al., 1991). When more than one of these residues is changed, a further decrease in secretion

efficiency is observed (69% decrease when 2 were changed; up to 100% decrease when all 6 were changed). It was proposed that this region would form two short β-sheets, separated by a β-turn. This motif was later shown to be critical by replacing the HlyA C-terminus with the *Pasteurella hemolytica* leukotoxin (LktA) C-terminus (Zhang et al., 1993). This protein could be secreted with the help of HlyB and HlyD, but examination of the C-terminal sequence revealed no homology with HlyA. Secondary structure analysis of the sequence did predict the β-sheet-β-turn-β-sheet motif at the extreme C-terminus, as well as the helix-turn-helix motif seen earlier for regions I and II. A second study, again using oligonucleotide-directed mutagenesis, identified four amino acids within the 46 residue C-terminus that are essential for maximal HlyA secretion (Kenny et al., 1992). It was postulated that these four residues (E-978, F-989, D-990 and D-1009) make contact with specific points on the HlyB/HlyD translocator complex and that they act co-operatively, since mutations to single residues resulted in only slight decreases in secretion. It was argued that the translocator complex may be able to recognize these specific residues and not a higher order structural motif as had been suggested by other groups. This point still remains to be clarified by further studies.

B) The hemolysin translocation machinery, HlyB, HlyD and TolC

It was known early on that HlyB and HlyD were required for HlyA secretion and likely interacted to form some kind of translocation complex (Wagner et al., 1983). Early reports that intracellular pools of hemolysin could be detected in wild type cells and in *hlyB* mutants suggested that hemolysin crossed the inner and outer membranes in two distinct steps. Deletion mutants of *hlyB* were originally found to accumulate in the periplasm while *hlyD* deletion mutants had a large amount of HlyA associated with the outer membrane (Wagner et al., 1983). This led to the theory that HlyB acted after HlyA had crossed the inner membrane and that HlyD was required for release of HlyA from the outer

membrane. However, it now appears that these two proteins act together to bridge both membranes.

Sequence analysis of HlyB predicted 8 membrane spanning regions in the N-terminal region and a cytoplasmic tail at the C-terminal end of the protein. This was confirmed using fusions between HlyB and the proteins PhoA, LacZ and β-lactamase (Wang et al., 1991; Gentschev and Goebel, 1992). An interesting feature of HlyB is that its C-terminus is homologous with the eukaryotic plasma membrane protein, P-glycoprotein (Gerlach et al., 1986) which has been implicated in multidrug resistance in tumor cells. It contains a nucleotide-binding domain, similar to those found in many other transporter proteins (Gerlach et al., 1986; Fath and Kolter, 1993). This suggests that HlyB is able to couple energy from ATP to the secretion of HlyA. A proton motive force had also been implicated in the initial steps of the secretion process, as the proton ionophore carbonylcyanide *m*-chlorophenylhydrazone (CCCP) and potassium ionophore valinomycin both inhibit secretion at a step before HlyA associates with the inner membrane (Koronakis et al., 1991). Later stages of the secretion process are not affected by these inhibitors, but metabolic inhibitors (KCN; Springer and Goebel, 1980) and specific P-type ATPase inhibitors (Koronakis et al., 1993) completely inhibit secretion at a later stage.

The C-terminal region of HlyB has been fused to glutathione S-transferase (GST) in order to examine the possibility that binding of ATP causes a conformational change (Koronakis et al., 1993). The GST-HlyB fusion alone was completely degraded by proteinase K over a 60 min period. However, addition of Mg^{2+} and ATP or ADP almost fully protected the fusion protein from proteinase K digestion. Non-hydrolyzable analogues of ATP were also able to protect the fusion protein, indicating that nucleotide binding to HlyB caused a conformational change which protected the protein from proteolytic degradation (Koronakis et. al., 1993).

While early cell fractionation studies suggested that HlyD was located in the outer membrane (Wagner et al., 1983), sequence analysis predicted an integral inner membrane protein with a single membrane spanning region between amino acids 60-80 (Schulein et al., 1992). Fusions to LacZ and PhoA were located in the inner membrane, confirming the predictions (Schulein et al., 1992). The N-terminus was found to be in the cytoplasm while the remaining 400 amino acids were located in the periplasm. The C-terminus is thought to span the periplasm, possibly interacting with TolC.

While the translocation machinery was first thought to consist of only the 54.6 kDa HlyD and the HlyB protein (believed to be 79.9 kDa from sequence data), a third component, the outer membraned protein TolC, was later shown to be involved in HlyA secretion. It was identified by its sequence homology with one of the *Erwinia chrysanthemi* protease secretion components. In this operon, *prtD* and *prtE* encode proteins that are homologous to HlyB and HlyD respectively. A third component of the operon, *prtF*, which is necessary for secretion of three proteases (PrtA, PrtB and PrtC), was shown to resemble *E. coli* TolC (Wandersman and Delepelaire, 1990). When *tolC* was mutated in *E. coli* cells containing the HlyA secretion machinery, release of HlyA was blocked and only an intracellular pool of hemolysin could be detected. *E. coli* mutants which are defective in synthesizing LPS were shown to secrete HlyA with decreased efficiency (Wandersman and Letoffe, 1993). TolC production was decreased in these mutants, leading to speculation that TolC requires LPS for insertion into the outer membrane, as has been speculated for the outer membrane porins (See section on integral membrane proteins above).

To better understand the translocation machinery and how HlyA interacts with it, the cellular localization of HlyA was identified in HlyB and HlyD mutants (Oropeza-Wekerle et al., 1990). Fractionation of cells lacking a functional HlyB showed that HlyA was associated with the inner membrane, whether or not there was a functional HlyD

present. A HlyA construct lacking amino acids 9-37 at the N-terminus was also found associated with the inner membrane. This suggested that any information at the N-terminus that is required for insertion in the inner membrane is located further along the protein (Erb et al., 1987). More recent immunological studies have also shown HlyB to be exclusively in the inner membrane fractions (Juranka et al., 1992). Since similar amounts of HlyA were found intracellularly in $HlyB^-$ $HlyD^-$ as in $HlyB^-$ $HlyD^+$ mutants, it was concluded that HlyA must first interact with HlyB.

A short 34 amino acid hydrophobic stretch from the tetracycline resistance determinant (TetC) was fused with the C-terminal 240 amino acids of HlyB to determine the importance of this region of HlyB. Secretion of HlyA still occurred, albeit with decreased efficiency. It depended on HlyD and TolC, as HlyA accumulated in the periplasm of $HlyD^-$ or $TolC^-$ mutants (Thomas et al., 1992). Since the small 34 amino acid N-terminal fragment from TetC was only long enough to anchor the fusion protein in the membrane, it was concluded that the C-terminus of HlyB is sufficient for HlyA secretion, while the N-terminus is required for optimal secretion levels. The results also suggested that the C-terminus is sufficient for interaction with other components of the translocation machinery. Since it appears that TolC and HlyD are required later in the translocation process, it has been proposed that they may form a pore in the outer membrane through which HlyA is secreted (Fath and Kolter, 1993). However, no experimental evidence yet exists which demonstrates an interaction between HlyD and TolC.

C) Other HlyA-like bacterial secretion systems

Outer membrane proteins similar to TolC have been shown to be necessary for the secretion of the *Bordetella pertussis* adenylate cyclase (CyaA; Glaser et al., 1988), the *Pseudomonas aeruginosa* alkaline protease (AprA; Guzzo et al., 1991) and the *P. vulgaris* α-hemolysin (HlyA; Koronakis et al., 1988). The third component of the cyclolysin and

alkaline protease translocators is linked closely to the other secretion genes (CyaE and AprF respectively) while the *P. vulgaris* hemolysin uses a TolC homologue which is not linked to the other translocator genes. An outer membrane protein has not yet been shown to be required for the secretion of the *M. morganii* α-hemolysin or the *P. haemolytica* leukotoxin (LktA). All of these secreted proteins lack a typical N-terminal signal sequence but contain an HlyA-like signal at the C-terminus. Proteins analagous to HlyB and HlyD have been identified in each of these species as well (Fath and Kolter, 1993). With the close similarities between these systems it was expected that *E. coli* containing the HlyA system would likely be able to secrete the other heterologous proteins. It was found that the HlyB/HlyD/TolC translocator was able to secrete CyaA (Masure et al., 1990), LktA (Chang et al., 1989) and the two α-hemolysins from *M. Morganii* and *P. vulgaris* (Koronakis et al., 1987) at normal levels. However, AprA was secreted with a decreased efficiency (Guzzo et al., 1991) while PrtB was only secreted at 2% of HlyA secretion (Delepelaire and Wandersman, 1990). This indicates that there is some species specificity in these systems.

3. The general secretion pathway

A) Secretion of *Klebsiella oxytoca* pullulanase

The Gram-negative bacteria *Klebsiella oxytoca* (originally *Klebsiella pneumoniae*) secretes the 145 kDa fatty acylated protein pullulanase (PulA; Pugsley et al., 1986). This enzyme catalyzes the hydrolysis of (1→6) α-linkages in starch. PulA contains a typical 19 amino acid signal sequence which directs it to the *sec* machinery for export across the inner membrane (Pugsley et al., 1991a). The amino acid sequence at the signal sequence cleavage site, Leu-Leu-Ser-Gly-Cys-Asp, is the consensus sequence for cleavage by lipoprotein signal peptidase (Pugsley and Schwartz, 1985). The lipoprotein signal

peptidase cleaves on the N-terminal side of the Cys residue after it has been fatty acylated (Pugsley et al., 1986). The acylation of pullulanase is not absolutely required for secretion of the protein, as non-acylated pullulanase is also secreted (Kornacker et al., 1989). Further, PulA mutants in which the Cys^{+1} residue is replaced by Leu (Kornacker et al., 1991) or Ser (Murooka and Ikeda, 1989) are not acylated but are secreted at reduced rates. After PulA crosses the outer membrane, it remains anchored to the membrane until it is proteolytically cleaved and released into the medium. Acylation appears to be necessary for anchoring of PulA to the membrane. Pronase is able to release PulA from the outer membrane, but it is not known which protease is used *in vivo* (Pugsley et al., 1990; Pugsley, 1993a).

When d'Enfert et al. (1987) cloned the structural gene for pullulanase (*pulA*) into *E. coli*, no secretion was observed and PulA was located inside the cell. However, when they used an 18.8 kb fragment containing *pulA* and its surrounding genes, PulA was secreted by *E. coli* (d'Enfert et al., 1987). Transposon mutagenesis of this fragment located 15 genes both upstream and downstream of the *pulA* gene which affected PulA secretion. Thirteen are located 5' to *pulA* and are transcribed in the opposite orientation (d'Enfert et al., 1987). A pair of genes are located at the 3' end of *pulA*. The *pulB* gene is located next to *pulA* , while *pulS* is located next to *pulB* but in the opposite orientation (d'Enfert and Pugsley, 1989). The outer membrane lipoprotein PulS, but not PulB, is necessary for PulA secretion in *E. coli.* (d'Enfert and Pugsley, 1989).

The first two genes characterized were *pulC* and *pulD* which are located 5' to *pulA* (d'Enfert et al., 1989). The *pulC* gene was found to encode a 31 kDa gene product, while the *pulD* gene product was found to be 70.6 kDa. Fusions of both were made to alkaline phosphatase and fractionation of cells containing the fusions were used to identify their locations. PulC fractionated with inner membrane fractions on sucrose density gradients,

while PulD was associated with outer membrane fractions (d'Enfert et al., 1989). In later studies, genes *pulF, pulK, pulL, pulM* and *pulN* were all found to encode integral inner membrane proteins ranging in size from 18-44 kDa (Pugsley and Reyss, 1990; Possot et al., 1992), while *pulE* encodes a 58 kDa cytoplasmic protein (Possot et al., 1992). PulE contains two sequences known as 'Walker boxes', which are common to proteins that bind ATP (Walker et al., 1982). They are similar to the ATP-binding motifs found in HlyB, the *E. coli* hemolysin transporter protein (see section on *E. coli* α-hemolysin above).

The gene products of *pulG, pulH, pulI*, and *pulJ* have also been shown to be integral membrane proteins (Reyss and Pugsley, 1990). These proteins differ from the other membrane components of the *pul* system in that they do not contain typical signal sequences. Instead, they have the consensus prepilin signal peptide sequence first identified in *Pseudomonas aeruginosa* (Pugsley and Dupuy, 1992). This species produces a type IV pilin composed of a protein (PilA) which has a 6 amino acid signal sequence (Johnson et al., 1986). This signal is positively charged, lacks the hydrophobic region of typical sequences and is cleaved on the N-terminal side of a Phe or Met in the consensus sequence Gly-(Phe or Met)-Thr-Leu-X-Glu-X_{16-18} (X = a hydrophobic amino acid; Nunn and Lory, 1991). A prepilin peptidase encoded by the *pilD* gene in *P. aeruginosa* specifically cleaves this signal and not the *sec* system signal sequence (Nunn and Lory, 1991). Strom et al. (1991) have shown that mutations in *pilD* not only affect the export of PilA but also the secretion of other extracellular proteins. Since PulG, PulH, PulI and PulJ contain type IV signal sequences, it was proposed that *K. oxytoca* must also have a PilD-like protein. In fact, the final gene in the *pul* operon, *pulO*, was found to encode such a protein. PulO is able to cleave the type IV prepilin signal sequence of the four *pul* operon proteins (Pugsley and Dupuy, 1992) and the signal peptide of a gonococcal type IV prepilin protein (Dupuy et al., 1992). As well, it can methylate the Phe residue at +1 of the mature

PulG protein (Pugsley, 1993b) in the same way PilD can methylate its pilin proteins (Strom et al., 1993). Thus PulO is required in the GSP for the correct processing and localization of four other Pul proteins.

The discovery that the GSP utilizes type IV pilin-like proteins led to speculation that a pilin-like structure may be involved in the secretion mechanism. Since pilin proteins oligomerize in the periplasm before being translocated outside the cell, Pugsley and Possot (1993) proposed that one of the type IV pilin-like Pul proteins may oligomerize into a structure which crosses the periplasm and plays a role in secretion. They tried cross-linking experiments in *E. coli* containing the *pul* operon to see if any PulG oligomers could be located. However, only homodimers of PulG were found, with no evidence for larger multiprotein complexes.

B) The general secretion pathway in other bacteria

A *pul*-like operon encoding the machinery for a GSP has now been found in a number of different bacteria. These include *P. aeruginosa, Erwinia carotovora, Erwinia chrysanthemi, Xanthomonas campestris, Aeromonas hydrophila* and *Vibrio cholerae.* While all contain many of the elements found in the *pul* system, each differs from this system to varying degrees.

P. aeruginosa contains secretion genes (called the *xcp* genes) which are homologous to the *pul* secretion genes (Bally et al., 1992). Genes *xcpR-Z* are related to *PulE-M* of *K. oxytoca*, but no homologues to *pulC*, *pulD* (the first two genes in the *pul* operon), *pulN* and *pulO* (the last two genes in the *pul* operon) are located in the *xcp* operon (Filloux et al., 1990; Bally et al., 1992). Homologues to *pulC* and *pulD* (*xcpP* and *xcpQ*)

have been found close to, but not in, the *xcp* operon (Akrim et al., 1993). As described above, the *pulO* homologue (originally identified as *xcpA* but now known as *pilD*; Nunn et al., 1990) was found to reside in an operon required for type IV pilin biogenesis. As with PulO, PilD functions to properly process the *P. aeruginosa* proteins XcpT-XcpW for their proper localization in the inner membrane and functioning in the GSP (Nunn and Lory, 1992, 1993). This explains why mutations to *pilD* result in pleiotropic secretion mutants (Bally et al., 1989, 1991). Other *P. aeruginosa pil* proteins also have similarities to various Pul proteins. PilB, located within the *pil* operon, was found to be homologous to the XcpR/PulE proteins and it contains the expected ATP-binding motif (Turner et al., 1993). PilQ, which is located outside the *pil* operon, shows homology to the outer membrane protein PulD (Martin et al., 1993). While both PilB and PilQ are required for pilin biogenesis, neither has been shown to have any role in the secretion of pilin or other proteins. No homologues to PulS or PulB have yet been found for *P. aeruginosa*.

To date, only *E. carotovora* has been found to contain homologues to all 15 of the *pul* secretion proteins (Reeves et al., 1993). The *outB-O* genes and *outS* gene of *E. carotovora* operon are homologous to the *pulB-O* and *pulS* genes. As well, *outO* is located at the end of the *out* operon. One difference in the *E. carotovora out* system is that OutB is required for extracellular secretion in *E. coli* (Condemine et al., 1992). It has been speculated that *E. coli* may contain a PulB homologue which can interact with the *pul* system but not the *E. carotovora outB* system (Pugsley, 1993a). As with *P. aeruginosa*, homologues to all the *pul* proteins except PulS, PulB and PulN have been identified in the *E. chrysanthemi* (Lindeberg and Collmer, 1992). However, the *E. chrysanthemi outC-O* genes are located within a single operon, with *outO* located next to *outM*. *E. chrysanthemi* and *E. carotovora* are the only two species found to date which contain a PulO-like prepilin peptidase gene at the end of their operons.

In *A. hydrophila* (Jiang and Howard, 1992; Howard et al., 1993) and *X. campestris* (Dums et al., 1991) the GSP operons have been partially characterized, with the *pulD-N* homologues (the *exe* genes) and *pulD-J* homologues (the *xps* genes) identified respectively. Howard et al. (1993) found no further open reading frames past *exeN* and speculate that the *pulO* homologue is located outside the *exe* operon, as was found with *P. aeruginosa pilD*. The *V. cholerae* PulE homologue has been identified (EpsE) and shown to contain the expected ATP-binding motif (Sandkvist et al., 1993).

Yersinia enterocolitica secretes a number of plasmid-encoded proteins known as Yops (Michiels and Cornelis, 1991). The genes required for secretion are in a locus known as *virC*, also located on these plasmids (Michiels et al., 1991). There are 13 open reading frames located in *virC*, and the gene product of one, YscC, shares significant homology with PulD. Based on this homology, Michiels et al. (1991) speculated that YscC has a role in the secretion of Yops, but this has yet to be proven. A protein homologous to YscC, MxiD, has also been found in a *Shigella flexneri* operon which is necessary for the secretion of invasin proteins (Allaoui et al., 1993). The only members of the *virC* operon which have been found to be involved in extracellular protein secretion are *yscD, yscJ* and *yscL,* although none shares any homology with any Pul protein (Michiels et al., 1991). Two proteins, YscN (Woestyn et al., 1994) and SycE (Wattiau and Cornelis, 1993), located outside the *virC* operon are also required for secretion. Because YscN contains two ATP-binding sites, Woestyn et al. (1994) believe it may have a role in energizing the secretion process in the same way that has been suggested for PulE. However, no homology has been found between YscN and PulE. SycE is believed to be a cytoplasmic chaperone specific for Yop proteins and does not appear to have a function homologous with any of the Pul proteins (Wattiau and Cornelis, 1993). As well, Yop proteins do not have typical signal sequences to direct them across the inner membrane

(Michiels and Cornelis, 1991). However, there appears to be a secretion signal located at the N-terminus of the YopH protein as fusion of its N-terminal 65 amino acids to either LacZ or PhoA results in secretion of the fusion proteins by *Y. enterocolitica* (Michiels and Cornelis, 1991). It would appear then that, in spite of the similarities of some of the components between the two systems, Yops are secreted by a system different from the GSP.

The fact that proteins secreted via the GSP by other Gram-negative bacteria accumulate in the periplasm when cloned into *E. coli* is not surprising since this species does not normally secrete proteins and it does not appear to contain a *pul*-like operon (Lindeberg and Collmer, 1992). When either the *pul* secretion operon or the *E. chrysanthemi out* operon are cloned into *E. coli*, secretion of PulA and PelE respectively are enabled (d'Enfert et al., 1987; He et al., 1991b). However, attempts to reconstitute the Out secretion system of *E. carotovora* in *E. coli* have been unsuccessful (Reeves et al., 1993). This may indicate that factors outside the *pul*-like operons are necessary for secretion. The *E. coli* homologue(s) of these factors may be able to interact with the *pul* and *E. chrysanthemi out* GSP systems but not with the *E. carotovora out* system.

The presence of a *pul*-like operon does not necessarily mean that a bacterium can secrete any protein requiring the GSP machinery. There appears to be some species-specificity involved in the recognition of secreted proteins by different GSP systems. *K. oxytoca* is unable to secrete the PelE protein of *E. chrysanthemi* (He et al., 1991b) while *P. aeruginosa* cannot secrete PulA (de Groot et al., 1991). Even more closely related bacteria are also unable to secrete heterologous proteins. Thus *E. chrysanthemi* cannot secrete the *E. carotovora* PelE (He et al., 1991b) or cellulase (EGZ; Py et al., 1991) while *E. carotovora* cannot secrete *E. chrysanthemi* cellulase (CelV; Py et al., 1991). Similarly, *Pseudomonas fluorescens* is unable to secrete the *P. aeruginosa* elastase protein (de Groot

et al., 1991). It is interesting to note that the *xcp* operon of *P. aeruginosa* can restore protein secretion in an *X. campestris* secretion mutant while the *xps* operon of *X. campestris* can only partially restore secretion in *P. aeruginosa* secretion mutants (de Groot et al., 1991). These results indicate that different components of each system might recognize signals specific for each organism. With homologies between the different species GSP genes varying from 30-80%, it is not surprising to find that the GSP system from one organism cannot complement a defect in another organism (Pugsley, 1993a).

C) The periplasm and the GSP

The identification of different components of the GSP machinery has not yet led to an explanation of how secreted proteins are translocated across the outer membrane. Much of the evidence points to a two-step mechanism in which crossing the inner membrane by the *sec* system occurs first. Both the *E. chrysanthemi* pectate lyase isozyme PelE and *K. oxytoca* PulA have been shown to require the *sec* export machinery for translocation across the inner membrane (He et al., 1991a; Pugsley et al., 1991a). Since other proteins secreted by the GSP also contain typical signal sequences, it is likely they all cross the inner membrane in a *sec*-dependent manner (Pugsley, 1993a). A number of observations have indicated that these proteins enter the periplasm at the end of the first step, before being translocated across the outer membrane.

The first evidence that the periplasm was part of the general secretion pathway was found by Howard and Buckley (1985a) who demonstrated that the precursor of an *A. hydrophila* toxin, preproaerolysin, could be trapped in the inner membrane by collapsing the PMF. Under these conditions, an intracellular pool of the leaderless proaerolysin was also found. While both forms of aerolysin were protected from proteolytic degradation

when protease was added to the culture supernatant, the proaerolysin alone was digested when protease was added to cells that had undergone osmotic shock. This showed that aerolysin did not cross the inner and outer membranes simultaneously. Pleiotropic secretion mutants of *A. hydrophila* were found to accumulate proaerolysin in the periplasm, further suggesting that this was the location of the intracellular pool in the wild type cells (Howard and Buckley, 1983, 1985a). Similar results were observed when mutations were made to the GSP of *E. chrysanthemi* (Andro et al., 1984). Expression of a *V. cholerae* haemolysin (Mercurio and Manning, 1985) and deoxyribonuclease (Focareta and Manning, 1987), the *P. aeruginosa* exotoxin A (Douglas et al., 1987; Lory et al., 1988), *E. chrysanthemi* PelE (Collmer et al., 1985) and *A. hydrophila* aerolysin (Howard and Buckley, 1986) in *E. coli* all resulted in the accumulation of the proteins in the *E. coli* periplasm, indirect evidence that they normally pass through this space in their native bacteria. A fusion protein made between PulA and MalE also accumulated in the periplasm of *E. coli* provided that the *pul* secretion genes were not expressed (Poquet et al., 1993a). When the *pul* secretion operon was cloned into this strain on a separate, inducible plasmid, the accumulated fusion protein was released into the culture supernatant when the secretion operon was expressed. As had been shown by Wong and Buckley (1989; see Results) this showed that GSP proteins directed to the periplasm are still able to cross the outer membrane. Pulse-chase experiments with the *V. cholerae* heat-labile enterotoxin (LT) revealed that the LT subunits quickly entered into the periplasm before slowly being released into the culture medium (Hirst and Holmgren, 1987a). Overproduction of PelE in *E. chrysanthemi* resulted in the accumulation of PelE in the bacterial periplasm (He et al., 1991a), while mutants of *P. aeruginosa* accumulated exotoxin A in the periplasm before it was slowly moved across the outer membrane (Lu et al., 1993). Originally it had been reported that exotoxin A did not enter the periplasm but rather, crossed the inner and outer membranes together in a single step (Lory et al., 1983). It may be that the efficiency of

translocation of wild type exotoxin A from the periplasm across the outer membrane is so great that no periplasmic intermediate could be observed in the earlier experiments (Lu et al., 1993).

D) The role of signals and protein folding in the GSP

Several groups have searched for signals within secreted proteins which could be recognized by the GSP machinery. While each group has located regions within their protein that are necessary for secretion, none of these regions has yet been found to be required in any other protein. Kornacker and Pugsley (1989) initially showed by deletion mutagenesis that the first 656 amino-terminal residues of PulA contain enough information for secretion of the protein across the outer membrane. Removal of more residues from the C-terminus results in the accumulation of the mutants in the periplasm. Further, fusion of the N-terminal 834 amino acids of PulA to β-lactamase leads to the release of the normally periplasmic protein from *E. coli* containing the *pul* secretion operon (Kornacker and Pugsley, 1990). A similar fusion protein made with PhoA is not secreted. However, the PhoA fusions are proteolytically degraded in the cells, and it is not clear if the inability to secrete the fusion proteins is due to this (Kornacker and Pugsley, 1990).

Deletion mutagenesis of the *P. aeruginosa* exotoxin A identified an even smaller region required for secretion, as the first 30 residues of the mature protein were found to contain enough information to cause secretion of the protein (Hamood et al., 1989). However, no other secreted proteins have been found which contain a similar 30 residue sequence, suggesting it is unlikely that a universal primary structural motif acts as the secretion signal. Site-directed mutagenesis of the Asp residue at position +2 of the mature PulA protein resulted in a secretion defect (Pugsley and Kornacker, 1991), but this does

not appear to affect the interaction between PulA and the GSP. Rather, it prevents the release of PulA from the outer membrane surface (Poquet et al., 1993b).

Deletion of the C-terminal cellulose-binding domain or the internal catalytic domain of the *E. chrysanthemi* EGZ cellulase prevents secretion of the protein (Py et al., 1993). Insertion of 8-18 residues between these two domains also results in a secretion defect, indicating that tertiary structure may play an important role in secretion signalling. However, no structures have yet been found which would confirm this theory.

Experiments have also been performed to look at the degree of folding of secreted proteins. The secretion of the B subunit of *V. cholerae* heat-labile enterotoxin was examined in these studies. While *V. cholerae* secretes this protein, *E. coli* does not. As well, *E. coli* does not secrete its own, similar enterotoxin (Neill et al., 1983). However, Neill et al. (1983) found that *V. cholerae* could secrete the *E. coli* toxin, likely because *V. cholerae* has the GSP machinery while *E. coli* does not. Hirst and Holmgren (1987b) performed pulse-chase experiments of the B subunit in *V. cholerae* to find out if the final pentamer is formed before or after secretion across the outer membrane. They found that monomeric B subunits were initially located in the periplasm, but pentamers were quickly assembled shortly after. These pentamers were identical to the pentamers isolated in the culture supernatant in their resistance to SDS, leading to the conclusion that the holotoxin was secreted across the outer membrane in its final folded form. A *V. cholerae* homologue of the *E. coli* DsbA protein (a periplasmic protein required for disulphide bond formation; Bardwell et al., 1991) was found to be required for the formation of both the A and B subunits of the enterotoxin. It was also required for the secretion of the enterotoxin complex and a *V. cholerae* protease (Yu et al., 1992; Peek and Taylor, 1992). A mutation in the *E. coli dsbA* gene decreased the rate of disulphide bond formation in PulA and also slowed the rate of its secretion (Pugsley, 1992). Site-directed mutagenesis of two cysteine

residues in *E. chrysanthemi* EGZ prevented formation of a disulphide bond and subsequent secretion of the protein (Bortoli-German et al., 1994). It is not clear though if EGZ secretion was prevented because of the lack of the disulphide bond or as a result of its increased susceptibility to proteolytic digestion. While not showing that proteins are secreted across the outer membrane in their final conformations, these observations indicate that some folding can be tolerated by the GSP machinery. This contrasts with the *sec* system, which requires proteins to be in an unfolded state for export across the inner membrane (see above) as well as the IgA protease secretion system, which does not translocate proteins with a high degree of tertiary structure across the outer membrane (see section on IgA protease secretion above).

4. The secretion of pertussis toxin by Bordetella pertussis

The secretion of pertussis toxin by *Bordetella pertussis* requires at least one gene product (PtlC) located downstream of the toxin (Covacci and Rappuoli, 1993). Six other genes are linked with the *ptlC* gene in an operon and their products are similar to the VirB protein of *Agrobacterium tumefaciens*, a protein believed to make a transmembrane structure which mediates transport of T-DNA to plant cells (Weiss et al., 1993). No homology has yet been found between these gene products and the *pul* operon proteins. However, this system has only recently been identified and the details involved in the secretion process remain to be determined.

5. The *Aeromonas hydrophila* toxin aerolysin

A. hydrophila is a facultatively anaerobic Gram-negative bacteria. It secretes a number of different proteins, including proteases (Rivero et al., 1990; Leung and Stevenson, 1988a, 1988b), amylases (Gobius and Pemberton, 1988; Chang et al., 1993), a glycerophospholipid:cholesterol acyltransferase (GCAT; MacIntyre and Buckley, 1978) and a number of hemolysins (Hirono and Aoki, 1991; Howard and Buckley, 1985a). *A. hydrophila* can cause diarrheal disease and wound infections in humans and fatal septicemia in fish and mice, and some of the secreted proteins play a role in the pathogenicity of the organism (Leung and Stevenson, 1988b; Chakraborty et al., 1987; Kindschuh et al., 1987).

The hemolytic toxin aerolysin is produced by a number of different *Aeromonas* species (Hirono and Aoki, 1991; Husslein et al., 1988). Aerolysin is synthesized as a 54 kDa precursor containing a typical 23 amino acid signal sequence (Howard et al., 1987; van der Goot et al., 1992). This form of the toxin is known as preproaerolysin. The signal sequence is removed after export across the inner membrane, which presumably occurs via the *sec* system (Howard and Buckley, 1985a). The resulting protoxin, known as proaerolysin, is composed of 470 amino acids (Figure 1). It is secreted into the extracellular milieu, where it is proteolytically converted to active aerolysin (Howard and Buckley, 1985b). Many proteases, including trypsin, chymotrypsin and an extracellular protease of *A. hydrophila*, can activate proaerolysin by removing approximately 40 amino acids at the C-terminal of the protoxin (Howard and Buckley, 1985b; van der Goot et al., 1992; see Figure 1). The active toxin is able to disrupt membranes by inserting into them and forming channels (Wilmsen et al., 1990; Chakraborty et al., 1990). Aerolysin must oligomerize before it can enter into the membranes (Garland and Buckley, 1988; van der

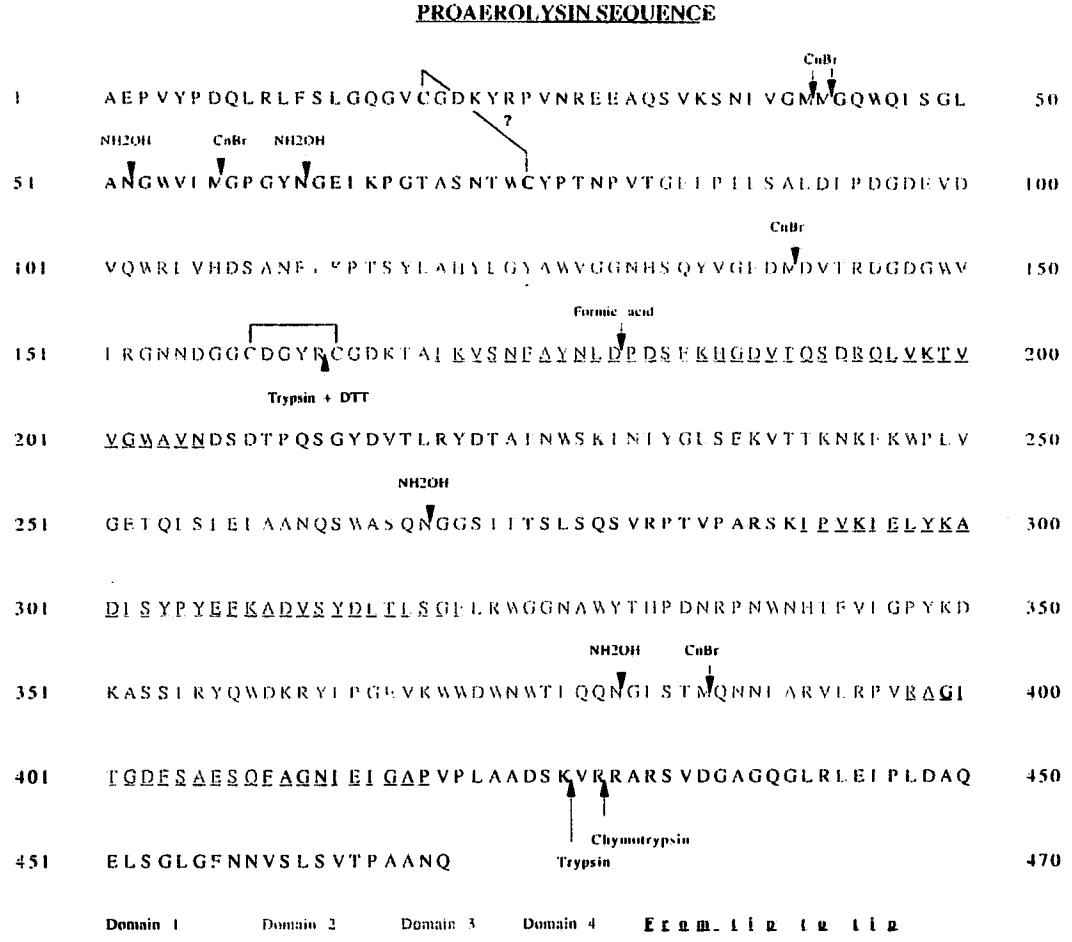

Figure 1. Amino acid sequence of proaerolysin. The four different domains are indicated in different colors. Protease and chemical cleavage sites are shown by arrows. Disulfide bonds are indicated by lines joining cysteine residues.

Goot et al., 1993). The X-ray crystallographic structure of proaerolysin has recently identified a region which may be involved in this process (Parker et al., 1994; see Figure 1). Proaerolysin is unable to oligomerize and therefore is inactive. A similar pattern of activation and aggregation has been observed with the *Clostridium septicum* alpha toxin (Ballard et al., 1993).

As described above, proaerolysin is secreted from *A. hydrophila* by means of a general secretion pathway analogous to the Pul system found in *K. oxytoca* (*exe* system; Jiang and Howard, 1991; Howard et al., 1993). However, identification of 11 of the Exe components has not led to an increased understanding of the secretion process. The experiments described in this thesis represent an attempt to reveal how aerolysin interacts with the GSP. A number of different approaches have been used. Aerolysin was initially cloned into 3 different heterologous hosts to see if the GSP of other Gram-negative bacteria could direct its secretion. Site-directed mutagenesis and the generation of AerA'-'PhoA fusion proteins were used to try to find signals within AerA which might direct the protein to the GSP. In order to determine the energy requirements for secretion, the effects of a proton ionophore and the pH of the growth medium on the secretion of a periplasmic pool of proaerolysin in *A. salmonicida* were studied. Finally, the importance in secretion of a periplasmic-spanning, energy-transducing protein that is required for macromolecule import (TonB), was investigated by creating mutants which are resistant to the antibiotic pirazmonam and potentially lack this protein.

MATERIALS AND METHODS

Bacterial strains

The genotypes and sources of the strains used in these studies are listed in Table 1. The strains Rif-1 and AH65-Rif^r were isolated by plating 100 µl of an overnight culture of AS440 and AH65 cultures respectively onto human blood agar (HBA) plates containing 40 µg ml^{-1} rifampicin. Plates were incubated at 30°C and individual colonies were picked and tested for their ability to secrete protease, glycerophospholipid:cholesterol acyltransferase (GCAT; see below) and aerolysin (for AH65; see below). The whole cell protein profiles of these resistant strains were also examined on sodium dodecyl sulphate-polyacrylamide gels. Colonies which maintained the same phenotype as wild type controls were kept and used in these studies. The pirazmonam resistant mutants SHJ2, SHJ17 and 2H$^-$ were isolated by N. Gletsu and S. Hemming-Julseth by plating 1:100 dilutions of overnight cultures of AH65 onto HBA plates containing 0.05 µM pirazmonam (Nikaido and Rosenberg, 1990). The two strains SHJ2 and 2H$^-$ produced non-hemolytic colonies while SHJ17 gave hemolytic colonies.

Media and reagents

Luria-Bertani (LB) medium was made as described by Sambrook et al. (1989), except that the pH was adjusted to 7.5. LB-Davis medium was made by mixing 9 parts LB medium with 1 part of 10 x modified Davis buffer (Ashton et al., 1980). Glucose was added to a final concentration of 0.2% when it was required in the medium. Riddle's medium was made essentially as described by Riddle et al. (1981), with 0.1% histidine and 0.1 % glutamic acid but no $CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$ or $MnCl_2 \cdot 4H_2O$ added to the medium. Low-phosphate Riddle's medium was made in the same way except that it contained 0.2 mM KH_2PO_4. Also, the K_2HPO_4 was omitted and 4-(2-hydroxyethyl)

Table I Bacterial strains used

Strain	Genotype or description	Source
Aeromonas hydrophila		
AH65	Wild-type	This laboratory
AH65-Rif^r	Rif^r strain of AH65	This laboratory
S9	Pleiotropic secretion mutant of AH65	Howard and Buckley, 1983
L1.97	AH65 strain with mutation in *exe*E	Jiang and Howard, 1991
SHJ2	Pirazmonam resistant, non-hemolytic strain of AH65	This study
2H$^-$	Pirazmonam resistant, non-hemolytic strain of AH65	This study
SHJ17	Pirazmonam resistant, hemolytic strain of AH65	This study
Aeromonas salmonicida		
AS440	Wild-type; ATCC 14174	ATCC
Rif-1	Rif^r strain of AS440	This laboratory
CB3	Rif-1::*Tn5*; deficient in secreted protease; Km^r and Rif^r	Buckley, 1990
Escherichia coli		
HB101	*supE*44*hsdS*20($r_B^-m_B^-$)*recA*13*ara*-14*proA*2*lacY*1*galK*2*rpsL*20*xyl*-5*mtl*-1	E. E. Ishiguro
CC118	*araD*139Δ(*ara leu*) 7697 Δ*lacX*74 *phoA*Δ20*galEgalKthi rpsE rpoB* $argE_{am}$*recA*1	C. Manoil
LE392	*supE*44*supF*58*hsdR*514*galK*2*galT*-22*metB*1*trpR*55*lacY*1	C. Manoil
JM105	*supEendAsbcB*15*hsdR*4*rpsLthi*Δ (*lac-proAB*)	Amersham
TG1	*supE hsd*Δ5 *thi* Δ(*lac-proAB*) F' [*traD*36 *proAB*$^+$*lacI*^{q} *lacZ*ΔM15]	Amersham
Vibrio sp.		
MVT606	wild-type marine *Vibrio sp.* strain 60; *str*-606	S. Mizushima
MVT1064	MVT606 *epr*-1064	S. Mizushima
MVT1181	MVT606 *epr*-1181	S. Mizushima
MVT1192	MVT606 *epr*-1192	S. Mizushima

piperazine-N'-ethanesulfonic acid (HEPES) was added in its place to a final concentration of 0.05 M. The pH of the low-phosphate Riddle's medium was adjusted to 7.4. YT medium was made as described by Sambrook et. al. (1989). Pulse labeling experiments were performed in Met$^-$ media, which was composed of M9 minimal media (Miller, 1972) containing 1 mM $MgSO_4$, 1 mg ml^{-1} thiamine, 0.2% glucose (w/v) and 0.3% methionine assay medium (Difco). Blood plates were prepared using tryptic soy agar (TSA; Difco) and 5% human blood (v/v) warmed to 50°C. When required, 5-bromo-4-chloro-3-indolyl-phosphate (XP) or 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal; Boehringer Mannheim), dissolved in dimethyl formamide, were added to plates at a final concentration of 40 μg ml^{-1} in order to detect alkaline phosphatase or β-galactosidase activity respectively. When necessary, antibiotics (Sigma) were added to plates and media at the following concentrations: kanamycin and rifampicin, 40 μg ml^{-1}; ampicillin, 100 μg ml^{-1}; chloramphenicol, 25 μg ml^{-1}; streptomycin, 400 μg ml^{-1}; pirazmonam (a kind gift from Bristol-Myers Squibb), 0.05 μM.

Growth of bacterial cultures

All *Escherichia coli* cultures were grown in a New Brunswick Scientific Gyrotary Water Bath Shaker (Model G-76) at 37°C and 250 rpm. All other bacterial 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 Gilford RESPONSE UV-VIS spectrophotometer. Cultures of cells containing plasmids with *tac* promoters were normally grown to an OD_{600} of 0.5 before adding isopropyl-β–D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM.

Cell fractionation

Culture supernatants were obtained by centrifuging cells in either a Beckman JA 20 or JA 14 rotor at approx. 9000 x g, or in a Fisher microcentrifuge for 10 min. Shock fluids were usually obtained by the sucrose-EDTA method of Willis et al. (1974). When shocking marine *Vibrio sp.* strain 60, the NaCl method described by Ichige et al. (1988) was used.

To prepare outer membranes, shocked cells were resuspended in a minimum of 5 ml of either 20% sucrose, 10 mM HEPES, pH 7.4 or 10 mM Tris(hydroxymethyl)-aminomethane (Tris)-HCl, pH 7.4. Ribonuclease A (RNase A) and deoxyribonuclease I (DNase I) from Sigma were added to the resuspended cells at final concentrations of 10 μg ml^{-1}. Cells were passed through a pre-chilled French Pressure cell three times at a pressure of 1100 kg cm^{-2}. Debris and unbroken cells were spun down at 3000 x g for 10 min at 4°C. For the samples resuspended in the sucrose/HEPES solution, EDTA was added to the supernatant to a final concentration of 5 mM. These samples were then layered onto a two-step discontinuous sucrose gradient previously described as the SG0 gradient by Ishidate et al. (1986). Samples were centrifuged in a Beckman SW55 rotor for 3 h at 4°C and 40 000 rpm. Bands were removed by puncturing the tubes from the side using a number 18 needle attached to a syringe. Samples were diluted to approx. 25% sucrose with 10 mM HEPES, layered onto a second discontinuous sucrose density gradient (previously described as SG1 by Ishidate et al.; 1986), and spun in a Beckman SW41 rotor for 16 h at 4°C and 39 000 rpm. Fractions (0.7 ml) were collected from the top of the gradient using a Pasteur pipette and the absorbance at 280 nm (A_{280}) was measured. In some cases, samples were floated by adding sucrose to 60% (w/w) and centrifuging in an SW41 rotor as above after overlaying with sucrose solutions of lower density (SG1). Samples were run on sodium dodecyl sulphate (SDS)-polyacrylamide gels (See below).

In order to prepare outer membranes according to the method of Filip et al. (1973), French pressed cells suspended in 10 mM Tris-HCl, pH 7.4 were spun at 3000 x g for 10 min at 4°C to remove debris, before being centrifuged at 48 000 x g and 4°C for 45 min. Pellets were resuspended in 20 mM Tris-HCl, pH 7.4, 0.5% (w/v) sodium lauryl sarcosinate (SLS) to give an approx. protein concentration of 1 mg/ml. The suspensions were incubated at 23°C for 25 min before being diluted 3.5-fold with Tris-SLS buffer and spun at 48 000 x g and 23°C for 30 min. Pellets were washed once with 20 mM Tris-HCl, pH 7.4 and resuspended in 100 µl of 20 mM Tris-HCl, pH 7.4. Lipopolysaccharide was removed by treating them with an equal volume of buffer saturated phenol at 70°C for 10 min. The aqueous layer was removed and an equal volume of buffer was added to the phenol layer to repeat the extraction. The phenol layer was then treated twice with 2 volumes of acetone and once with 2 volumes of ether. The final pellet was dried, resuspended in 2% SDS, 5% β-mercaptoethanol, 10 % glycerol, 27 mM H_2SO_4, 55 mM Tris-HCl, pH 6.1 (1 x protein sample buffer) and boiled for 3 min before being loaded onto an SDS-polyacrylamide gel. Whole cell fractions used in enzyme assays were obtained by resuspending cell pellets in 20 mM Tris-HCl, pH 7.4 and freeze/thawing the samples three times using an ethanol/dry ice bath and a 37°C water bath.

Protein electrophoresis and immunoblotting

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in 12% slabs with 3% stacking gels by the method of Neville (1971) using a Bio-Rad mini-PROTEAN II system. Samples were prepared in 1 x protein sample buffer and boiled for 3 min before being loaded. Gels were run at 16 mA constant current through the stacking gel and 40 mA through the separating gel. Proteins were then either stained with Coomasie Blue or transferred to nitrocellulose by the method of Towbin et al. (1979) using a Bio-Rad mini Trans-blot electrophoretic transfer cell. Nitrocellulose was blocked in 1% bovine serum

albumin (BSA; fraction V, Sigma), 1 % fetal calf serum (HyClone), 0.5% polyoxyethylenesorbitan monolaurate (Tween 20) in 0.85% NaCl, 10 mM NaH_2PO_4, pH 7.4 (PBS) at 37°C for 1 h. The blocking solution was removed and 30 ml of PBS containing 1 M glucose, 10% glycerol, 10% fetal calf serum, 0.05% Tween 20 was poured over the nitrocellulose. An anti-aerolysin monoclonal or polyclonal antibody, made in this laboratory, was usually used as the primary antibody in the next step. Also used were polyclonal antibodies made against alkaline phosphatase (made by R. Beecroft) and the *E. coli* TonB (a gift from K. Postle). A 1:4000 dilution of the primary antibody was added to the solution and the blot was incubated overnight at 4°C on a shaking table. The primary antibody was removed and the nitrocellulose was washed 5 x 5 min with PBS containing 0.5% Tween 20. The nitrocellulose was then covered in a solution of PBS containing 1% BSA, 1% fetal calf serum and 0.5% Tween 20 and a 1:4000 dilution of either goat α-mouse IgG (Caltag; for monoclonals) or goat α-rabbit IgG (Caltag; for polyclonals) antibodies linked to either alkaline phosphatase or horseradish peroxidase. Blots were incubated for 1 h at room temperature on a shaking table before being washed 3 x 5 min with PBS containing 0.5% Tween 20. When alkaline phosphatase antibodies were used, they were washed an extra 3 x 5 min in 100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris-HCl, pH 9.8, while horseradish peroxidase antibody blots were washed 3 x 5 min with 10 mM Tris-HCl, pH 7.4. Blots were stained as described by Sambrook et al. (1989) or they were developed using the Amersham ECL system as described by the manufacturer.

Plasmid construction and purification

A list of plasmids used in these studies is given in Table 2. Restriction digests of plasmids were performed at 37°C for 90 min with restriction enzymes from Pharmacia or Boehringer Mannheim. Digestions were stopped by the addition of 40% sucrose, 60 mM

Table 2: Plasmids and phage used in this study

Plasmid or Phage	Description	Source
pMMB66EH	RSF1010 Δ (*Pst*I-*Pvu*II, 2.8 kb) Ω (*lacI*^{q} *tacP rrnB bla Nru*I-*Aha*III, 3.0 kb) Ap^r	Furste et al., 1986
pMMB66HE	Same as pMMB66EH, with orientation of multi-cloning site reversed	Furste et al., 1986
pMMB208	IncQ, *lacI*^{q}, *cat*(Cm^r), *Ptac*, *rrnB* with polylinker from M13mp19	Morales et al., 1991
pCH2	*phoA*::*bla* promoter and signal sequence of pKT287	S. Lory
pHI.1	*E. coli phoA* in pBR322	S. Lory
pAP208	pMMB208 Ω (pHI.1, *HindIII XhoI*, 2.5 kb) Cm^r	This study
pFUS208	pMMB208 Ω (pCH2, *HindIII-XhoI*, 2.9 kb) Cm^r	This study
pPH501	*aerA* in pBR322; Tc^r	S. P. Howard
pKW2	pMMB66EH Ω (pPH501, *EcoRI-PstI*, 3.4 kb) Ap^r	This study
pNB5	pMMB66HE Ω (pPH501, *NsiI-BclI*, 1.7 kb) Ap^r	This laboratory
pNK1	pNB5::*Tn10;* Ap^r, Km^r	This study
pKM2	pKW2::*Tn10*; Ap^r, Km^r	This study
pRK2013	Conjugative helper plasmid· Km^r	E. W. Nester
pJT2	pMMB66EHΩ(pJT-1, *Eco*RI-*Hind*III, 1.2 kb) Ap^r	Thornton et al., 1988
pUW964	pRK2013 kan::Tn7xyz::Tn5; Km^r	Weiss et al., 1983
λTn*PhoA*-1	λTn*5*::*phoA*	C. Manoil
λ1105	λptac-transposase mini kan	Way et al., 1984
mp18/mp19	M13 sequencing phage	Pharmacia

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 (Pharmacia or BRL) and 1 x ligation buffer supplied by the companies. Ligations were incubated overnight at 16°C before being used to transform competent cells (See below). The wide-host-range vectors pMMB66HE and EH (Furste et al., 1986) were used to move *aerA* into *A. salmonicida* and *Vibrio spp.* strain 60. The wide-host-range plasmid pMMB208 (Morales et al., 1991) was used to move different constructs into *A. hydrophila*. Plasmid pPH501 (Howard and Buckley, 1986) was the source of the *aerA* gene. Digestion of pPH501 with *EcoRI* and *PstI* released a 3.4 kb fragment composed of the 1.5 kb *aerA* gene, a 0.5 kb upstream region which contains the *aerA* promoter, and a 1.4 kb region downstream. This was ligated into pMMB66EH to make plasmid pKW2. Plasmid pPH501 was also digested with *NsiI* and *BclI* by M. Green (Wong et al., 1989), to release a 1.6 kb fragment which lacked the *aerA* promoter and contained only 180 bp downstream of *aerA*. This was ligated into pMMB66HE to make plasmid pNB5.

It was found that the marine *Vibrio spp.* strain 60 and its pleiotropic mutants were all resistant to ampicillin, making it difficult to select for clones containing any of the pMMB66 constructs. To solve this problem, the modified Tn*10kan* from λ1105 was inserted into pKW2 and pNB5 by D. McLean (Wong et al., 1990) to give pKM2 and pNK1 respectively. Insertion of the transposon did not affect *aerA* or β-lactamase expression.

Plasmids pHI.1 (Inouye et al., 1981) and pCH2 (Hoffman and Wright, 1985) were used as sources of *phoA* containing the native signal sequence and a *bla* signal sequence respectively. These plasmids were digested with *HindIII* and *XhoI* to release the *phoA* inserts. They were ligated into pMMB208 to give pAP208 and pFUS208 (*bla* signal sequence).

Mini plasmid preparations followed the alkali lysis method described by Sambrook et al. (1989). The plasmid preparations were always extracted with phenol:chloroform. Minipreparations supplied enough DNA for most cloning experiments, but when larger plasmid preparations were required, the method of Birnboim and Doly (1979) was used. *E. coli* strains containing the desired plasmid were always 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 100 mM boric acid, 100 mM Tris, 2 mM EDTA. Submerged miniature electrophoresis tanks (Fotodyne) were used, normally at a constant voltage between 80-100 V.

Plasmid transformation and mobilization techniques

Plasmids and ligation mixtures were transformed into *E. coli* strains HB101 or CC118 using the $CaCl_2$ method described by Sambrook et al. (1989) with the following changes: 50 mM $CaCl_2$ was used; cell pellets were resuspended 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 spp.* and *Vibrio spp.* 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 optimum temperatures for each culture. The *E. coli* helper strain MM297-pRK2013 (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. Culture supernatants were removed by applying a vacuum to the filter funnel flask. Filters were incubated on TSA plates containing no antibiotics at 30°C (when *A. salmonicida* or *Vibrio spp.* were used as recipients) or 37°C (when *A. hydrophila* was used as the recipient) for at

least 3 h. Filters were then suspended in 5 ml of LB medium and vortexed to remove cells. Dilutions of the mating mixes were plated onto antibiotic-selective HBA plates to select for transconjugates.

Preparation of phage stocks, replicative form DNA and single stranded template DNA

The *Hind*III-*Eco*RI fragment of pNB5 containing the *aerA* gene was cloned into M13mp19 and transformed into *E. coli* strain TG1 as described above. After heat shocking the cells at 42°C for 1.5 min, 1 x YT melted top agar cooled to 50°C was added along with X-gal, IPTG and TG1 cells grown to log phase, and the mixture was poured over 2 x YT agar plates. White plaques were picked with sterile toothpicks and placed into 2 ml cultures of TG1 grown in 2 x YT medium to an OD_{600} of 0.2. Cultures were incubated at 37°C and 250 rpm for 5 h before being centrifuged at 4°C for 15 min at 6 000 x g. Supernatants were collected and stored at 4°C as phage stocks. Preparations of replicative form (RF) DNA were made from the remaining cells by resuspending them in 200 µl of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0, and continuing the miniplasmid procedure as described under "Plasmid construction and purification". Restriction digestions were carried out on the resulting RF DNA preparations to confirm the presence of the *aerA* insert.

Single stranded template DNA was prepared by adding 100 µl of phage stocks to 10 ml samples of TG1 culture grown to an OD_{600} of 0.5. Cultures were grown at 37°C and 250 rpm for 5 h before being spun down at 2500 rpm at room temperature in an International clinical centrifuge (model CL) for 15 min. The supernatants were carefully transferred to a second tube and a second centrifugation done. A total of 7.5 ml of each supernatant was transferred to a third tube, which was chilled on ice and contained 2 ml of 20% polyethylene glycol (PEG) and 2.5 M ammonium acetate. Samples were left on ice

overnight before being spun down at room temperature and 2800 rpm for 15 min in the clinical centrifuge. As much of the supernatant was aspirated off as possible, and a second centrifugation done to remove the remaining PEG solution. Pellets were resuspended in 700 µl of 1 mM EDTA, 10 mM Tris-HCl, pH 7.4 (low TE buffer), and extracted twice with buffer-saturated phenol (BRL), followed by a 1:1 mixture of phenol: $CHCl_3$/ isoamyl alcohol (24/1) and finally with $CHCl_3$/ isoamyl alcohol (24/1). An equal volume of reagent to sample was used in all extractions. The template DNA was precipitated with 2 volumes of 95% ethanol containing 125 mM ammonium acetate. The samples were left at -20°C for 1 h, then spun in a microfuge for 15 min. The ethanol was aspirated off, and the pellets were washed in 70% ethanol before being dried in a vacuum desiccator. They were then resuspended in 25 µl of sterile distilled water.

Site-directed mutagenesis

The oligonucleotides used to replace amino acids in *aerA* and the changes that were made are listed in Table 3. All oligonucleotides were synthesized by the Regional DNA Synthesis Laboratory at the University of Calgary. Oligonucleotides were 5'-phosphoryl-ated using 2 units of T4 polynucleotide kinase per 50 pmole of DNA. Phosphorylated oligonucleotides were annealed to single stranded templates derived from M13mp19 containing the *aerA* insert from pNB5 (see above) using reagents contained in the Amersham oligonucleotide-directed *in vitro* mutagenesis system, version 2.1. The mutagenesis reactions used followed the methods of Eckstein (Taylor et al., 1985; Nakamaye et al., 1986; Sayers et al., 1988) with materials from Amersham. The mutant oligonucleotide was extended using the Klenow polymerase and T4 DNA ligase to form a mutant heteroduplex. The use of thionucleotide dCTPαS in the extension reaction prevented the mutant strand from being nicked by the restriction endonuclease *Nci*I. The template DNA could be nicked by *Nci*I, allowing the digestion of this strand by exo-

Table 3. Primers used in the site-directed mutagenesis of aerolysin

Oligonucleotide[a]	DNA codon change	Amino acid change
C ACC AAC GGG TCC AAG A	TGG to GGG	Trp227 to Gly
C ACC AAC TTG TCC AAG A	TGG to TTG	Trp227 to Leu
C ACC AAC TTC TCC AAG A	TTG to TTC[b]	Trp227 to Phe
T GAA GTG CAG TGG TGG G	AAG to CAG	Lys369 to Gln
G AAG TGG TTG GAC TGG A	TGG to TTG	Trp371 to Leu
G TGG TGG AAC TGG AAC T	GAC to AAC	Asp372 to Asn
G TGG GAC TTG AAC TGG A	TGG to TTG	Trp373 to Leu
C CGT GGC GAC AAT GAC G	AAC to GAC	Asn154 to Asp
AC AAT GAC GCC GGC TGT	GGC to GCC	Gly157 to Ala
C GGC GGC AGT GAC GGC T	TGT to AGT	Cys159 to Ser

[a]Sequences are written 5' to 3'. The codon that is changed is underlined.

[b]This codon change was made in two steps, TGG to TTG followed by TTG to TTC.

nuclease III. The exonuclease was allowed to digest past the mutation site before the strand was repolymerized using DNA polymerase I and T4 DNA ligase. The final reaction mixture was used to transform competent TG1 cells which were plated onto 2 x YT plates to obtain plaques as described above for making phage stocks. White colonies were picked, inoculated into TG1 cells in log phase and template preparations were made for use in hybridization and sequencing experiments. Cells were resuspended in 200 µl of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0 and RF preparations were made. Restriction digests of RF preparations identified potential mutants for sequencing. The complete *aerA* gene of these mutants was sequenced to ensure that only the desired change had occurred during the mutagenesis reactions. Once a clone had been identified in this way, the mutant *aerA* insert was ligated into pMMB66HE, transformed into HB101 and filter mated into CB3. Since all mutants were derived from the *aerA* insert from pNB5, none contained the *aerA* promoter and all were under the control of the *tac* promoter.

Purification of the Leu227 and Phe227 mutants required concentrating 250 ml of culture to approx. 20 ml using a Sartorius Mini Sartocon concentrator (Model SM17521) and a cellulose triacetate membrane with a nominal molecular weight cutoff of 30 000. The concentrated mutant protoxins were then purified by N. Gletsu as previously described (Wong and Buckley, 1991).

DNA hybridization and sequencing techniques

A piece of GeneScreen Plus (GS+; New England Nuclear) was soaked in 0.9 M NaCl, 0.09 M sodium citrate (6 x SSC) and placed in a Bio-Dot apparatus (Bio-Rad). The wells were washed twice with 6 x SSC before 100 µl of phage preparations were placed in the sample wells. The samples were drawn down by applying a vacuum and then washed with 100 µl of 6 x SSC. The GS+ was dried at room temperature, baked at 67°C for 2 h, and then prehybridized in 6 x SSC containing 0.2% SDS and 10 x Denhardt's solution

(0.2% Ficoll [type 40; Pharmacia], 0.2% polyvinylpyrrolidone, 0.2% BSA [fraction V]) at room temperature for 1 h. The mutant oligonucleotides were end-labeled by mixing 20 pmol of DNA with 2 units of T4 polynucleotide kinase and 15 μCi γ-^{32}P ATP in 10 mM $MgCl_2$, 7 mM dithiothreitol, 100 mM Tris-HCl, pH 8.0 and incubating for 30 min at 37°C. The GS+ was washed 3 times in 6 x SSC buffer before being placed in 10 ml of 6 x SSC, 10 x Denhardt's solution. The labeled oligonucleotides were placed on top of the GS+ and left to incubate at 67°C for 15 min. This was followed by a 1 h incubation at room temperature. The GS+ was washed 3 x 5 min in 6 x SSC, blotted dry, and exposed to Kodak XAR-5 X-ray film for 2.5 h at - 70°C. The film was developed and the GS+ subsequently washed with 6 x SSC at higher temperatures until the probe was washed from the control, wild type phage preparation. Samples giving positive results were sequenced by the dideoxy termination method of Sanger et al. (1977) using [α-^{35}S]-dATP (10 μCi μl^{-1}; Amersham) and the modified T7 DNA polymerase (Tabor and Richardson, 1989) supplied in the Sequenase version 2.0 sequencing kit (United States Biochemical). Primers used were 16-18 base oligonucleotides which were complementary to portions of the aerolysin DNA near the region to be sequenced.

Tn*phoA* insertions

Stocks of the phage λTn*phoA* (Gutierrez et al., 1987) were propagated as described above. Cultures of CC118 containing pNB5 were grown to an OD_{600} of 0.5 The cultures were spun down in 1 ml aliquots, and each aliquot was resuspended in 400 μl of ice cold 10 mM $MgSO_4$ and 100 μl of λTn*phoA* phage stock (4.0 x 10^9 pfu ml^{-1}). Samples were initially incubated at 30°C for 3.5 h with no shaking before adding 1 ml of LB medium. They were then incubated for a further 4 h at 30°C and 250 rpm. Aliquots of 100 μl were spread onto LB agar containing 100 μg ml^{-1} ampicillin, 300 μg ml^{-1} kanamycin and XP. More than 1000 colonies grew on each plate. A volume of 2.5 ml of LB media was added

and a sterile glass rod was used to scrape all the colonies from each plate. The resuspended cells were spun down in a microfuge and used for miniplasmid preparations. The plasmids were used to transform CC118 cells and fusions were selected on HBA plates containing ampicillin, kanamycin and XP. Blue colonies were picked, regrown, and plasmids isolated. Restriction digests were used to determine the approximate location of the fused *phoA*. Fusions which appeared to be in the *aerA* gene were cloned into M13mp19 to produce templates which were sequenced to locate the fusion sites.

Pulse labelling and immunoprecipitation experiments

Cultures of CB3 containing mutant aerolysin constructs or *phoA* fusions were grown to an OD_{600} of 0.5 and then induced by adding IPTG to 1 mM. After a further 15 min incubation, 300 µl samples were spun down in a microfuge, washed once in Met^- medium and resuspended in 300 µl of the same medium containing 54 µCi [^{35}S]-methionine (612 Ci $mmol^{-1}$; New England Nuclear). The samples were incubated for 5, 10, 15 or 30 min at 30°C before being fractionated as described above. Shocked cells were resuspended in 300 µl of Met^- medium. All fractions were precipitated by adding 0.25 volumes of 1 mg ml^{-1} BSA, followed by 1 volume of ice cold 20% trichloroacetic acid. After centrifugation, pellets were washed with acetone, air dried and resuspended in 1/5 the original volume of 1% SDS, 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride and boiled for 2 min. Triton buffer (1.25% *t*-octylphenoxypolyethoxyethanol [Triton X-100], 190 mM NaCl, 6 mM EDTA, 60 mM Tris-HCl, pH 7.4) was added to make up to the original volume, followed by 20 µl of cold 10% *Staphylococcus aureus* cells (SAC; Calbiochem). After 2 h on ice, samples were centrifuged and the supernatants were transferred into tubes containing 3.25 µl of monoclonal aerolysin antibody, alkaline phosphatase antiserum or GCAT antiserum. They were left overnight on ice. Antibody complexes were recovered by incubating the samples

with 20 µl of 10% SAC (washed in 100 mg ml^{-1} BSA) for 1 h on ice and centrifuging. Pellets were washed twice in Triton buffer and once in 10 mM Tris-HCl, pH 7.4. They were resuspended in 1/10 the original volume of 1 x protein sample buffer and electrophoresed as described above. Gels were fixed in 7% acetic acid, 30 % methanol for 30 min, then soaked in AMPLIFY (Amersham) for 30 min. The gels were finally dried and exposed to Kodak X-Omatic X-ray film for 24-48 h at -70°C.

Detection of hemolytic activity

Hemolytic titres were used to measure the activity of aerolysin. Samples were made up to 200 µl with PBS, and unless otherwise noted, 1 µg of trypsin was added to each. After incubating for 5 min at room temperature, the samples were serially diluted 1:2 with PBS in microtitre plates. Human erythrocytes that had been washed in PBS were added to a final concentration of 0.4% (v/v) and the plates were incubated at 37°C for 1 h. Hemolytic activity is expressed as the inverse of the largest dilution with which complete lysis occurred.

To quantitate amounts of aerolysin, samples (normally 20 µl) were diluted to 85 µl with PBS containing 1 µg of trypsin. Supernatant samples which had been buffered below pH 7 had 5 µl of 1 M Tris-HCl, pH 8 added to raise the pH. After incubating 5 min at room temperature, 90 µl of PBS containing 0.1% BSA was added to each sample before serially diluting 1:2 as above in microtitre plates with V-shaped bottoms. Each well had 90 µl of 0.8% (v/v) human erythrocytes in PBS added to it and the plates were incubated at 37°C for 1 h. The plates were centrifuged and 135 µl of the supernatants were removed from the wells containing partially hemolyzed cells. The supernatants were diluted with 850 µl of PBS and the absorbance at 413 nm 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. Neither CCCP nor any of the buffers used in any of the media interfered with this assay system.

Enzyme assays

Alkaline phosphatase activity was measured essentially as described by Brickman and Beckwith (1975). Samples were made up to 1 ml with distilled water and 100 µl of a 0.4% solution of nitrophenyl phosphate (Boehringer Mannheim) in 1 M Tris-HCl, pH 8.0 was added to each. The mixtures were incubated at 37°C for 5-20 min, until a yellow color had developed. The absorbance at 420 nm (A_{420}) was measured and the activity reported as ΔA_{420} min^{-1} ml^{-1} of sample.

GCAT activity was measured using a modified version of the procedure described by Buckley et al. (1982). Small unilamellar vesicles were formed from mixtures of 1 mM cholesterol, [4-^{14}C] cholesterol (50 mCi $mmol^{-1}$; Amersham) and 1 mM egg phosphatidylcholine (Sigma) dispersed in 0.4 ml of 0.25% Triton X-100, 20 mM Tris-HCl, pH 7.4, 33 mM KCl. Enzyme samples were made up 100 µl with distilled water, added to the vesicle mixture and incubated at 37°C for 15 min. Reactions were stopped by the addition of 2.5 ml of $CHCl_3$:methanol (2:1). After the samples had been centrifuged, 1 ml portions of the lower organic phase were transferred to new tubes and the organic solvent was evaporated with nitrogen. Residues were redissolved in a minimum volume of $CHCl_3$:methanol (4:1) and applied to silica gel 60 thin layer plates (Merck) which were developed in chromatography tanks containing petroleum ether:ether:acetic acid (70:30:1). They were air dried and placed in an iodine tank until bands could be observed. The bands corresponding to cholesterol ester and cholesterol were cut from the plate, placed in scintillation vials containing 2 ml of scintillation cocktail, and counted in a Beckman LS 8100 scintillation counter.

Protease activities were determined essentially as described by Young and Broadbent (1982). A solution of 17 mg ml^{-1} Hide powder azure (Sigma) in 20 mM Tris-HCl, pH 7.4 was homogenized to get a homogeneous suspension. The samples (10-100 µl) and Hide powder mixture (950 µl) were prewarmed to 37°C before mixing. After incubating at 37°C for 15 min, they were centrifuged for 5 min and the A_{595} of the supernatants was read.

β-lactamase was assayed using the chromogenic substrate {7-(thienyl -2-acetamido)-3-[2-(4-N,N-dimethylaminophenylazo)pyridinium methyl]-3-cephem-4 carboxylic acid} (PADAC; Calbiochem). Samples of 1-20 µl were added to cuvettes containing 900 µl of 20 mM Tris-HCl, pH 8.0, followed by 100 µl of 200 µM PADAC dissolved in 20 mM Tris-HCl, pH 8.0. The ΔA_{572} was then measured in a Gilford recording spectrophotometer for 5 min.

A modified protocol of the α-ketoglutarate reductive amination method of Halpern and Lupo (1965) was used to measure the cytoplasmic marker glutamate dehydrogenase. Samples (5-20 µl) were added to 835 µl of 0.1 M imidazole-HCl, pH 7.9, 70 µl 0.2 M α-ketoglutarate, 35 µl of 26 mM EDTA, 50 µl of 6.4 M ammonium acetate, 15 µl of 0.1 M adenosine diphosphate and 10 µl of 12 mM NADH. The ΔA_{340} was measured over a 10 min period in a Gilford spectrophotometer.

RESULTS

Cloning of *aerA* into a wide-host-range vector and expression in *E. coli*

A number of groups have expressed proteins requiring a GSP in heterologous hosts, but none have been successful in demonstrating secretion (for a review, see Pugsley, 1993a). In order to see if aerolysin could be secreted by other Gram-negative bacteria, *aerA* first had to be inserted into a wide-host-range vector (pMMB66; Furste et al., 1986), so that it could be transferred into other Gram-negative bacteria using a triparental conjugation technique (see Materials and Methods). Two *aerA* constructs were made: i) pKW2, which contains the *aerA* promoter and 1.4 kb of DNA downstream from *aerA* and ii) pNB5, which contains no promoter and only 180 bp downstream (see Materials and Methods). In both constructs, *aerA* was inserted downstream of a *tac* promoter, allowing induction by the addition of IPTG.

Both constructs were first transformed into *E. coli* HB101 to produce suitable plasmid donors for the transconjugations. Induction of both pKW2 and pNB5 in HB101 resulted in the production of aerolysin. As shown in Figure 2A, the protein was located in the cells early after induction. However, within 5 h, hemolytic activity began to appear outside the cells. As shown in Figure 2C, this occurred at the same time as the periplasmic marker β-lactamase was released into the culture supernatant and corresponded to a decrease in growth of the cultures (Figure 2B). These observations led to the same conclusion made earlier by Howard and Buckley (1986), namely that aerolysin produced in HB101 disrupts the cells because they can't secrete the protein across the outer membrane. Since proaerolysin, but not preproaerolysin, was found in HB101-pKW2 cell shockates run on immunoblots (not shown here), it is likely that the protoxin was exported across the inner membrane via the *sec* system, but became trapped in the periplasm due to the lack of a

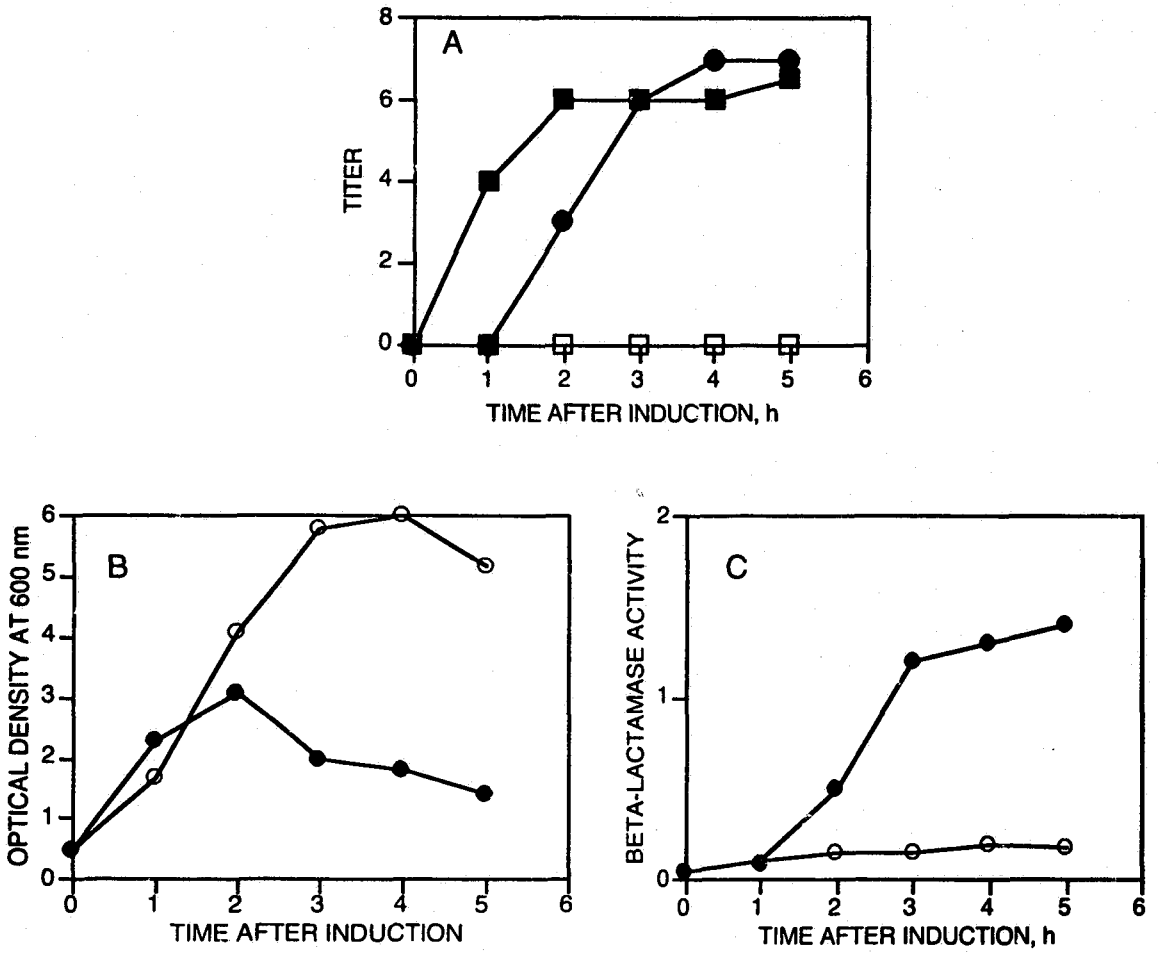

Figure 2. Production of aerolysin by *E. coli* containing pKW2. Cultures were grown in LB medium containing 0.2% glucose and induced at an OD_{600} of 0.5 by the addition of IPTG to a final concentration of 1 mM. (A) Hemolytic activity in cells (■) and culture supernatants (●) of induced cells. All samples were initially treated with 0.5 μg/ml trypsin for 5 min to activate any protoxin. No aerolysin was detected in uninduced cells or culture supernatants (□). (B) Growth of induced (●) and uninduced (○) cultures. (C) β-lactamase in culture supernatants of induced (●) and uninduced (○) cultures. β-lactamase activity is expressed in arbitrary units.

GSP. Eventually, proteases within the periplasm presumably activated the protoxin to aerolysin, which caused cell lysis.

Release of aerolysin by *A. salmonicida*

The inability of *E. coli* to secrete aerolysin is not surprising. All previous attempts to clone GSP proteins in *E. coli* without also cloning in the *pul*-like operon genes have resulted in the periplasmic accumulation of the proteins (Howard and Buckley, 1986; d'Enfert et al., 1987). As well, *E. coli* secretes very few of its native proteins (Oropeza-Wekerle et al., 1990). However, *A. salmonicida* secretes a number of different proteins including hemolysins, proteases and GCAT (Hirono and Aoki, 1993; MacIntyre et al., 1979). For this reason, and since it is closely related to *A. hydrophila*, *A. salmonicida* was chosen to receive the *aerA* constructs.

In contrast to the results seen for *E. coli*, aerolysin induced in *A. salmonicida* was secreted into the extracellular medium. When pKW2 and pNB5 were transferred to Rif-1, and the transconjugates were grown up in LB media containing 0.2% glucose, hemolytic activity began to appear in the culture supernatants of both strains shortly after induction and there was no cell-associated aerolysin detected in either until late in growth (Figure 3). The production of the toxin did not result in cell lysis as there was no effect on the growth of the induced cells (Figure 4), and less than 20% of the total β-lactamase activity was extracellular (Figure 5). Proaerolysin was the major protein observed in induced supernatant samples run on SDS-polyacrylamide gels (Figure 6). There was no evidence of a larger protein corresponding to the preprotoxin, indicating that the signal sequence had been correctly removed by *A. salmonicida*. Thus the results suggest that aerolysin is first exported across the inner membrane of *A. salmonicida* in a *sec*-dependent fashion before it is translocated across the outer membrane.

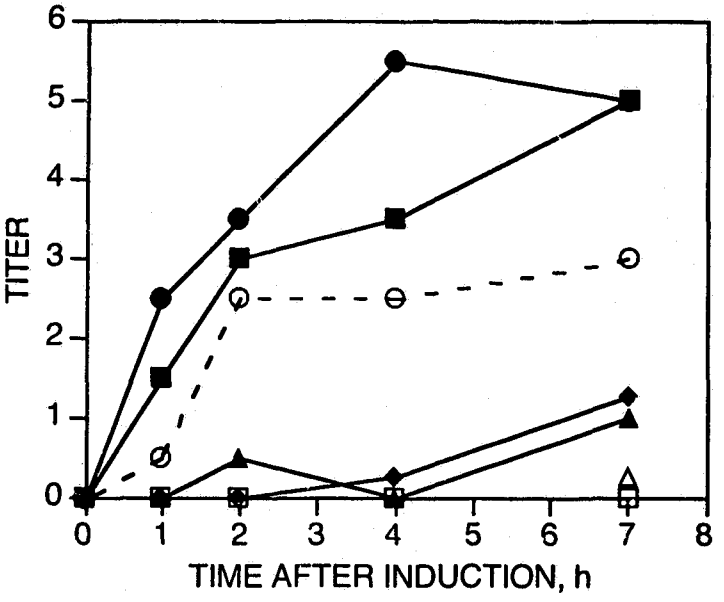

Figure 3. Production of aerolysin by *A. salmonicida*. The hemolytic activity of culture supernatant samples from Rif-1 strains containing pNB5 (■,□) and pKW2 (●,○), and cell samples from strains containing pNB5 (▲,△) and pKW2 (◆) are shown. Cultures were grown up and induced as described in Figure 2. Solid symbols refer to induced cultures; open symbols to uninduced cells.

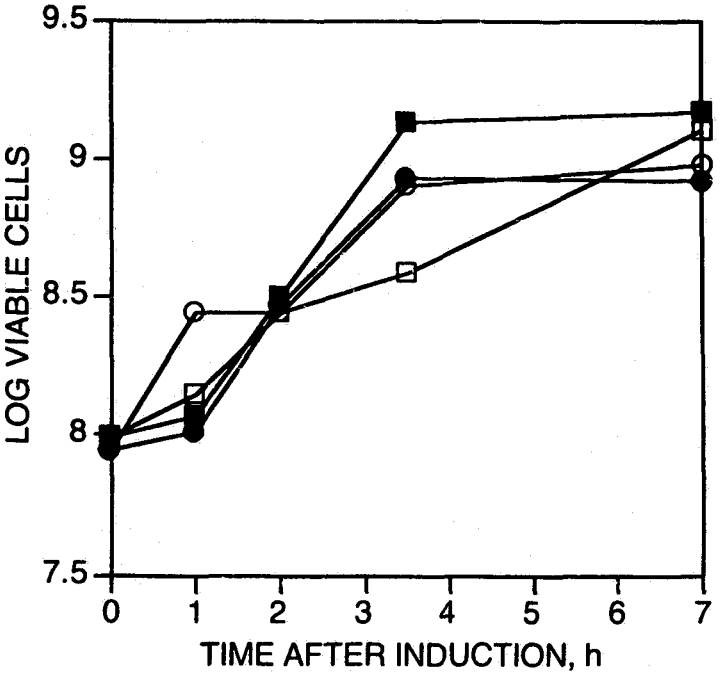

Figure 4. Effect of aerolysin production on the growth of *A. salmonicida*. This is the same experiment as the one described in Figure 3. Shown are Rif-1 strains containing pNB5 (●,○) and pKW2 (■,□). Solid symbols refer to induced cultures; open symbols to uninduced cultures.

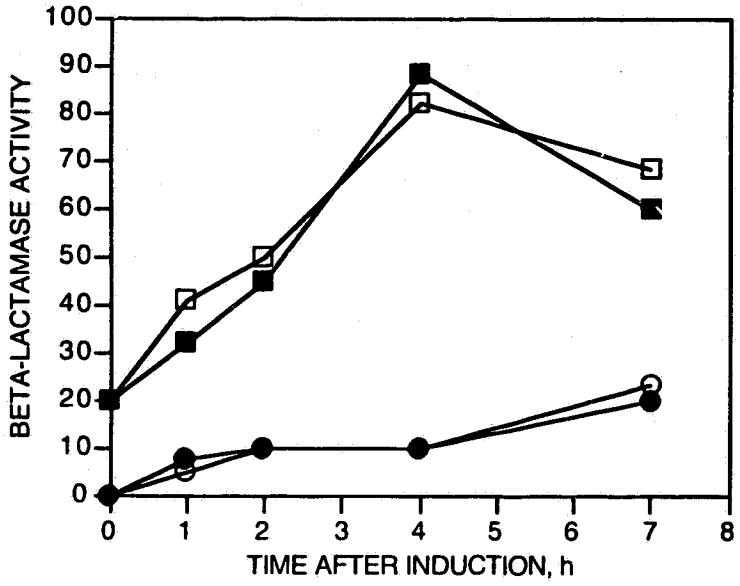

Figure 5. Effect of aerolysin production on β-lactamase release by *A. salmonicida*. Only the results for pKW2 are shown. A similar pattern was observed with pNB5 plasmid-bearing strains. This is the same experiment as described in Figures 3 and 4. Shown are activities in the induced cells (■), uninduced cells (□), induced culture supernatants (●) and uninduced culture supernatants (○). Enzyme activity is expressed in arbitrary units.

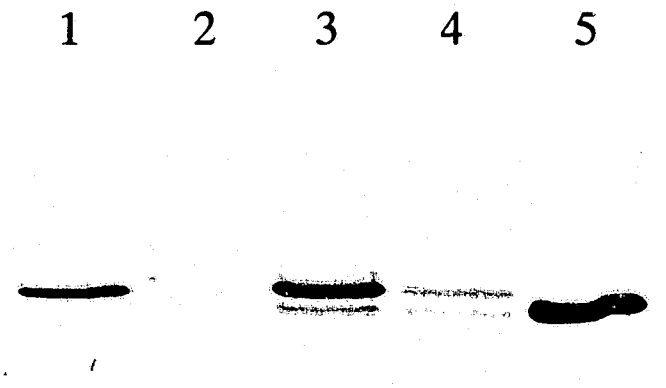

Figure 6. SDS-polyacrylamide gel electrophoresis of *A. salmonicida* culture supernatants before and after induction of plasmid-coded aerolysin. Culture supernatants were obtained from cells grown to an OD_{600} of approx. 2.5, after being induced with IPTG at an OD_{600} of 0.5. After the addition of appropriate volumes of 4 x sample buffer, 20-µl samples were applied to the gel. Lanes 1 and 2, induced and uninduced Rif-1-pNB5 respectively; lanes 3 and 4, induced and uninduced Rif-1-pKW2 respectively; lane 5, purified aerolysin.

It can also be seen in Figure 3 and Figure 6 that proaerolysin was synthesized and secreted in uninduced cultures of Rif-1-pKW2. This indicates that the *aerA* promoter can be recognized by *A. salmonicida*. HB101 is not able to recognize this promoter, as no aerolysin was produced in uninduced cultures of HB101-pKW2 (Figure 2A).

A. salmonicida does not appear to produce aerolysin itself, as subcellular fractions of Rif-1 did not cross-react with monoclonal α-aerolysin antibodies on Western blots (data not shown). This made it suitable to use as an *aerA*⁻ host to study the secretion of mutant *aerA* gene products by the *Aeromonas* spp. GSP.

Production and secretion of GCAT by *E. coli* and *A. salmonicida*

A. hydrophila also secretes the lipase GCAT (MacIntyre and Buckley, 1978). To see if it could also be secreted by a heterologous host, experiments similar to those described above were performed with HB101 and Rif-1 strains containing the cloned *A. hydrophila* GCAT gene. As with aerolysin, in *E. coli*, GCAT was found in the periplasm up until there was evidence of cell lysis, 3-5 h after induction (Figure 7A). Thus there was a decrease in viable cell count 2-3 h after induction with IPTG (Figure 7B) and an increase in β-lactamase activity in the culture supernatants. β-lactamase remained in the periplasm of uninduced cells (data not shown). In Rif-1 though, GCAT activity quickly appeared in the culture supernatant (Figure 8B). As was observed with aerolysin, neither an increase in β-lactamase activity in the culture supernatant nor a decrease in the viable cells was found at any time after induction (Figures 8C and 8A). These results show that at least two very different *A. hydrophila* proteins can be secreted by *A. salmonicida*, but not by *E. coli*.

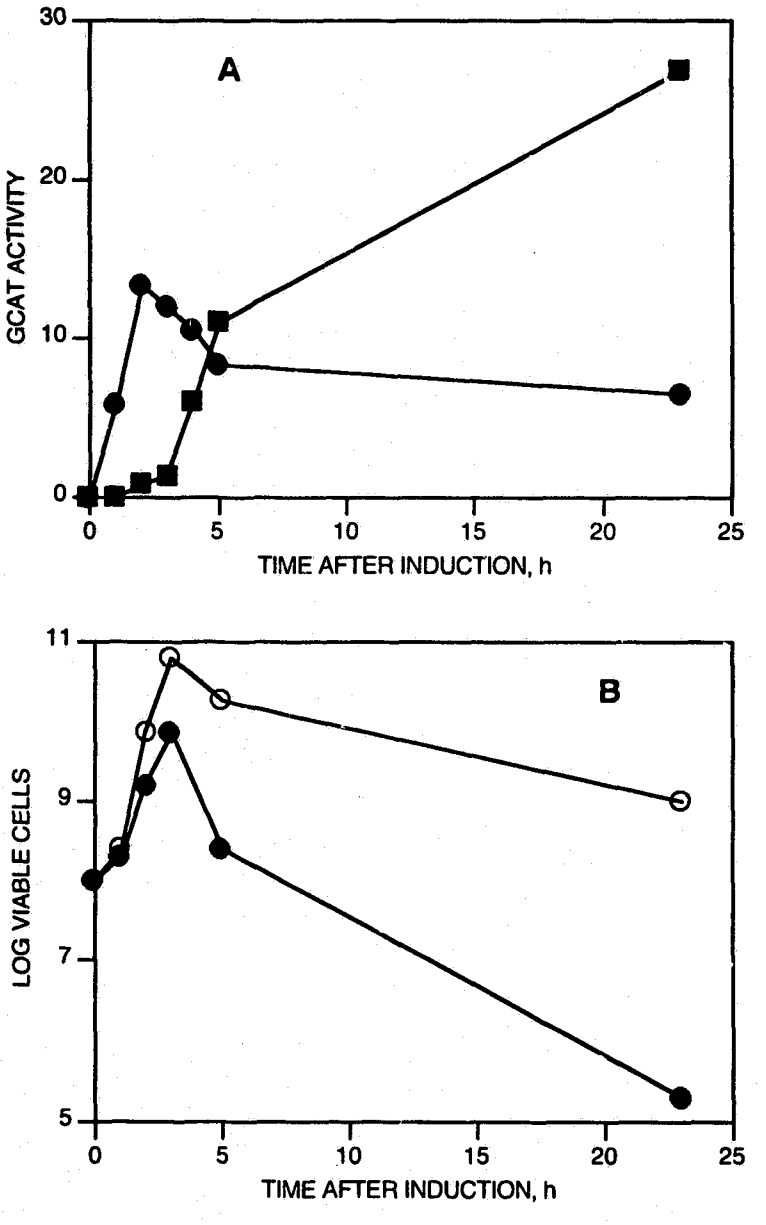

Figure 7. Expression of GCAT in HB101. HB101 was grown in LB medium containing 0.2% glucose and induced at an OD_{600} of 0.5. (A) GCAT activity measured in 50 µl of culture supernatant samples (■) and 10 µl of periplasmic shockates (●) of induced HB101-pJT22. (B) Viable cells in cultures of induced (●) HB101-pJT22 and uninduced (O) HB101-pJT22.

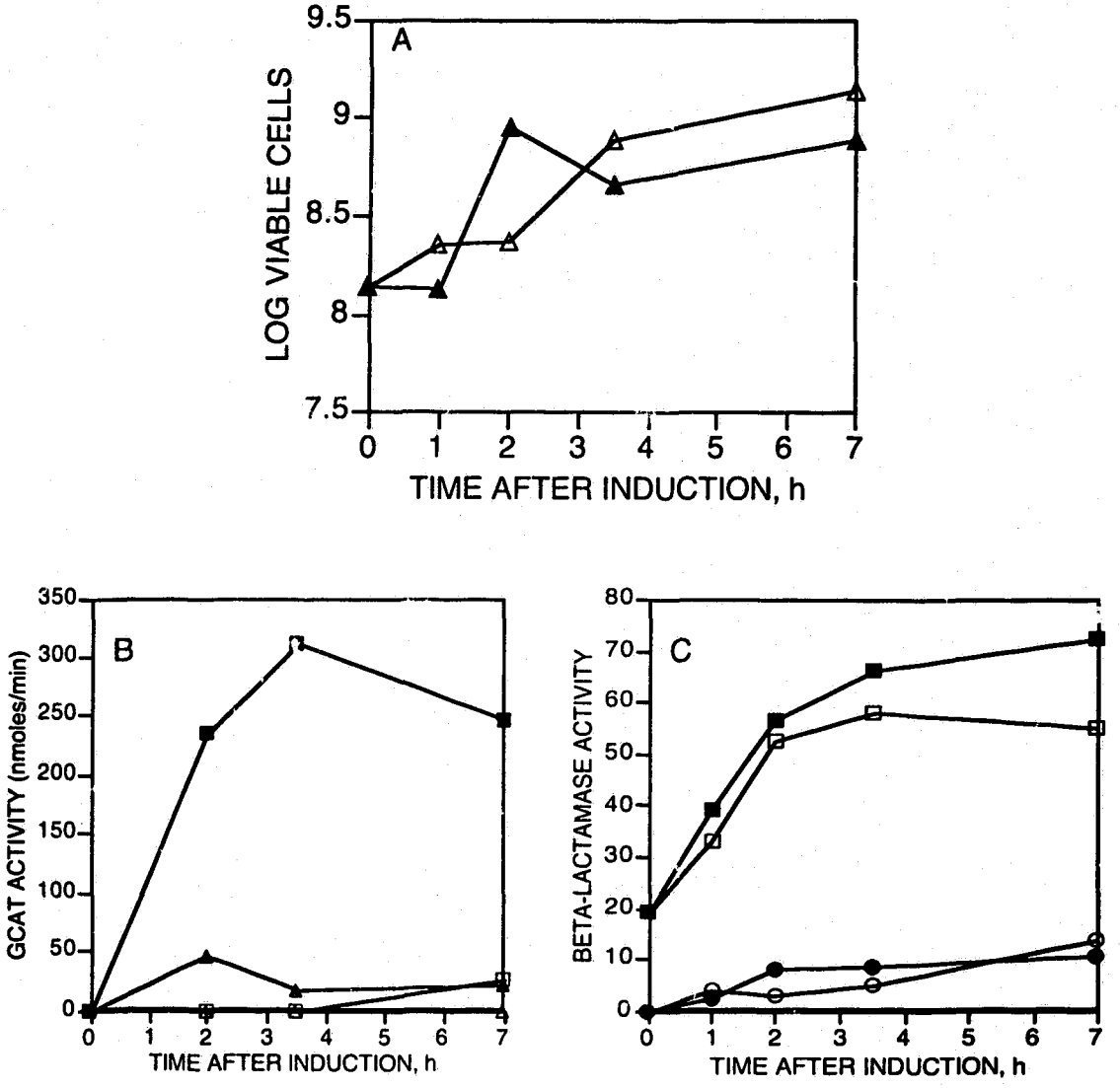

Figure 8. Production of GCAT by *A. salmonicida* containing pJT2. Cultures of Rif-1-pJT2 were grown and induced as described in Figure 2. (A) Growth of induced (▲) and uninduced (△) cultures. (B) GCAT activity in the culture supernatants (■) and whole cells (▲) of induced cultures. Little activity was detected in uninduced cells (△) or culture supernatants (□). (C) β-lactamase in culture supernatants of induced (●) and uninduced (○) cultures and in the cells of induced (■) and uninduced (□) cultures.

Effect of aerolysin expression on the extracellular secretion of protease and GCAT by *A. salmonicida*

In order to determine if plasmid-encoded aerolysin could compete with endogenous *A. salmonicida* proteins for components of the GSP, protease and GCAT assays were performed on Rif-1-pNB5 culture supernatant samples to see if they were secreted at normal levels when aerolysin was overexpressed. As shown in Figure 9, the induction of aerolysin synthesis by pNB5 had no effect on the secretion of the *A. salmonicida* protease. A small decrease in the GCAT activity was found in induced culture supernatant samples, however the extent of the reduction varied in a number of different experiments.

It is possible that neither the GCAT nor the protease of *A. salmonicida* is secreted by the GSP. Previous experiments demonstrated that secretion mutants of *A. hydrophila* could not secrete aerolysin or protease (Howard and Buckley, 1983). We have also found that GCAT activity in *A. hydrophila* pleiotropic mutants S9 and L1.97, the latter containing a lesion in the *exeE* GSP gene, was found primarily in the periplasm (data not shown). This is evidence that the *A. hydrophila* GCAT is secreted by the GSP. It seems likely then, that the *A. salmonicida* GCAT and protease may also use the GSP. If this is the case, then it would appear that the GSP is an extremely efficient system as neither protease nor GCAT secretion was greatly affected by the overexpression of aerolysin.

Location of the intracellular pool of proaerolysin in *A. salmonicida*

Figure 3 shows that a small amount of proaerolysin could be detected in the whole cells of Rif-1-pKW2 and Rif-1-pNB5. To determine its location, the cells were osmotically shocked. The resulting shockates contained most of the recovered hemolytic activity. Little or no activity remained with the shocked cells (data not shown). The

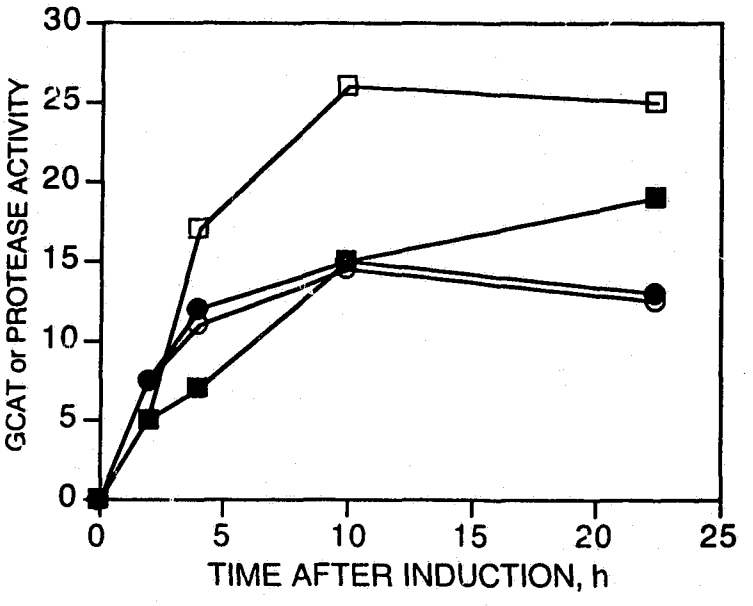

Figure 9. Influence of aerolysin production on the export of chromosomal GCAT and protease by *A. salmonicida*. Rif-1-pNB5 cells were grown in LB medium. Shown are protease activities in the culture supernatants of uninduced (○) and induced (●) cultures and GCAT activities in culture supernatants of uninduced (□) and induced (■) cultures. Activities are in arbitrary units.

shockates were run on an SDS-polyacrylamide gel along with a culture supernatant sample, transferred to nitrocellulose and screened for aerolysin with anti-aerolysin monoclonal antibodies. The resulting Western blot (Figure 10) shows that proaerolysin was present in the shockable fraction while processed aerolysin was found in the culture supernatant. This is evidence that proaerolysin normally passes through the periplasm before being secreted across the outer membrane. However, it is possible that overexpression results in more proaerolysin than the secretion machinery can handle and that the excess protein could be diverted to the periplasm as a result. This would appear to be unlikely though as the overexpression of aerolysin did not affect the secretion of protease and only a small decrease in GCAT secretion was observed (see above).

In order to see if proaerolysin detected in the periplasm could still be translocated across the outer membrane, experiments were set up to follow this pool of proaerolysin. If the protoxin was diverted to the periplasm it might be expected that it could no longer enter the GSP. Rif-1-pKW2 cells were grown up and induced to a point where proaerolysin was detected in the periplasm. The cells were transferred into LB media containing chloramphenicol, thereby preventing the synthesis of *de novo* aerolysin. The results shown in Figure 11 show that the shockable proaerolysin pool decreased with time while the amount of proaerolysin in the culture supernatant increased. No β-lactamase was located in the culture supernatant, indicating that the toxin was not released by cell lysis. Thus the periplasmic pool of proaerolysin is available for secretion. Since the pleiotropic secretion mutants S9 and L1.97 are unable to secrete their periplasmic pools of protoxin, it would appear that secretion from the periplasm proceeds via the GSP. This is also further evidence that aerolysin normally passes through the periplasm as it is secreted out of the cell.

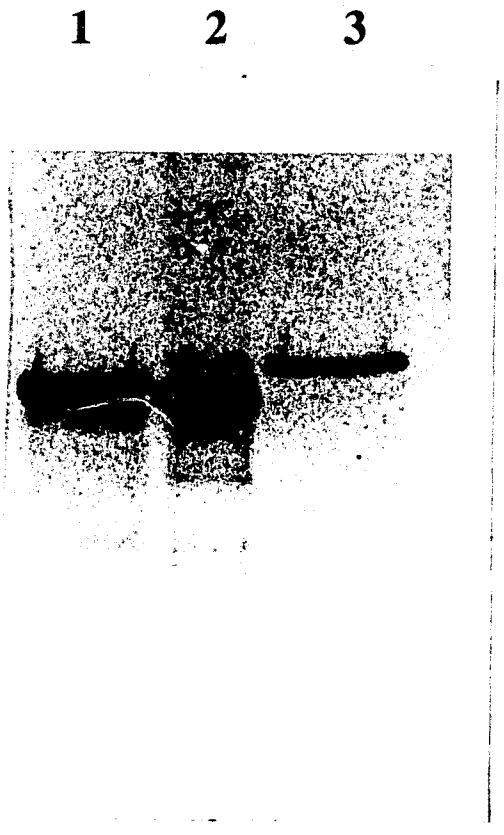

Figure 10. Presence of proaerolysin in the periplasm of *A. salmonicida*. Rif-1 containing pKW2 was grown overnight in LB medium with no glucose. Equivalent amounts of culture supernatant and shock fluid were separated by SDS-polyacrylamide gel electrophoresis and immunoblotted using an α-aerolysin monoclonal antibody. Lane 1, purified aerolysin; lane 2, culture supernatant; lane 3, shock fluid.

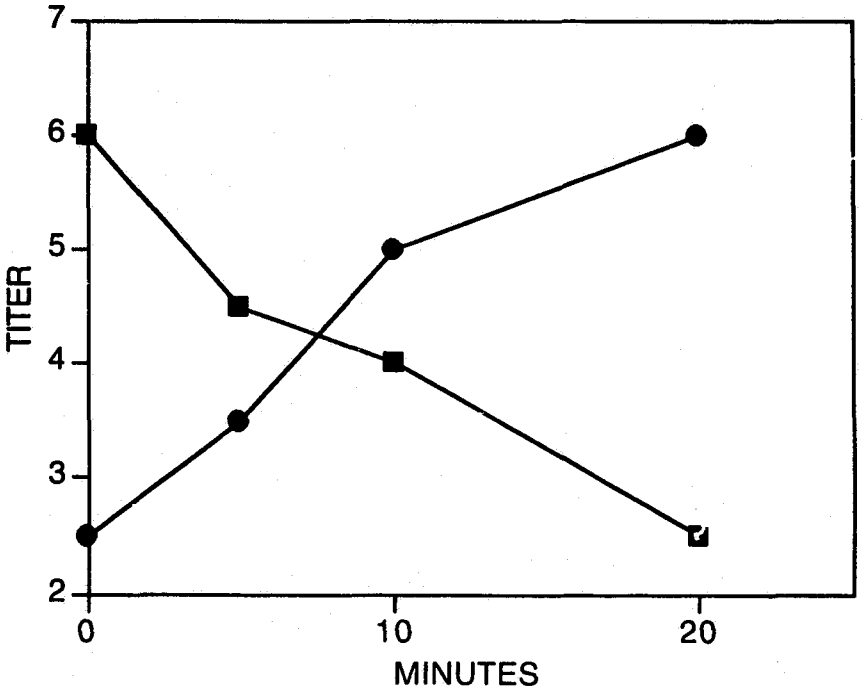

Figure 11. Release of proaerolysin from the periplasm of *A. salmonicida*. Cells were grown as described in the legend to Figure 10, washed, and transferred to fresh medium containing 100 μg ml^{-1} of chloramphenicol. Presented are titers of 100 μl volumes of supernatant (●) and equivalent volumes of shock fluids (■).

Secretion of aerolysin by a marine *Vibrio* spp.

The results obtained with aerolysin in *A. salmonicida* were the first to demonstrate that a GSP protein could be secreted by a heterologous host. In order to see if aerolysin could be secreted by a more distantly related Gram-negative organism, *aerA* was moved into the marine *Vibrio* spp. strain MVT606, which Ichige et al. (1988) have described. This strain secretes a number of different proteins, including a metalloprotease. While MVT606 was not hemolytic on HBA plates, transconjugates containing either pKM2 or pNK1 produced zones of clearing around individual colonies. As shown in Figure 12A, induction of aerolysin expression did not affect the growth of MVT606. High levels of hemolytic activity were detected in the culture supernatants of induced cultures, with approx. 60-fold more activity located extracellularly compared to in the periplasm (Figure 12B). Release was not due to cell lysis as β-lactamase activity remained intracellular (data not shown here) and cell growth was unaffected by expression of aerolysin (Figure 12A). Thus MVT606, like the *Aeromonas* spp., is able to secrete aerolysin.

As shown in Table 4, both induced and uninduced cultures of MVT606-pKM2 produced aerolysin, while only induced cultures of MVT606-pNK1 synthesized aerolysin. This indicates that this *Vibrio* spp., like *A. salmonicida*, is capable of utilizing the *aerA* promoter and that *aerA* is completely under the control of the *tac* promoter in pNK1.

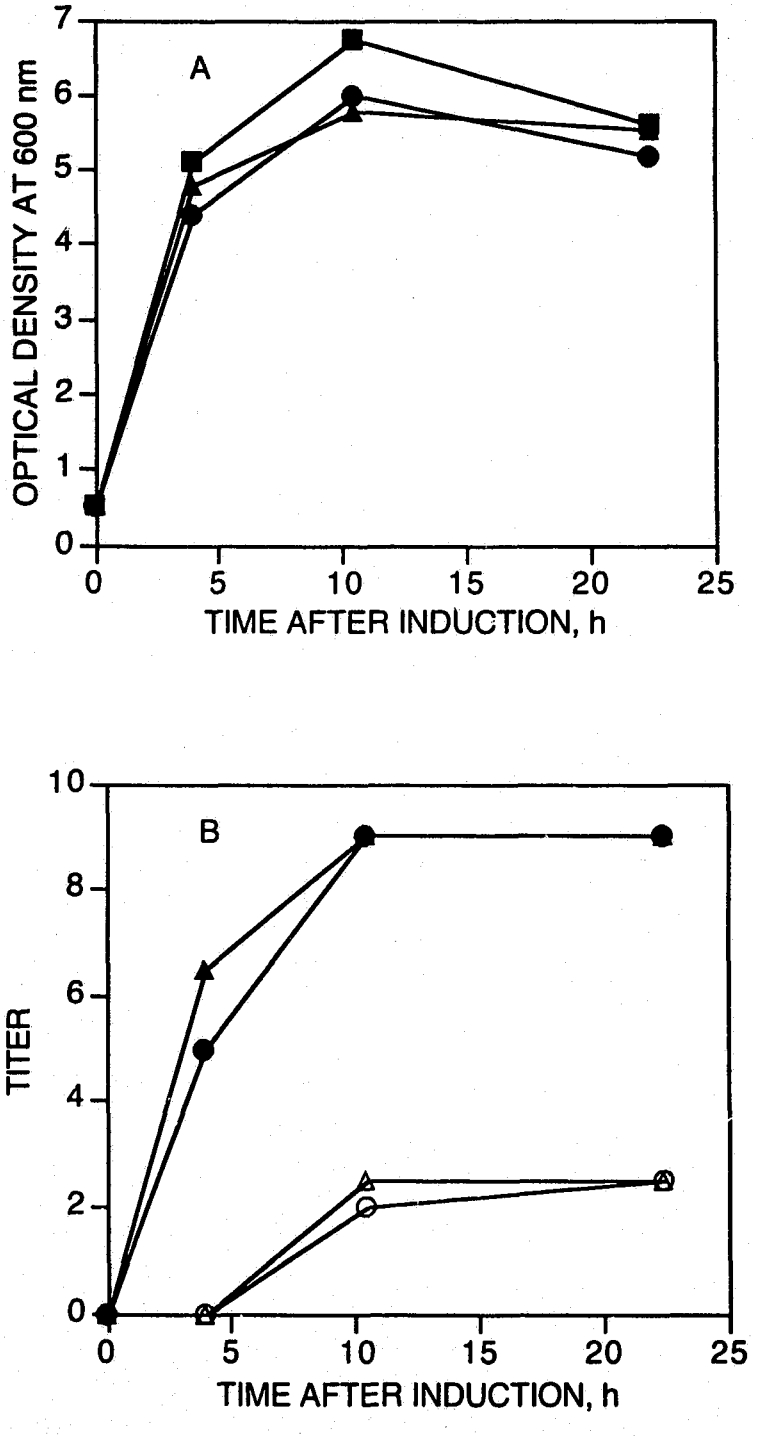

Figure 12. Cell growth and aerolysin production by MVT606 containing pKM2 or pNK1 grown in LB medium. (A) Growth of MVT606 (■), MVT606-pKM2 (▲), and MVT606-pNK1 (●). (B) Aerolysin produced in culture supernatants (▲) and in shockates (Δ) by MVT606-pKM2 and MVT606-pNK1 (●,O). Cultures were induced with IPTG after reaching an OD_{600} of 0.5.

Table 4. Production of aerolysin by the parent *Vibrio* strain MVT606.

Strain and growth medium[a]	Optical density at 600 nm	Titer[b]
MVT606-pKM2		
LB	5.2	7.5
LB + IPTG	5.9	9.0
MVT606-pNK1		
LB	5.3	0.0
LB + IPTG	5.5	7.0

[a] Bacteria were grown overnight in LB medium, with or without 1 mM IPTG.

[b] Titers of the culture supernatants were measured as described in Materials and Methods.

Secretion of proaerolysin from pleiotropic secretion mutants of MVT606

Ichige et al. (1988) produced several pleiotropic secretory mutants of MVT606 by random transposon mutagenesis of the chromosomal DNA. Plasmid pKM2 was transferred to three of them, MVT1064, MVT1181 and MVT1192, to see if they were also unable to secrete aerolysin. Growth of all the mutant *Vibrio* strains containing the plasmid was slower than the growth of MVT606-pKM2, with MVT1181-pKM2 demonstrating the most pronounced reduction (Figure 13). While culture supernatants of all the mutant strains containing pKM2 exhibited some hemolytic activity, there was 4-30 fold less than in the culture supernatant of the MVT606-pKM2 at each time point (Figure 14A). More importantly, all the mutant strains containing pKM2 accumulated large amounts of aerolysin in the periplasm (Figure 14B), as had been found for the pleiotropic secretion mutants of *A. hydrophila* (Howard and Buckley, 1983; Jiang and Howard, 1991). The amounts in the mutant periplasms were at least 100-fold higher than that observed in the periplasm of MVT606-pKM2 3 h after induction. The similarity between these results and those found with the pleiotropic secretion mutants of *A. hydrophila* suggests that *Aeromonas* spp. and the marine *Vibrio* spp. use a similar secretion pathway.

Molecular size of the aerolysin produced in the marine *Vibrio* spp.

To further examine the similarities between the *Aeromonas* spp. and *Vibrio* spp. secretion pathways, samples of culture supernatants of the different *Vibrio* strains containing pKM2 were run on Western blots to determine if the secreted toxin had been processed. Figure 15 shows that MVT606-pKM2 (lane 1) processed the protoxin into active aerolysin. In contrast, small amounts of proaerolysin were detected in the culture supernatant of MVT1181-pKM2 (lane 4). As shown in Figure 14A, culture supernatants

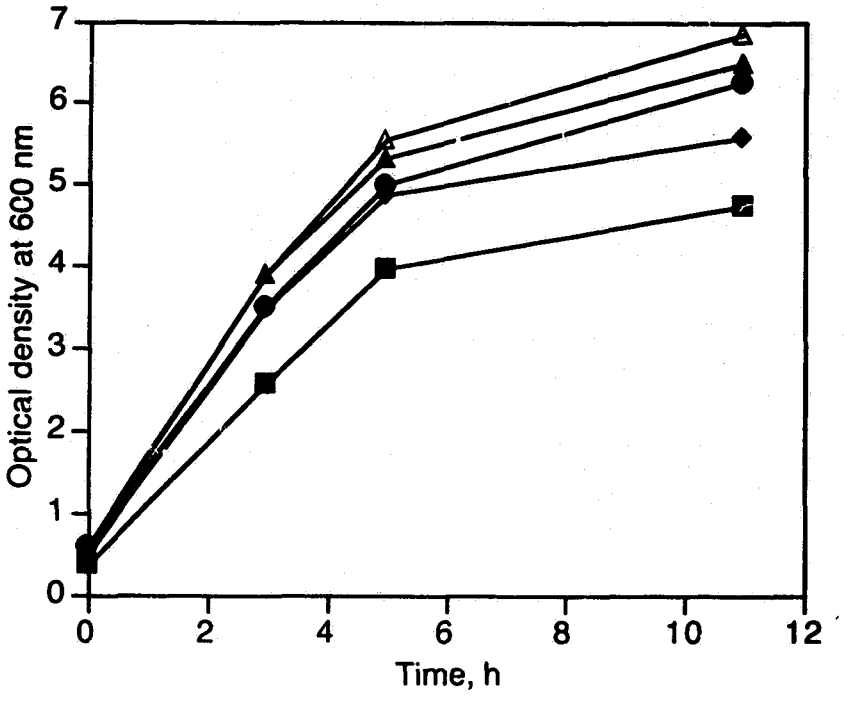

Figure 13. Growth of MVT606 and the pleiotropic secretion mutants containing pKM2. Cultures of the marine *Vibrio* spp. were grown in LB medium and induced with IPTG at an OD_{600} of 0.5. Growth was followed by measuring the OD_{600} of the cultures over the next 11 h. Shown are MVT606 (Δ), MVT606-pKM2 (▲), MVT1064-pKM2 (◆), MVT1181-pKM2 (■), and MVT1192-pKM2 (●).

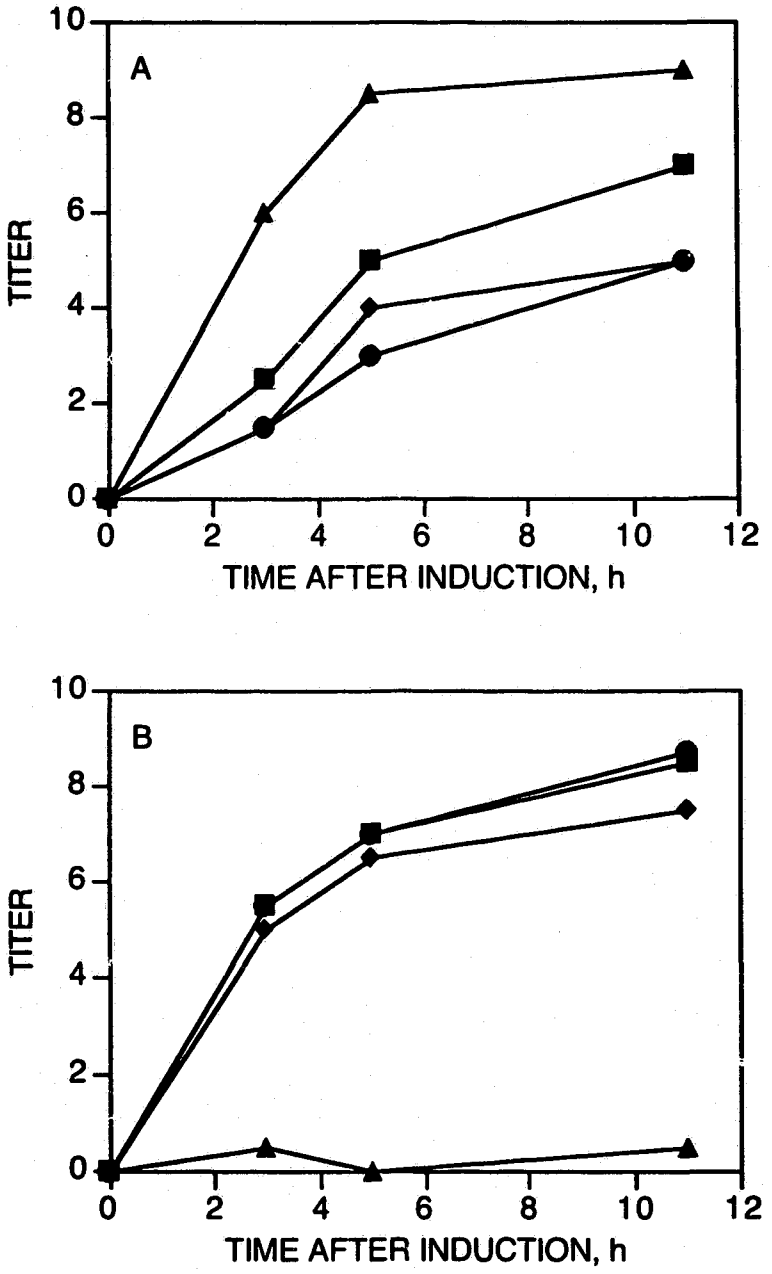

Figure 14. Distribution of aerolysin in the culture supernatants (A) and shockates (B) of the *Vibrio* strains containing pKM2. Cultures were grown as described in Figure 11. Shown are MVT606-pKM2 (▲), MVT1064-pKM2 (◆), MVT1181-pKM2 (■), and MVT1192-pKM2 (●).

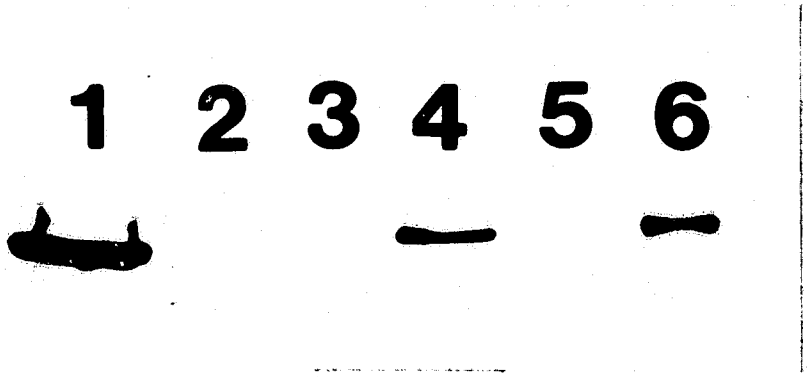

Figure 15. Characterization of aerolysin in the culture supernatants of the *Vibrio* strains containing pKM2 by immunoblotting. Lane 1, MVT606-pKM2; lane 2, MVT1192-pKM2; lane 3, MVT1064-pKM2; lane 4, MVT1181-pKM2; lane 5, MVT606; lane 6, purified proaerolysin.

of this strain have the highest titers. Jiang and Howard (1991) have found that some mutations to the GSP secretion operon also affect the biogenesis of the outer membrane. The results with MVT1181-pKM2 may indicate that a similar mutation has occurred, resulting in a "leaky" outer membrane.

Another surprising observation made with MVT1181-pKM2 and MVT1192-pKM2 was that active aerolysin was released by osmotic shock (data not shown). This indicated that the proaerolysin was being proteolytically processed in these mutants. Additionally, processed toxin was observed on immunoblots of periplasmic shockates of these two *Vibrio* mutants (Figure 16, lanes 3 and 1). Only proaerolysin was located in the periplasm of MVT1064-pKM2 (lane 5) while neither was observed in MVT606-pKM2 shocks (lane 6). When *o*-phenanthroline, an inhibitor of the MVT606 protease (Ichige et al., 1988), was present during the shocking procedure, only proaerolysin was recovered from the periplasm of MVT1181-pKM2 (lane 4) and MVT1192-pKM2 (lane 2). This demonstrated that proaerolysin is not activated in the periplasm of the mutants. Apparently, in the absence of a protease inhibitor, the shockate procedure releases the periplasmic pools of both proaerolysin and the *Vibrio* metalloprotease, resulting in activation of the toxin. It would then appear that proaerolysin and the metal protease can apparently remain in the periplasm together without causing the activation of the proaerolysin. This could mean that the protease is inactive in the periplasm or that the two proteins pass through different compartments in the periplasm to prevent activation of the proaerolysin.

Secretion of Trp227 aerolysin mutants

It has been speculated that there are signals within secreted proteins which direct them to the secretion machinery (Kornacker and Pugsley, 1990; Hamood et al., 1989). However, none have yet been found in any of the proteins known to use the GSP. In an

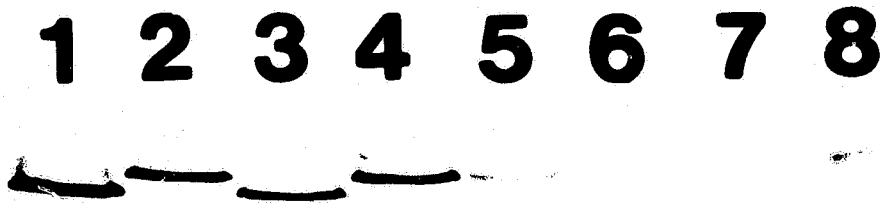

Figure 16. Characterization of aerolysin in the shockates of the *Vibrio* strains containing pKM2 by immunoblotting: inhibition of activation during shocking of MVT1181-pKM2 and MVT1192-pKM2 by *o*-phenanthroline. Cells from the experiment described in Figure 11 were fractionated by osmotic shock as described in Materials and Methods. Lane 1, MVT1192-pKM2; lane 2, MVT1192-pKM2 plus *o*-phenanthroline; lane 3, MVT1181-pKM2; lane 4, MVT1181-pKM2 plus *o*-phenanthroline; lane 5, MVT1064-pKM2; lane 6, MVT606-pKM2; lane 7, MVT606; lane 8, purified proaerolysin.

attempt to locate such a signal within aerolysin, site-directed mutagenesis was used to change specific amino acids in the toxin (see Table 3).

Howard et al. (1987) had previously found a 10 amino acid region in aerolysin which is similar to a 10 amino acid sequence in *Staphylococcal aureus* α-toxin. A Trp residue is found in this sequence at position 227. Because of the similarity of this region to α-toxin, and because Trp residues have been postulated to play a role in membrane interaction of other toxins (de Kruijff, 1990), Trp227 was chosen as the first amino acid to change by site-directed mutagenesis. It was changed to three different amino acids, Gly, Leu and Phe, and the resulting mutant *aerA* genes were inserted into pMMB66. As with wild type proaerolysin, each of the three mutant protoxins was expressed in *E. coli* and could be detected in the periplasmic shockates (data not shown). When the constructs were transferred to CB3, different amounts of each of the mutant protoxins were found in the culture supernatants of the induced cultures (Figure 17). Only a small amount of Leu227 (lane 4) or Gly227 (lane 5) was observed in the culture supernatant samples run on an SDS-polyacrylamide gel. Even the Phe227 mutant showed a decreased amount of supernatant protoxin as compared to wild type (lane 3 vs lane 2). This demonstrated that even the relatively conservative change from a Trp to a Phe at this position of aerolysin results in a decreased secretion efficiency by CB3.

Intracellular accumulations of Trp227 aerolysin mutants in CB3

While the mutant aerolysins were greatly reduced in the culture supernatants, they could be detected in the whole cells of CB3 by Western blotting (data not shown). This suggested that they might be getting stuck at some point in the secretion pathway. In order to closely follow the proteins as they passed through the cells, CB3 cultures containing either wild type proaerolysin or one of the Trp227 mutants were induced with IPTG,

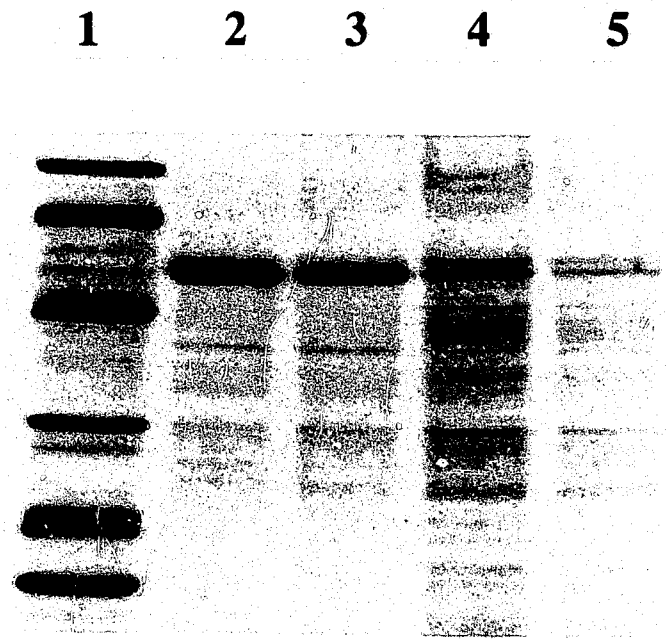

Figure 17. SDS-polyacrylamide gel electrophoresis of Trp227 mutants in *A. salmonicida* culture supernatants. Cultures were induced at an optical density at 600 nm of 0.5. Culture supernatants were obtained from cells grown to an optical density of approximately 6.0 in LB-Davis medium containing 0.2% glucose and concentrated 25-fold as described under Materials and Methods. Amounts corresponding to approximately 50 µl of original culture supernatants were applied. Lane 1, molecular mass standards (97, 66, 43, 31, 21.5, 14.4 kDa); lane 2, wild type (Trp227); lane 3, Phe227; lane 4, Leu227; lane 5, Gly227.

pulsed with [^{35}S]-methionine, and immunoprecipitated with α-aerolysin monoclonal antibodies. The results presented in Figure 18 show that wild type proaerolysin appeared in the culture supernatant of CB3-pNB5 less than 15 min after pulse labelling with [^{35}S]-methionine (lane 16), while none was observed in the periplasmic shockate or shocked cell fractions. Howard and Buckley (1985a) found similar results for aerolysin secretion in *A. hydrophila*, although they did detect small amounts of proaerolysin in the periplasmic shockates. However, their AH65 cells had been treated with CCCP which prevented the periplasmic pool of proaerolysin from being secreted. In contrast, the Gly227 (lane 2), Leu227 (lane 8) and Phe227 (lane 14) proaerolysins initially appeared in the periplasmic shockate fractions. While a small amount of Phe227 appeared in the culture supernatant (lane 13) none of the Gly227 (lane 1) or Leu227 (lane7) was detected outside the cells. After a further 15 min, the Leu227 (lane 12) and Gly227 (lane 6) began to accumulate in the shocked cell fractions, although their concentrations in the shocks were still higher. There was still none in the culture supernatants.

These results indicate that the mutations did not affect export across the inner membrane into the periplasm, or processing by signal peptidase, as all the aerolysin seen in the autoradiograph corresponded in size to proaerolysin. Thus the intracellular accumulations of the mutant proteins which were observed must have resulted from a block in secretion at a later step. It appears that the mutant proteins are unable to associate with a component of the GSP because of the changes at position 227. The observed accumulation of Gly227 and Leu227 in the shocked cell fractions suggest that the proteins are associated with either the inner or outer membrane, or in the cytoplasm. However, the latter is unlikely since no preproaerolysin was observed.

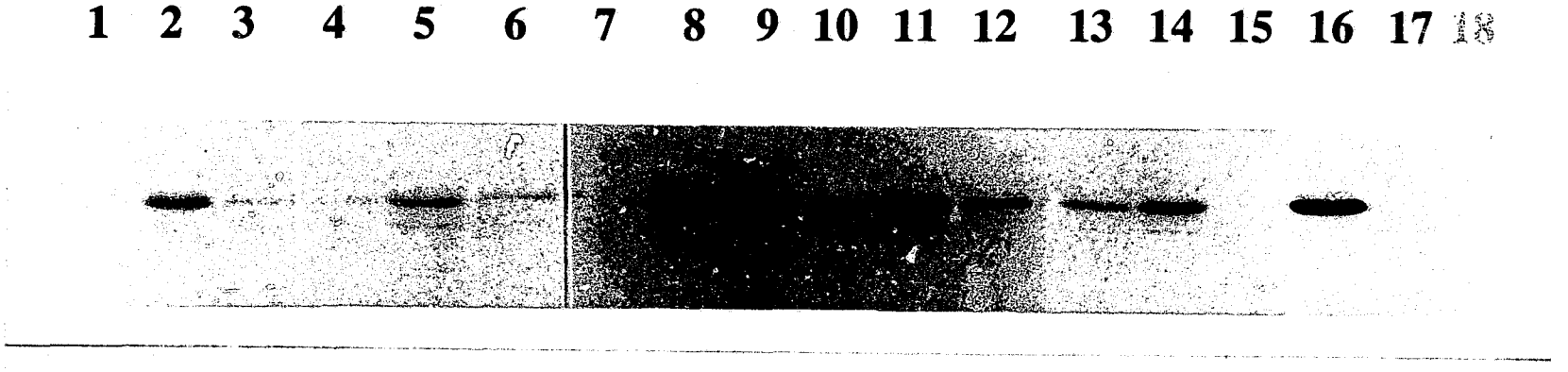

Figure 18. Subcellular distribution of mutant proaerolysins. Cells were induced with IPTG, then pulsed with [^{35}S]-methionine, and fractions were isolated and immunoprecipitated as described under Materials and Methods. Lanes 1-3, Gly227 pulsed 15 min; lanes 4-6, Gly227 pulsed 45 min; lanes 7-9, Leu227 pulsed 15 min; lanes 10-12, Leu227 pulsed 45 min; lanes 13-15, Phe227 pulsed 15 min; lanes 16-18, wild type Trp227 pulsed 15 min. Lanes 1, 4, 7, 10, 13, and 16, culture supernatants; lanes 2, 5, 8, 11, 14, and 17, shockates; lanes 3, 6, 9, 12, 15, and 18, shocked cells.

Subcellular localization of the Trp227 aerolysin mutants

To determine where the Leu227 and Gly227 mutant proteins were getting stuck in the cell, cultures of CB3 containing the mutant genes in LB medium containing Davis buffer and glucose were induced and grown overnight. The membrane fractions were isolated on sucrose density gradients as described in Materials and Methods. Fractions of 0.7 ml were taken from the top of the gradients and screened at 280 nm. Peak fractions were mixed with sample buffer, boiled for 3 min and loaded onto SDS-polyacrylamide gels. As shown in Figure 19, most of the cell-associated Gly227 (lane 9) and Leu227 (lane 6) were detected on Western blots in the fractions containing outer membranes. In contrast, none of the wild type proaerolysin was found in either the inner or outer membrane fractions (lanes 2 and 3). In order to determine if these results were due to an artifact which resulted from unlysed cells, membrane preparations of Leu227 were floated from the bottom of a sucrose density gradient (Figure 20). Again, the Leu227 protein was only detected in the outer membrane fractions. It is possible that the mutant proteins enter the membrane but are then unable to interact with one of the secretion components. Alternatively, they could be diverted into the outer membrane because they are unable to interact with the secretion machinery.

Effect of expression of mutant aerolysins on the secretion of chromosomal extracellular protease

If the Leu227 and Gly227 mutants are getting stuck in the outer membrane, it would not be surprising to find that they could block the secretion of other *A. salmonicida* proteins which require the GSP. The mutant proteins may be stuck in a position which prevents other secreted proteins from interacting with components of the secretion pathway. As mentioned earlier, we have assumed that the *A. salmonicida* protease, like the *A. hydrophila* and MVT606 proteases, utilizes the GSP. When Leu227 was expressed in

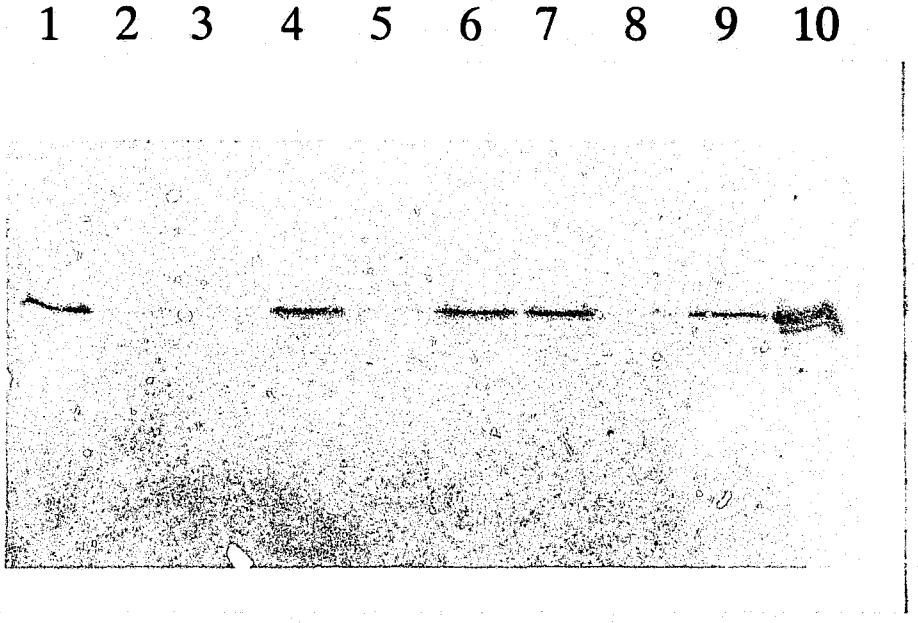

Figure 19. Association of mutant proaerolysins with *A. salmonicida* membrane fractions. Cultures of CB3 containing the different mutants were grown up in LB-Davis medium containing 0.2% glucose. Cells were harvested 16 h after induction with IPTG and fractionated as described under Materials and Methods. After separation on sucrose density gradients, inner and outer membrane fractions were electrophoresed in an SDS-polyacrylamide gel and immunoblotted with α-aerolysin monoclonal antibodies. Lanes 1-3, wild type; lanes 4-6, Gly227; lanes 7-9, Leu227; lane 10 proaerolysin standard. Lanes 1,4, and 7, samples applied to sucrose density gradients; lanes 2, 5, and 8, inner membrane fractions; lanes 3, 6, and 9, outer membrane fractions.

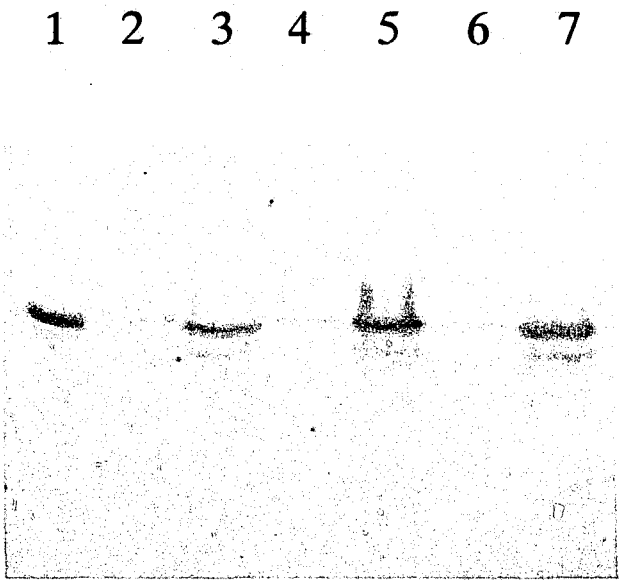

Figure 20. Isolation of Leu227 proaerolysin with the outer membrane of *A. salmonicida*. Outer membranes obtained as in Figure 17 were made to 60% (w/w) sucrose and recentrifuged after overlaying as described under Materials and Methods. Fractions containing outer membranes were electrophoresed and immunoblotted as in Figure 17. Lane 1, proaerolysin standard; lanes 2, 4, and 6, membranes from uninduced cells; lanes 3, 5, and 7, membranes from induced cells. Lanes 2 and 3 contain cell envelopes before separation; lanes 4 and 5, outer membrane fractions after the first sucrose density gradient; lanes 6 and 7, outer membrane fractions after the second sucrose gradient.

Rif-1, no effect was observed in the amount of protease found on the culture supernatant compared to uninduced cultures (Figure 21). It would appear then that there are either a large number of sites able to transport proteins across the outer membrane by the GSP, or that the mutant proteins are diverted into the outer membrane because they are unable to associate with certain components of the secretion machinery. It may also be possible that proteins using the GSP share components of the secretion machinery at an early stage in the translocation process, but use different components at later stages of secretion. If this is the case, then the mutant protoxins may be getting stuck at one of the components required for the later stages of aerolysin secretion, but not for secretion of the protease.

Proteolytic digestion of purified Trp227 aerolysin mutants

Proaerolysin can be activated by trypsin to the active toxin. The C-terminal 43 amino acids are proteolytically removed by trypsin to form active aerolysin (Howard and Buckley, 1985b). Aerolysin is quite resistant to further proteolysis and forms oligomers (Garland and Buckley, 1988). We wished to see if the mutant proteins behaved in a similar way. As shown in Figure 22, both Phe227 (lane 2) and Leu227 (lane 3) were correctly processed by trypsin to a species similar in size to aerolysin. This indicates that the changes made do not drastically alter the conformation of the protein. These mutants also form high molecular weight oligomers, seen at the top of each lane, in the same way as wild type. However, neither of the processed mutant proteins was as active as wild type. Table 5 shows that Phe227 had approx. 12-25% of wild type activity while the leucine mutant had about 1%. Neither of the mutants was active before treatment with trypsin. Thus the mutations to Trp227 affected both the secretion and the hemolytic activity of the proteins, but did not greatly change their conformation.

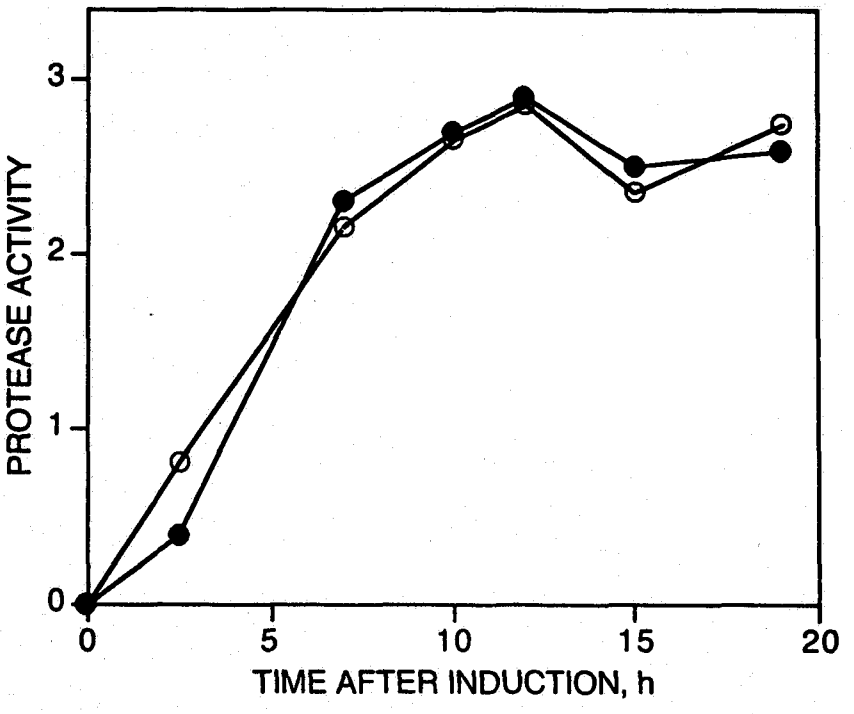

Figure 21. Influence of Leu227 proaerolysin production on the secretion of the extracellular *A. salmonicida* chromosomal protease. Protease activity was measured in the culture supernatants of induced (O) and uninduced (●) cells. The results are the means of duplicate determinations.

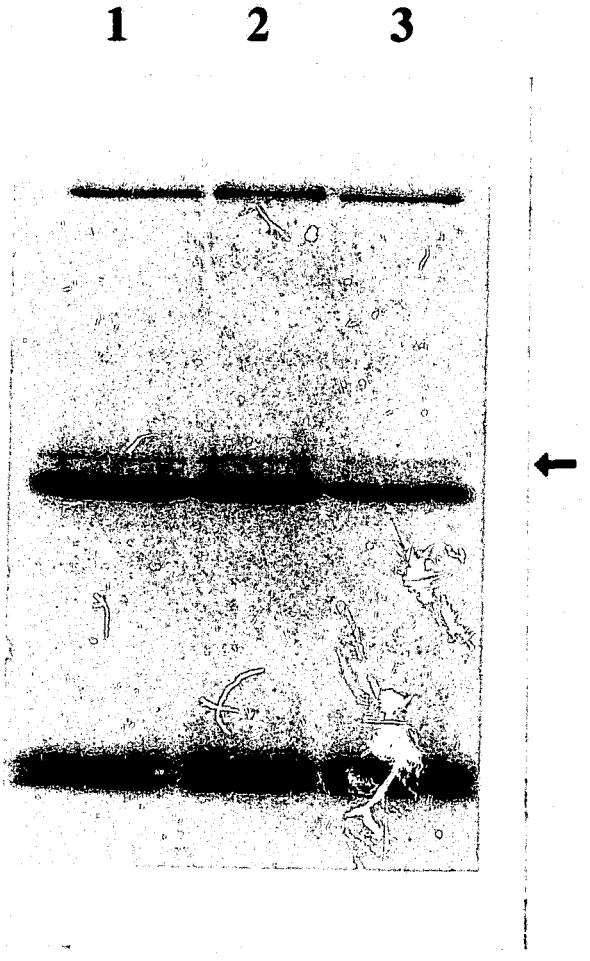

Figure 22. Effect of trypsin treatment on purified Leu227 and Phe227 proaerolysins. Samples were digested with 10 μg ml^{-1} trypsin for 30 min before separation. **Lane 1,** proaerolysin; lane 2, Phe227; lane 3, Leu227. The arrow marks the position of all three proteins before trypsin treatment.

Table 5. Hemolytic activity of the Trp227 mutant proteins.

Amino acid at position 227	Titer before trypsin	Titer after trypsin
Trp	0	10
Phe	0	7
Leu	0	3

Wild-type (Trp227), Phe 227 and Leu227 proaerolysin (10 μg) were titered before and after incubation with trypsin (0.1 μg) for 10 min.

Proteolytic degradation of outer membrane-associated Leu227 proaerolysin

The results presented in Figure 22 show that purified Leu227 is as resistant to trypsin as wild type aerolysin. It is possible though, that the protein trapped in the outer membrane is in a different trypsin-sensitive conformation. To check this possibility, outer membrane fractions containing the Leu227 protoxin were digested with different concentrations of trypsin and run on a Western blot. The results show a different pattern from that seen with purified Leu227. At 10 μg/ml trypsin, the Leu227 in the outer membrane fractions began to disappear, and there were no signs of oligomers forming (lane 4, Figure 23). At 1 mg/ml, all the Leu227 in the outer membrane had disappeared after a 30 min incubation (lane 5). In contrast, 1 mg/ml of trypsin converted purified proaerolysin to the active toxin but did not degrade it further (lane 6). The degradation of the membrane associated Leu227 required trypsin, as outer membrane associated Leu227 was not degraded if it was incubated alone for 30 min (lane 3). These results suggest that the membrane associated form of Leu227 is in a different conformation than wild type proaerolysin or Leu227 in solution. This leads to the intriguing possibility that proaerolysin may have to undergo a conformational shift during its transport across the outer membrane. Trypsin added to intact CB3 cells containing Leu227 did not result in a decrease in outer membrane associated Leu227 (data not shown), indicating that the trypsin-sensitive region of the proaerolysin is not exposed to the exterior. The addition of trypsin to cultures of induced CB3-pNB5 also did not result in a decrease in the amount of aerolysin found in the culture supernatant. If the protein crosses the outer membrane in an unfolded conformation, it might have been expected that it would be more sensitive to trypsin as it initially became exposed to the surface of the cell. However, these results indicate that it crosses into the supernatant in a trypsin resistant conformation, or that folding into its native conformation occurs very quickly.

1 2 3 4 5 6

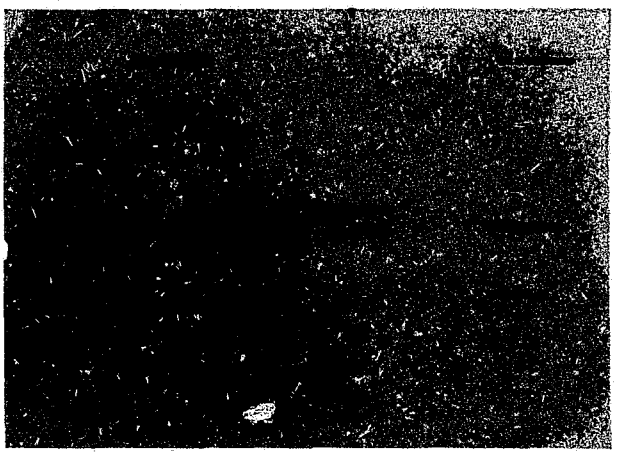

Figure 23. Effect of trypsin digestion on Leu227 proaerolysin in outer membranes. Outer membranes obtained from sucrose density gradients as in Figure 17 were incubated for 30 min with 10 μg ml^{-1} or 1 mg ml^{-1} trypsin. They were then electrophoresed and immunoblotted. Lane 1, proaerolysin; lane 2, proaerolysin and 10 μg ml^{-1} trypsin; lane 3, Leu227 proaerolysin in outer membranes; lane 4, Leu227 outer membranes and 10 μg ml^{-1} trypsin; lane 5, Leu227 outer membranes and 1 mg ml^{-1} trypsin; lane 6, proaerolysin and 1 mg ml^{-1} trypsin.

Site-directed mutagenesis of a tryptophan rich region and a cysteine residue in aerolysin

Since inhibition of aerolysin secretion was successfully accomplished by changing a single tryptophan residue, we wished to see if changing other tryptophan residues would have the same effect. One region in aerolysin contains three Trp residues within a 5 amino acid sequence. This sequence (^{369}Lys-Trp-Trp-Asp-Trp) is similar to one found in the oxygen-labile toxins that has been proposed to play a role in membrane penetration (de Kruijff, 1990). Two of the Trp residues (371 and 373) were changed to Leu while the Lys and Asp were changed to Gln and Asn respectively. In contrast to the reduction in secretion that occurred when Trp227 was changed to Leu, no reduction was observed when either Trp371 or Trp373 was changed in a similar manner (lanes 3 and 5, Figure 24). These residues have now been shown to play a role in the oligomerization of the active toxin (van der Goot et al., 1993b). Both the Gln369 and Asn373 mutants were also secreted as efficiently as wild type protoxin in CB3 (lanes 2 and 4).

Aerolysin contains 4 cysteine residues. The Cys at amino acid 159 was changed to a serine to see if disrupting a disulfide bond and producing a free cysteine would affect the conformation of the protein, and thus disrupt secretion. Two amino acids near Cys159, Asn154 and Gly157, were also changed, to an aspartic acid and alanine respectively. All three of these mutants were secreted as efficiently as wild type protoxin (data not shown). It had previously been shown that mutating any of the six histidine residues in aerolysin had no effect on their secretion (Green and Buckley, 1990). As well, approx. 15 other individual amino acid changes have now been made to aerolysin and only the Trp227 changes have resulted in reduced secretion (unpublished results, this lab). They are also the only single amino acid changes found to date for any GSP protein which affect translocation across the outer membrane. It is possible that this Trp directly interacts with one of the secretion components. However, proaerolysin is a dimer in solution (van der

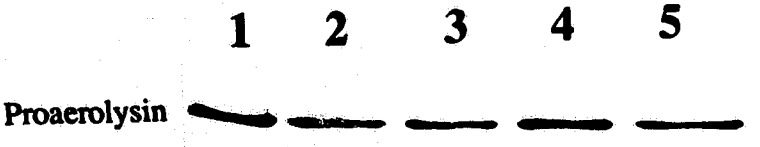

Figure 24. Secretion of Leu371, Leu373, Asn372 and Gln369 by *A. salmonicida*. CB3 containing the cloned modified *aerA* structural genes was grown in LB-Davis media containing 0.2% glucose and induced with IPTG at an OD_{600} of 0.5. Equivalent volumes of culture supernatant from cells grown to an OD_{600} of approx. 4.5 were concentrated 40-fold by membrane filtration, boiled in 1 x sample buffer and applied directly to a 12% SDS-polyacrylamide gel. Lane 1, wild type proaerolysin; lane 2, Gln369; lane 3, Leu371; lane 4, Asn372; lane 5, Leu373.

Goot et al., 1993a) and the X-ray crystal structure of proaerolysin predicts that Trp227 is not exposed on the surface of the dimer (Parker et al., 1994). Since the purified Trp mutants are as resistant to trypsin as wild type, it is unlikely that the Trp plays a large role in the dimer conformation. However, since the membrane-associated mutants are sensitive to trypsin, Trp227 may play a role in a conformational shift as the protoxin crosses the outer membrane.

Location of AerA'-'PhoA fusion proteins

A technique which has been used in the past to locate translocation domains in exported proteins has been to fuse portions of the proteins to a leaderless PhoA (Hoffman and Wright, 1985). PhoA is a periplasmic protein in *E. coli* which can be detected once it has crossed the inner membrane. Thus fusion of leaderless PhoA to appropriate signals allows the detection of correctly exported fusion proteins. To see if there are any domains in aerolysin which can direct PhoA across the inner and outer membranes, fusions of varying lengths of AerA were made to PhoA. This was done by introducing λTnPhoA into *E. coli* containing pNB5. Plasmids containing the PhoA transposon were obtained and used to transform CC118. Of the 1200 transformants that grew on each plate, about 15-20 were blue colonies. Sequencing of the plasmids obtained from these identified three containing genes for fusion proteins which were used in subsequent experiments (Figure 25). The largest fusion, in plasmid pAB2, has nearly all of the proaerolysin structural gene, coding for 435 of the 470 amino acids of proaerolysin. The intermediate fusion, pAA2, contains approximately half of the *aerA* gene, coding for 234 amino acids of proaerolysin. The smallest fusion, pAD3, contains only the sequence of *aerA* which codes for the signal peptide and the first two amino acids of the protoxin molecule.

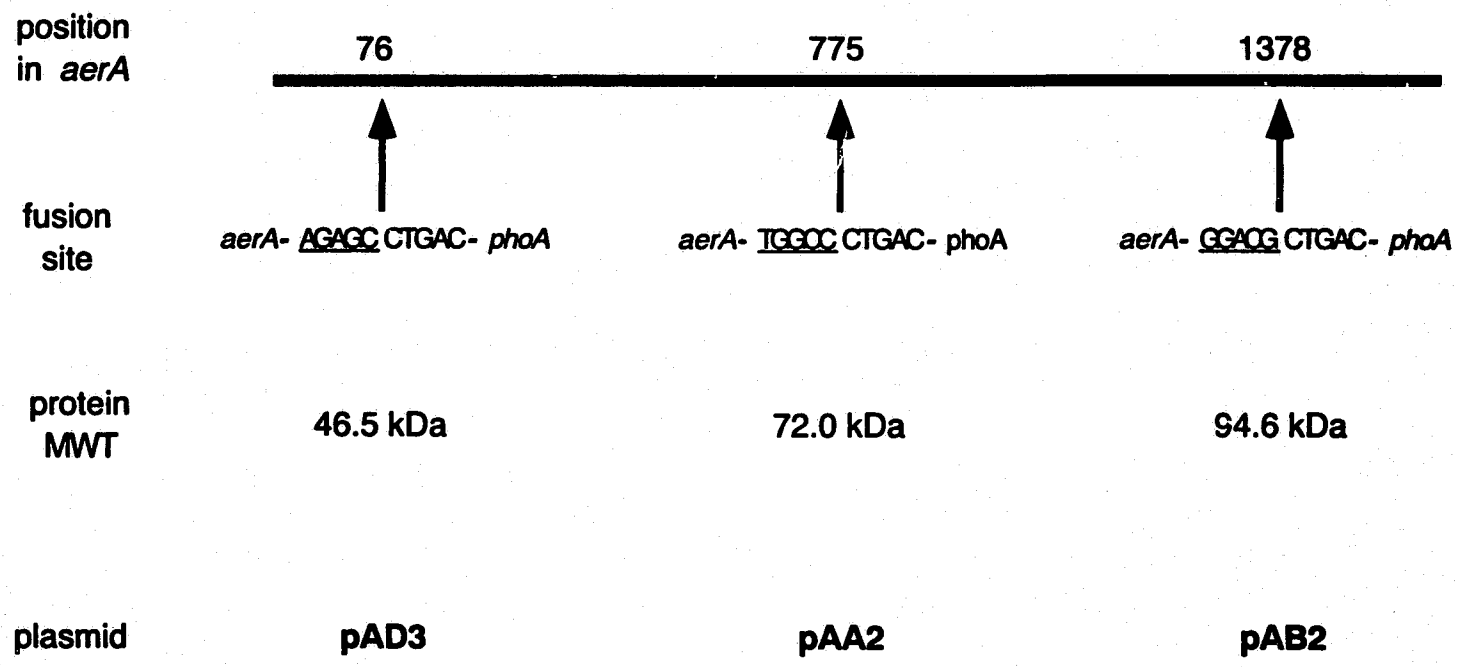

Figure 24. Location of the *aerA'-'phoA* fusion sites. Nucleotides in *aerA* are numbered from the beginning of the structural gene of preproaerolysin. Molecular masses of the predicted fusion proteins (in kDa) are calculated with the assumption that the signal sequence is correctly removed.

Size of the AerA'-'PhoA fusion proteins expressed in *E. coli*

The calculated molecular masses of the fusion proteins pAD3, pAA2, and pAB2 were calculated from their nucleotide sequences to be 46.5, 72.0 and 94.6 kDa respectively (Figure 25). To see if CC118 containing the fusion plasmids produced fusion proteins of this size, induced cell samples were run on Western blots and screened with α-AerA and α-PhoA antibodies. Proteins of the correct sizes were identified in *E. coli* shocked cells (Figure 26) but not in culture supernatant fractions (data not shown). The smallest, pAD3, was nearly identical in size to PhoA itself and was only detected by the α-PhoA polyclonal antibody. Not surprisingly, this fusion protein was not detected by the α-aerolysin monoclonal antibody, as not enough of the aerolysin protein was present to cross-react. However, the two larger fusion proteins were detected by both antibodies. A number of smaller sized bands were detected by the α-PhoA polyclonal in the pAA2 and pAB2 cells, indicating that the fusion proteins may be degraded by an intracellular protease. Since these bands were only observed using the α-PhoA antibody, and bands similar in size to PhoA were observed as degradation products, it would appear that the aerolysin portion of the fusion proteins was degraded.

Size and cellular location of the AerA'-'PhoA fusion proteins expressed in *A. salmonicida*

While it was not surprising to find that the fusion proteins were not secreted by *E. coli* because it lacks a GSP, it was hoped that enough information was contained in the portions of AerA to direct the fusion proteins across the outer membrane in *A. salmonicida*. The two larger fusion proteins were not detected in CB3 grown up in LB/0.2% glucose medium using immunoblots with either an α-PhoA or α-AerA antibodies (data not shown). In all three cases, only a band the size of PhoA itself could be detected in the intracellular fractions using the α-PhoA polyclonal antibody. This suggested that the fusion proteins

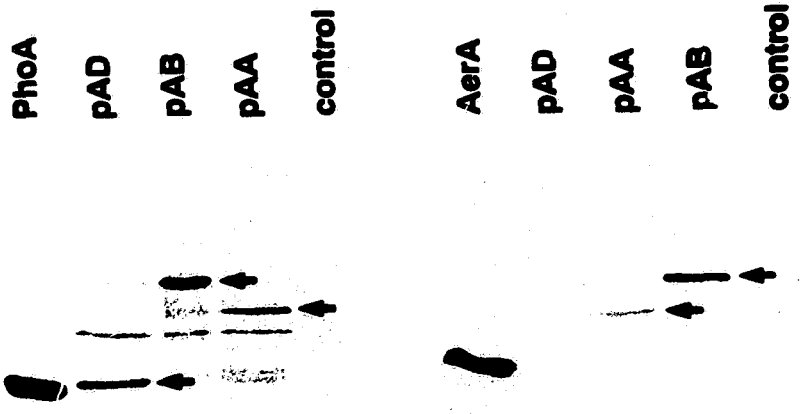

Figure 26. Immunoblot of fusion proteins expressed by plasmids in *E. coli*. Cultures were grown in LB medium containing 0.2% glucose, and induced with IPTG at an OD_{600} of 0.5. Proteins detected with PhoA antiserum are shown in the left panel. Proteins detected with the aerolysin antiserum are shown in the right panel. The apparent molecular masses, obtained by comparison with the positions of standard proteins, were 45.2, 74.1, and 97.5 kDa for the products of pAD3, pAA2, and pAB2 respectively. The lanes marked PhoA and AerA contained purified alkaline phosphatase and aerolysin respectively.

were being degraded in the CB3 cells as well. To try to detect the fusion proteins in the *A. salmonicida* cells before they were degraded, cultures of CB3 containing the fusions were pulse labelled with [^{35}S]-methionine for short time periods and immunoprecipitated with α-PhoA antiserum (Figure 27). All three fusions were predominantly located in the shocked cell and periplasmic fractions. Only in the case of pAD3 was any of the fusion protein seen in the culture supernatant (Figure 27). For both pAA2 and pAB2, smaller sized bands were located in both intracellular fractions, indicating that the fusion proteins were being degraded intracellularly. Therefore, under these conditions, the fusion proteins did not appear to be secreted by *A. salmonicida*.

The distribution of alkaline phosphatase activities in cells grown in LB medium containing glucose paralleled the distribution of fusion proteins observed in the pulse labelling experiment (Figure 28A). The highest level of PhoA activity was found in the periplasm of CB3-pAD3, with over 60% of the activity found in the shocks and 30% in the shocked cells. This was reversed for the two larger fusion proteins, with higher amounts located in the shocked cells. Little or no enzyme activity was observed in any of the culture supernatant fractions. Under these same conditions, aerolysin was secreted by *A. salmonicida* (Figure 2A).

Effect of growth media on the distribution of alkaline phosphatase in *A. salmonicida*

A drastic change in the distribution of alkaline phosphatase activities was observed in all the strains when the composition of the growth media was changed. When glucose was not added to the growth media, the culture supernatant of CB3-pAD3 had most of the PhoA activity after 8 h growth (Figure 28B). This was also found for CB3-pAA2 and CB3-pAB2. In no case was there evidence of non-selective leakage through the outer membrane, as two marker enzymes, β-lactamase and RNase, remained associated with the

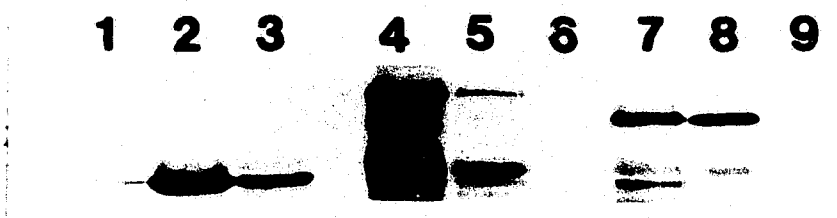

Figure 27. Autoradiograph of PhoA fusion proteins expressed in *A. salmonicida*. Proteins were labelled with [^{35}S]-methionine and precipitated with PhoA antiserum as described in the text. Lanes 1, 6, and 9, supernatants; lanes 2, 5, and 8, shockates; and lanes 3, 4, and 7, shocked cells. Lanes 1-3, CB3-pAD3; lanes 4-6, CB3-pAB2; lanes 7-9, CB3-pAA2.

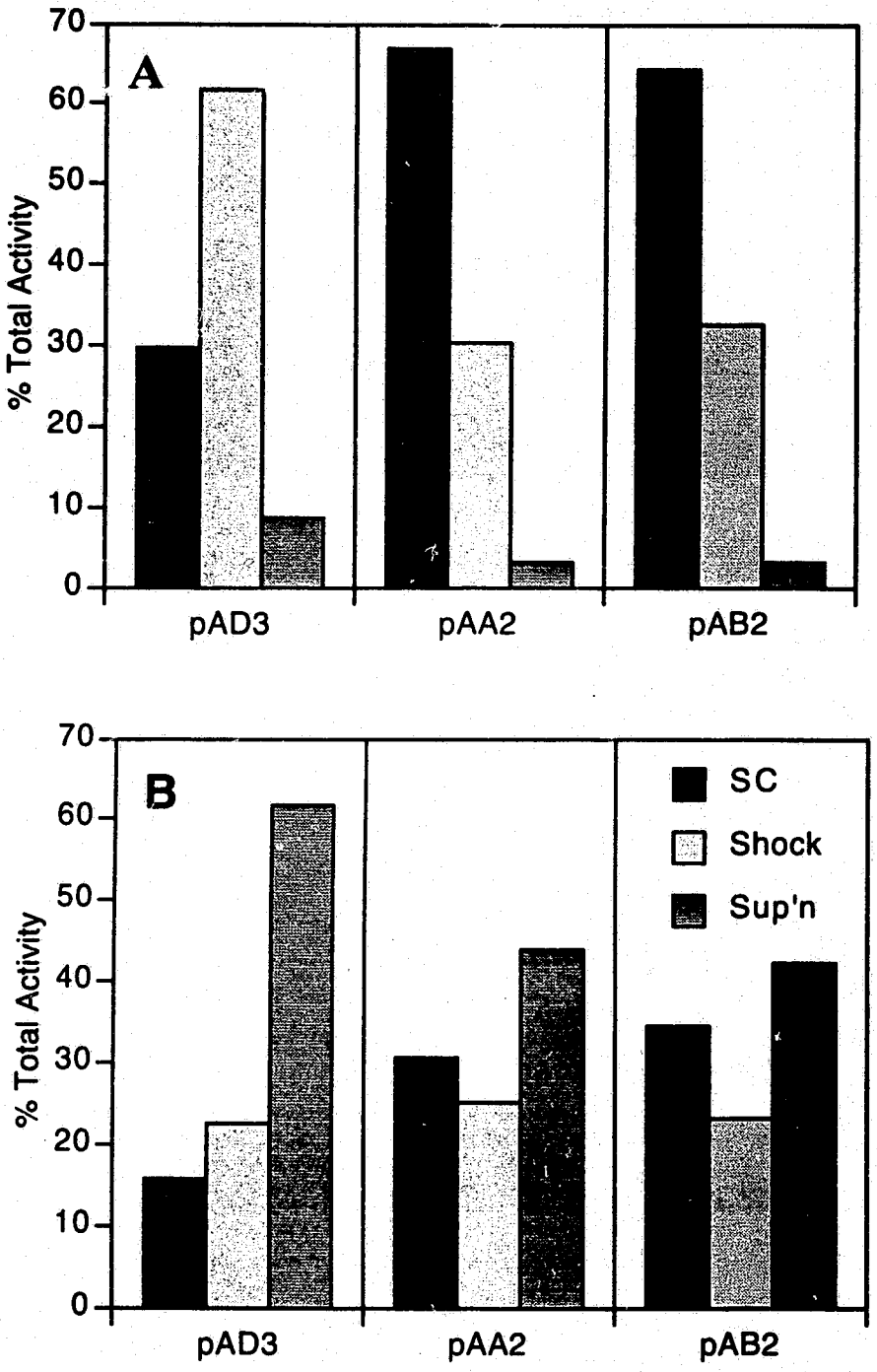

Figure 28. Distribution of alkaline phosphatase activity in fusion strains of *A. salmonicida*. Cells were induced at an OD_{600} of 0.5 and fractionated after 7.75 h. (A) Cells grown in LB medium containing 0.2% glucose; (B) cells grown in LB medium. Values are percentage of recovered alkaline phosphatase activity. Uninduced cells with *phoA* fusions produced less than 5% of the enzyme activity of induced cells. In all cases, most of the RNase and β-lactamase were recovered in the shockates.

cells (data not shown). The total recovered PhoA activity was 10-50% greater in the LB-glucose samples. The results in Figure 29A show that 2 h after induction, a culture of CB3-pAD3 grown in LB medium alone contained twice as much PhoA in the culture supernatant as the cells grown in LB medium containing glucose. This increased to a 4-5 fold difference at 6-8 h after induction. Measurement of the cell free culture supernatants over time showed that the pH of those containing glucose dropped to 5.5 during the 8 h period, while the pH of the supernatants containing no glucose rose to 8.5 over the same time period (Figure 29B). Similar results were found with CB3-pAA2 and CB3-pAB2 (data not shown). These results indicate that PhoA can be secreted by *A. salmonicida* at high pH. It is possible that the complete fusion proteins were secreted under these conditions. However, it is also possible that the fusions were still degraded in the CB3 cells and that it was PhoA alone that was secreted.

To see if any of the fusion proteins could be detected in the culture supernatants of cultures grown in the absence of glucose, samples were taken at mid-log phase and run on SDS-polyacrylamide gels. As shown in Figure 30, the only band that was observed for any of the fusions corresponded in size to PhoA itself. The N-terminal sequence of the extracellular pAD3 protein was determined to be Thr-Glu-Pro-Phe-Pro-Phe. This sequence corresponds to the C-terminal portion of the linker region in Tn*phoA* (Manoil and Beckwith, 1985) which connects the first two amino acids of AerA to PhoA in the D3 fusion protein. Thus the AerA portion of the fusion protein had been removed. While N-terminal sequences were not determined for pAA2 and pAB2 fusion proteins, because all three strains secreted similar sized proteins, it was assumed that they were also degraded to a similar PhoA species. Figure 30 also shows that the amount of PhoA released into the culture supernatants is substantial, reaching levels similar to those observed for proaerolysin secreted by *A. salmonicida* (Figure 6). Few if any other proteins were seen in the culture supernatants (Figure 30), a sign that cell lysis had not occurred.

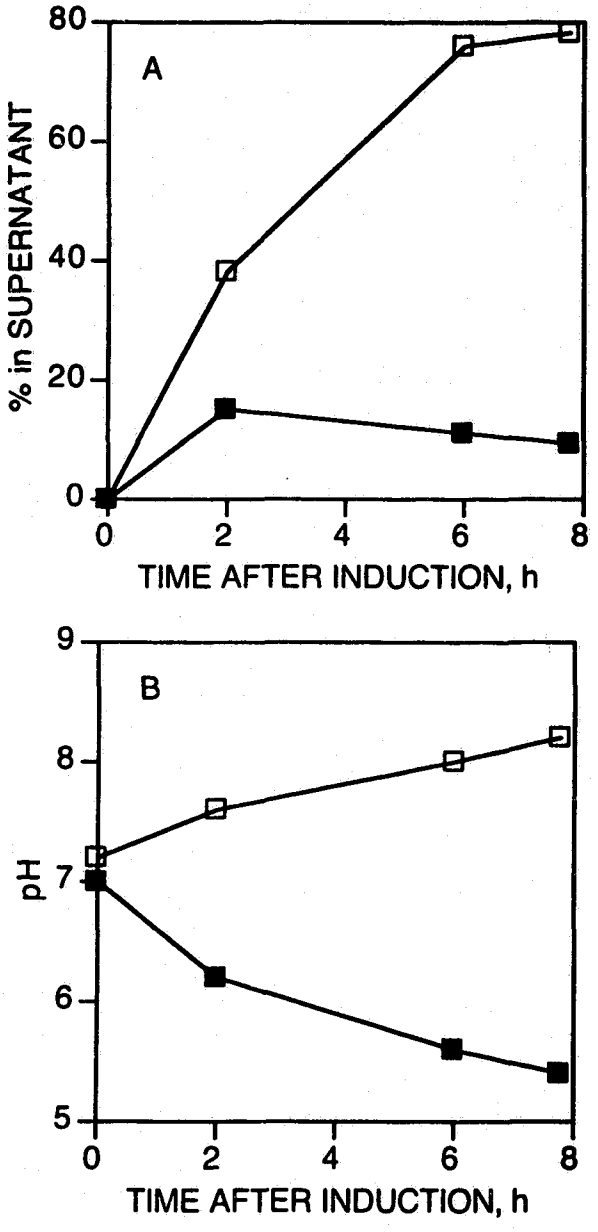

Figure 29. Effect of the change in medium pH on the distribution of alkaline phosphatase activity. Cultures of CB3-pAD3 were grown up in either LB (□) or LB + 0.2% glucose (■) medium, induced at an OD_{600} of 0.5 and followed over an 8 h period. PhoA activity was measured in culture supernatants and shockates and expressed as a percentage of the total activity recovered. PhoA activity in the supernatant (A) and the pH of the supernatant (B) are shown. This is one of three similar experiments. Nearly identical results were obtained with strains containing pAA2 or pAB2.

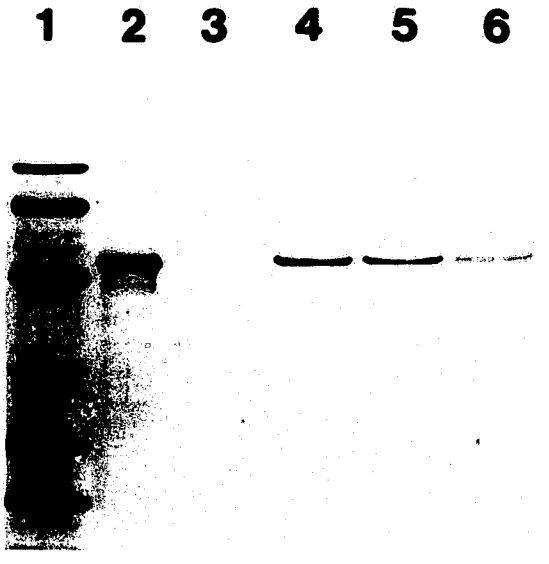

Figure 30. Alkaline phosphatase in the culture supernatants of *A. salmonicida*. Culture supernatants were obtained from cells grown in LB medium, induced at an OD_{600} of approx. 0.5 and grown for 16 h to an OD_{600} of approx. 7.0. Sample buffer was added and 10 µl aliquots were applied to the gel. Lane 1, molecular mass standards (97, 66, 43, 31, 21.5, and 14. 4 kDa); lane 2, *E. coli* alkaline phosphatase; lane 3, uninduced pAD3; lanes 4-6, induced pAD3, pAA2, and pAB2 respectively.

Distribution of alkaline phosphatase produced by wild type *A. hydrophila* and by pleiotropic export mutants containing cloned pAD3

The large amounts of alkaline phosphatase released by *A. salmonicida* containing the fusion plasmids suggested the possibility that when the pH is high, PhoA may follow the same secretion pathway as aerolysin out of the cell. To check this, the pAD3 fusion was transferred into plasmid pMMB208 (pAD3.1) to allow its transconjugation into wild type *A. hydrophila*, AH65, and the two *A. hydrophila* pleiotropic secretion mutants, L1.97 and S9. Table 6 shows that wild type AH65 grown in LB medium was able to release the pAD3.1-encoded PhoA activity into the culture supernatant as did CB3. The release of PhoA was not a result of non-specific leakage due to cell lysis, as the periplasmic marker β-lactamase remained in the shockate fractions. Interestingly, unlike CB3, AH65 was able to secrete PhoA in both LB and LB-glucose media (data not shown). This was apparently due to a difference in metabolism between the two species. While the pH of the culture supernatant dropped initially when AH65-pAD3.1 was grown in LB-glucose media, it eventually rose to the same level as cultures grown in LB media alone. Neither of the two pleiotropic mutants were able to secrete PhoA in LB medium (Table 6). Instead, both L1.97 and S9 accumulated PhoA activity in their periplasms along with aerolysin. This indicates that the *E. coli* periplasmic protein PhoA is able to use the *Aeromonas* spp. GSP to be secreted into the extracellular milieu.

Distribution of cloned *E. coli* alkaline phosphatase expressed in *A. hydrophila* and *A. salmonicida*

While the earlier results suggest that PhoA itself was secreted by *Aeromonas spp.* after removal of the aerolysin portion of the fusion protein, it was still possible that the complete fusion proteins were being translocated across the outer membrane and then

Table 6. Distribution of alkaline phosphatase in *A. hydrophila* and in pleiotropic secretion mutant strains containing pAD3.1[a].

	Alkaline Phosphatase			β-lactamase			Aerolysin		
Strain	SN	shock	SC	SN	shock	SC	SN	shock	SC
AH65-pAD3.1	51	9	40	3	77	20	100	0	0
S9-pAD3.1	3	75	22	0	73	27	0	100	0
L1.97-pAD3.1	3	66	31	0	89	11	0	80	20

[a] All strains were induced at an OD_{600} of 0.5 and grown to an OD_{600} of 5-7 in LB medium. Each strain produced approximately the same amounts of the two enzymes and aerolysin. All values are expressed as percentages of the total recovered activity. SN = culture supernatants; shock = periplasmic shockates; SC = shocked cells.

degraded to PhoA outside the cell, even though the *A. salmonicida* strain used (CB3) does not secrete an extracellular protease. An outer membrane protease like *E. coli* OmpT could degrade the fusion proteins outside the cell (Baneyx and Georgiou, 1990). However, it seems unlikely that a portion of AerA as small as the two N-terminal amino acids, as found in pAD3, could direct PhoA to the GSP machinery. To rule out this possibility, two different constructs containing PhoA alone were cloned into both CB3 and AH65. One encoded *E. coli* PhoA containing its own signal sequence (pHI.1; Inouye et al., 1981) while the other contained the β-lactamase signal sequence fused to the *E. coli* PhoA structural gene (pCH2; Hoffman and Wright, 1985). The product of the latter construct has been shown to be located in the *E. coli* periplasm like wild type PhoA (Hoffman and Wright, 1985). Both constructs were cloned into pMMB208 (Morales et al., 1991) to facilitate transfer into the *Aeromonas spp.* The plasmid containing the PhoA signal sequence was called pAP208 while the β-lactamase signal sequence containing construct was named pFUS208. CB3 was found to release PhoA produced from the pFUS208 construct in the same way as it released the fusion proteins (data not shown). However, the CB3-pFUS208 cells aggregated in LB medium and this made it difficult to follow the growth of these cultures by measuring the OD_{600}. The results for AH65 were similar, as the majority of PhoA activity, but not β-lactamase activity, was found in the culture supernatant (Figure 31). The same distribution was obtained when pAP208 was transferred into AH65 (data not shown). However, when pFUS208 was transferred into the two pleiotropic export mutants S9 and L1.97, over 75% of the PhoA activity was located in the periplasm of the mutants and less than 5% was recovered in the culture supernatants (Figure 31),. The location of PhoA in these strains was confirmed by immunoblotting (Figure 32). Thus L1.97-pFUS208 (lane 2) and S9-pFUS208 (lane 5) show accumulations of PhoA in the periplasmic shockates while AH65-

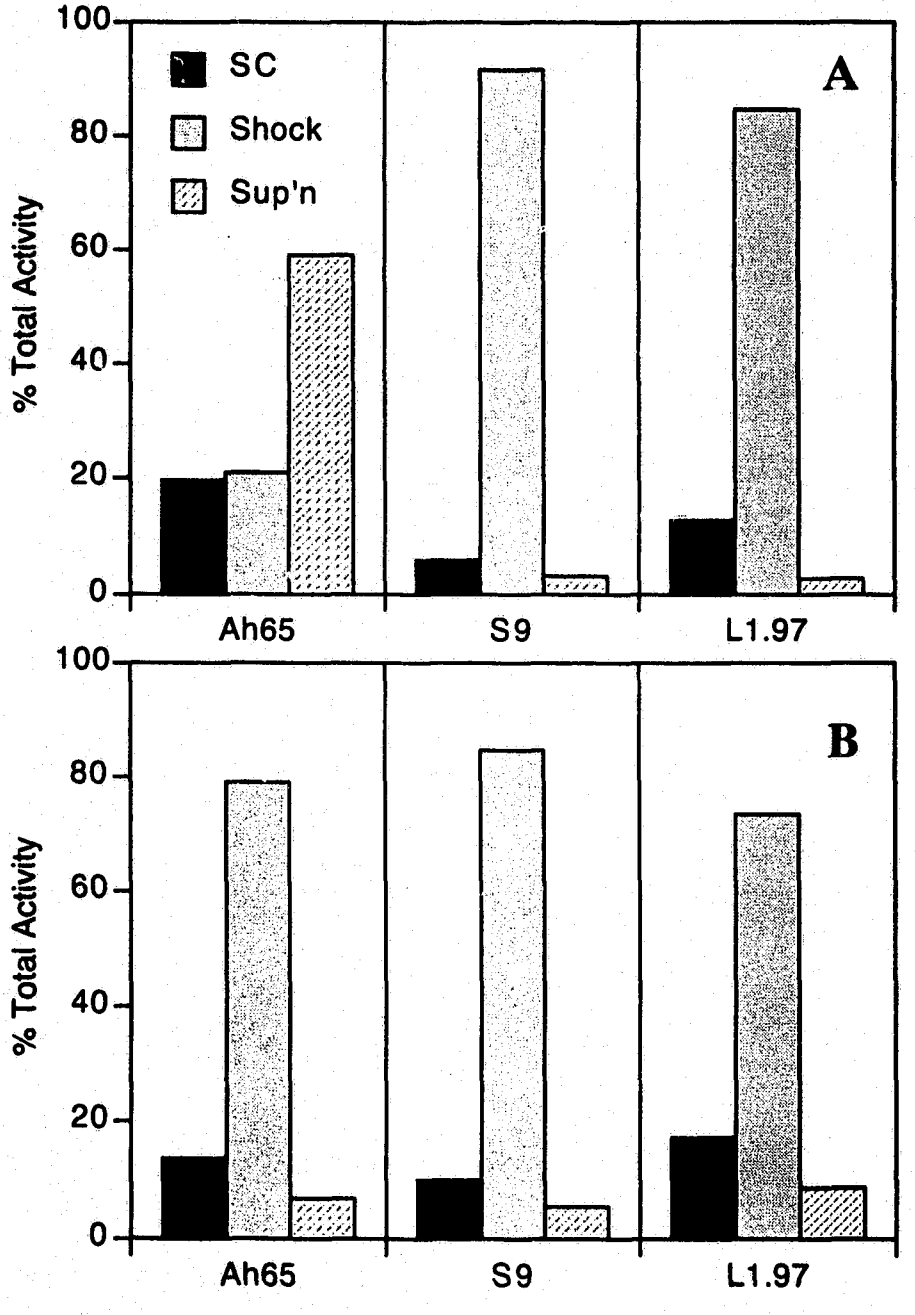

Figure 31. Distribution of cloned *E. coli* alkaline phosphatase and β-lactamase expressed by wild type *A. hydrophila* and by pleiotropic export mutants. Cells containing pFUS208 were grown in LB medium and induced at an OD_{600} of 0.5. Samples of each strain were taken at late-log phase. Similar results were obtained at other times. (A) shows alkaline phosphatase activity; (B) shows β-lactamase activity. Activity is expressed as percentages of total recovered activity.

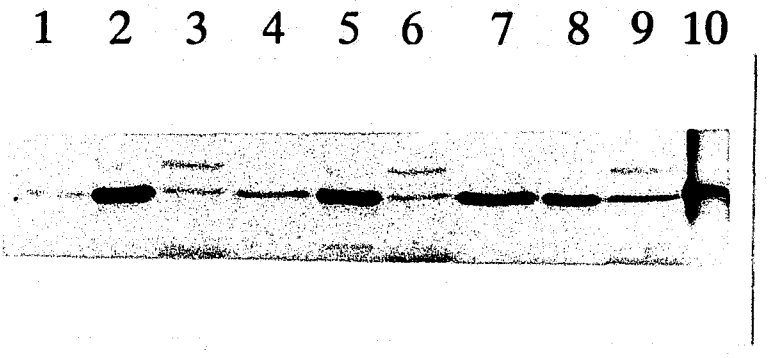

Figure 32. Location of *E. coli* alkaline phosphatase in *A. hydrophila* strains determined by immunoblotting. Supernatant, shockate and shocked cells of S9 (lanes 1-3), L1.97 (lanes 4-6), and wild type (lanes 7-9) are shown. Lane 10 shows the alkaline phosphatase standard. The samples came from the experiment in Figure 32.

pFUS208 shows a PhoA band in the culture supernatant (lane 7). This also demonstrated that the PhoA activities observed were due to a protein corresponding in size to the native *E. coli* PhoA. These results show that the *E. coli* PhoA can be recognized and secreted by the *Aeromonas spp.* GSP.

Effect of CCCP on the secretion of proaerolysin across the outer membrane of *A. salmonicida*

While export of proteins across the inner membrane has been shown to utilize both ATP and a PMF as sources of energy (Geller et al., 1986), the energy requirements for secretion across the outer membrane have yet to be determined. Howard and Buckley (1985a) demonstrated that the proton ionophore CCCP prevented the export of preproaerolysin across the inner membrane of *A. hydrophila.* The precursor became trapped in the inner membrane, where it could be identified by pulse-labelling. This indicated that a PMF plays a role in aerolysin export across the inner membrane, further evidence that a *sec*-like system is involved in this step. It was also noted that the release of proaerolysin from the periplasm appeared to be affected by the addition of CCCP. To further investigate this, the fate of the periplasmic pool of proaerolysin in Rif-1-pKW2 was studied in the presence of CCCP.

Cultures of Rif-1-pKW2 were grown up in LB medium to an OD_{600} of 2.4 to 2.8. As shown in Figure 3 and Figure 6, proaerolysin is expressed in Rif-1-pKW2 without induction by IPTG and some of the protoxin is found in the periplasmic shockates (Figure 10). The cultures were spun down and resuspended in either fresh LB medium or LB medium supplemented with 60 μM CCCP. In both cases, 100 μg/ml of chloramphenicol (Cm) was added to prevent the synthesis of new protein. The samples were left at 27°C and subsamples were taken for osmotic shocking over 20 min. The cells transferred to LB medium released approx. half of their periplasmic proaerolysin into the culture supernatant

over this time (Figure 33). The addition of CCCP to the medium prevented this release, and the levels of proaerolysin in the periplasm remained constant over the 20 min incubation period. These results suggest that secretion of proaerolysin across the outer membrane also requires a proton motive force. It is possible that *A. salmonicida* utilizes the PMF across the inner membrane to help power translocation across the outer membrane. This would be analogous to the mechanism employed by *E. coli* for import of colicins, vitamin B_{12} and iron siderophores. With the help of the inner membrane protein TonB (see below and Postle, 1990) the PMF of the inner membrane is used to power the translocation of these macromolecules across the outer membrane . Another possibility is that a proton gradient across the outer membrane helps to power the secretion of proteins. However, this seems unlikely since the outer membrane porins would be expected to form holes large enough to allow free passage of protons. Nevertheless, it has been speculated that the pH of the periplasm is significantly lower than that of the supernatant (Stock et al., 1977). If there were a gradient across the outer membrane, then it would be expected that decreasing it by lowering the pH of the medium would inhibit secretion of proaerolysin. As shown in Figure 33, this is exactly what happens. When the pH of the media was lowered to 5.5 with acetate buffer, proaerolysin remained in the periplasm of Rif-1-pKW2 over the 20 min incubation period and did not move into the culture supernatant. This effect was reversible, as cells incubated in the pH 5.5 medium began to secrete the pool of periplasmic protoxin when transferred into pH 7.0 medium (Figure 34). The amount of protoxin released from the cells increased as the pH of the medium rose above 5.5 (Figure 35). This is what would be expected to happen if a PMF existed across the outer membrane that drove secretion.

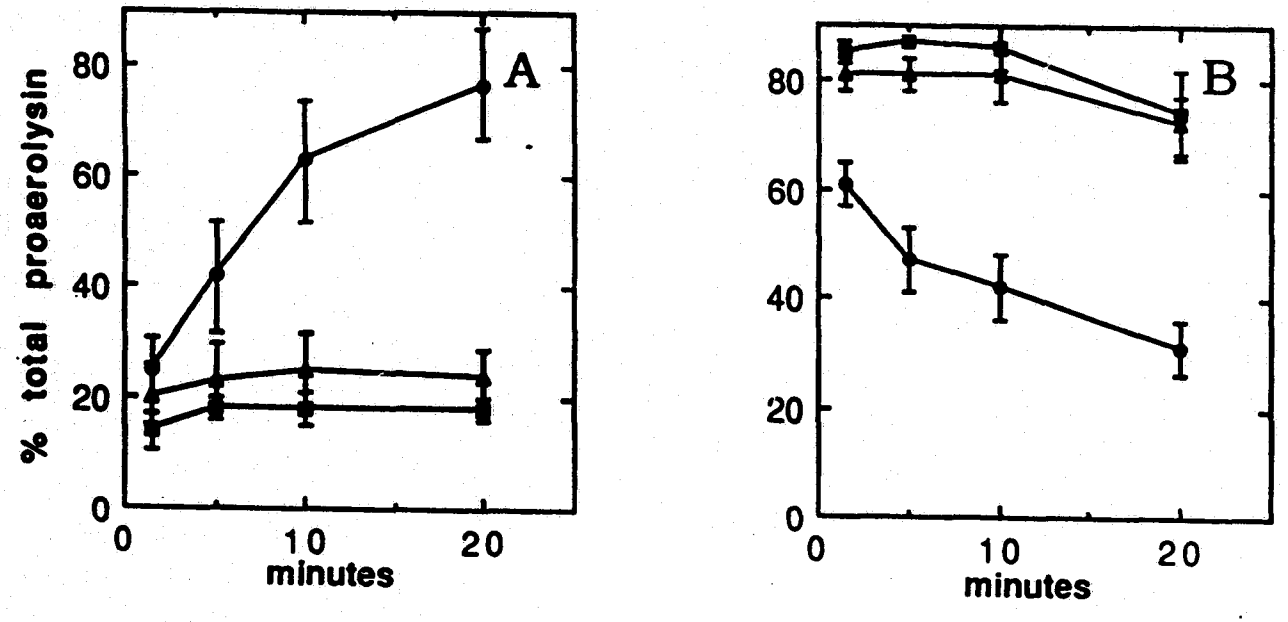

Figure 33. Time dependence of proaerolysin released by *A. salmonicida* treated with CCCP or low pH medium. Rif-1-pKW2 was grown uninduced to an OD_{600} of 2.4 to 2.8 in LB medium. Cells were obtained by centrifugation and resuspended in (●) LB medium containing 20 mM Pipes, pH 7.0; (▲) LB medium containing 20 mM sodium acetate, pH 5.5; (■) LB medium containing 20 mM Pipes and 60 μM CCCP. Chloramphenicol was added to all samples to prevent synthesis of new protein. Cells were fractionated at the indicated times and the subcellular fractions titered for hemolytic activity. Results are the means (± SEM) of five experiments. (A) Proaerolysin released into the media. (B) Proaerolysin remaining in the periplasm.

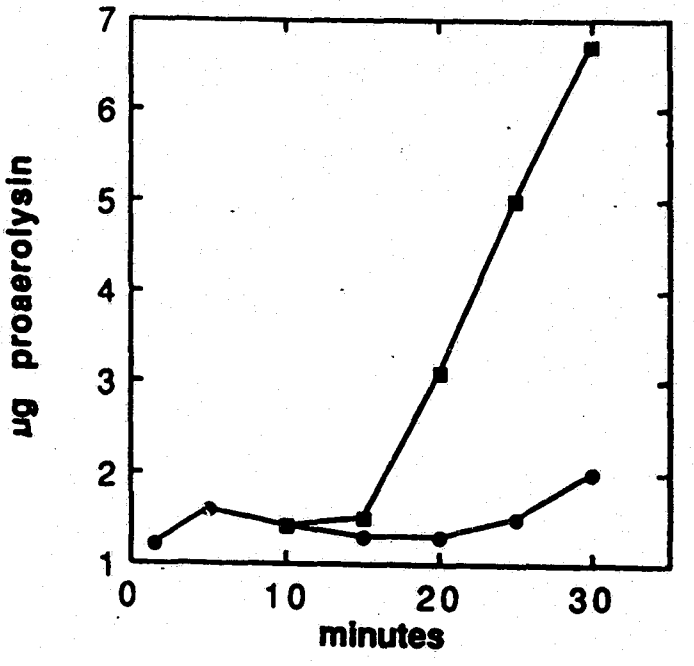

Figure 34. Release of proaerolysin from *A. salmonicida* after transfer from pH 5.5 media back to pH 7.0. Cells were grown as described in Figure 33 and resuspended in pH 5.5 media as described in Figure 35. They were incubated at 27°C and samples taken at the time points shown. After 10 min, half the remaining suspension was repelleted and resuspended in LB media buffered at pH 7.0 with 20 mM Pipes. Release of proaerolysin was followed for an additional 20 min. This is one of three similar experiments. (●) pH 5.5 throughout; (■) switched to pH 7.0.

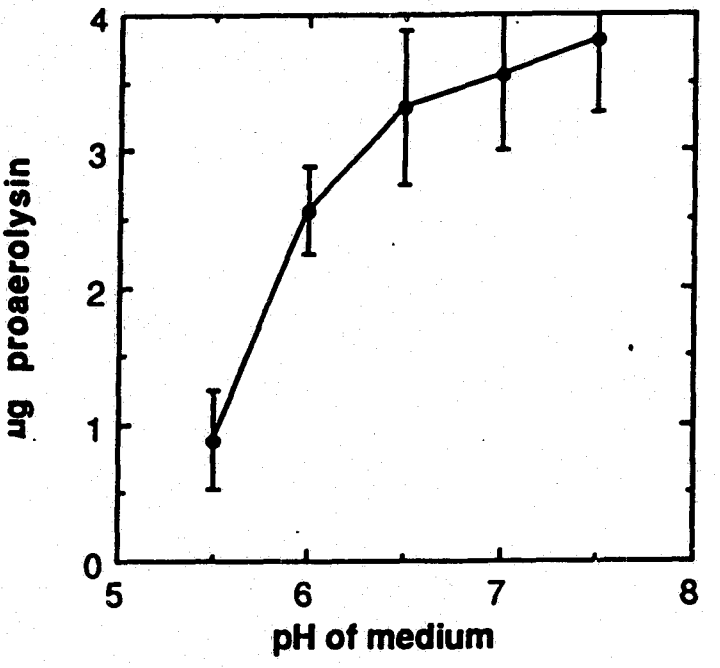

Figure 35. Dependence of aerolysin release on pH. Cells were grown as in Figure 34. They were pelleted by centrifugation and rapidly resuspended in LB medium buffered to the pH values shown (20 mM acetate for pH 5.5; 20 mM 2-[*N*-morpholino] ethanesulfonic acid (MES) for pH 6.0; 20 mM Pipes for pH 6.5 and 7.0). Chloramphenicol (100 µg/ml) was present in all samples. Proaerolysin was measured as described in Methods and Materials. Results are the means (± SEM) of six experiments.

Isolation of pirazmonam resistant *A. hydrophila*

The previous results demonstrated that an electrochemical gradient is required for secretion of proaerolysin from the periplasm via the GSP. While they fit a model in which a PMF across the outer membrane energizes the translocation process, it is still more likely that the CCCP affected the inner membrane PMF, and that the pH effect is unrelated. Thus, changing the pH of the medium could lead to reversible changes to the outer membrane or to a component of the GSP. To utilize the inner membrane PMF for secretion, some system would have to exist which transfers this energy to the outer membrane. As mentioned above, such a system exists in the import of macromolecules in Gram-negative bacteria. TonB is an inner membrane protein that is necessary to power the import of macromolecules across the outer membrane. It is anchored in the cytoplasmic membrane and is believed to span the length of the periplasm, with its C-terminus making contact with outer membrane receptors (Hannavy et al., 1990). It has been postulated that it couples the energy from the PMF across the inner membrane to the outer membrane receptors, with a conformational change in TonB possibly opening gated channels formed by the receptors (Rutz et al., 1992). It is possible that a similar system exists in *Aeromonas spp.* and is responsible for energizing the secretion of proaerolysin.

In an effort to see if proaerolysin secretion involves TonB, pirazmonam resistant mutants of AH65 were isolated. In *E. coli*, resistance to this antibiotic is due to a mutation in *tonB*, as pirazmonam mimics the iron siderophores and enters the cells by using the TonB-dependent outer membrane siderophore receptors (Nikaido and Rosenberg, 1990). TonB mutants are unable to energize the import of the antibiotic into the cell and thus survive. It was hoped that pirazmonam-resistant mutants of AH65 would also be TonB mutants and, if TonB is required for secretion, that they would have pleiotropic secretion defects.

Plating of 1:10 dilutions of overnight cultures of AH65 onto HBA plates containing 0.05 μM pirazmonam resulted in the growth of 100-150 colonies per plate. Of these, 4-6 were non-hemolytic (approx. 1 in 2×10^5 of the plated bacteria). Two of these colonies (SHJ2 and 2H$^-$), along with one pirazmonam-resistant hemolytic colony (SHJ17), were selected for further study. The results in Figure 36 show that the growth of wild type AH65 was inhibited by as little as 0.05 μM pirazmonam, while all the pirazmonam resistant mutants grew in concentrations up to 10 μM.

To screen these mutants for the presence of TonB, whole cell fractions were separated by SDS-PAGE and immunoblotted with an *E. coli* α-TonB polyclonal antibody. Unfortunately the antibody was found to cross-react with a major outer membrane protein of AH65 that corresponded to the *E. coli* OmpF protein (Jeanteur et al., 1992). It was not clear if this protein masked an AH65 TonB-like protein on the gels (not shown). In any case, there was no apparent difference between the mutants and wild type. These results were thus, unable to reveal whether the pirazmonam-resistant mutants lacked, or had an altered, TonB-like protein.

Expression of secreted proteins by *A. hydrophila* pirazmonam mutants

As mentioned earlier, we had hoped to find that a TonB-like protein was involved in secretion. Since the Western blot analysis described above could neither confirm nor rule out the existence of TonB-like proteins in *A. hydrophila* wild type and mutant strains, we proceeded to determine if the mutants could secrete GSP proteins. The non-hemolytic phenotype of the pirazmonam resistant mutants suggested that they were either unable to secrete aerolysin or they did not produce any aerolysin. A third possibility was that the strains produced an inactive form of aerolysin. To determine if wild type aerolysin was expressed but not secreted, culture supernatants, periplasmic shocks and shocked cell fractions of the pirazmonam mutants were obtained and titered. While SHJ17 secreted as

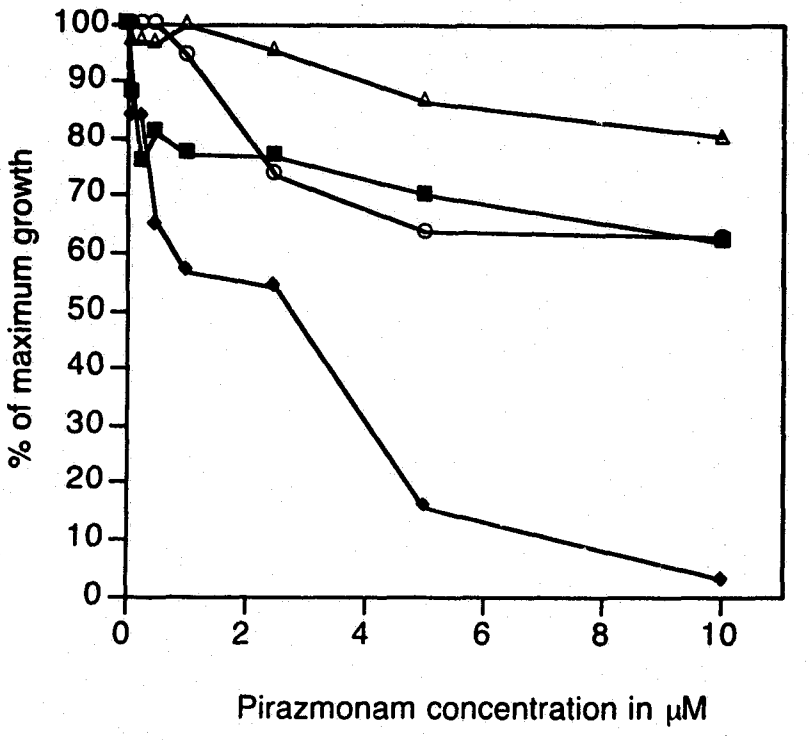

Figure 36. Effect of pirazmonam concentration on the growth of AH65 and pirazmonam-resistant mutants. Cultures were grown overnight in LB medium (containing 0.05 μM pirazmonam for the mutants) and used to inoculate fresh medium containing varying concentrations of pirazmonam. Growth was determined by measuring the OD_{600} 7 h later. Inhibition was expressed as the percentage of growth compared to the culture containing no pirazmonam. Shown are (■) SHJ2; (○) $2H^-$; (△) SHJ17; (◆) AH65.

Table 7. Activity of secreted proteins in the culture supernatants of AH65 and the pirazmonam mutants.

Strain	OD_{600}	Aerolysin	GCAT[a]	Protease[b]
AH65	7.1	7	3.50	100
SHJ2	7.4	0	4.14	7.0
SHJ17	6.5	7	4.08	ND
$2H^-$	4.0[c]	0	2.68	2.8

[a] GCAT activity is expressed in nmoles of cholesterol ester formed per ml sample per min.

[b] Protease activity is expressed as the percentage of the wild type activity.

[c] $2H^-$ had not grown to an equivalent point of growth after 16 h growth

much aerolysin as wild type AH65, no aerolysin was detected in the culture supernatant fractions of SHJ2 or 2H$^-$ (Table 7). In fact, no aerolysin was detected in any of the non-hemolytic mutant subcellular fractions. Immunoblotting of the fractions confirmed the absence of aerolysin in both SHJ2 and 2H$^-$ (data not shown).

To determine whether other secreted proteins were affected in the same way as aerolysin, the subcellular fractions were also assayed for GCAT and protease activities. While the mutants secreted amounts of GCAT into the supernatant similar to those seen for AH65, the levels of extracellular protease in the culture supernatants of the non-hemolytic mutants were reduced by 90-98% compared to wild type (Table 7). Thus the pirazmonam-resistant mutants do not appear to be producing aerolysin or secreted protease, but are producing and secreting GCAT. The latter observation also indicates that the GSP in the mutants is still functioning.

The expression of outer membrane proteins in *A. hydrophila* pirazmonam mutants

When whole cell samples of the non-hemolytic pirazmonam-resistant mutants were run on SDS-polyacrylamide gels and compared to wild type AH65, it was observed that the mutants were missing a 45 kDa protein, with a 43.8 kDa protein taking its place. Additionally, they had decreased amounts of a 52.4 kDa protein. A similar pattern was observed in outer membrane preparations of 2H$^-$ (Figure 37) and SHJ2 (data not shown), demonstrating that these were outer membrane proteins. The hemolytic mutant SHJ17 on the other hand, had an outer membrane profile similar to AH65 (data not shown). Jeanteur et al. (1992) had previously found that the 45 and 52.4 kDa proteins were similar to the *E. coli* OmpF and LamB porin proteins respectively. Thus the mutation in 2H$^-$ and SHJ2 also seems to affect the expression of these two porins. Like the secreted proteins though, not all the outer membrane proteins are similarly affected. A third AH65 porin, a PhoE-like

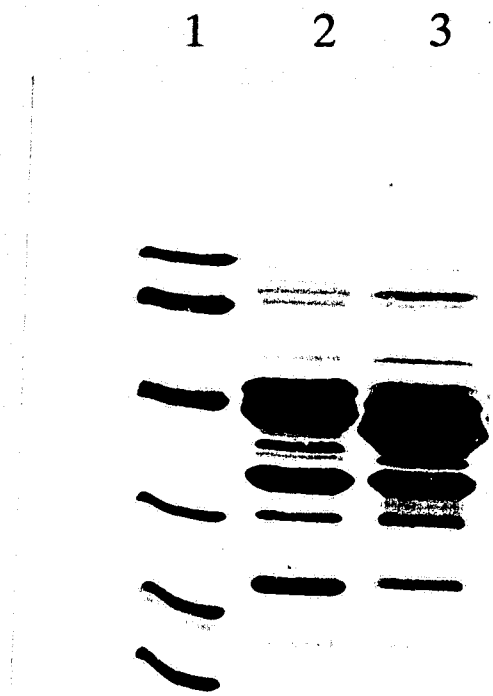

Figure 37. Expression of outer membrane proteins by the *A. hydrophila* pirazmonam mutants. Cultures were grown overnight in LB medium and pelleted by centrifugation. Outer membranes were prepared by the SLS-extraction method described in Materials and Methods. Samples were run on a 12 % SDS-polyacrylamide gel. Lane 1, molecular mass standards (97, 66, 43, 31, 21.5 and 14.4 kDa); lane 2, wild type AH65; lane 3, $2H^-$.

protein of 40 kDa, was produced in both the wild type AH65 and non-hemolytic mutants to similar levels by growing them in low phosphate Riddle's media (data not shown). These results suggest that the mutations in SHJ2 and 2H$^-$ affect not only the expression of some secreted proteins but also two outer membrane proteins.

Secretion of cloned aerolysin in non-hemolytic *A. hydrophila* pirazmonam mutants

While the earlier results demonstrating GCAT secretion in the pirazmonam-resistant mutants had suggested that the GSP was still functional in the mutants, it was possible that the GCAT was non-specifically leaking out of the cells. In order to determine whether or not the mutant strains were capable of secreting GSP proteins, the *aerA*-containing plasmid pNK1 was mated into a rif^r strain of SHJ2 (SHJ2-rif^r). SHJ2-rif^r-pNK1 expressed no aerolysin when grown in LB media until induced with IPTG. The appearance of hemolytic activity was first seen during late log/early stationary phase. The amount rose from late log phase through stationary phase and was about 16 times higher than observed in AH65-rif^r alone (Table 8). Aerolysin could be detected in the periplasm at the same time, but at amounts approx. 30-fold lower than seen in the supernatant (Table 8). The periplasmic marker β-lactamase was found almost exclusively in the periplasmic shockates (more than 90% of the total recovered activity) indicating that the cells had not lysed and were not allowing the aerolysin to leak out. Glutamate dehydrogenase (GDH) assays of the subcellular fractions revealed that less than 5% of the total activity was located in the culture supernatants, further evidence that the cells were not lysing. These results strongly suggest that the cloned aerolysin was being released via a functioning GSP and that the non-hemolytic phenotype of SHJ2 is due to a defect in the expression of aerolysin, and not an inability to secrete the toxin.

Table 8. Hemolytic titers of cloned aerolysin secreted by AH65 and SHJ2-rif^r-pNK1 grown in LB media.

Strain[a]	OD_{600}	Hemolytic Titer in Culture Supernatants	Hemolytic Titer in Periplasmic Shocks
AH65-rif^r	9.53	6	0
SHJ2-rif^r	8.56	0	0
SHJ2-rif^r-pNK1	8.76	10	5

[a]Strains were grown up to an OD_{600} of 0.5 - 0.7 and induced with IPTG. Cultures were grown for a further 10 h before measuring the OD_{600}, spinning down the cells and assaying 100 µl of culture supernatants for hemolytic activity. Cells were shocked and 20 µl of the periplasmic shocks were titered.

The effect of glucose on secretion of cloned aerolysin from SHJ2

While aerolysin was expressed and secreted from SHJ2-rif^r-pNK1 when grown and induced in LB medium alone, little was released into the culture supernatants when glucose was added to the medium (Figure 38). No aerolysin was detected intracellularly when these cultures were induced with IPTG (data not shown). In contrast, wild type AH65 expressed and secreted aerolysin normally under these conditions (Figure 38). It appears that the SHJ2-rif^r-pNK1 cultures stop growing after 3-4 h in the LB-glucose medium (Figure 39), whereas the same strains grown in LB medium alone, and wild type grown in LB-glucose medium, continue to grow past this point. Examination of the pH of the culture supernatants revealed a drop over a 16 h growth period for the mutant grown in media containing glucose. While there was an initial drop in pH for the wild type strain grown in this medium, after 16 h there was a net increase observed. This suggests that the mutant continues to release acidic metabolites into the growth medium while wild type AH65 does not. This was not an effect caused by the plasmid as a drop in pH was also observed in SHJ2 cultures grown in LB-glucose medium (data not shown). The inability to express the plasmid encoded aerolysin does not appear to be due to the presence of glucose in the medium, as SHJ2-rif^r-pNK1 grown in LB-glucose medium buffered with Davis buffer was able to express and secrete the toxin (data not shown). In all cases where aerolysin was found in the culture supernatants, β-lactamase remained intracellular. Again, this is evidence that the GSP is functional under these conditions. The results appear to indicate that the pirazmonam mutants have a lesion in some global regulatory gene, as aerolysin, extracellular protease and selected outer membrane protein synthesis, as well as some aspect of metabolism in the cells, are all affected.

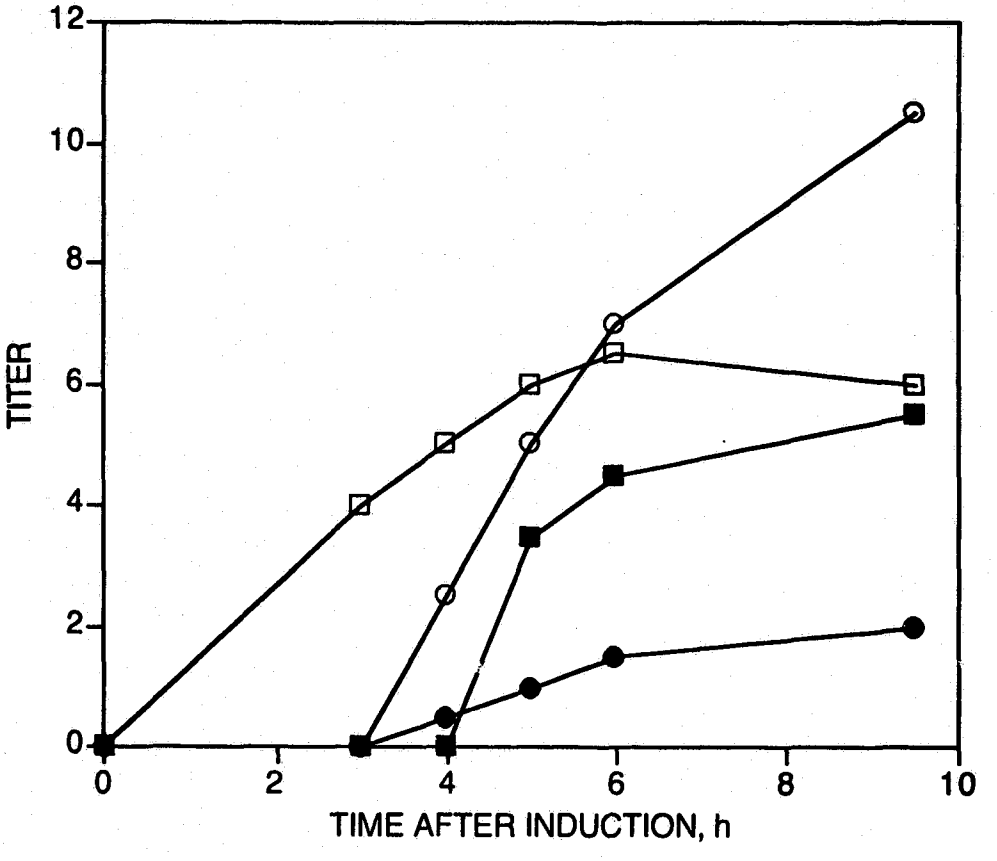

Figure 38. Secretion of aerolysin by AH65 and SHJ2-rif^r-pNK1 grown in LB medium and LB medium containing 0.2% glucose. Cultures were grown to an OD_{600} of 0.5 and induced. Culture supernatants were titered for hemolytic activity by treating with 0.5 μg/ml trypsin before incubating with 0.4% human red blood cells. Shown are AH65 grown in LB (□) and LB-0.2% glucose (■) media; SHJ2-rif^r-pNK1 grown in LB (○) and LB-0.2% glucose (●) media.

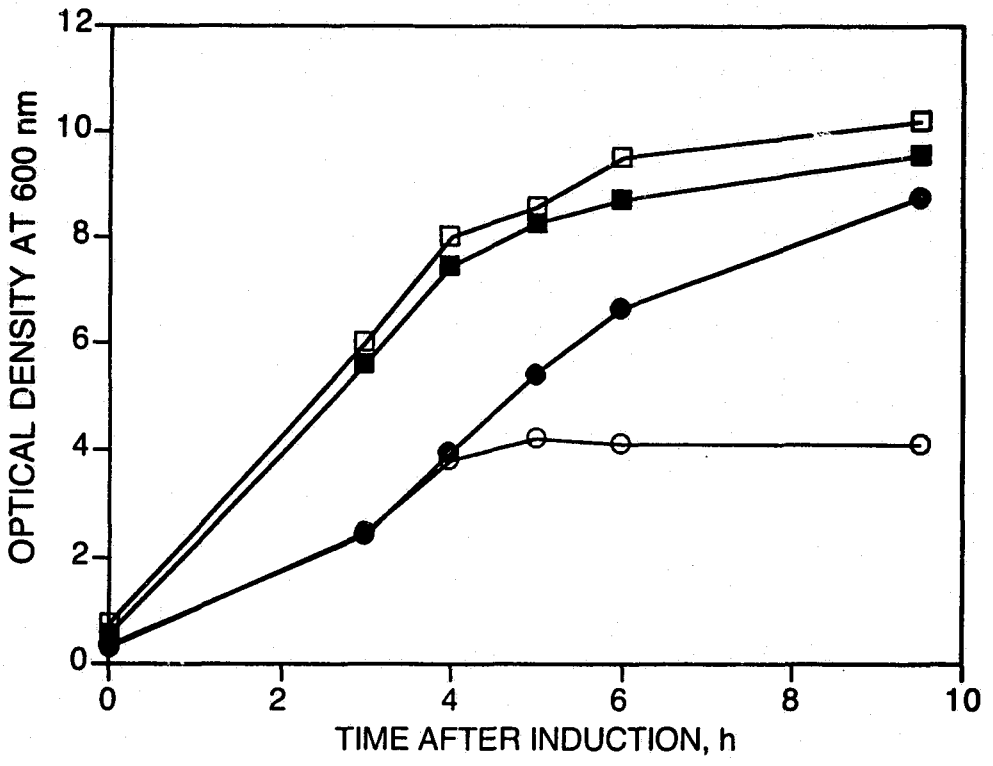

Figure 39. Effect of glucose on the growth of SHJ2-rif^r-pNK1. Cultures of SHJ2-rif^r-pNK1 (○,●) and AH65 (□,■) were grown up in LB media with (open symbols) and without (filled symbols) 0.2% glucose. When the cultures reached an OD_{600} of 0.5, IPTG was added to a final concentration of 1 mM. Growth was followed over the next 10 h by reading the OD_{600} of the cultures.

Isolation of *A. hydrophila* pirazmonam-resistant mutants using transposon mutagenesis

An effort was made to duplicate the phenotype of SHJ2 and 2H$^-$ by transposon mutagenesis. Cultures of AH65 were filter mated with HB101 containing the Tn5 plasmid pUW964 (Weiss et al., 1983) and transconjugates were selected on HBA plates containing 40 μg/ml kanamycin and 0.05 μM pirazmonam. Although pirazmonam-resistant transposon mutants were easy to obtain (generated at a frequency of approx. 1 in 2 x 10^4; non-hemolytic colonies at approx. 1 in 1 x 10^5), none of the non-hemolytic colonies showed the same outer membrane profile as 2H$^-$ or SHJ2 (data not shown). This could indicate that the mutations in SHJ2 and 2H$^-$ are point mutations or that the affected genes are part of an operon. If point mutations are responsible for the observed phenotype, it would be expected that revertants could be found if SHJ2 and 2H$^-$ were grown in medium lacking pirazmonam. Under such conditions though, no revertants of SHJ2 or 2H$^-$ were found.

The results described above suggest that the non-hemolytic pirazmonam-resistant mutants have a defect in some global regulatory system. While they do not directly answer the original question as to whether a TonB-like protein is involved in secretion, there is some evidence that this is not the case. The great majority of pirazmonam-resistant mutants observed were hemolytic (see above). If it is assumed that pirazmonam will select for *tonB*$^-$ mutants in *A. hydrophila* as in *E. coli*, then many of these hemolytic colonies likely represent such mutants. The hemolytic phenotype indicates they all produce and secrete aerolysin, suggesting that TonB does not have a role in the GSP.

DISCUSSION

Secretion of GSP proteins by heterologous hosts

Several other groups have expressed GSP proteins in heterologous systems, but none of the proteins were secreted. Even in the case of two closely related species, *E. carotovora* and *E. chrysanthemi*, it has been shown that secreted proteins from one cannot use the GSP system of the other (Reeves et al., 1993). While *E. carotovora* has a PulN homologue (OutN), a homologous gene has not been identified in *E. chrysanthemi*. It was originally proposed that this difference in the GSP systems could account for the inability of the two species to secrete each other's proteins. In the studies described in this thesis, both *A. salmonicida* and the marine *Vibrio* spp. were found to be capable of secreting *A. hydrophila* aerolysin. This therefore is the first time that it has been demonstrated that a GSP protein can be secreted by a heterologous host.

While the PulD-PulN homologues have been identified in *A. hydrophila* (Howard et al., 1993), none of the GSP components from either *A. salmonicida* or the marine *Vibrio* spp. used in this study have yet been described. Therefore, it remains to be seen how similar the GSP components of the three bacteria might be. Both the EpsE protein of *V. cholerae* (Sandkvist et al., 1993) and the *A. hydrophila* ExeE protein (Jiang and Howard, 1992) have approx. 60% identity with *pulE*. Additionally, it has been found that the *V. cholerae epsE* gene can complement a defect in the *A. hydrophila exeE* gene (Howard, personal communication). This suggests that *A. hydrophila* and *V. cholerae* have similar secretion systems, and supports the view that the marine *Vibrio* spp. strain MVT606 may contain a GSP similar to both. This would explain the observation that MVT606 is able to secrete aerolysin (Figure 12) but pleiotropic secretion mutants cannot. Instead, the mutants accumulate proaerolysin in the periplasm (Figure 16), in the same way as *A. hydrophila* pleiotropic secretion mutants (Howard and Buckley, 1983). In agreement with these

results, Leece and Hirst (1992) have subsequently found that the *E. coli* enterotoxin B subunit can be secreted by MVT606, but not by pleiotropic secretion mutants. Now that the GSP components from a number of different species have been identified and characterized, it will be possible to start testing whether individual components are interchangeable. Aside from Howard's studies with *epsE*, all other attempts at complementing secretion defects in one species with the GSP genes from another species have involved the use of the entire *pul*-like operon, and not individual genes (de Groot et al., 1991). In situations where GSP proteins from one species cannot be secreted by a heterologous system, as is the case between *E. carotovora* and *E. chrysanthemi* (Reeves et al., 1993), it seems likely that some of the Pul-like components will be interchangeable, while those that are not may be responsible for recognizing species-specific secretion signals.

Entry of GSP proteins into the periplasm

Several observations led to the proposal that proaerolysin transiently enters the periplasm before being translocated across the outer membrane. Protoxin is detected in periplasmic shocks of *A. hydrophila* cells that have been treated with CCCP and pulse labeled with ^{35}S-methionine (Howard and Buckley, 1985a), and pleiotropic secretion mutants accumulate protoxin in the periplasm (Howard and Buckley, 1983, 1985a). Hirst and Holmgren (1987a) have also shown that heat-labile enterotoxin enters the *V. cholerae* periplasm before being released into the extracellular milieu. A number of observations made in the present studies further support the existence of a periplasmic pool of secreted proteins. As described earlier, pleiotropic secretion mutants of *Vibrio* spp. MVT606 accumulate protoxin in the periplasm (Figure 14B). In addition, overexpression of aerolysin in *A. salmonicida* results in an easily detectable periplasmic pool of protoxin (Figure 10). It could be that overexpression produces more protein than the secretion

system can handle, leading to the diversion of protoxin to the periplasm. Such a situation could lead to a block in the secretion system, preventing the translocation of other normally secreted proteins. However, overexpression of aerolysin did not affect the secretion of the endogenous *A. salmonicida* proteins GCAT and protease (Figure 9). This suggests that the secretion apparatus is not being overloaded. Evidence that the periplasmic pool of protoxin represents a pool of protein that will be secreted was obtained by treating Rif-1-pKW2 cells with CCCP. Under these conditions, the periplasmic pool of proaerolysin was prevented from leaving the periplasm (Figure 33). However, when the CCCP was removed, the periplasmic pool was released into the culture supernatant. This demonstrates that the protoxin in the periplasm is still able to enter the secretion pathway, and that it is not simply shunted there as a result of overproduction. Additional evidence supporting this view comes from studies with the Trp227 aerolysin mutants. All three mutant proteins were able to cross the inner membrane, and their signal sequences were removed correctly before they were released into the periplasm. However, all of the proteins had difficulty leaving the cell, and large amounts of the Leu227 and Gly227 proaerolysins were recovered bound to the outer membrane. These results suggest that the mutations affected the second step in secretion, preventing the periplasmic proaerolysin from crossing the outer membrane.

Secretion of the periplasmic *E. coli* protein PhoA by the *Aeromonas* spp. GSP

Perhaps the most surprising results in this thesis are those showing that normally periplasmic *E. coli* PhoA can be secreted by the *A. hydrophila* GSP. Neil et al. (1983) found that heat-labile enterotoxin, which is also periplasmic in *E. coli*, could be secreted by *V. cholerae*. Later it was shown that this release required the GSP (Sandkvist et al., 1993). Perhaps this is less surprising than the result obtained with alkaline phosphatase because the *E. coli* enterotoxin is functionally and structurally very similar to cholera toxin

(Dallas and Falkow, 1980). The fact that some *E. coli* periplasmic proteins can be secreted by other Gram-negative bacteria could indicate that *E. coli* may have had its own GSP at one time. The observation that there are genes with homology to the *P. aeruginosa xcp* genes located on the *E. coli* chromosome could support this idea (Whitchurch et al., 1991). The loss of one or more of the secretion components would have resulted in previously secreted proteins being retained in the periplasm. Expression of such proteins in a host retaining a functional GSP would result in their secretion.

There have been two other reports of PhoA release from Gram-negative cells. Cheng et al. (1970) observed that PhoA is released from *P. aeruginosa* when the pH of the medium rises above 7.2, but that it is retained in the periplasm at lower pH values (Ingram et al., 1973). Additionally, Taylor et al. (1989) found that *V. cholerae* releases PhoA activity into the culture supernatant when it expresses fusion proteins made between PhoA and the *V. cholerae* outer membrane protein OmpV. Unfortunately, they did not check to see if the entire fusion protein was released or only the PhoA portion. Because many fusion proteins have been found to be proteolytically degraded in the periplasm of their hosts (Strauch and Beckwith, 1988; Manoil and Beckwith, 1986; Gunter and Braun, 1988), including the AerA'-'PhoA fusions described here, one would predict that only the PhoA portion of the OmpV'-'PhoA fusion protein would be secreted into the culture supernatant. Thus the secretion of PhoA by *Aeromonas* spp. does not appear to be unique, and PhoA may be a periplasmic protein in *E. coli* simply because it lacks a GSP.

It is not possible to determine from the results in this thesis if the entire secretory pathway is required for PhoA release or if only some components are necessary. The fact that two independently isolated pleiotropic mutants, L1.97 and S9, failed to secrete the enzyme (Figure 32) suggests that at least two of the gene products are required. In addition to L1.97 used in this study (and S9, which is only partly characterized), strains with

defects in two other GSP genes, *exeK* and *exeC,* are available (Howard et al., 1993). It remains to be determined if *E. coli* PhoA can be secreted by either of these mutants.

The role of protein folding in protein secretion

The transient appearance of secreted proteins in the periplasm before they exit the cells has led to speculation about the spatial relationship of the GSP proteins to this compartment. Twelve of the fifteen Pul secretory proteins are associated with the inner membrane, while only PulD and PulS may be associated with the outer membrane (Pugsley, 1993a). Secreted proteins may be exported across the inner membrane at sites which are associated with these inner membrane proteins. The Pul proteins and their homologues may form complexes which recognize a signal in secreted proteins as they cross the inner membrane, and therefore, before they have much secondary structure. However, there are several reasons to believe that secreted proteins fold in the periplasm. Sequence comparisons of a number of proteins secreted via the GSP have not revealed the presence of any stretches of amino acids that could act as a secretion signal (Pugsley, 1993a). This indicates that a secretion signal would have to be produced in the tertiary structure of the proteins. Hirst and Holmgren (1987b) demonstrated that heat-labile enterotoxin (which is composed of 5 B subunits and 1 A subunit) assembles into its multimeric form in the periplasm before crossing the outer membrane. In addition, Pugsley (1992) showed that mutations to the periplasmic protein DsbA prevent disulfide bond formation in PulA and consequently delay its secretion. Finally, in the present studies it has been shown that the proaerolysin recovered from periplasmic shockates is as resistant to trypsin as the protoxin recovered from culture supernatants.

Active PhoA is a dimer in the *E. coli* periplasm (Kim and Wyckoff, 1991). Since PhoA activity was detected in the periplasms and culture supernatants of AH65 and CB3 containing cloned *E. coli* PhoA or the AerA'-'PhoA fusions, it seems reasonable to assume

it is secreted as a dimer by the *Aeromonas* spp. Interestingly, it has also been found that proaerolysin exists as a dimer in solution (van der Goot et al., 1993). In the case of proaerolysin, it appears that the dimers form in the periplasm before being secreted across the outer membrane (Hardie and Buckley, unpublished results). PulA is also found in the extracellular milieu as large multimer complexes (Pugsley et al., 1986), although it is not known if these large complexes form in the periplasm or after the monomers have been released from the cell. It is possible that these dimers and multimers form some sort of tertiary structure that is recognized by the secretion apparatus (see section on signals within proteins below). However, multimerization of proteins in the periplasm is not a requirement for secretion of a GSP protein, as GCAT of *A. hydrophila* is secreted as a monomer (Ausio et al., 1993). The GCAT monomer must obtain a structure that allows its secretion via the GSP.

Although the complete PhoA fusion proteins expressed by *A. salmonicida* could initially be detected in the periplasm (Figure 27), they were all rapidly degraded by removal of the aerolysin portion. Like *E. coli*, *A. salmonicida* must contain periplasmic proteases that recognize and degrade proteins with foreign or abnormal conformations. Both proaerolysin and alkaline phosphatase appear to be dimers in the periplasm (see above). Perhaps dimerization of the partial aerolysin portions in the fusion proteins cannot occur, whereas the intact portions of PhoA can dimerize. This could allow for the survival of the PhoA, but lead to proteolytic degradation of the protoxin portions. The observation that the proaerolysin portion of even the largest fusion protein was degraded may mean that the C-terminal 35 amino acids of proaerolysin are needed for dimerization, or that the presence of PhoA blocks its dimerization. The observation that proaerolysin is not degraded in the periplasm of *Aeromonas* spp. (Figure 10) or in *A. hydrophila* pleiotropic secretion mutants (Howard and Buckley, 1985a) indicates that proaerolysin is resistant to periplasmic proteases, perhaps because it dimerizes.

It is not possible to tell whether proteolysis of the aerolysin portions of the fusion proteins was a consequence or a cause of the bacteria's inability to secrete them. On the one hand, had they remained intact, one or more of the proteins could have found their way outside the cells. Since both PhoA and AerA could have similar secretion signals (see below), this is not an unlikely possibility. On the other hand, the fusions may have all been unable to pass through the pathway, and as a result they may have accumulated in the periplasm where the aerolysin portions were removed, resulting in a product (PhoA itself) which then could leave the cell.

Signals within secreted proteins

In order to be selectively secreted from the periplasm, a protein must contain information in its structure that directs it to the secretion machinery. Alternatively, it could lack information which would cause it to be retained in the periplasm. While deletion mutagenesis of exotoxin A (Hamood et al., 1989) and fusions of *pulA* to the β-lactamase gene (Kornacker and Pugsley, 1990) had located regions which potentially contain secretion-directing signals, no specific signals have been identified. Originally, the region of AerA containing Trp227, which was found to be similar with the *S. aureus* α-toxin, was proposed to play a role in toxin interaction with the target membrane. Since α-toxin is made by a Gram-positive bacteria, there should be no need for a signal to direct it across an outer membrane. Surprisingly then, the single amino acid changes to Trp227 have been the only ones made in any protein secreted by the GSP which have affected secretion. Interestingly, Trp110 of *E. coli* PhoA is found in a region which has some similarity to the the region surrounding Trp227 of aerolysin (Chang et al., 1986). It could be that these Trp residues are part of a secretion signal. However, a search of protein sequence data bases revealed that over 1500 proteins from widely different sources contain sequences similar to this region (M. Parker, personal communication). Additionally, the X-ray crystallographic

structure of the proaerolysin dimer (Parker et al., 1994) predicts that the Trp227 residue is not exposed on the surface of the molecule. This would appear to rule out a direct interaction between Trp227 and the secretion machinery. On the other hand, a slight conformational change in the protein could expose Trp227 to the surface of the protein (M. Parker, personal communication), possibly allowing it to play a role in a 3-dimensional structural secretion signal.

As discussed above, it appears that GSP proteins are folded before they leave the cell. It would be unlikely that they could change to a less stable conformation as they crossed the outer membrane, only to fold back again. Such a change could only occur if a net increase in thermodynamic stability of the proteins resulted. Perhaps the periplasmic and extracellular forms of secreted proteins are not the same. An interaction with the secretion machinery could cause a conformational shift in secreted proteins as they cross the outer membrane. The observation that the Leu227 mutant becomes more sensitive to trypsin treatment when it is trapped in the outer membrane (Figure 22) could be evidence that just such a conformational shift does occur during aerolysin secretion. Without the proper Trp residue at position 227, the mutant protein may be unable to interact with part of the translocation machinery, trapping it in a protease-sensitive conformation in the outer membrane. Perhaps all secreted bacterial proteins must undergo conformational shifts during translocation to allow proper interaction with the secretion machinery. Trp227 and the amino acids surrounding it may play an important role in such a process. Site-directed mutagenesis of other amino acids in this region might help to determine if this is the case. Such mutations may result in similar secretion defects to the Trp227 mutants if these residues are also part of a secretion signal or if they are needed to position Trp227 in the correct spatial configuration during a conformational shift. It is also possible that the increased trypsin sensitivity of the membrane-associated Leu227 mutant is not a result of a conformational shift during secretion. The mutation itself may have caused the

conformational change. The mutant protoxin could still be resistant to periplasmic proteases and trypsin, but may have become more sensitive to an outer membrane associated protease like the *E. coli* protease OmpT (Baneyx and Georgiou, 1990). Trypsin may activate such a protease, as the outer membrane associated Leu227 was not degraded without the addition of trypsin (Figure 23, lane 3). Since such an outer membrane protease would not be present in purified samples of Leu227 proaerolysin, purified Leu227 would not be degraded by the addition of trypsin, as was observed in Figure 22.

Since 12 of the Pul secretion proteins have been located in the inner membrane, it may be possible to determine if there is an interaction between proaerolysin and these proteins by making inner membrane vesicles from *E. coli* strains containing the cloned *exe* operon. These vesicles could contain the correctly folded inner membrane Exe components. Addition of proaerolysin and a cross-linking reagent may result in the identification of specific Exe proteins. If no specific interactions are found, it may be because no PulO homologue is found in the *exe* operon (Howard et al., 1993). Without a prepilin peptidase in the system, the Exe proteins containing prepilin signal sequences (ExeG-ExeJ) may be unable to correctly localize into the inner membrane, possibly resulting in the formation of an incomplete secretion complex . It might be possible to clone a prepilin peptidase from a heterologous system (e.g. PulO or PilB) along with the *exe* operon to properly process the prepilin signal sequences.

Another approach to detect interactions between aerolysin and the secretion machinery would be to look for suppressor mutants. If Trp227 does play a role in a secretion signal, it may be possible to find *A. hydrophila* mutants which can secrete the Trp227 mutants normally by mutating one or more of the secretory components required to recognize such a signal. The *aerA*$^-$ mutant I2.66 (Howard, unpublished results) could be used to locate such spontaneous suppressor mutants. The different *exe* genes of these mutants would then have to be screened or sequenced to see if any were mutated.

It is also possible that the region surrounding Trp227 is not part of a secretion signal, but is instead directly involved in the translocation of aerolysin across the outer membrane. The lower secretion rates of the Phe227 and Leu227 proaerolysins observed in Figure 17 appear to parallel the lower hemolytic activities of their activated forms (Table 5). Thus, the Phe227 mutant was released at about one-fourth the rate of native proaerolysin, and after processing, the purified protein was about one-fourth as active as aerolysin itself. This suggests that the ability of these proteins to cross the outer membrane may depend on a part of the protein that is also involved in channel formation. Thus the Trp227-containing region may play a role in insertion of proaerolysin into the outer membrane during secretion. The corresponding region in *S. aureus* α -toxin (Howard et al., 1987), a protein that does not have to cross an outer membrane, may have a similar role in membrane penetration of its target cells. However, the studies in this thesis have shown that PhoA can be secreted by the *Aeromonas* spp. GSP, and there is no reason to believe that PhoA can insert into lipid bilayers. If insertion of secreted proteins into the outer membrane is a requirement for secretion via the GSP, perhaps an interaction between the Pul-like proteins and the secreted protein is required to expose the amino acids required for this process. Thus PhoA may contain the regions necessary for membrane insertion, but without the Pul-like proteins in *E. coli*, it may be unable to insert into the outer membrane and remains in the periplasm as a result.

Finally, it is possible that the Trp227 region is not required for secretion, and that the GSP proteins are secreted because they lack a signal which causes them to be retained in the periplasm. The Trp227 mutations may have fortuitously changed the protein in such a way that a periplasmic retention signal was partially created, preventing them from leaving the cell as efficiently as wild type. *E. coli* PhoA could then be secreted simply because it lacks an *Aeromonas* spp. periplasmic retention signal.

The energy requirements of secretion

The results in these studies are the first to implicate a PMF in the secretion of a protein across the outer membrane. Koronakis et al. (1991) have since found that a PMF is also required in the secretion of HlyA from *E. coli*. Interestingly, both the GSP (Possot and Pugsley, 1994) and HlyA (Gerlach et al., 1986) secretion systems utilize components which contain consensus ATP-binding sites. However, neither HlyB, nor the PulE homologues have been shown to hydrolyze ATP as an integral step in the secretion process. It has been proposed that the *P. aeruginosa* PulE homologue XcpR is only necessary for energizing the assembly of the secretion apparatus, and not the actual secretion process (Tommassen et al., 1992). This comes from the observation that XcpR is homologous to PilB, which is required for pilin biogenesis, but not protein secretion, in *P. aeruginosa* (Nunn et al., 1990; Turner et al., 1993). If this turns out to be the case, the PMF could be the sole energy source required for outer membrane translocation of proteins via the GSP. Another possibility is that the folding of a secreted protein plays a role in energizing the secretion process. If a conformational change is required for exposing the Trp227 region to the secretion machinery, this change in protein folding could be used as a source of energy. Jacob-Dubuisson et al. (1994) have proposed that the *E. coli* pilin protein PapC is driven across the outer membrane by such a change in protein conformation. They speculated that periplasmic chaperones could keep the pilin subunits in high energy conformations until they are secreted. This appears to be the only energy source required for pilin transport, as no ATP-binding proteins are necessary in PapC pilin biogenesis and a PMF is not necessary for its secretion (Jacob-Dubuisson et al., 1994). The fact that we apparently find proaerolysin in its final mature conformation in the periplasm suggests that similar periplasmic chaperones are not involved in its secretion. As mentioned above though, some components of the GSP may be required to alter the conformation of proaerolysin during its translocation across the outer membrane.

As proposed in the Results section, the existence of a periplasmic-spanning energy transducing system like TonB would be the most likely explanation of the need for a PMF to drive the second step in secretion. However, the high number of pirazmonam-resistant, hemolytic mutants of *A. hydrophila* isolated in these studies indirectly suggests that TonB itself does not participate in such a scheme. Pugsley (1993a) has also reported that TonB does not appear to play a role in PulA secretion. It is possible that another structure that traverses the periplasmic space energizes the translocation of proteins out of the cell. Since 12 of the 15 Pul secretion proteins are believed to be associated with the periplasmic face of the inner membrane (d'Enfert and Pugsley, 1989; Reyss and Pugsley, 1990; Pugsley and Reyss, 1990; Pugsley and Dupuy, 1992), it is possible they interact together to form such a structure in *K. oxytoca*. Four of the Pul proteins (PulG-PulJ) are thought to form a pilin-like structure because they contain type IV pilin signal sequences. These Pul proteins could form a periplasmic-spanning energy transducer in a manner analogous to TonB. However, attempts to find an oligomeric PulG-containing complex have been unsuccessful (Pugsley, 1993b).

It has been proposed that TonB energizes the opening of pores formed by outer membrane receptors (Rutz et al., 1992). One of the *E. chrysanthemi* Out proteins, OutD, has recently been shown to form mixed multimers in the outer membrane with a homologous phage protein (Kazmierczak et al., 1994). These results suggest that PulD and its homologues could form gated channels large enough to allow the passage of proteins. This would fit a model in which other Pul proteins form a TonB-like structure that uses the PMF to open and close the outer membrane channel. However, such a channel would have to be unidirectional, allowing only the flow of specific proteins out of the cell, and preventing the non-specific leakage of other periplasmic molecules. While such channels are now believed to exist across both the endoplasmic reticulum (Simon and Blobel, 1991) and prokaryotic inner membrane (Simon and Blobel, 1992), there is no

evidence of their presence in the bacterial outer membrane. Outer membrane channels would have to be larger than the inner membrane pores of the *sec* system as they would have to allow passage of folded proteins. It might be expected that these channels could be blocked by the overexpression of secreted proteins. This was not observed in the present studies, as the overexpression of aerolysin did not reduce the secretion of endogenous GCAT and protease in *A. salmonicida* (Figure 9). The Trp227 mutant proteins also did not reduce secretion of the *A. salmonicida* protease (Figure 21). Such a decrease would have been expected if the Leu227 mutant was blocking such a channel.

Although it seems unlikely, there is no direct evidence to refute the existence of a PMF across the outer membrane. The observed inhibition of proaerolysin secretion by lowering the media pH (Figure 33) is consistent with such a possibility, as is the inhibition of PhoA secretion under similar conditions (Figure 28). While porins in the outer membrane probably make the outer membrane permeable to protons, they may be missing from localized regions in the membrane, perhaps allowing for a build up of a proton gradient. The observation that proaerolysin found in the periplasms of *A. salmonicida* and the pleiotropic mutants of *A. hydrophila* is somehow protected from periplasmic proteases could support the idea that the periplasm is divided into different compartments or pools. While specific proteases which activate proaerolysin outside the cell may be inactive in the periplasm, it is also possible that proaerolysin is in a compartment which lacks proteases. Secretion compartments may then lack both porins and proteases. The observation from electron microscopy studies that there are inner and outer membrane junctions in Gram-negative bacteria could support the idea of membrane enclosed regions in the periplasm (Bayer, 1979). However, recent results have suggested that Bayer bridges are artifacts resulting from the fixation methods used in preparing cells for the electron microscope (Kellenberger, 1990).

The effect of medium pH could also be due to inhibition of a component of the secretion machinery or to a pH-sensitive change in the outer membrane. In either case the PMF across the inner membrane would not be affected.

SUMMARY

The observations made in this thesis have revealed a number of features of secretion via the GSP. For the first time it has been demonstrated that a protein which utilizes this pathway can be secreted by a heterologous host. Future studies of the different GSP systems will hopefully explain why the systems in *Vibrio* and *Aeromonas* species are interchangeable while those of some other bacteria appear not to be. The demonstration of a pool of proaerolysin in the periplasm of Rif-1-pKW2 which was secreted further supports a two-step model of secretion in which proteins transiently enter the periplasm after export across the inner membrane. This periplasmic proaerolysin is as resistant to trypsin as the purified protoxin, indicating that the proteins fold into their mature conformations in the periplasm before being translocated across the outer membrane. A Trp residue was identified in a region of proaerolysin which may be involved in secretion. This residue was changed to three different amino acids, resulting in decreased secretion of the protein in all three cases. The periplasmic *E. coli* protein PhoA was shown to be secreted in *A. salmonicida* and *A. hydrophila*. The *A. hydrophila* GSP appeared to be required for its secretion, as a strain containing a mutation in *exeE* was unable to secrete PhoA. Secretion also requires a proton motive force. While there is a possibility that this may be across the outer membrane, it is more likely that the PMF across the inner membrane is used. The protein TonB does not appear to be involved in this process, but a similar energy-transducing complex could be formed by the GSP secretion machinery.

REFERENCES

Akita, M., S. Sasaki, S. Matsuyama, and S. Mizushima. 1990. SecA interacts with secretory proteins by recognizing the positive charge at the amino terminus of the signal peptide in *Escherichia coli.* J. Biol. Chem. **265**:8164-8169.

Akiyama, Y., and K. Ito. 1987. Topology analysis of the SecY protein, an integral membrane protein involved in protein export in *Escherichia coli.* EMBO J. **6**:3465-3470.

Akrim, M., M. Bally, G. Ball, J. Tommassen, H. Teerink, A. Filloux, and A. Lazdunski. 1993. Xcp-mediated protein secretion in *Pseudomonas aeruginosa*: identification of two additional genes and evidence for regulation of *xcp* gene expression. Mol. Microbiol. **10**:431-443.

Allaoui, A., P. J. Sansonetti, and C. Parsot. 1993. MxiD, an outer membrane protein necessary for the secretion of the *Shigella flexneri* Ipa invasins. Mol. Microbiol. **7**:59-68.

Altman, E., C. A. Kumamoto, and S. D. Emr. 1991. Heat-shock proteins can substitute for SecB function during protein export in *Escherichia coli.* EMBO J. **10**:239-245.

Altman, E. V., A. Bankaitis, and S. D. Emr. 1990a. Characterization of a region in mature LamB protein that interacts with a component of the export machinery of *Escherichia coli.* J. Biol. Chem. **265**:18148-18153.

Altman, E. V., S. D. Emr, and C. A. Kumamoto. 1990b. The presence of both the signal sequence and a region of mature LamB is required for the interaction of LamB with the export factor SecB. J. Biol. Chem. **265**:18154-18160.

Andersson, G., and G. von Heijne. 1991. A 30-residue-long "export initiation domain" adjacent to the signal sequence is critical for protein translocation across the inner membrane of *Escherichia coli.* Proc. Natl. Acad. Sci. USA **88**:9751-9754.

Andro, T., J. -P. Chambost, A. Kotoujansky, J. Cattaneo, Y. Bertheau, F. Barras, F. van Gijsegem, and A. Coleno. 1984. Mutants of *Erwinia chrysanthemi* defective in secretion of pectinase and cellulase. J. Bacteriol. **160**:1199-1203.

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

Arkowitz, R. A., J. C. Joly, and W. Wickner. 1993. Translocation can drive the unfolding of a preprotein domain. EMBO J. **12**:243-253.

Ashton, D. M., G. D. Sweet, J. M. Somers, and W. W. Kay. 1980. Citrate transport in *Salmonella typhimurium:* studies with 2-fluoro-L-erythrocitrate as a substrate. Can. J. Biochem. **58**:797-803.

Ausio, J., F. G. van der Goot, and J. T. Buckley. 1993. Physical and chemical characterization of the oligomerization state of the *Aeromonas hydrophila* lipase/acyltransferase. FEBS Lett. **333**:296-300.

Bakker, E. P., and L. L. Randall. 1984. The requirement for energy during export of β-lactamase in *Escherichia coli* is fulfilled by the total protonmotive force. EMBO J. **3**:895-900.

Ballard, J., Y. Sokolov, W. -L. Yuan, B. L. Kagan, and R. K. Tweten. 1993. Activation and mechanism of *Clostridium septicum* alpha toxin. Mol. Microbiol. **10**:627-634.

Bally, M., A. Filloux, M. Akrim, G. Ball, A. Lazdunski, and J. Tommassen. 1992. Protein secretion in *Pseudomonas aeruginosa*: characterization of seven *xcp* genes and processing of secretory apparatus components by prepilin peptidase. Mol. Microbiol. **6**:1121-1131.

Bally, M., G. Ball, A. Badere, and A. Lazdunski. 1991. Protein secretion in *Pseudomonas aeruginosa*: the *xcpA* gene encodes an integral inner membrane protein homologous to *Klebsiella pneumoniae* secretion function protein PulO. J. Bacteriol. **173**:479-486.

Bally, M., B. Wretlind, and A. Lazdunski. 1989. Protein secretion in *Pseudomonas aeruginosa*: Molecular cloning and characterization of the *xcp-1* gene. J. Bacteriol. **171**:4342-4348.

Baneyx, F., and G. Georgiou. 1990. In vivo degradation of secreted fusion proteins by the *Escherichia coli* outer membrane protease OmpT. J. Bacteriol. **172**:491-494.

Bankaitis, V. A., and P. J. Bassford, Jr. 1985. Proper interaction between at least two components is required for efficient export of proteins to the *Escherichia coli* cell envelope. J. Bacteriol. **161**:169-178.

Bardwell, J. C. A., K. McGovern, and J. Beckwith. 1991. Identification of a protein required for disulfide bond formation *in vivo*. Cell **65**:581-589.

Bassford, P., and J. Beckwith. 1979. *Escherichia coli* mutants accumulating the precursor of a secreted protein in the cytoplasm. Nature **277**:538-541.

Batenburg, A. M., R. A. Demel, A. J. Verkleij, and B. de Kruijff. 1988. Penetration of the signal sequence of *Escherichia coli* PhoE protein into phospholipid model membranes leads to lipid-specific changes in signal peptide structure and alterations of lipid organization. Biochemistry **27**:5678-5685.

Bayer, M. E. 1979. The fusion sites between outer and cytoplasmic membrane of bacteria: their role in membrane assembly and virus infection, p. 167-202. *In* M. Inouye (ed.), **Bacterial outer membranes**. John Wiley & Sons Inc., New York.

Bedouelle, H., P. J. Bassford Jr., V. Fowler, I. Zabin, J. Beckwith, and M. Hofnung. 1980. Mutations which alter the function of the signal sequence of the maltose binding protein of *Escherichia coli*. Nature **285**:78-81.

Bernstein, H. D., M. A. Poritz, K. Strub, P. J. Hoben, S. Brenner, and P. Walter. 1989. Model for signal sequence recognition from amino-acid sequence of 54K subunit of signal recognition particle. Nature **340**:482-486.

Bieker-Brady, K., and T. J. Silhavy. 1992. Suppressor analysis suggests a multistep, cyclic mechanism for protein secretion in *Escherichia coli.* EMBO J. **11**:3165-3174.

Bieker, K. L., and T. J. Silhavy. 1990. PrlA (SecY) and PrlG (SecE) interact directly and function sequentially during protein translocation in *E. coli.* Cell **61**:833-842.

Birnboim, H. C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Biochim. Biophys. Acta. **299**:516-520.

Bishop, R. W., and R. M. Bell. 1985. Assembly of the endoplasmic reticulum phospholipid bilayer: The phosphatidylcholine transporter. Cell **42**:51-60.

Blobel, G., and B. Dobberstein. 1975. Transfer of proteins across membranes I. Presence of proteolytically processed and unprocessed nascent immunoglobulin light chains on membrane-bound ribosomes of murine myeloma. J. Cell Biol. **67**:835-851.

Bochkareva, E. S., N. M. Lissin, and A. G. Girshovich. 1988. Transient association of newly synthesized unfolded proteins with the heat-shock GroEL protein. Nature **336**:254-257.

Bortoli-German, I., E. Brun, B. Py, M. Chippaux, and F. Barras. 1994. Periplasmic disulphide bond formation is essential for cellulase secretion by the plant pathogen *Erwinia chrysanthemi.* Mol Microbiol. **11**:545-553.

Bosch, D., P. de Boer, W. Bitter, and J. Tommassen. 1989. The role of the positively charged N-terminus of the signal sequence of *E. coli* outer membrane protein PhoE in export. Biochim. Biophys. Acta. **979**:69-76.

Brass, J. M., C. F. Higgins, M. Foley, P. A. Rugman, J. Birmingham, and P. B. Garland. 1986. Lateral diffusion of proteins in the periplasm of *Escherichia coli.* J. Bacteriol. **165**:787-794.

Breukink, E., R. A. Demel, G. de Korte-Kool, and B. de Kruijff. 1992. SecA insertion into phospholipids is stimulated by negatively charged lipids and inhibited by ATP: A monolayer study. Biochemistry **31**:1119-1124.

Brickman, E., and J. Beckwith. 1975. Analysis of the regulation of *Escherichia coli* alkaline phosphatase synthesis using deletions and ϕ80 transducing phages. J. Mol. Biol. **96**:307-316.

Briggs, M. S., D. G. Cornell, R. A. Dluhy, and L. M. Gierasch. 1986. Conformations of signal peptides induced by lipids suggest initial steps in protein export. Science **233**:206-208.

Bruch, M. D., C. J. McKnight, and L. M. Gierasch. 1989. Helix formation and stability in signal sequences. Biochemistry **28**:8554-8561.

Brundage, L., C. J. Fimmel, S. Mizushima, and W. Wickner. 1992. SecY, SecE, and band 1 form the membrane-embedded domain of *Escherichia coli* preprotein translocase. J. Biol. Chem. 267:4166-4170.

Brundage, L., J. P. Hendrick, E. Schiebel, A. J. M. Driessen, and W. Wickner. 1990. The purified *E. coli* integral membrane protein SecY/E is sufficient for reconstitution of SecA-dependent precursor protein translocation. Cell 62:649-657.

Buckley, J. T. 1992. Crossing three membranes. Channel formation by aerolysin. FEBS Lett. 307:30-33.

Buckley, J. T. 1990. Purification of cloned proaerolysin released by a low protease mutant of *Aeromonas salmonicida*. Bioc. Cell Biol. 68:221-224.

Buckley, J. T., L. N. Halasa, and S. MacIntyre. 1982. Purification and partial characterization of a bacterial phospholipid:cholesterol acyl-transferase. J. Biol. Chem. 257:3320-3325.

Burnell, E., L. van Alphen, A. Verkleij, and B. de Kruijff. 1980. ^{31}P-Nuclear magnetic resonance and freeze-fracture electron microscopy studies in *Escherichia coli*. I. Cytoplasmic membrane and total phospholipids. Biochim. Biophys Acta 597:492-501.

Chakraborti, A. S., K. Ishidate, W. R. Cook, J. Zrike, and L. I. Rothfield. 1986. Accumulation of a murein-membrane attachment site fraction when cell division is blocked in *lkyD* and *cha* mutants of *Salmonella typhimurium* and *Escherichia coli*. J. Bacteriol. 168:1422-1429.

Chakraborty, T., A. Schmid, S. Notermans, and R. Benz. 1990. Aerolysin of *Aeromonas sobria*: Evidence for formation of ion-permeable channels and comparison with alpha-toxin of *Staphylococcus aureus*. Infect. Immun. 58:2127-2132.

Chakraborty, T., B. Huhle, H. Hof, H. Bergbauer, and W. Goebel. 1987. Marker exchange mutagenesis of the aerolysin determinant in *Aeromonas hydrophila* demonstrates the role of aerolysin in *A. hydrophila*-associated systemic infection. Infect. Immun. 55:2274-2280.

Chambert, R., F. Benyahia, and M. -F. Petit-Glatron. 1990. Secretion of *Bacillus subtilis* levansucrase. Fe(III) could act as a cofactor in an efficient coupling of the folding and translocation processes. Biochem J. 265:375-382.

Chang, M. C., J. C. Chang, and J. P. Chen. 1993. Cloning and nucleotide sequence of an extracellular α-amylase gene from *Aeromonas hydrophila* MCC-1. J. Gen. Microbiol. 139:3215-3223.

Chang, Y.-F., R. Young, T. L. Moulds, and D. K. Struck. 1989. Secretion of the *Pasteurella* leukotoxin by *Escherichia coli*. FEMS Microbiol. Lett. 60:169-174.

Chang, C. N., W.-J. Kuang, and E. Y Chen. 1986. Nucleotide sequence of the alkaline phosphatase gene of *Escherichia coli*. Gene 44:121-125.

Chen, L., and P. C. Tai. 1985. ATP is essential for protein translocation into *Escherichia coli* membrane vesicles. Proc. Natl. Acad. Sci. USA **82**:4384-4388.

Cheng, K.-J., J. M. Ingram, and J. W. Costerton. 1970. Release of alkaline phosphatase from cells of *Pseudomonas aeruginosa* by manipulation of cation concentration and pH. J. Bacteriol. **104**:748-753.

Chou, M. M., and D. A. Kendall. 1990. Polymeric sequences reveal a functional relationship between hydrophobicity and length of signal peptides. J. Biol. Chem. **265**:2873-2880.

Click, E. -M., K. S. Anderson, M. J. Androlewicz, M. L. Wei, and P. Cresswell. 1992. Transport and expression of class I MHC glycoproteins in an antigen-processing mutant cell line. Cold Spring Harbor Symposia on Quantitative Biology **57**:571-577.

Collier, D. N., V. A. Bankaitis, J. B. Weiss, and P. J. Bassford Jr. 1988. The antifolding activity of SecB promotes the export of the *E. coli* maltose-binding protein. Cell **53**:273-283.

Collmer, A., C. Schoedel, D. L. Roeder, J. L. Reid, and J. F. Rissler. 1985. Molecular cloning in *Escherichia coli* of *Erwinia chrysanthemi* genes encoding multiple forms of pectate lyase. J. Bacteriol. **161**:913-920.

Condemine, G., C. Dorel, N. Hugouvieux-Cotte-Pattat, and J. Robert-Baudouy. 1992. Some of the out genes involved in the secretion of pectate lyases in *Erwinia chrysanthemi* are regulated by *kdgR*. Mol. Microbiol. **6**:3199-3211.

Connolly, T., and R. Gilmore. 1993. GTP hydrolysis by complexes of the signal recognition particle and the signal recognition particle receptor. J. Cell Biol. **123**:799-807.

Connolly, T., and R. Gilmore. 1989. The signal recognition particle receptor mediates the GTP-dependent displacement of SRP from the signal sequence of the nascent polypeptide. Cell **57**:599-610.

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

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

Cowan, S. W., T. Schirmer, G. Rummel, M. Steiert, R. Ghosh, R. A. Pauptit, J. N. Jansonius, and J. P. Rosenbusch. Crystal structures explain properties of two *E. coli* porins. 1992. Nature **358**:727-733.

Crooke, E., L. Brundage, M. Rice, and W. Wickner. 1988. ProOmpA spontaneously folds in a membrane assembly competent state which trigger factor stabilizes. EMBO J. **7**:1831-1835.

Cronan, J. E., Jr., R. B. Gennis, and S. R. Maloy. 1987. Cytoplasmic membrane, p. 31-55. *In* F. C. Neidhardt (ed.), ***Escherichia coli* and *Salmonella typhimurium***

cellular and molecular biology. American Society for Microbiology Publications, Washington, D. C.

Crowley, K. S., G. D. Reinhart, and A. E. Johnson. 1993. The signal sequence moves through a ribosomal tunnel into a noncytoplasmic aqueous environment at the ER membrane early in translocation. Cell **73**:1101-1115.

Cunningham, K., and W. Wickner. 1989. Specific recognition of the leader region of precursor proteins is required for the activation of translocation ATPase of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **86**:8630-8634.

Cunningham, K., R. Lill, E. Crooke, M. Rice, K. Moore, W. Wickner, and D. Oliver. 1989. SecA protein, a peripheral protein of the *Escherichia coli* plasma membrane, is essential for the functional binding and translocation of proOmpA. EMBO J. **8**:955-959.

Dallas, W. S., and S. Falkow. 1980. Amino acid sequence homology between cholera toxin and *Escherichia coli* heat-labile toxin. Nature **288**:499-501.

de Cock, H., W. Overeem, and J. Tommassen. 1992. Biogenesis of outer membrane protein PhoE of *Escherichia coli*. Evidence for multiple SecB-binding sites in the mature portion of the PhoE protein. J. Mol. Biol. **224**:369-379.

de Cock, H., and J. Tommassen. 1992. SecB-binding does not maintain the translocation-competent state of prePhoE. Mol Microbiol. **6**:599-604.

de Groot, A., A. Filloux, and J. Tommassen. 1991. Conservation of *xcp* genes, involved in the two-step protein secretion process, in different *Pseudomonas* species and other gram-negative bacteria. Mol. Gen. Genet. **229**:278-284.

de Kruijff, B. 1990. Cholesterol as a target for toxins. Biosci. Rep. **10**:127-130.

Delepelaire, P., and C. Wandersman. 1990. Protein secretion in Gram-negative bacteria. J. Biol. Chem. **265**:17118-17125.

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

d'Enfert, C., I. Reyss, C. Wandersman, and A. P. Pugsley. 1989. Protein secretion by Gram-negative bacteria. Characterization of two membrane proteins required for pullulanase secretion by *Escherichia coli* K-12. J. Biol. Chem. **264**:17462-17468.

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

Derman, A. I., J. W. Puziss, P. J. Bassford, Jr., and J. Beckwith. 1993. A signal sequence is not required for protein export in *prlA* mutants of *Escherichia coli*. EMBO J. **12**:879-888.

de Vrije, G. J., A. M. Batenburg, J. A. Killian, and B. de Kruijff. 1990. Lipid involvement in protein translocation in *Escherichia coli*. Mol. Microbiol. **4**:143-150.

de Vrije, G. J., R. L. de Swart, W. Dowhan, J. Tommassen, and B. de Kruijff. 1988. Phosphatidylglycerol is involved in protein translocation across *Escherichia coli* inner membranes. Nature **334**:173-175.

Dommair, K., H. Kiefer, and F. Jahnig. 1990. Refolding of an integral membrane protein. OmpA of *Escherichia coli*. J. Biol. Chem. **265**:18907-18911.

Douglas, C. M., C. Guidi-Rontani, and R. J. Collier. 1987. Exotoxin A of *Pseudomonas aeruginosa*: Active, cloned toxin is secreted into the periplasmic space of *Escherichia coli*. J. Bacteriol. **169**:4962-4966.

Driessen, A. J. M. 1992. Precursor protein translocation by the *Escherichia coli* translocase is directed by the protonmotive force. EMBO J. **11**:847-853.

Driessen, A. J. M., and W. Wickner. 1991. Proton transfer is rate-limiting for translocation of precursor proteins by the *Escherichia coli* translocase. Proc. Natl. Acad. Sci. USA **88**:2471-2475.

Doud, S. K., M. M. Chou, and D. A. Kendall. 1993. Titration of protein transport activity by incremental changes in signal peptide hydrophobicity. Biochemistry **32**:1251-1256.

Dums, F., J. M. Dow, and M. J. Daniels. 1991. Structural characterization of protein secretion genes of the bacterial phytopathogen *Xanthomonas campestris* pathovar *campestris*: relatedness to secretion systems of other gram-negative bacteria. Mol. Gen. Genet. **229**:357-364.

Dupuy, B., M. -K. Taha, O. Possot, C. Marchal, and A. P. Pugsley. 1992. PulO, a component of the pullulanase secretion pathway of *Klebsiella oxytoca*, correctly and efficiently processes gonococcal type IV prepilin in *Escherichia coli*. Mol. Microbiol. **6**:1887-1894.

Eisele, J. -L., and J. P. Rosenbusch. 1990. *In vitro* folding and oligomerization of a membrane protein. Transition of bacterial porin from random coil to native conformation. J. Biol. Chem. **265**:10217-10220.

Emr, S., S. Hanley-Way, and T. J. Silhavy. 1981. Suppressor mutations that restore export of a protein with a defective signal sequence. Cell **23**:79-88.

Emr, S. D., J. Hedgpeth, J.-M. Clement, T. J. Silhavy, and M. Hofnung. 1980. Sequence analysis of mutations that prevent export of λ receptor, an *Escherichia coli* outer membrane protein. Nature **285**:82-85.

Erb, K., M. Vogel, W. Wagner, and W. Goebel. 1987. Alkaline phosphatase which lacks its own signal sequence becomes enzymatically active when fused to N-terminal sequences of *Escherichia coli* haemolysin (HlyA). Mol. Gen. Genet. **208**:88-93.

Fath, M. J., and R. Kolter. 1993. ABC transporters: Bacterial exporters. Microbiol. Rev. **57**:995-1017.

Felmlee, T., S. Pellett, E. Lee, and R. A. Welch. 1985a. *Escherichia coli* hemolysin is released extracellularly without cleavage of a signal peptide. J. Bacteriol. **163**:88-93.

Felmlee, T., S. Pellett, and R. A. Welch. 1985b. Nucleotide sequence of an *Escherichia coli* chromosomal hemolysin. J. Bacteriol. **163**:94-105.

Figurski, D. H., and D. R. Helinski. 1979. Replication of an origin-containing derivative of plasmid RK2 dependent on a plasmid function provided in *trans*. Proc. Natl. Acad. Sci. USA **76**:1648-1652.

Fikes, J. D., and P. J. Bassford, Jr. 1989. Novel *secA* alleles improve export of maltose-binding protein synthesized with a defective signal peptide. J. Bacteriol. **171**:402-409.

Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart. 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. **115**:717-722.

Filloux, A., M. Bally, G. Ball, M. Akrim, J. Tommassen, and A. Lazdunski. 1990. Protein secretion in Gram-negative bacteria: transport across the outer membrane involves common mechanisms in different bacteria. EMBO J. **9**:4323-4329.

Focareta, T., and P. A. Manning. 1987. Extracellular proteins of *Vibrio cholerae*: molecular cloning, nucleotide sequence and characterization of the deoxyribonuclease (DNase) together with its periplasmic location in *Escherichia coli* K-12. Gene **53**:31-40.

Francisco, J. A., C. F. Earhart, and G. Georgiou. 1992. Transport and anchoring of β-lactamase to the external surface of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **89**:2713-2717.

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

Gannon, P. M., P. Li, and C. A. Kumamoto. 1989. The mature portion of *Escherichia coli* maltose-binding protein (MBP) determines the dependence of MBP on SecB for export. J. Bacteriol. **171**:813-818.

Gardel, C., K. Johnson, A. Jacq, and J. Beckwith. 1990. The *secD* locus of *E. coli* codes for two membrane proteins required for export. EMBO J. **9**:3209-3216.

Gardel, C., S. Benson, J. Hunt, S. Michaelis, and J. Beckwith. 1987. *secD*, a new gene involved in protein export in *Escherichia coli*. J. Bacteriol. **169**:1286-1290.

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

Geller, B. L. 1991. Energy requirements for protein translocation across the *Escherichia coli* inner membrane. Mol. Microbiol. **5**:2093-2098.

Geller, B. L., and H. M. Green. 1989. Translocation of proOmpA across inner membrane vesicles of *Escherichia coli* occurs in two consecutive energetically distinct steps. J. Biol. Chem. **28**:16465-16469.

Geller, B. L., N. R. Movva, and W. Wickner. 1986. Both ATP and the electrochemical potential are required for optimal assembly of pro-OmpA into *Escherichia coli* inner membrane vesicles. Proc. Natl. Acad. Sci. USA **83**:4219-4222.

Gentschev, I., and W. Goebel. 1992. Topological and functional studies on HlyB of *Escherichia coli*. Mol. Gen. Genet. **232**:40-48.

Gentschev, I., J. Hess, and W. Goebel. 1990. Change in the cellular localization of alkaline phosphatase by alteration of its carboxy-terminal sequence. Mol. Gen. Genet. **222**:211-216.

Gerlach, J. H., J. A. Endicott, P. F. Juranka, G. Henderson, F. Sarangi, K. L. Deuchars, and V. Ling. 1986. Homology between P-glycoprotein and a bacterial haemolysin transport protein suggests a model for multidrug resistance. Nature **324**:485-489.

Gierasch, L. M. 1989. Signal sequences. Biochemistry **28**:923-930.

Gilmore, R. 1993. Protein translocation across the endoplasmic reticulum: A tunnel with toll booths at entry and exit. Cell **75**:589-592.

Gilmore, R., P. Walter, and G. Blobel. 1982. Protein translocation across the endoplasmic reticulum II. Isolation and characterization of the signal recognition particle receptor. J. Cell Biol. **95**:470-477.

Glaser, P., H. Sakamoto, J. Bellalou, A. Ullmann, and A. Danchin. 1988. Secretion of cyclolysin, the calmodulin-sensitive adenylate cyclase-haemolysin bifunctional protein of *Bordetella pertussis*. EMBO J. 7:3997-4004.

Gobius, K. S., and J. M. Pemberton. 1988. Molecular cloning, characterization, and nucleotide sequence of an extracellular amylase gene from *Aeromonas hydrophila*. J. Bacteriol. **170**:1325-1332.

Goebel, W., and J. Hedgpeth. 1982. Cloning and functional characterization of the plasmid-encoded hemolysin determinant of *Escherichia coli*. J. Bacteriol. **151**:1290-1298.

Gonzalez-Carrero, M. I., J. C. Zabala, F. de la Cruz, and J. M. Ortiz. 1985. Purification of α-hemolysin from an overproducing *E. coli* strain. Mol. Gen. Genet. **199**:106-110.

Gorlich, D., and T. A. Rapoport. 1993. Protein translocation into proteoliposomes reconstituted from purified components of the endoplasmic reticulum membrane. Cell **75**:615-630.

Gorlich, D., E. Hartmann, S. Prehn, and T. A. Rapoport. 1992a. A protein of the endoplasmic reticulum involved early in polypeptide translocation. Nature **357**:47-52.

Gorlich, D., S. Prehn, E. Hartmann, K. Kalies, and T. A. Rapoport. 1992b. A mammalian homolog of SEC61p and SECYp is associated with ribosomes and nascent polypeptides during translocation. Cell **71**:489-503.

Gorlich, D., S. Prehn, E. Hartmann, J. Herz, A. Otto, R. Kraft, M. Wiedmann, S. Knespel, B. Dobberstein, and T. A. Rapoport. 1990. The signal sequence receptor has a second subunit and is part of a translocation complex in the endoplasmic reticulum as probed by bifunctional reagents. J. Cell Biol. **111**:2283-2294.

Graham, L. L., R. Harris, W. Villiger, and T. J. Beveridge. 1991. Freeze-substitution of Gram-negative eubacteria: General cell morphology and envelope profiles. J. Bacteriol. **173**:1623-1633.

Gray, G. L., D. H. Smith, J. S. Baldridge, R. N. Harkins, M. L. Vasil, E. Y. Chen, and H. L. Heyneker. 1984. Cloning, nucleotide sequence, and expression in *Escherichia coli* of the exotoxin A structural gene of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci. USA **81**:2645-2649.

Green, M. J., and J. T. Buckley. 1990. Site-directed mutagenesis of the hole-forming toxin aerolysin: Studies on the roles of histidines in receptor binding and oligomerization of the monomer. Biochemistry **29**:2177-2180.

Gunter, K., and V. Braun. 1988. Probing FhuA'-'PhoA fusion proteins for the study of FhuA export into the cell envelope of *Escherichia coli* K12. Mol. Gen. Genet. **215**:69-75.

Gutierrez, C., J. Barondess, C. Manoil, and J. Beckwith. 1987. The use of transposon Tn*phoA* to detect genes for cell envelope proteins subject to a common regulatory stimulus. Analysis of osmotically regulated genes in *Escherichia coli*. J. Mol. Biol. **195**:289-297.

Guzzo, J., F. Duong, C. Wandersman, M. Murgier, and A. Lazdunski. 1991. The secretion genes of *Pseudomonas aeruginosa* alkaline protease are functionally related to those of *Erwinia chrysanthemi* proteases and *Escherichia coli* α-haemolysin. Mol. Microbiol. **5**:447-453.

Halpern, Y. S., and M. Lupo. 1965. Glutamate transport in wild-type and mutant strains of *E. coli*. J. Bacteriol. **90**:1288-1295.

Hamood, A. N., J. C. Olson, T. S. Vincent, and B. H. Iglewski. 1989. Regions of toxin A involved in toxin A excretion in *Pseudomonas aeruginosa*. J. Bacteriol. **171**:1817-1824.

Hancock, R. E. W. 1991. Bacterial outer membranes: Evolving concepts. ASM News **57**:175-182.

Hannavy, K., G. C. Barr, C. J. Dorman, J. Adamson, L. R. Mazengera, M. P. Gallagher, J. S. Evans, B. A. Levine, I. P. Taylor, and C. F. Higgins. 1990. TonB protein of *Salmonella typhimurium*. A model for signal transduction between membranes. J. Mol. Biol. **216**:897-910.

Hardie, K. R., J.-P. Issartel, E. Koronakis, C. Hughes, and V. Koronakis. 1991. *In vitro* activation of *Escherichia coli* prohaemolysin to the mature membrane-targeted toxin requires HlyC and a low molecular-weight cytosolic polypeptide. Mol. Microbiol. **5**:1669-1679.

Hardy, S. J. S., and L. L. Randall. 1991. A kinetic partitioning model of nonnative proteins by the bacterial chaperone SecB. Science **251**:439-443.

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

Hartmann, E., T. Sommer, S. Prehn, D. Gorlich, S. Jentsch, and T. A. Rapoport. 1994. Evolutionary conservation of components of the protein translocation complex. Nature **367**:654-657.

Hartmann, E., M. Wiedmann, and T. A. Rapoport. 1989. A membrane component of the endoplasmic reticulum that may be essential for protein translocation. EMBO J. **8**: 2225-2229.

Haryama, S., M. Tsuda, and T. Iino. 1980. High frequency mobilization of the chromosome of *Escherichia coli* by a mutant of plasmid RP4 temperature-sensitive for maintenance. Mol. Gen. Genet. **180**:47-56.

He, S. Y., C. Schoedel, A. Chatterjee, and A. Collmer. 1991a. Extracellular secretion of pectate lyase by the *Erwinia chrysanthemi* Out pathway is dependent upon sec-mediated export across the inner membrane. J. Bacteriol. **173**:4310-4317.

He, S. Y., M. Lindeberg, A. K. Chatterjee, and A. Collmer. 1991b. Cloned *Erwinia chrysanthemi* out genes enable *Escherichia coli* to selectively secrete a diverse family of heterologous proteins to its milieu. Proc. Natl. Acad. Sci. USA **88**:1079-1083.

Hendrick, J. P., and W. Wickner. 1991. SecA protein needs both acidic phospholipids and SecY/E protein for functional high-affinity binding to the *Escherichia coli* plasma membrane. J. Biol. Chem. **266**:24596-24600.

Hess, J., I. Gentschev, W. Goebel, and T. Jarchau. 1990. Analysis of the haemolysin secretion system by PhoA-HlyA fusion proteins. Mol. Gen. Genet. **224**:201-208.

Hess, J., W. Wels, M. Vogel, and W. Goebel. 1986. Nucleotide sequence of a plasmid-encoded hemolysin determinant and its comparison with a corresponding chromosomal hemolysin sequence. FEMS Microb. Lett. **34**:1-11.

Hilton, S., W. D. McCubbin, C. M. Kay, and J. T. Buckley. 1990. Purification and spectral study of a microbial fatty acyltransferase: Activation by limited proteolysis. Biochemistry **29**:9072-9078.

Hirono, I., and T. Aoki. 1993. Cloning and characterization of three hemolysin genes from *Aeromonas salmonicida*. Microb. Pathogen. **15**:269-282.

Hirono, I., and T. Aoki. 1991. Nucleotide sequence and expression of an extracellular hemolysin gene of *Aeromonas hydrophila*. Microb. Pathogen. **11**:189-197.

Hirst, T. R., and R. A. Welch. 1988. Mechanisms for secretion of extracellular proteins by Gram-negative bacteria. Trends Biochem. Sci. **13**:265-269.

Hirst, T. R., and J. Holmgren. 1987a. Transient entry of enterotoxin subunits into the periplasm occurs during their secretion from *Vibrio cholerae*. J. Bacteriol. **169**:1037-1045.

Hirst, T. R., and J. Holmgren. 1987b. Conformation of protein secreted across bacterial outer membranes: A study of enterotoxin translocation from *Vibrio cholerae*. Proc. Natl. Acad. Sci. USA **84**:7418-7422.

Hobot, J. A., E. Carlemalm, W. Villiger, and E. Kellenberg. 1984. Periplasmic gel: New concept resulting from the reinvestigation of bacterial cell envelope ultrastructure by new methods. J. Bacteriol. **160**:143-152.

Hoffman, C. S., and A. Wright. 1985. Fusions of secreted proteins to alkaline phosphatase: an approach for studying protein secretion. Proc. Natl. Acad. Sci. USA **82**:5107-5111.

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

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

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

Howard, S. P., and J. T. Buckley. 1985b. Activation of the hole-forming toxin aerolysin by extracellular processing. J. Bacteriol. **163**:336-340.

Howard, S. P., and J. T. Buckley. 1983. Intracellular accumulation of extracellular proteins by pleiotropic export mutants of *Aeromonas hydrophila*. J. Bacteriol. **154**:413-418.

Howard, S. P., W. J. Garland, M. J. Green, and J. T. Buckley. 1987. Nucleotide sequence of the gene for the hole-forming toxin aerolysin of *Aeromonas hydrophila*. J. Bacteriol. **169**:2869-2871.

Husslein, V., B. Huhle, T. Jarchau, R. Lurz, W. Goebel, and T. Chakraborty. 1988. Nucleotide sequence and transcriptional analysis of the *aerCaerA* region of *Aeromonas sobria* encoding aerolysin and its regulatory region. Mol. Microbiol. **2**:507-517.

Ichige, A., K. Oishi, and S. Mizushima. 1988. Isolation and characterization of mutants of a marine *Vibrio* strain that are defective in the production of extracellular proteases. J. Bacteriol. **170**:3537-3542.

Ingram, J. M., K.-J. Cheng, and J. W. Costerton. 1983. Alkaline phosphatase of *Pseudomonas aeruginosa*: the mechanism of secretion and release of the enzyme from whole cells. Can. J. Microbiol. **19**:1407-1415.

Inouye, H., S. Michaelis, A. Wright, and J. Beckwith. 1981. Cloning and restriction mapping of the alkaline phosphatase structural gene (*phoA*) of *Escherichia coli* and generation of deletion mutants in vitro. J. Bacteriol. **146**:668-675.

Ishidate, K., E. S. Creeger, J. Zrike, S. Deb., B. Glauner, T. J. MacAlister, and L. I. Rothfield. 1986. Isolation of differentiated membrane domains from *Escherichia coli* and *Salmonella typhimurium*, including a fraction containing attachment sites between the inner and outer membranes and the murein skeleton of the cell envelope. J. Biol. Chem. **261**:428-443.

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

Jacob-Dubuisson, F., R. Striker, and S. J. Hultgren. 1994. Chaperone-assisted self-assembly of pili independent of cellular energy. J. Biol. Chem. **269**:12447-12455.

Jain, M. K., and R. C. Wagner. 1980. **Introduction to biological membranes.** John Wiley & Sons Inc., New York.

Jeanteur, D., N. Gletsu. F. Pattus, and J. T. Buckley. 1992. Purification of *Aeromonas hydrophila* major outer-membrane proteins: *N*-terminal sequence analysis and channel-forming properties. Mol. Microbiol. **6**:3355-3363.

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

Jiang, B., and S. P. Howard. 1991. Mutagenesis and isolation of *Aeromonas hydrophila* genes which are required for extracellular secretion. J. Bacteriol. **173**:1241-1249.

Johnson, K., M. L. Parker, and S. Lory. 1986. Nucleotide sequence and transcriptional initiation site of two *Pseudomonas aeruginosa* pilin genes. J. Biol. Chem. **261**:15703-15708.

Joly, J. C., and W. Wickner. 1993. The SecA and SecY subunits of translocase are the nearest neighbors of a translocating preprotein, shielding it from phospholipids. EMBO J. **12**:255-263.

Kato, M., H. Tokuda, and S. Mizushima. 1992. *In vitro* translocation of secretory proteins possessing no charges at the mature domain takes place efficiently in a protonmotive force-dependent manner. J. Biol. Chem. **267**:413-418.

Kazmierczak, B. I., D. L. Mielke, M. Russel, and P. Model. 1994. pIV, a filamentous phage protein that mediates phage export across the bacterial cell envelope, forms a multimer. J. Mol. Biol. **238**:187-198.

Kellenberger, E. 1990. The 'Bayer bridges' confronted with results from improved electron microscopy methods. Mol. Microbiol. 4:697-705.

Kendall, D. A., and E. T. Kaiser. 1988. A functional decaisoleucine-containing signal sequence. J. Biol. Chem. **265**:2873-2880.

Kendall, D. A., S. C. Bock, and E. T. Kaiser. 1986. Idealization of the hydrophobic segment of the alkaline phosphatase signal sequence. Nature 321:706-708.

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

Kenny, B., R. Haigh, and I. B. Holland. 1991. Analysis of the haemolysin transport process through the secretion from *Escherichia coli* of PCM, CAT or β-galactosidase fused to the Hly *C*-terminal signal domain. Mol. Microbiol. 5:2557-2568.

Killian, J. A., A. M. Ph. de Jong, J. Bijvelt, A. J. Verkleij, and B. de Kruijff. 1990. Induction of non-bilayer lipid structures by functional signal peptides. EMBO J. 8:815-819.

Kim, E. E., and H. W. Wyckoff. 1991. Reaction mechanism of alkaline phosphatase based on crystal structures. Two-metal ion catalysis. J. Mol. Biol. 218:449-464.

Kimura, E., M. Akita, S. Matsuyama, and S. Mizushima. 1991. Determination of a region in SecA that interacts with presecretory proteins in *Escherichia coli.* J. Biol. Chem. 266:6600-6606.

Kindschuh, M., L. K. Pickering, T. G. Clearly, and G. Ruiz-Palacios. 1987. Clinical and biochemical significance of toxin production by *Aeromonas hydrophila.* J. Clin. Microbiol. 25:916-921.

Klappa, P., M. Zimmerman, and R. Zimmerman. 1994. The membrane proteins TRAMp and sec61αp may be involved in post-translational transport of presecretory proteins into mammalian microsomes. FEBS Lett. 341:281-287.

Klauser, T., J. Kramer, K. Otzelberger, J. Pohlner, and T. F. Meyer. 1993a. Characterization of the *Neisseria* Iga$_\beta$-core. The essential unit for outer membrane targeting and extracellular protein secretion. J. Mol. Biol. 234:579-593.

Klauser, T., J. Pohlner, and T. F. Meyer. 1993b. The secretion pathway of IgA protease-type proteins in Gram-negative bacteria. Bioessays 15:799-805.

Klauser, T., J. Pohlner, and T. F. Meyer. 1992. Selective extracellular release of cholera toxin B subunit by *Escherichia coli*: dissection of *Neisseria* Iga$_\beta$-mediated outer membrane transport. EMBO J. 11:2327-2335.

Klauser, T., J. Pohlner, and T. F. Meyer. 1990. Extracellular transport of cholera toxin B subunit using *Neisseria* IgA protease β-domain: conformation-dependent outer membrane translocation. EMBO J. 9:1991-1999.

Klose, M., H. Schwarz, S. MacIntyre, R. Freudl, M. -L. Eschbach, and U. Henning. 1988a. Internal deletions in the gene for an *Escherichia coli* outer membrane protein define an area possibly important for recognition of the outer membrane by this polypeptide. J. Biol Chem. 264:13291-13296.

Klose, M., S. MacIntyre, H. Schwarz, and U. Henning. 1988b. The influence of amino substitutions within the mature part of an *Escherichia coli* outer membrane protein (OmpA) on assembly of the polypeptide into its membrane. J. Biol. Chem. **263**:13297-13302.

Kontinen, V. P., P. Saris, and M. Sarvas. 1991. A gene (*prsA*) of *Bacillus subtilis* involved in a novel, late stage of protein export. Mol. Microbiol. **5**:1273-1283.

Kornacker, M. G., and A. P. Pugsley. 1990. The normally periplasmic enzyme β-lactamase is specifically and efficiently translocated through the *Escherichia coli* outer membrane when it is fused to the cell-surface enzyme pullulanase. Mol. Microbiol. **4**:1101-1109.

Kornacker, M. G., and A. P. Pugsley. 1989. Molecular characterization of *pulA* and its product, pullulanase, a secreted enzyme of *Klebsiella pneumoniae* UNF5023. Mol. Microbiol. **4**:73-85.

Kornacker, M. G., D. Faucher, and A. P. Pugsley. 1991. Outer membrane translocation of the extracellular enzyme pullulanase in *Escherichia coli* K12 does not require a fatty acylated N-terminal cysteine. J. Biol. Chem. **266**:13842-13848.

Kornacker, M. G., A. Boyd, A. P. Pugsley, and G. S. Plastow. 1989. *Klebsiella pneumoniae* strain K21: Evidence for the rapid secretion of an unacylated form of pullulanase. Mol. Microbiol. **3**:497-503.

Koronakis, V., C. Hughes, and E. Koronakis. 1993. ATPase activity and ATP/ADP-induced conformational change in the soluble domain of the bacterial protein translocator HlyB. Mol. Microbiol. **8**:1163-1175.

Koronakis, V., C. Hughes, and E. Koronakis. 1991. Energetically distinct early and late stages of HlyB/HlyD-dependent secretion across both *Escherichia coli* membranes. EMBO J. **10**:3263-3272.

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

Koronakis, V., M. Cross, B. Senior, E. Koronakis, and C. Hughes. 1987. The secreted hemolysins of *Proteus mirabilis, Proteus vulgaris*, and *Morganella morganii* are genetically related to each other and to the alpha-hemolysin of *Escherichia coli*. J. Bacteriol. **169**:1509-1515.

Krieg, U. C., A. E. Johnson, and P. Walter. 1989. Protein translocation across the endoplasmic reticulum membrane: Identification by photocross-linking of a 39-kD integral membrane glycoprotein as part of a putative translocation tunnel. J. Cell Biol. **109**:2033-2043.

Kumamoto, C. A., and J. Beckwith. 1985. Evidence for specificity at an early step in protein export in *Escherichia coli*. J. Bacteriol. **163**:267-274.

Kurzchalia, T. V., M. Wiedmann, A. S. Girshovich, E. S. Bochkareva, H. Bielka, and T. A. Rapoport. 1986. The signal sequence of nascent preprolactin interacts with the 54K polypeptide of the signal recognition particle. Nature **320**:634-636.

Kusters, R., E. Breukink, A. Gallusser, A. Kuhn, and B. de Kruijff. 1994. A dual role for phosphatidylglycerol in protein translocation across the *Escherichia coli* inner membrane. J. Biol. Chem. **269**:1243-1248.

Kusters, R., R. Huijbregts, and B. de Kruijff. 1992. Elevated cytosolic concentrations of SecA compensate for a protein translocation defect in *Escherichia coli* cells with reduced levels of negatively charged phospholipids. FEBS Lett. **208**:97-100.

Kusters, R., T. de Vrije, E. Breukink, and B. de Kruijff. 1989. SecB protein stabilizes a translocation-competent state of purified prePhoE protein. J. Biol. Chem. 264:20827-20830.

Lauffer, L., P. D. Garcia, R. N. Harkins, L. Coussens, A. Ullrich, and P. Walter. 1985. Topology of signal recognition particle receptor in endoplasmic reticulum membrane. Nature **318**:334-337.

Lecker, S. H., A. J. M. Driessen, and W. Wickner. 1990. ProOmpA contains secondary and tertiary structure prior to translocation and is shielded from aggregation by association with SecB protein. EMBO J. **9**:2309-2314.

Lecker, S., R. Lill, T. Ziegelhoffer, C. Georgopoulos, P. J. Bassford Jr., C. A. Kumamoto, and W. Wickner. 1989. Three pure chaperone proteins of *Escherichia coli*-SecB, trigger factor and GroEL-form soluble complexes with precursor proteins in vitro. EMBO J. **8**:2703-2709.

Leece, R., and T. R. Hirst. 1992. Expression of the B subunit of *Escherichia coli* heat-labile enterotoxin in a marine *Vibrio* and in a mutant that is pleiotropically defective in the secretion of extracellular proteins. J. Gen. Microbiol. **138**:719:724.

Leung, K. Y., and R. M. W. Stevenson. 1988a. Characteristics and distribution of extracellular proteases from *Aeromonas hydrophila*. J. Gen. Microbiol. **134**:151-160.

Leung, K. Y., and R. M. W. Stevenson. 1988b. Tn5-induced protease-deficient strains of *Aeromonas hydrophila* with reduced virulence for fish. Infect. Immun. **56**:2639-2644.

Lill, R., W. Dowhan, and W. Wickner. 1990. The ATPase activity of SecA is regulated by acidic phospholipids, SecY, and the leader and mature domains of precursor proteins. Cell **60**:271-280.

Lill, R., K. Cunningham, L. A. Brundage, K. Ito, D. Oliver, and W. Wickner. 1989. SecA hydrolyzes ATP and is an essential component of the protein translocation ATPase of *Escherichia coli*. EMBO J. **8**:961-966.

Lindeberg, M., and A. Collmer. 1992. Analysis of eight *out* genes in a cluster required for pectic enzyme secretion by *Erwinia chrysanthemi*: sequence comparison with secretion genes from other Gram-negative bacteria. J. Bacteriol. **174**:7385-7397.

Lory, S., M. S. Strom, and K. Johnson. 1988. Expression and secretion of the cloned *Pseudomonas aeruginosa* exotoxin A by *Escherichia coli*. J. Bacteriol. **170**:714-719.

Lory, S., P. C. Tai, and B. D. Davis. 1983. Mechanism of excretion of proteins by Gram-negative bacteria: *Pseudomonas aeruginosa* exotoxin A. J. Bacteriol. **156**:695-702.

Lu, H.-M., S. Mizushima, and S. Lory. 1993. A periplasmic intermediate in the extracellular secretion pathway of *Pseudomonas aeruginosa* exotoxin A. J. Bacteriol. **175**:7463-7467.

Lu, H.-M., H. Yamada, and S. Mizushima. 1991. A proline residue near the amino terminus of the mature domain of secretory proteins lowers the level of the proton motive force required for translocation. J. Biol. Chem. **266**:9977-9982.

Luirink, J., and B. Dobberstein. 1994. Mammalian and *Escherichia coli* signal recognition particles. Mol. Microbiol. **11**:9-13.

Luirink, J., S. High, H. Wood, A. Giner, D. Tollervey, and B. Dobberstein. 1992. Signal-sequence recognition by an *Escherichia coli* ribonucleoprotein complex. Nature **359**:741-743.

MacIntyre, S., B. Mutschler, and U. Henning. 1991. Requirement of the SecB chaperone for export of a non-secretory polypeptide in *Escherichia coli*. Mol. Gen. Genet. **227**:224-228.

MacIntyre, S., T. J. Trust, and J. T. Buckley. 1979. Distribution of glycerophospholipid-cholesterol acyltransferase in selected bacterial species. J. Bacteriol. **139**:132-136.

MacIntyre, S., and J. T. Buckley. 1978. Presence of glycerophospholipid:cholesterol acyltransferase and phospholipase in culture supernatant of *Aeromonas hydrophila*. J. Bacteriol. **135**:402-407.

Mackman, N., K. Baker, L. Gray, R. Haigh, J. M. Nicaud, and I. B. Holland. 1987. Release of a chimeric protein into the medium from *Escherichia coli* using the C-terminal secretion signal of haemolysin. EMBO J. **6**:2835-2841.

Mackman, N., and I. B. Holland. 1984a. Secretion of a 107K dalton polypeptide into the medium from a haemolytic *E. coli* K12 strain. Mol. Gen. Genet. **193**:312-315.

Mackman, N., and I. B. Holland. 1984b. Functional characterization of a cloned haemolysin determinant from *E. coli* of human origin, encoding information for the secretion of a 107K polypeptide. Mol. Gen. Genet. **196**:129-134.

Manoil, C., and J. Beckwith. 1986. A genetic approach to analyzing membrane topology. Science **233**:1403-1408.

Manoil, C., and J. Beckwith. 1985. Tn*phoA*: A transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82**:8129-8133.

Martin, P. R., M. Hobbs, P. D. Free, Y. Jeske, and J. S. Mattick. 1993. Characterization of *pilQ*, a new gene required for the biogenesis of type 4 fimbriae in *Pseudomonas aeruginosa*. Mol. Microbiol. **9**:857-868.

Masibay, A. S., P. V. Balaji, E. E. Boeggeman, and P. K. Qasba. 1993. Mutational analysis of the Golgi retention signal of bovine β-1,4-galactosyltransferase. J. Biol. Chem. **268**:9908-9916.

Masure, H. R., D. C. Au, M. K. Gross, M. G. Donovan, and D. R. Storm. 1990. Secretion of the *Bordetella pertussis* adenylate cyclase from *Escherichia coli* containing the hemolysin operon. Biochemistry **29**:140-145.

Matsuyama, S., Y. Fujita, and S. Mizushima. 1993. SecD is involved in the release of translocated secretory proteins from the cytoplasmic membrane of *Escherichia coli*. EMBO J. **12**:265-270.

Matsuyama, S., Y. Fujita, K. Sagara, and S. Mizushima. 1992. Overproduction, purification and characterization of SecD and SecF, integral membrane components of the protein translocation machinery of *Escherichia coli*. Biochim. Biophys. Acta. **1122**:77-84.

Matsuyama, S., J. Akimaru, and S. Mizushima. 1990. SecE-dependent overproduction of SecY in *Escherichia coli*. Evidence for interactions between two components of the secretory machinery. FEBS Lett. **269**:96-100.

Mercurio, A., and P. A. Manning. 1985. Cellular localization and export of the soluble haemolysin of *Vibrio cholerae* El Tor. Mol. Gen. Genet. **200**:472-475.

Meyer, D. I., E. Krause, and B. Dobberstein. 1982. Secretory protein translocation across membranes - the role of the 'docking protein'. Nature **297**:647-650.

Michaelis, S., H. Inouye, D. Oliver, and J. Beckwith. 1983. Mutations that alter the signal sequence of alkaline phosphatase in *Escherichia coli*. J. Bacteriol. **154**:366-374.

Michiels, T., and G. R. Cornelis. 1991. Secretion of hybrid proteins by the *Yersinia* Yop export system. J. Bacteriol. **173**:1677-1685.

Michiels, T., J. -C. Vanooteghem, C. Lambert de Rouvroit, B. China, A. Gustin, P. Boudry, and G. R. Cornelis. 1991. Analysis of *virC*, an operon involved in the secretion of Yop proteins by *Yersinia enterocolitica*. J. Bacteriol. **173**:4994-5009.

Miller, J. D., H. D. Bernstein, and P. Walter. 1994. Interaction of *E. coli* Ffh/4.5S ribonucleoprotein and FtsY mimics that of mammalian signal recognition particle and its receptor. Nature **367**:657-659.

Miller, J. H. 1972. **Experiments in Molecular Genetics**, p 431, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Milstein, C., G. G. Brownlee, T. M. Harrison, and M. B. Mathews. 1972. A possible precursor of immunoglobulin light chains. Nature New Biol. **239**:117-121.

Mitchell, C., and D. Oliver. 1993. Two distinct ATP-binding domains are needed to promote protein export by *Escherichia coli* SecA ATPase. Mol. Microbiol. **10**:483-497.

Miyazaki, H., N. Yanagida, S. Horinouchi, and T. Beppu. 1989. Characterization of the precursor of *Serratia marcescens* serine protease and COOH-terminal processing of the precursor during its excretion through the outer membrane of *Escherichia coli*. J. Bacteriol. **171**:6566-6572.

Morales, V. M., A. Backman, and M. Bagdasarian. 1991. A series of wide-host-range low-copy-number vectors that allow direct screening for recombinants. Gene **97**:39-47.

Munro, S., and H. R. B. Pelham. 1987. A C-terminal signal prevents secretion of luminal ER proteins. Cell **48**:899-907.

Murooka, Y., and R. Ikeda. 1989. Biosynthesis and secretion of pullulanase, a lipoprotein from *Klebsiella aerogenes*. J. Biol. Chem. **264**:17524-17531.

Nakamaye, K., and F. Eckstein. 1986. Inhibition of restriction endonuclease NciI cleavage by phosphorothioate groups and its application to oligonucleotide-directed mutagenesis. Nucl. Acids Res. **14**:9679-9698.

Nakamura, K., Y. Imai, A. Nakamura, and K. Yamane. 1992. Small cytoplasmic RNA of *Bacillus subtilis*: Functional relationship with human signal recognition particle 7S RNA and *Escherichia coli* 4.5S RNA. J. Bacteriol. **174**:2185-2192.

Nakamura, K., H. Takamatsu, Y. Akiyama, K. Ito, and K. Yamane. 1990. Complementation of the protein transport defect of an *Escherichia coli secY* mutant (*secY24*) by *Bacillus subtilis secY* homologue. FEBS Lett. **273**:75-78.

Neill, R. J., B. E. Ivins, and R. K. Holmes. 1983. Synthesis and secretion of the plasmid-encoded heat-labile enterotoxin of *Escherichia coli* in *Vibrio cholerae*. Science **221**:289-291.

Neville, D. M. 1971. Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. J. Biol. Chem. **246**:6328-6334.

Nicaud, J. M., N. Mackman, L. Gray, and I. B. Holland. 1986. The C-terminal, 23 kDa peptide of *E. coli* haemolysin 2001 contains all the information necessary for its secretion by the haemolysin (Hly) export machinery. FEBS Lett. **204**:331-335.

Nicchitta, C. V., and G. Blobel. 1990. Assembly of translocation-competent proteoliposomes from detergent-solubilized rough microsomes. Cell **60**:259-269.

Nikaido, H. 1994. Porins and specific diffusion channels in bacterial outer membranes. J. Biol. Chem. **269**:3905-3908.

Nikaido, H. 1992. Porins and specific channels of bacterial outer membranes. Mol. Microbiol. **6**:435-442

Nikaido, H., and M. H. Saier, Jr. 1992. Transport proteins in bacteria: Common themes in their design. Science **258**:936-942.

Nikaido, H., and E. Y. Rosenberg. Cir and Fiu proteins in the outer membrane of *Escherichia coli* catalyze transport of monomeric catechols: Study with β-lactam antibiotics containing catechol and analogous groups. J. Bacteriol. **172**:1361-1367.

Nikaido, H., and M. Vaara. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol. Rev. **49**:1-32.

Noegel, A., U. Rdest, and W. Goebel. 1981. Determination of the functions of hemolytic plasmid pHly152 of *Escherichia coli*. J. Bacteriol. **145**:233-247.

Noegel, A., U. Rdest, W. Springer, and W. Goebel. 1979. Plasmid cistrons controlling synthesis and excretion of the exotoxin α-hemolysin of *Escherichia coli*. Mol. Gen. Genet. **175**:343-350.

Nothwehr, S. F., C. J. Roberts, and T. H. Stevens. 1993. Membrane protein retention in the yeast Golgi apparatus: Dipeptidyl aminopeptidaseA is retained by a cytoplasmic signal containing aromatic residues. J. Cell Biol. **121**:1197-1209.

Nunn, D. N., and S. Lory. 1993. Cleavage, methylation, and localization of the *Pseudomonas aeruginosa* export proteins Xcp T, -U, -V, and -W. J. Bacteriol. **175**:4375-4382.

Nunn, D. N., and S. Lory. 1992. Components of the protein-excretion apparatus of *Pseudomonas aeruginosa* are processed by the type IV prepilin peptidase. Proc. Natl. Acad. Sci. USA **89**:47-51.

Nunn, D. N., and S. Lory. 1991. Product of the *Pseudomonas aeruginosa* gene *pilD* is a prepilin leader peptidase. Proc. Natl. Acad. Sci. USA **88**:3281-3285.

Nunn, D., S. Bergman, and S. Lory. 1990. Products of three accessory genes, *pilB*, *pilC*, and *pilD*, are required for biogenesis of *Pseudomonas aeruginosa* pili. J. Bacteriol. **172**:2911-2919.

Oliver, D. B. 1987. Periplasm and protein secretion, p. 56-69. *In* F. C. Neidhardt (ed.), ***Escherichia coli* and *Salmonella typhimurium* cellular and molecular biology**. American Society for Microbiology Publications, Washington, D. C.

Oliver, D. B., and J. Beckwith. 1982. Regulation of a membrane component required for protein secretion in *Escherichia coli*. Cell **30**:311-319.

Oliver, D. B., and J. Beckwith. 1981. *E. coli* mutant pleiotropically defective in the export of secreted proteins. Cell **25**:765-772.

Ondraczek, R., S. Hobbie, and V. Braun. 1992. In vitro activation of the *Serratia marcescens* hemolysin through modification and complementation. J. Bacteriol. **174**:5086-5094.

Oropeza-Wekerle, R. L., W. Speth, B. Imhof, I. Gentschev, and W. Goebel. 1990. Translocation and compartmentalization of *Escherichia coli* hemolysin (HlyA). J. Bacteriol. **172**:3711-3717.

Palade, G. 1975. Intracellular aspects of the process of protein synthesis. Science. **189**:347-358.

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

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

Peek, J. A., and R. K. Taylor. 1992. Characterization of a periplasmic thiol:disulfide interchange protein required for the functional maturation of secreted virulence factors of *Vibrio cholerae*. Proc. Natl. Acad Sci. USA **89**:6210-6214.

Petit-Glatron, M. -F., L. Grajcar, A. Munz, and R. Chambert. 1993. The contribution of the cell wall to a transmembrane calcium gradient could play a key role in *Bacillus subtilis* protein secretion. Mol. Microbiol. **9**:1097-1106.

Phillips, G. J., and T. J. Silhavy. 1992. The *E. coli ffh* gene is necessary for viability and efficient protein export. Nature **359**:744-746.

Pogliano, K. J., and J. Beckwith. 1994a. Genetic and molecular characterization of the *Escherichia coli secD* operon and its products. J. Bacteriol. **176**:804-814.

Pogliano, J. A., and J. Beckwith. 1994b. SecD and SecF facilitate protein export in *Escherichia coli*. EMBO J. **13**:554-561.

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

Poole, K., E. Schiebel, and V. Braun. 1988. Molecular characterization of the hemolysin determinant of *Serratia marcescens*. J. Bacteriol. **170**:3177-3188.

Poquet, I., D. Faucher, and A. P. Pugsley. 1993a. Stable periplasmic secretion intermediate in the general secretory pathway of *Escherichia coli*. EMBO J. **12**:271-278.

Poquet, I., M. G. Kornacker, and A. P. Pugsley. 1993b. The role of the lipoprotein sorting signal (aspartate +2) in pullulanase secretion. Mol. Microbiol. **9**:1061-1069.

Poritz, M. A., H. D. Bernstein, K. Strub, D. Zopf, H. Wilhelm, and P. Walter. 1990. An *E. coli* ribonucleoprotein containing 4.5*S* RNA resembles mammalian signal recognition particle. Science **250**:1111-1117.

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

Possot, O., C. d'Enfert, I. Reyss, and A. P. Pugsley. 1992. Pullulanase secretion in *Escherichia coli* K-12 requires a cytoplasmic protein and a putative polytopic cytoplasmic membrane protein. Mol. Microbiol. **6**:95-105.

Postle, K. 1990. TonB and the Gram-negative dilemma. Mol. Microbiol. **4**:2019-2025.

Poulsen, K., J. P. Hjorth, and M. Killian. 1988. Limited diversity of the immunoglobulin A1 protease gene (*iga*) among *Haemophilus influenzae* serotype b strains. Infect. Immun. **56**:987-992.

Pugsley, A. P. 1993a. The complete general secretory pathway in Gram-negative bacteria. Microbiol. Rev. **57**:50-108.

Pugsley, A. P. 1993b. Processing and methylation of PulG, a pilin-like component of the general secretory pathway of *Klebsiella oxytoca*. Mol. Microbiol. **9**:295-308.

Pugsley, A. P. 1992. Translocation of a folded protein across the outer membrane in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **89**:12058-12062.

Pugsley, A. P., and O. Possot. 1993. The general secretion pathway of *Klebsiella oxytoca*: no evidence for relocalization or assembly of pilin-like PulG protein into a multiprotein complex. Mol. Microbiol. **10**:665-674.

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

Pugsley, A. P., and M. G. Kornacker. 1991. Secretion of the cell surface lipoprotein pullulanase in *Escherichia coli*. Cooperation or competition between the specific secretion pathway and the lipoprotein sorting pathway. J. Biol. Chem. **266**:13640-13645.

Pugsley, A. P., and I. Reyss. 1990. Five genes at the 3' end of the *Klebsiella pneumoniae pulC* operon are required for pullulanase secretion. Mol. Microbiol. **4**:365-379.

Pugsley, A. P., and M. Schwartz. 1985. Export and secretion of proteins by bacteria. FEMS Microbiol. Rev. **32**:3-38.

Pugsley, A. P., M. B. Kornacker, and I. Poquet. 1991a. The general protein-export pathway is directly required for the extracellular pullulanase secretion in *Escherichia coli* K12. Mol. Microbiol. **5**:343-352.

Pugsley, A. P., I. Poquet, and M. G. Kornacker. 1991b. Two distinct steps in pullulanase secretion by *Escherichia coli* K12. Mol. Microbiol. **5**:865-873.

Pugsley, A. P., M. G. Kornacker, and A. Ryter. 1990. Analysis of the subcellular location of pullulanase produced by *Escherichia coli* carrying the *pulA* gene from *Klebsiella pneumoniae* strain UNF5023. Mol Microbiol. **4**:59-72.

Pugsley, A. P., C. Chapon, and M. Schwartz. 1986. Extracellular pullulanase of *Klebsiella pneumoniae* is a lipoprotein. J. Bacteriol. **166**:1083-1088.

Py, B., M. Chippaux, and F. Barras. 1993. Mutagenesis of cellulase EGZ for studying the general protein secretory pathway in *Erwinia chrysanthemi*. Mol. Microbiol. **7**:785-793.

Py, B., G. P. C. Salmond, M. Chippaux, and F. Barras. 1991. Secretion of cellulases in *Erwinia chrysanthemi* and *E. carotovora* is species-specific. FEMS Microbiol. Lett. 79:315-322.

Randall, L. L. 1992. Peptide binding by chaperone SecB: Implications for recognition of nonnative structure. Science 257:241-245.

Randall, L. L., T. B. Topping, and S. J. S. Hardy. 1990. No specific recognition of leader peptide by SecB, a chaperone involved in protein export. Science 248:860-863.

Rapiejko, P., and R. Gilmore. 1992. Protein translocation across the ER requires a functional GTP binding site in the α subunit of the signal recognition particle receptor. J. Cell Biol. 117:493-503.

Rapoport, T. A. 1992. Transport of proteins across the endoplasmic reticulum membrane. Science 258:931-936.

Reeves, P. J., D. Whitcombe, S. Wharam, M. Gibson, G. Allison, N. Bunce, R. Barallon, P. Douglas, V. Mulholland, S. Stevens, D. Walker, and G. P. C. Salmond. 1993. Molecular cloning and characterization of 13 *out* genes from *Erwinia carotovora* subspecies *carotovora*: genes encoding members of a general secretion pathway (GSP) widespread in Gram-negative bacteria. Mol. Microbiol. 8:443-456.

Reyss, I., and A. P. Pugsley. 1990. Five additional genes in the *pulC-O* operon of the gram-negative bacterium *Klebsiella oxytoca* UNF5023 which are required for pullulanase secretion. Mol. Gen. Genet. 222:176-184.

Rivero, O., J. Anguita, C. Paniagua, and G. Naharro. 1990. Molecular cloning and characterization of an extracellular protease gene from *Aeromonas hydrophila*. J. Bacteriol. 172:3905-3908.

Rothman, J. E. 1987. Protein sorting by selective retention in the endoplasmic reticulum and Golgi stack. Cell 50:521-522.

Rothman, J. E., and L. Orci. 1992. Molecular dissection of the secretory pathway. Nature 355:409-415.

Rusch, S. L., and D. A. Kendall. 1994. Transport of an export-defective protein by a highly hydrophobic signal peptide. J. Biol. Chem. 269:1243-1248.

Rutz, J. M., J. Liu, J. A. Lyons, J. Goranson, S. K. Armstrong, M. A. McIntosh, J. B. Feix, and P. E. Klebba. 1992. Formation of a gated channel by a ligand-specific transport protein in the bacterial outer membrane. Science 258:471-475.

Sadaie, Y., H. Takamatsu, K. Nakamura, and K. Yamane. 1991. Sequencing reveals similarities of the wild-type *div*$^+$ gene of *Bacillus subtilis* to the *Escherichia coli secA* gene. Gene 98:101-105.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning. A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sandkvist, M., V. Morales, and M. Bagdasarian. 1993. A protein required for secretion of cholera toxin through the outer membrane of *Vibrio cholerae*. Gene 123:81-86.

Sanger, F., S. Niklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463-5467.

Sasaki, S., S. Matsuyama, and S. Mizushima. 1990. *In vitro* kinetic analysis of the role of the positive charge at the amino-terminal region of signal peptides in translocation of secretory proteins across the cytoplasmic membrane in *Escherichia coli*. J. Biol. Chem. 265:4358-4363.

Sayers, J. R., W. Schmidt, and F. Eckstein. 1988. 5'-3' Exonucleases in phosphorothioate-based oligonucleotide-directed mutagenesis. Nucl. Acids Res. 16:791-802.

Schatz, P. J., and J. Beckwith. 1990. Genetic analysis of protein export in *Escherichia coli*. Ann. Rev. Genet. 24:215-248.

Schatz, P. J., P. D. Riggs, A. Jacq, M. L. Fath, and J. Beckwith. 1989. The *secE* gene encodes an integral membrane protein required for protein export in *Escherichia coli*. Genes Dev. 3:1035-1044.

Schechter, I., D. J. McKean, R. Guyer, and W. Terry. 1974. Partial amino acid sequence of the precursor of immunoglobulin light chain programmed by messenger RNA *in vitro*. Science 188:160-162.

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

Schiebel, E., and V. Braun. 1989. Integration of the *Serratia marcescens* haemolysin into human erythrocyte membranes. Mol. Microbiol. 3:445-453.

Schiebel, E., H. Schwarz, and V. Braun. 1989. Subcellular location and unique secretion of the hemolysin of *Serratia marcescens*. J. Biol. Chem. 264:16311-16320.

Schlenstedt, G., G. H. Gudmundsson, H. G. Boman, and R. Zimmerman. 1990. A large presecretory protein translocates both cotranslationally, using signal recognition particle and ribosome, and post-translationally, without these ribonucleoparticles, when synthesized in the presence of mammalian microsomes. J. Biol. Chem. 265:13960-13968.

Schlenstedt, G., and R. Zimmerman. 1987. Import of frog prepropeptide GLa into microsomes requires ATP but does not involve docking protein or ribosomes. EMBO J. 6:699-703.

Schonherr, R., R. Tsolis, T. Focareta, and V. Braun. 1993. Amino acid replacements in *Serratia marcescens* haemolysin ShlA define sites involved in activation and secretion. Mol. Microbiol. 9:1229-1237.

Schulein, R., I. Gentschev, H. Mollenkopf, and W. Goebel. 1992. A topological model for the haemolysin translocator protein HlyD. Mol. Gen. Genet. **234**:155-163.

Sen, K., and H. Nikaido. 1991. Lipopolysaccharide structure required for in vitro trimerization of *Escherichia coli* OmpF porin. J. Bacteriol. **173**:926-928.

Shinkai, A., L. H. Mei, H. Tokuda, and S. Mizushima. 1991. The conformation of SecA, as revealed by its protease sensitivity, is altered upon interaction with ATP, presecretory proteins, everted membrane vesicles, and phospholipids. J. Biol. Chem. **266**:5827-5833.

Shiozuka, K., K. Tani, S. Mizushima, and H. Tokuda. 1990. The proton motive force lowers the level of ATP required for the *in vitro* translocation of a secretory protein in *Escherichia coli*. J. Biol. Chem. **265**:18843-18847.

Siegel, V., and P. Walter. 1988. Each of the activities of signal recognition particle (SRP) is contained within a distinct domain: Analysis of biochemical mutants of SRP. Cell **52**:39-49.

Simon, S., C. S. Peskin, and G. F. Oster. 1992. What drives the translocation of proteins? Proc. Natl. Acad. Sci. USA **89**:3770-3774.

Simon, S. M., and G. Blobel. 1992.Signal peptides open protein-conducting channels in *E. coli*. Cell **69**:677-684.

Simon, S. M., and G. Blobel. 1991. A protein-conducting channel in the endoplasmic reticulum. Cell **65**:371-380.

Simonen, M., and I. Palva. 1993. Protein secretion in *Bacillus* species. Microbiol. Rev. **57**:109-137.

Singer, S. J. 1990. The structure and insertion of integral proteins in membranes. Ann. Rev. Cell Biol. **6**:247-296.

Springer, W., and W. Goebel. 1980. Synthesis and secretion of hemolysin by *Escherichia coli*. J. Bacteriol. **144**:53-59.

Stader, J., L. J. Gansheroff, and T. J. Silhavy. 1989. New suppressors of signal-sequence mutations, *prlG*, are linked tightly to the *secE* gene of *Escherichia coli*. Genes Dev. **3**:1045-1052.

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

Stock, J. B., B. Rauch, and S. Roseman. 1977. Periplasmic space in *Salmonella typhimurium* and *Escherichia coli*. J. Biol. Chem. **252**:7850-7861.

Strauch, J. L., and J. Beckwith. 1988. An *E. coli* mutation preventing degradation of abnormal periplasmic proteins. Proc. Natl. Acad. Sci. USA **85**:1576-1580.

Strom, M. S., D. N. Nunn, and S. Lory. 1993. A single bifunctional enzyme, PilD, catalyzes cleavage and N-methylation of proteins belonging to the type IV pilin family. Proc. Natl. Acad. Sci. USA 90:2404-2408.

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

Struck, J. C. R., D. W. Vogt, N. Ulbrich, and V. A. Erdmann. 1988. The *Bacillus subtilis* scRNA is related to the 4.5S RNA from *Escherichia coli*. Nucleic Acids Res. 16:2719.

Struyve, M., M. Moons, and J. Tommassen. 1991. Carboxy-terminal phenylalanine is essential for the correct assembly of a bacterial outer membrane protein. J. Mol. Biol. 218:141-148.

Sugai, M., and H. C. Wu. 1992. Export of the outer membrane lipoprotein is defective in *secD*, *secE*, and *secF* mutants of *Escherichia coli*. J. Bacteriol. 174:2511-2516.

Suh, J. -W., S. A. Boylan, S. M. Thomas, K. M. Dolan, D. B. Oliver, and C. W. Price. 1990. Isolation of a *secY* homologue from *Bacillus subtilis*: evidence for a common protein export pathway in eubacteria. Mol. Microbiol. 4:305-314.

Summers, R. G., and J. R. Knowles. 1989. Illicit secretion of a cytoplasmic protein into the periplasm of *Escherichia coli* requires a signal peptide plus a portion of the cognate secreted protein. J. Biol. Chem. 264:20074-20081.

Tabor, S., and C. C. Richardson. 1989. Selective inactivation of the exonuclease activity of bacteriophage T7 DNA polymerase by *in vitro* mutagenesis. J. Biol. Chem. 264:6447-6458.

Tajima, S., L. Lauffer, V. L. Rath, and P. Walter. 1986. The signal recognition particle receptor is a complex that contains two distinct polypeptide chains. J. Cell Biol. 103:1167-1178.

Talmadge, K., S. Stahl, and W. Gilbert. 1980. Eukaryotic signal sequence transports insulin antigen in *Escherichia coli*. Proc. Natl. Acad. Sci. USA 77:3369-3373.

Tani, K., H. Tokuda, and S. Mizushima. 1990. Translocation of proOmpA possessing an intramolecular disulfide bridge into membrane vesicles of *Escherichia coli*. J. Biol. Chem. 265:17341-17347.

Tani, K., K. Shiozuka, H. Tokuda, and S. Mizushima. 1989. *In vitro* analysis of the process of translocation of OmpA across the *Escherichia coli* cytoplasmic membrane. J. Biol. Chem. 264:18582-18588.

Taura, T., T. Baba, Y. Akiyama, and K. Ito. 1993. Determinants of the quantity of the stable SecY complex in the *Escherichia coli* cell. J. Bacteriol. 175:7771-7775.

Taylor, R. K., C. Manoil, and J. J. Mekalanos. 1989. Broad host range vectors for the delivery of Tn*phoA*: use in genetic analysis of secreted virulence determinants of *Vibrio cholerae*. J. Bacteriol. 171:1870-1878.

Taylor, J. W., J. Ott, and F. Eckstein. 1985. The rapid generation of oligonucleotide-directed mutations at high frequency using phosphorothioate-modified DNA. Nucl. Acids Res. 13:8765-8785.

Thomas, Jr., W. D., S. P. Wagner, and R. A. Welch. 1992. A heterologous membrane protein domain fused to the C-terminal ATP-binding domain of HlyB can export *Escherichia coli* hemolysin. J. Bacteriol. 174:6771-6779.

Thornton, J., S. P. Howard, and J. T. Buckley. 1988. Molecular cloning of a phospholipid-cholesterol acyltransferase from *Aeromonas hydrophila*. Sequence homologies with lecithin-cholesterol acyltransferase and other lipases. Biochim. Biophys. Acta 959:153-159.

Tokuda, H., K. Shiozuka, and S. Mizushima. 1990. Reconstitution of translocation activity for secretory proteins from solubilized components of *Escherichia coli*. Eur. J. Biochem. 192:583-589.

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

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc. Natl. Acad. Sci. USA 76:4350-4354.

Turner, L. R., J. C. Lara, D. N. Nunn, and S. Lory. 1993. Mutations in the consensus ATP-binding sites of XcpR and PilB eliminate extracellular protein secretion and pilus biogenesis in *Pseudomonas aeruginosa*. J. Bacteriol. 175:4962-4969.

Ulbrandt, N. D., E. London, and D.B. Oliver. 1992. Deep penetration of a portion of *Escherichia coli* SecA protein into model membranes is promoted by anionic phospholipids and by partial unfolding. J. Biol. Chem. 267:15184-15192.

Uphoff, T. S., and R. A. Welch. 1990. Nucleotide sequencing of the *Proteus mirabilis* calcium-independent hemolysin genes (*hpmA* and *hpmB*) reveals sequence similarity with the *Serratia marcescens* genes (*shlA* and *shlB*). J. Bacteriol. 172:1206-1216.

van der Goot, F. G., J. Ausio, K. R. Wong, F. Pattus, and J. T. Buckley. 1993a. Dimerization stabilizes the pore-forming toxin aerolysin in solution. J. Biol. Chem. 268:18272-18279.

van der Goot, F. G., F. Pattus, K. R. Wong, and J. T. Buckley. 1993b. Oligomerization of the channel-forming toxin aerolysin precedes insertion into lipid bilayers. Biochemistry 32:2636-2642.

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

Verner, K., and G. Schatz. 1988. Protein translocation across membranes. Science 241:1307-1313.

Vlasuk, G. P., S. Inouye, H. Ito, K. Itakura, and M. Inouye. 1983. Effects of the complete removal of basic amino acid residues from the signal peptide on secretion of lipoprotein in *Escherichia coli.* J. Biol. Chem. 258:7141-7148.

von Heijne, G. 1990. The signal peptide. J. Membr. Biol. 115:195-201.

von Heijne, G. 1984. Analysis of the distribution of charged residues in the N-terminal region of signal sequences: Implications for protein export in prokaryotic and eukaryotic cells. EMBO J. 3:2315-2318.

von Heijne, G. 1983. Patterns of amino acids near signal-sequence cleavage sites. Eur. J. Biochem. 133:17-21.

Wagner, W., M. Vogel, and W. Goebel. 1983. Transport of hemolysin across the outer membrane of *Escherichia coli* requires two functions. J. Bacteriol. 154:200-210.

Walker, J. E., M. Saraste, M. J. Runswick, and N. J. Gay. 1982. Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. 1:945-951.

Walter, P., and G. Blobel. 1983. Disassembly and reconstitution of signal recognition particle. Cell 34:525-533.

Walter, P., and G. Blobel. 1982. Signal recognition particle contains a 7S RNA essential for protein translocation across the endoplasmic reticulum. Nature 299:691-698.

Walter, P., and G. Blobel. 1981. Translocation of proteins across the endoplasmic reticulum III. Signal recognition protein (SRP) causes signal sequence-dependent and site-specific arrest of chain elongation that is released by microsomal membranes. J. Cell Biol. 91:557-561.

Walter, P., and G. Blobel. 1980. Purification of a membrane-associated protein complex required for protein translocation across the endoplasmic reticulum. Proc. Natl. Acad. Sci. USA 77:7112-7116.

Wandersman, C., and S. Letoffe. 1993. Involvement of lipopolysaccharide in the secretion of *Escherichia coli* α-haemolysin and *Erwinia chrysanthemi* proteases. Mol. Microbiol. 7:141-150.

Wandersman, C., and P. Delepelaire. 1990. TolC, an *Escherichia coli* outer membrane protein required for hemolysin secretion. Proc. Natl. Acad. Sci. USA 87:4776-4780.

Wang, R., S. J. Seror, M. Blight, J. M. Pratt, J. K. Broome-Smith, and I. B. Holland. 1991. Analysis of the membrane organization of an *Escherichia coli* protein translocator, HlyB, a member of a large family of prokaryote and eukaryote surface transport proteins. J. Mol. Biol. 217:441-454.

Wang, L. -F., A. A. Kortt, and D. J. Stewart. 1993a. Use of a Gram⁻ signal peptide for protein secretion by Gram⁺ hosts: basic protease of *Dichelobacter nodosus* is produced and secreted by Bacillus subtilis. Gene 131:97-102.

Wang, Z., J. D. Jones, J. Rizo, and L. M. Gierasch. 1993b. Membrane-bound conformation of a signal peptide: A transferred nuclear Overhauser effect analysis. Biochemistry 32:13991-13999.

Watanabe, M., and G. Blobel. 1989. Site-specific antibodies against the PrlA (SecY) protein of *Escherichia coli* inhibit export by interfering with plasma membrane binding of preproteins. Proc. Natl. Acad. Sci. USA 86:1895-1899.

Wattiau, P., and G. R. Cornelis. 1993. SycE, a chaperone-like protein of *Yersinia enterocolitica* involved in the secretion of YopE. Mol. Microbiol. 8:123-131.

Way, J. C., M. A. Davis, D. Morisato, D. E. Roberts, and N. Kleckner. 1984. New Tn10 derivatives for transposition mutagenesis and for construction of *lacZ* operon fusions by transposition. Gene 32:369-379.

Weiss, A. A., F. D. Johnson, and D. L. Burns. 1993. Molecular characterization of an operon required for pertussis toxin secretion. Proc. Natl. Acad. Sci. USA 90:2970-2974.

Weiss, A. A., E. L. Hewlett, G. A. Myers, and S. Falkow. 1983. Tn5-induced mutations affecting virulence factors of *Bordetella pertussis*. Infect. Immun. 42:33-41.

Weiss, M. S., U. Abele, J. Weckesser, W. Welte, E. Schiltz, and G. E. Schulz. 1991. Molecular architecture and electrostatic properties of a bacterial porin. Science 254:1627--1629.

Welch, R. A., E. P. Dellinger, B. Minshew, and S. Falkow. 1981. Haemolysin contributes to virulence of extra-intestinal *E. coli* infections. Nature 294:665-667.

Whitchurch, C. B., M. Hobbs, S. P. Livingston, V. Krishnapillai, and J. S. Mattick. 1991. Characterization of a *Pseudomonas aeruginosa* twitching motility gene and evidence for a specialised protein export system widespread in eubacteria. Gene 101:33-44.

Wiedmann, M., T. V. Kurzchalia, E. Hartmann, and T. A. Rapoport. 1987. A signal sequence receptor in the endoplasmic reticulum. Nature 328:830-833.

Wild, J., E. Altman, T. Yura, and C. A. Gross. 1992. DnaK and DnaJ heat shock proteins participate in protein export in *Escherichia coli*. Genes Dev. 6:1165-1172.

Willems, R. J. L., C. Geuijen, H. G. J. van der Heide, G. Renauld, P. Bertin, W. M. R. van den Akker, C. Locht, and F. R. Mooi. 1994. Mutational analysis of the *Bordetella pertussis fim/fha* gene cluster: identification of a gene with sequence similarities to hemolysin accessory genes involved in export of FHA. Mol. Microbiol. 11:337-347.

Willis, R. C., R. G. Morris, C. Cirakoglu, G. D. Schellenberg, N. H. Gerber, and C. E. Furlong. 1974. Preparation of the periplasmic binding proteins from *Salmonella typhimurium* and *Escherichia coli*. Arch. Biochim. Biophys. 161:64-75.

Wilmsen, H. U., F. Pattus, and J. T. Buckley. 1990. Aerolysin, a hemolysin from *Aeromonas hydrophila*, forms voltage-gated channels in planar lipid bilayers. J. Membrane Biol. 115:71-81.

Woestyn, S., A. Allaoui, P. Wattiau, and G. R. Cornelis. 1994. YscN, the putative energizer of the *Yersinia* Yop secretion machinery. J. Bacteriol. 176:1561-1569.

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

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

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

Yamada, H., S. Matsuyama, H. Tokuda, and S. Mizushima. 1989. A high concentration of SecA allows proton motive force-independent translocation of a model secretory protein into *Escherichia coli* membrane vesicles. J. Biol. Chem.264:18577-18581.

Yamamoto, T., T. Gojobori, and T. Yokota. 1987. Evolutionary origin of pathogenic determinants in enterotoxigenic *Escherichia coli* and *Vibrio cholerae* O1. J. Bacteriol. 169:1352-1357.

Young, D. B., and D. A. Broadbent. 1982. Biochemical characterization of extracellular proteases from *Vibrio cholerae*. Infect. Immun. 37:875-883.

Yu, J., H. Webb, and T. R. Hirst. 1992. A homologue of the *Escherichia coli* DsbA protein involved in disulphide bond formation is required for enterotoxin biogenesis in *Vibrio cholerae*. Mol. Microbiol. 6:1949-1958.

Zhang, F., D. I. Greig, and V. Ling. 1993. Functional replacement of the hemolysin A transport signal by a different primary sequence. Proc. Natl. Acad. Sci. USA 90:4211-4215.

Zopf, D., H. D. Bernstein, and P. Walter. 1993. GTPase domain of the 54-kD subunit of the mammalian signal recognition particle is required for protein translocation but not for signal sequence binding. J. Cell. Biol. 120:1113-1121.

Zopf, D., H. D. Bernstein, A. E. Johnson, and P. Walter. 1990. The methionine-rich domain of the 54 kd protein subunit of the signal recognition particle contains an RNA binding site and can be crosslinked to a signal sequence. EMBO J. 9:4511-4517.

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Wong, K. R., and J. T. Buckley. 1993. *Aeromonas* spp. can secrete ***Escherichia coli*** **alkaline phosphatase into the culture supernatant, and its release requires a functional general secretion pathway.** Mol. Microbiol. **9**:955-963.

van der Goot, F. G., J. Ausio, K. R. Wong, F. Pattus, and J. T. Buckley. 1993. Dimerization stabilizes the pore-forming toxin aerolysin in solution. J. Biol. Chem. 268:18272-18279.

van der Goot, F. G., F. Pattus, K. R. Wong, and J. T. Buckley. 1993. Oligomerization of the channel-forming toxin aerolysin precedes insertion into lipid bilayers. A tryptophan-rich region is important in oligomerization. Biochemistry **32**:2636-2642.

Wong, K. R., and J. T. Buckley. 1991. Site-directed mutagenesis of a single tryptophan near the middle of the channel-forming toxin aerolysin inhibits its transfer across the outer membrane of *Aeromonas salmonicida*. J. Biol. Chem. **266**:14451-14456.

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

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

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

Wong, K. R., and J. S. Laskowski. 1984. Effect of humic acids on the properties of graphite aqueous suspensions. Colloids Surfaces **12**:319-332.

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