This is to certify that the

dissertation entitled

PROTEINS OF THE GENERAL SECRETION PATHWAY IN *VIBRIO CHOLERAE*: INDICATIONS FOR A MULTIPROTEIN COMPLEX

presented by

Lloyd Patrick Hough

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology

Major professor

Date November 29, 1999

PROTEINS OF THE GENERAL SECRETION PATHWAY IN *VIBRIO CHOLERAE*: INDICATIONS FOR A MULTIPROTEIN COMPLEX

By

Lloyd Patrick Hough

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology

1999

ABSTRACT

PROTEINS OF THE GENERAL SECRETION PATHWAY IN *VIBRIO CHOLERAE*: INDICATIONS FOR A MULTIPROTEIN COMPLEX

By

Lloyd Patrick Hough

The type II protein secretion pathway, or general secretion pathway (GSP), is responsible for the transport of proteases, cholera toxin and the related heat-labile enterotoxin, and other putative virulence factors, across the outer membrane of *Vibrio cholerae*. The function of the GSP in Gram-negative bacteria is dependent on the combined function of 14-16 genes and their associated gene products. In this study the production of polypeptides from several of those genes is demonstrated using the T7 promoter/polymerase system in *E. coli*. The processing of EpsG by a prepilin peptidase has been shown to occur in a *V. cholerae tcp*J mutant, which is defective in the peptidase that processes the TcpA pilin precursor. This predicted the existence in *V. cholerae* of a second prepilin peptidase specific for the Eps prepilin-like proteins. Four proteins, EpsD, EpsG, EpsF, and EpsM have been purified as C-terminal fusion proteins with an oligohistidine-tag. Two of the Eps proteins have been characterized by gel filtration analysis, revealing that both purified EpsM$(His)_6$ and wild-type EpsL present in 1% Triton X-100 extract are dimeric proteins. In addition, the subcellular localization of EpsC, EpsD, EpsG, EpsL, and EpsM is determined by sucrose gradient separation of the inner and outer membranes.

The EpsD protein is shown to fractionate with the outer membrane while the bitopic cytoplasmic membrane proteins EpsC, EpsG, EpsL, and EpsM are found to predominantly sediment with the cytoplasmic membrane, but peaks of EpsC, EpsG, and EpsD also sedimented with outer membrane vesicles. The EpsC and EpsD proteins can be coimmunoprecipitated with Anti-EpsD antiserum, and with Anti-EpsC antiserum after *in vivo* crosslinking with the cleavable, homobifunctional crosslinker dithiobis(succinimidyl propionate) (DSP). Furthermore, a *in vivo* stabilization of EpsD and a C-terminal truncation of EpsD, is demonstrated in the presence of EpsC, indicating that the interaction of EpsC and EpsD is likely to be direct, and that the interaction occurs through the N-terminal domain. Additionally, the coimmunoprecipitation, and crosslinking and coimmunoprecipitation of the integral CM protein, EpsL, and the OM secretin, EpsD, is reported. This evidence suggests that not only do EpsC and EpsD interact, but EpsL and EpsD are associated in a complex. Finally, gel filtration fractionation of Triton X-100 and Triton X-100/EDTA solubilized proteins shows that 4 integral CM proteins elute together in the same fraction. Since EpsL has been previously shown to interact directly with EpsE, an autophosphorylating peripheral cytoplasmic membrane protein, and with EpsM, another integral CM protein a hypothesis that each of the CM proteins can be found together in a single complex with the OM components of the GSP has been formulated.

To my wife Kimberly Hough.
Thank you for your love, support, and patience.

ACKNOWLEDGMENTS

Special thanks go to the following people for their assistance:

Dr. Michael Bagdasarian, my advisor, for the many hours of discussion and guidance.

Dr. Wendy Champness, Dr. Rawle Hollingsworth, Dr. Martha Mulks, and Dr. Pat Oriel, my guidance committee members, for their advice and time.

The Biotechnology Training Program, for the support, educational opportunities, and industrial exposure it provided.

The Institute of Genomic Research, for access to the preliminary genomic sequences of *Vibrio cholerae*.

Charles and Elizabeth Hough, my parents, for the support and encouragement they have given me over the years.

Finally, special thanks go to Kimberly Hough for your unconditional love, support, and understanding, without whom I would not have made it through this.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Ap	Ampicillin
Ap^R	Ampicillin Resistance
ATP	Adenosine Triphosphate
bp	base pair(s)
Cm	Chloramphenicol
Cm^R	Chloramphenicol resistance
CM	Cytoplasmic Membrane
CT	Cholera Toxin
DSP	dithiobis(succinimidyl propionate)
GEP	General Export Pathway
GSP	General Secretion Pathway
IPTG	Isopropyl-β-D-thiogalactopyranoside
kb	kilobase(s) or 1000 bp
kDa	kilodalton(s)
Km^R	Kanamycin resistance
LB	Luria-Bertani medium
LDAO	Lauryldimethylamine Oxide
LT	*Escherichia coli* Heat Labile Enterotoxin
MCS	Multiple Cloning Site
OD_{280}	Optical Density at 280 nm
OD_{650}	Optical Density at 650 nm
OG	Octyl-β-D-glucopyranoside
OM	Outer Membrane
ORF(s)	Open Reading Frame(s)
PAGE	Polyacrylamide Gel Electrophoresis
PBS	10 mM Phosphate Buffer pH 7.4/136 mM NaCl
PCR	Polymerase Chain Reaction
Pmx	Polymyxin B Sulfate
RBS	Ribosome binding site
Rif	Rifampicin
RT	Room Temperature
Sm	Streptomycin
Sm^R	Streptomycin Resistance
SDS	Sodium Dodecyl Sulfate
Tc	Tetracycline
Tc^R	Tetracycline Resistance
WT	Wild Type
[ ]	denotes plasmid carrier state
β-ME	β-Mercaptoethanol

CHAPTER 1

INTRODUCTION

Mechanisms of Protein Secretion

The process of protein secretion across the cell envelope plays a critical role in the phenomenon of life. The process involves the transport of macromolecules of various sizes, shapes, and biochemical features, across a lipid bilayer that separates the cell from the environment. While this lipid bilayer is responsible for separating and protecting a cell from its environment, it poses a significant problem for the cell: how to transport macromolecules and effect change across the cell envelope without destroying the integrity of the membrane. This problem is compounded in Gram-negative bacteria, in that they have not one, but two membranes separating the cytoplasm from the extracellular environment.

Saprophytic and pathogenic Gram-negative bacteria secrete a diverse array of proteins across the cell envelope, for an equally diverse number of reasons. The transport of degradative enzymes is crucial to the procurement of nutrients which are often found in insoluble macromolecules that cannot be transported across the cell envelope. The transport of degradative enzymes and toxins plays a significant role in the pathogenesis and dissemination of these organisms. Protein secretion also involves the transport and assembly of some outer membrane (OM) proteins in Gram-negative bacteria, which permit the cell to respond and adapt to changes in the cell's environment (Jiang and Howard, 1992; Sandkvist *et al.*, 1997).

The General Export Pathway (GEP)

Protein export has been defined as the transport of proteins that are retained partially or wholly within the cell boundary (Pugsley, 1993a). The proteins required for this function are encoded by the *sec* genes. Each protein known to be targeted to the periplasm and outer membrane, and in some cases to the extracellular environment, is synthesized in the cytoplasm with an amino-terminal (N-terminal) signal sequence (reviewed in Danese and Silhavy, 1998). Bacterial signal peptides are exclusively N-terminal, and consist of a long hydrophobic H-domain preceded by one or more positively charged amino acid residues in a generally hydrophilic N-domain (Pugsley, 1993a). As a group signal peptides have essentially the same structural features, but no two signal peptides from distinct presecretory proteins have the exact same sequence (Pugsley, 1993a). Signal peptides direct precursor polypeptides to the *sec* translocation apparatus in the cytoplasmic membrane, and are then removed during translocation (Danese and Silhavy, 1998). For this reason, they are believed to have no influence on downstream targeting (Pugsley, 1993a).

Type I Protein Secretion

Originally described for a group of highly homologous toxins and proteases in Gram-negative bacteria, transport mechanisms with similarities to type I protein secretion are widespread in eukaryotic and prokaryotic organisms (reviewed in Schneider and Hunke, 1998). Type I secretion refers to a *sec*-independent pathway that transports proteins lacking an N-terminal signal sequence from the cytoplasm to the extracellular environment in Gram-negative

bacteria in one step (Delepelaire and Wandersman, 1989; Gentschev *et al.*, 1990). Secretion is generally dedicated to the secretion of one or more closely related toxins, proteases, or lipases through an ABC-protein mediated exporter. The exporter consists of an ABC (ATP-Binding Cassette) transporter and two accessory proteins that are located in both membranes. (Binet *et al.*, 1997).

The genes are generally clustered with the secreted protein, consistent with their specificity. However, they can be promiscuous in that the secretion systems will often transport heterologous exoproteins from the same family (Binet *et al.*, 1997).

Type III Protein Secretion

The type III secretion mechanism is the most recently discovered system for the transport of proteins across the outer membrane of Gram-negative bacteria. It has been described in a variety of plant and animal pathogens, including *Pseudomonas*, *Salmonella*, *Shigella*, and *Yersinia*. Sometimes also referred to as the contact-dependant secretion pathway, the type III protein secretion systems described to date mediate the transport of proteins directly into the cytoplasm of a target cell and has been shown to share a common mechansim with flagellar assembly pathways (for recent reviews, see Hueck, 1998; Galán and Collmer, 1999). The type III pathway appears to be a specialized mechanism for the transport of virulence factors directly into the cytoplasm of potential host cells. Analysis of genetic elements harbored by pathogenic variants of Gram-negative bacteria have revealed the presence of pathogenicity islands, clusters of genes required for successful initiation of

infection often with features characteristic of mobile genetic elements. Contained within these pathogenicity islands are a collection of approximately 20 genes which encode the proteins of the type III protein secretion pathway. Many of the virulence factors delivered by the type III pathway resemble proteins involved in signal transduction pathways. This pathway permits the pathogenic bacterium to persuade a plant or animal cell, through these signal transduction pathways, to lower their defenses, or to undergo cytoskeletal rearrangements that result in the colonization or invasion by the pathogen (Hueck, 1998).

The type III secretion pathway consists of a variety of proteins, including an OM protein similar to the secretin family of protein transporters (Genin and Boucher, 1994), several lipoproteins, and a group of integral membrane proteins with a high degree of similarity to components of the flagellar export apparatus. This collection of proteins form a supramolecular structure in the cytoplasmic and outer membranes of Gram-negative bacteria that has recently been isolated from *Salmonella typhimurium* and visualized by electron microscopy (Kubori *et al.*, 1998). The supramolecular structure that was visualized closely resembles the flagellar basal body, and contains a long needle-like structure that extends from the basal body. This needle-like structure is proposed to be the channel through which secreted proteins are transported, and may be directly involved in the delivery of virulence factors to the cytoplasm of a target cell (Kubori *et al.*, 1998).

Autotransporters

The Type IV protein secretion mechanism is another example of how Gram-negative bacteria have evolved mechanisms for getting macromolecules to

the extracellular milieu. Proteins transported by this mechanism, often termed 'autotransporters', are unique in that the transport machinery is completely encoded within the precursor of the secreted protein itself (reviewed in Henderson *et al.*, 1998). The IgA1 protease of *Neisseria gonorrhoeae* is the classic example of the growing number of proteins known to be transported through this mechanism.

The autotransporters are typically proteases that have a fairly conserved structure. They consist of three domains, an N-terminal leader peptide, the surface localized mature protein (the α- or passenger domain), and a C-terminal β-domain (Henderson *et al.*, 1998). The N-terminal leader peptide, which shares many characteristics of prototypical *sec*-dependent signal peptides, mediates transport across the CM into the periplasmic space using the GEP. Upon reaching the periplasm, it is believed that the β-domain spontaneously inserts the polypeptide chain into the OM by adopting a β-barrel configuration, and the passenger domain is then translocated across the OM in an unfolded state.

Evidence supporting this model is provided by the predicted structure of the β-domain and the transport of a recombinant IgA1 protease passenger domain containing cysteine. The predicted structure of the β-domains of the 31 described autotransporters results in the prediction of an even number, 10 to 18, of anti-parallel amphipathic β-sheets, consistent with a porin-like structure (Henderson *et al.*, 1998). The evidence for a periplasmic intermediate and transport of an unfolded domain comes from studies involving the transport of recombinant proteins in which cysteine residues have been introduced. The

introduction of cysteine residues results in the formation of disulfide bonds, presumably by the periplasmic enzyme DsbA, and subsequently inhibits passage through the outer membrane (Jose *et al.*, 1996).

Type II Protein Secretion

The type II protein secretion system, or general secretory pathway (GSP), is a two-step mechanism that offers the Gram-negative bacterium several advantages over the aforementioned secretion systems. A protein secreted to the extracellular milieu through the GSP is first synthesized in the cytoplasm as a precursor with a traditional N-terminal signal sequence. The N-terminal signal sequence directs the protein to the GEP, provided by the Sec proteins in *Escherichia coli* and presumably by homologs in other Gram-negative bacteria, which translocates the precursor polypeptide to the periplasm. Upon reaching the periplasm the N-terminal signal sequence is cleaved from the precursor by a signal peptidase, and the protein is permitted to fold and assemble before being secreted.

The folding and assembly of exoproteins in the periplasm is the greatest advantage that the GSP offers the bacterium. The periplasmic space of the Gram-negative bacterium is one of the compartments which the bacterium may use to contain and segregate certain chemical reactions. The periplasm is an oxidizing environment, compared to the reducing environment of the cytoplasm, which favors the formation of disulfide bonds (Rietsch and Beckwith, 1998). The formation of disulfide bonds is often critical to the tertiary and quaternary structure of a protein (Missiakas and Raina, 1997). In the case of the *E. coli*

heat-labile enterotoxin (LT), a multi-subunit toxin which is secreted through the GSP of *Vibrio cholerae* (Sandkvist *et al.*, 1993), the protein requires the formation of disulfide bonds in both the A and B subunits for its biogenesis (Yu *et al.*, 1992), and enzymatic activity (Hol *et al.*, 1995; Orlandi, 1997). In addition to an environment favorable to the formation of disulfide bonds, the periplasm offers the cell a way to increase the relative concentration of a protein. This may be critical in the case of LT, since it has been shown that the LT B subunits will not spontaneously assemble *in vitro* until a high concentration of subunits has been reached (Sandkvist and Bagdasarian, 1993).

The first step of protein secretion through the GSP is the translocation of the protein precusors into the periplasm by the GEP. In *E. coli* the GEP is provided by the combined actions of the Sec proteins. To date only, two type II secretion systems have been reconstituted in *E. coli*, the pullulanase secretion system of *Klebsiella oxytoca* and the *out* system of *Erwinia chrysanthemi* and *Erwinia carotovora* (d'Enfert *et al.*, 1987; He *et al.*, 1991a; Lindeberg *et al.*, 1996). In both instances it was demonstrated that secretion of the heterologous exoproteins specific for each system required a functional Sec mediated export pathway (He *et al.*, 1991b; Pugsley *et al.*, 1991). It is generally assumed the GEP is similar to the Sec system of *E. coli* functions in *V. cholerae* and other Gram-negative bacteria. In support of this assumption, an ORF with 72% identity to both the *E. coli* SecA and SecB proteins can be located by BLAST in the unfinished *V. cholerae* genome (unpublished observations using preliminary sequence information made available from The Institute for Genomic Research).

The Secretion Genes.

The second step of protein secretion through the GSP depends on the combined function of at least 12, and possibly up to 16 gene products. In most systems these genes are designated A-O and S. In *V. cholerae*, a fragment containing the *eps*C-N genes has been described and demonstrated to be involved in the translocation of LT across the OM (Overbye *et al.*, 1993; Sandkvist *et al.*, 1993; Sandkvist *et al.*, 1997). Recently, a prepilin peptidase similar in function to the product of the *K. oxytoca pul*O was predicted (Sandkvist *et al.*, 1997), and subsequently cloned by Marsh and Taylor (1998), as the *vcp*D gene, bringing the number of genes involved in type II secretion in *V. cholerae* to at least 13.

Several genes identified by analysis of homologous secretion systems have not yet been described in *V. cholerae*. The genes *exe*A and *exe*B have been identified and demonstrated to be required for secretion of aerolysin in *Aeromonas hydrophila* (Howard *et al.*, 1996). Homologs of *exe*B have been described within the cluster of secretion genes in both the *K. oxytoca* pullulanase and *E. chrysanthemi* pectinase secretion systems, but mutations in these genes reportedly had no effect on pullulanase secretion, and decreased the secretion of pectinase by only 30%, suggesting that they may be dispensable in some systems (d'Enfert and Pugsley, 1989; Condemine *et al.*, 1992). Additionally, another gene that encodes an outer membrane lipoprotein, the S protein, encoded by *pul*S in *K. oxytoca* and *out*S in *E. chrysanthemi*, has been identified

and demonstrated to be essential for the function of the GSP (d'Enfert and Pugsley, 1989; Lindeberg *et al.*, 1996).

The Secretion Substrates

Each of the secretion systems is specific for a particular set of exoproteins. In addition to the CT and LT molecules, *V. cholerae* has been shown to secrete protease(s), lipase, and chitinase through the *eps* encoded GSP (Overbye *et al.*, 1993; Sandkvist *et al.*, 1997), while the secretion of at least some DNases and amylases occurs through other pathways (Sandkvist *et al.*, 1997, and unpublished observations). Other proteins secreted through type II secretion pathways include aerolysin in *Aeromonas* (Howard and Buckley, 1985), pullulanase in *Klebsiella* (d'Enfert *et al.*, 1987), pectinases and cellulases in *E. chrysanthemi* and *E. carotovora* (Murata *et al.*, 1990), alkaline phosphatase and elastase in *Pseudomonas aeruginosa* (Lazdunski *et al.*, 1990), and proteases and pectinases in *Xanthomonas campestris* (Dums *et al.*, 1991).

While all of these proteins are secreted through type II secretion systems, they are generally not secreted through heterologous systems. For example, *E. chrysanthemi* secretes a pectate lyase, but is unable to secrete a similar pectate lyase from *E. carotovora* (He *et al.*, 1991a; Py *et al.*, 1991), and *the K. oxytoca* Pul system cannot secrete *P. aeruginosa* or *E. chrysanthemi* proteins (de Groot *et al.*, 1991; He *et al.*, 1991a), suggesting that there must be species-specific recognition signal encoded within an exoprotein. However, no common secretion amino acid sequences in proteins secreted from any one organism have been identified, thereby implying that secretion signals must be a discontinuous, or

structural signal, contained within the folded protein (Pugsley, 1993a; Connell *et al.*, 1995). The nature of the discontinuous patch signal, or structural signal, is unknown. Indications for a discontinuous secretion signal have been shown in pullulanase, in which two regions consisting of 78 amino acids at the N-terminus and 80 amino acids near the C-terminus, were shown to be necessary for secretion of pullulanase, and sufficient for secretion of β-lactamase (Sauvonnet and Pugsley, 1996). However, a single 60 amino acid region of *P. aeruginosa* exotoxin A, was found to be sufficient for secretion of a β-lactamase fusion protein through the Xcp system (Lu and Lory, 1996), but then a region including these 60 amino acids could be deleted from exotoxin A without effecting secretion (McVay and Hamood, 1995). The complete lack of sequence homology between any of the identified regions, and the lack of strucutural information about the folding of truncated or fusion proteins, will make identification of any signal difficult. Furthermore, addition, or even substitution of various reporters to proteins which contain the appropriate signals can also inhibit secretion. Sauvonnet *et al.* have shown that while most small insertions had no effect on secretion of pullulase, additions of β-lactamase, alkaline phosphatase, or other domains to the C-terminus usually prevented their secretion (Sauvonnet *et al.*, 1995). Similarly, the insertion of β-lactamase or alkaline phosphatase to the A subunit of CT which properly assembled into a holotoxin like molecules, were not secreted by *V. cholerae* (Jobling and Holmes, 1992). Since these fusions are to a chain which is not required in the secretion of CT,

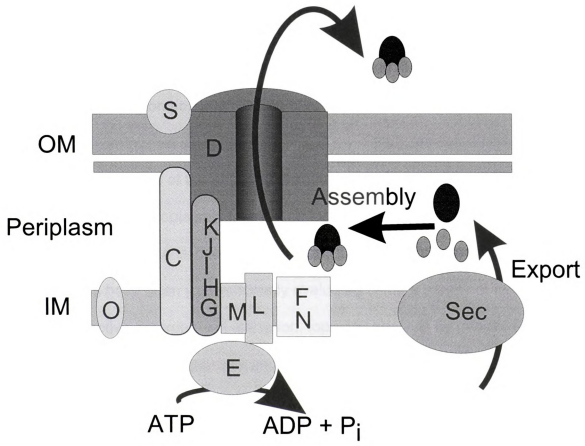


Figure 1.1 Hypothetical model of the type II secretion apparatus in Gram-negative bacteria.

and should not have affected the folding and consequently the signals contained within the B subunit, steric hindrances by a passenger domain will also hamper efforts to identify a signal.

The Proteins, the Interactions, and the Model.

The current model of Type II protein secretion in Gram-negative bacteria (Figure 1.1) is based upon the subcellular locations and identified interactions of the 12-14 secretion components. Subcellular locations of each of the 14 proteins identified thus far in most secretion systems, were initially predicted from computerized analyses of the amino acid sequences. Most of the proteins were predicted to have a cytoplasmic membrane location, with two proteins localized to the OM, and one localized to the cytoplasm. The subcellular location of many of the proteins have been experimentally evaluated using a variety of techniques including selective detergent solubilization, topology analysis by genetic fusions with reporter domains β-lactamase and alkaline phosphatase, and sucrose density gradient separation. However, many of the initial studies of subcellular localization have suffered from analysis of highly expressed protein(s), production in isolation from other GSP proteins, or production at non-stoichiometric amounts. The membrane location of the C protein was initially determined by detergent solubilization of a highly expressed protein (Bleves *et al.*, 1996). Subsequent analysis by genetic fusions with β-lactamase (BlaM) has determined the membrane topology of the C protein (Thomas *et al.*, 1997), and more recently, evidence obtained by sucrose gradient fractionation of membranes showed that the C protein is actually distributed between both the

cytoplasmic and outer membrane (Possot *et al.*, 1999). Antibodies have been used to demonstrate that the D protein separates with the outer membrane by sucrose gradient fractionation (Hardie *et al.*, 1996a). The E protein is the only GSP protein located exclusively in the cytoplasm, it is however associated with the membrane through other GSP proteins (Possot and Pugsley, 1994; Sandkvist *et al.*, 1995). The F protein is one of two proteins predicted to be a polytopic cytoplasmic membrane protein, and its membrane topology has been determined through fusions to BlaM (Thomas *et al.*, 1997). Nunn and Lory performed analyses of both precursor forms and processed and N-methylated forms of the prepilin-like proteins G, H, I, and J from the Xcp system of *P. aeruginosa*. Their analysis of the localization of the G, H, I, and J, proteins revealed that the proteins are solubilized in detergents that selectively solubilize CM proteins. Sucrose gradient separation of the CM and OM fractions demonstrated an association with both membranes, with each protein predominantly in the CM fractions. Their analysis also revealed that processing resulted in little change in the overall distribution of the prepilin-like proteins (Nunn and Lory, 1993) . However analysis of the G protein from *K. oxytoca* suggested that both the precursor and mature forms of the protein were predominantly located in the OM (Pugsley and Dupuy, 1992; Pugsley, 1993b; Pugsley and Possot, 1993). This observation was supported by both detergent solubilization and sucrose gradient fractionations. Localization of the K, L, M, N, and O proteins to the CM was done exclusively by genetic fusions to BlaM or alkaline phosphatase (Reeves *et al.*, 1994; Thomas *et al.*, 1997). Subcellular

location of the last of the 14 proteins included in this model, the S protein, was determined by palmitate labeling and sucrose gradient fractionation (d'Enfert and Pugsley, 1989).

The model presented in (Figure 1.1) presents a hypothetical organization of the type II secretion appratatus, or secreton, using only the 14 proteins known to be involved in the secretion of pullulanase and pectinase from reconsititution of the appropriate secretion pathways in *E. coli*. This model does not include the A or B proteins of *Aeromonas*, since these may not be required for the secretion of all proteins (Jahagirdar and Howard, 1994). The N protein is included in this model even though no N homolog has been identified in the reconstituted *E. chrysanthemi* Out secretion system.

The model presented in Figure 1.1 demonstrates some of the interactions that have been identified, and some that have been proposed, between proteins of the type II secretion pathway. Interactions between the E protein and L protein have been identified through studies with the *V. cholerae* EpsE and EpsL proteins (Sandkvist *et al.*, 1995). Interactions between the S protein and the D protein have been demonstrated through studies with the *K. oxytoca* PulD and PulS (Hardie *et al.*, 1996a; Hardie *et al.*, 1996b), and with the *E. chrysanthemi* OutD and OutS (Shevchik and Condemine, 1998). Interactions between the E protein and the G proteins have been suggested by isolation of suppressors with the *P. aeruginosa* XcpR and XcpT proteins (Kagami *et al.*, 1998). Indications of interactions between the prepilin-like proteins have also been reported (Lu *et al.*, 1997).

While the molecular architecture of the type II secretion pathway is slowly being worked out through biochemical and genetic analyses, precious little is currently known about the functions played by each of these proteins. Roles have been identified for the S proteins, the D proteins, and the O proteins. Parital roles have been identified for the E proteins, and the L proteins. The only component for which a certain enzymatic function has been assigned is the O protein, a prepilin peptidase. The O proteins encoded by *pil*D in *P. aeruginosa*, by *tap*D in *A. hyrdophila*, and *vcp*D in *V. cholerae*, have been shown to be functional in both pilus biogenesis and in protein secretion (Strom *et al.*, 1993; Pepe *et al.*, 1996; Marsh and Taylor, 1998). These proteins are responsible for the cleavage and N-methylation of the prepilin-like G, H, I, J, and possibly K proteins of the type II secretion pathway at a cleavage site that resembles the type IV-A prepilin subunit processing site (Nunn and Lory, 1992; Nunn and Lory, 1993; Bleves *et al.*, 1998; Fullner and Mekalanos, 1999). This function is also performed by the PulO and OutO genes of *K. oxytoca* and *E. chrysanthemi*, respectively (Nunn and Lory, 1991; Pugsley and Dupuy, 1992; Pugsley, 1993b).

A second component for which a function has been identified is the S protein. The S protein has a piloting and chaperone-like function in its interaction with the D protein (Hardie *et al.*, 1996a). Expression of the *K. oxytoca* D protein, PulD, in *E. coli* resulted in multimeric PulD that was not efficiently inserted into the outer membrane (Hardie *et al.*, 1996a). However, when PulD was expressed together with the S protein, the D became protected from proteolysis and efficiently inserted into the OM. Initially, it was suspected that the S protein was

exclusively a piloting protein responsible for transporting assembled secretin to the OM, however, when Hardie *et al.* replaced the lipoprotein-type signal peptide of PulS with the signal peptide of maltose binding protein, the resulting recombinant protein was able to protect the D protein from proteolysis , but no longer properly localized the D protein in the OM (Hardie *et al.*, 1996b). Daefler *et al.* then identified an S protein binding domain in the D protein by constructing hybrid proteins between N-terminal fragments from the filamentous bacteriophage pIV protein and C-terminal fragments of PulD (Daefler *et al.*, 1997). However, the S protein has only been identified in the *Klebsiella* Pul and the *Erwinia* Out secretion systems. It remains to be determined whether S homologs or analogs exist in other Gram-negative bacteria.

The D proteins have been putatively assigned the function as the pore though which secreted proteins cross the OM and as such are referred to as secretins. The function of the pore was initially proposed because the D protein is the only integral OM component of the type II secretion pathway, and because it shared homology with OM proteins involved in filamentous phage biogenesis and type III secretion systems. It was initially demonstrated that the M13 pIV protein could be found in *E. coli* as a homomultimers of approximately 10-12 subunits (Kazmierczak *et al.*, 1994), that were resistant to denaturation by SDS (Linderoth *et al.*, 1996). It has since been determined that D protein multimers of pIV, PulD, PilQ, or XcpQ consist of approximately 14 monomeric subunits by visualization of purified multimers under an electron microscope (Linderoth *et al.*, 1997; Bitter *et al.*, 1998; Nouwen *et al.*, 1999). The dimensions of the putative

pore formed by various secretins has also been estimated from the EM visualization of the mutlimeric structures (Linderoth *et al.*, 1997; Bitter *et al.*, 1998; Nouwen *et al.*, 1999). Images of the bacteriophage M13 pIV protein suggested that the central pore was approximately 8 nm, large enough to permit extrusion of an M13 phage (Linderoth *et al.*, 1997).

The existence of an open pore with a diameter of 8 nm in the OM of any Gram-negative bacterium would more than likely have deleterious effects. Thus, such a pore must be gated. Evidence in support of this hypothesis has been reported by Shevchick *et al.* The authors demonstrated that the *E. chrysanthemi* OutD expressed in *E. coli* in the presence of an appropriate pectinase resulted in the lysis of the host (Shevchik *et al.*, 1997), that was not observed when each gene was expressed in the same host separately. Additionally, the toxicity of the *E. chrystanthemi* OutD expressed in *E. coli*, was not caused by the pectinase from *E. carotovora*, which cannot be secreted by *E. chrysanthemi* (Shevchik *et al.*, 1997). This finding implies that the gate may normally be closed, and is opened upon substrate binding. This finding also suggests that the species specificity of the secretion apparatus may be limited to the N-terminal, or periplasmic, portion of the D protein.

The last protein for which a function is beginning to be understood is the E protein. Analysis of the amino acids sequences of several E homologs revealed a common Walker A motif, a motif commonly associated with nucleotide binding proteins. A functional Walker A motif has been demonstrated to be required for secretion through the GSP in the E homologs of *K. oxytoca* (Possot and Pugsley,

1994), *V. cholerae* (Sandkvist *et al.*, 1995), *P. aeruginosa* (Turner *et al.*, 1993), and *A. hydrophilia* (Howard *et al.*, 1996). The presence of the Walker A motif suggested that these proteins were likely to be ATP-binding proteins, and potentially ATPases. Purified protein of the *V. cholerae* E protein, EpsE, was demonstrated to be an autophosphorylase (Sandkvist *et al.*, 1995). However, the authors were unable to detect any catalytic ATPase activity of the purified EpsE protein, even when incubated in the presence of *V. cholerae* membranes (Sandkvist *et al.*, 1995). Additionally, it was demonstrated that the EpsE protein became membrane associated in the presence of EpsL, and integral bitopic membrane protein, an interaction that requires the N-terminus of EpsE to occur.

It has been proposed that the role of the E protein is to energize the process of secretion through the hydrolysis of ATP. However, there are indications that the process of secretion may only require ATP hydrolysis for certain secretion systems or possibly substrates. Letellier *et al.* found that both ATP and proton motive force (PMF) were required for the secretion of aerolysin from *A. hydrophila* (Letellier *et al.*, 1997). On the other hand, Possot *et al.* determined that ATP, when reduced to less than 10% of cellular pools, had no effect on secretion of mature proteins already in the periplasm (Possot *et al.*, 1997). Additionally, it was also noticed by Possot *et al.* that the presence of the E protein is required while the remaining 13 or so proteins are being produced, and presumably assembled, and cannot be added to the system later (Possot *et al.*, 1992). This would suggest that the role of the E protein is not in the energetics of secretion, but perhaps in the assembly of the secretion apparatus.

No function has yet been determined for the remaining proteins, however it is likely that many may play purely structural roles, while still others may be involved in assembly of the secretion apparatus. The prepilin-like proteins G, H, I, and J are hypothesized to form a pilus-like structure in the periplasm, but no evidence for such a structure has been reported. Limited evidence for a higher order structure formed by the prepilin-like proteins stems from *in vivo* crosslinking experiments which permitted the detection of a homodimer of the G protein (Pugsley, 1996), and heterodimers of the H, I, and J proteins copurifying with the G protein (Lu *et al.*, 1997). The same experiments identified a crosslinking product between the G (XcpT) protein of the type II secretion pathway, and PilA, the type IV pilus subunit of *P. aeruginosa*, suggesting that there may be some cross-talk or an intimate relationship between piliation and exoprotein transport systems in *Pseudomonas*.

No function has been reported for the C, F, M, and N proteins in the functioning of the type II secretion pathway. Although, two recent reports on the C protein have provided indications that the C protein may interact with the D protein in the OM. Bleves et. al. reported a proteolytic protection conferred upon the C protein by the presence of D (Bleves *et al.*, 1999). Additional, indications for function of the E protein come from Possot *et al.* in which they determined that the C protein fractionates with both the CM and OM fractions in sucrose gradient separations. However, they also report that the fractionation pattern of the C protein is not dependent on the presence of the D or any other protein from the GSP. *In vivo* crosslinking results also suggest that the C protein can form

high-order oligomers, that may depend on the presence of the D protein (Possot *et al.*, 1999).

Thus, the dissection of the interactions between proteins in the secretion pathway has not yet been completed. The implications of membrane association of the autophosphorylating E protein with the integral cytoplasmic membrane protein have yet to be defined. Is the E protein responsible for energizing the transport of secreted proteins across the outer membrane? Does the C protein interact with proteins in the OM? Do any of the other CM proteins with extended periplasmic domains interact with proteins in the OM?

The present study addressed primary the question of protein-protein interactions in the type II secretion pathway of *V. cholerae*. The indication of multiple interactions suggests that a multiprotein complex involving all Eps proteins may be the functional unit of the type II secretion pathway.

References

Binet, R., S. Létoffé, J. M. Ghigo, P. Delepelaire and C. Wandersman (1997). Protein secretion by Gram-negative bacterial ABC exporters--a review. *Gene* **192**: 7-11.

Bitter, W., M. Koster, M. Latijnhouwers, H. de Cock and J. Tommassen (1998). Formation of oligomeric rings by XcpQ and PilQ, which are involved in protein transport across the outer membrane of *Pseudomonas aeruginosa*. *Mol. Microbiol.* **27**: 209-219.

Bleves, S., M. Gérard-Vincent, A. Lazdunski and A. Filloux (1999). Structure-function analysis of XcpP, a component involved in general secretory pathway-dependent protein secretion in *Pseudomonas aeruginosa*. *J. Bacteriol.* **181**: 4012-4019.

Bleves, S., A. Lazdunski and A. Filloux (1996). Membrane topology of three Xcp proteins involved in exoprotein transport by *Pseudomonas aeruginosa*. *J. Bacteriol.* **178**: 4297-4300.

Bleves, S., R. Voulhoux, G. Michel, A. Lazdunski, J. Tommassen and A. Filloux (1998). The secretion apparatus of *Pseudomonas aeruginosa*: identification of a fifth pseudopilin, XcpX (GspK family). *Mol. Microbiol.* **27**: 31-40.

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

Connell, T. D., D. J. Metzger, M. Wang, M. G. Jobling and R. K. Holmes (1995). Initial studies of the structural signal for extracellular transport of cholera toxin and other proteins recognized by *Vibrio cholerae*. *Infect. Immun.* **63**: 4091-4098.

Daefler, S., I. Guilvout, K. R. Hardie, A. P. Pugsley and M. Russel (1997). The C-terminal domain of the secretin PulD contains the binding site for its cognate chaperone, PulS, and confers PulS dependence on pIV^{f1} function. *Mol. Microbiol.* **24**: 465-475.

Danese, P. N. and T. J. Silhavy (1998). Targeting and assembly of periplasmic and outer-membrane proteins in *Escherichia coli*. *Annu Rev Genet* **32**: 59-94.

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.

Delepelaire, P. and C. Wandersman (1989). Protease secretion by *Erwinia chrysanthemi*. Proteases B and C are synthesized and secreted as zymogens without a signal peptide. *J. Biol. Chem.* **264**: 9083-9089.

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

Dums, F., G. 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.

Fullner, K. J. and J. J. Mekalanos (1999). Genetic characterization of a new type IV-A pilus gene cluster found in both classical and El Tor biotypes of *Vibrio cholerae. Infect. Immun.* **67**: 1393-1404.

Galán, J. E. and A. Collmer (1999). Type III secretion machines: bacterial devices for protein delivery into host cells. *Science* **284**: 1322-1328.

Genin, S. and C. A. Boucher (1994). A superfamily of proteins involved in different secretion pathways in gram-negative bacteria: modular structure and specificity of the N-terminal domain. *Mol. Gen. Genet.* **243**: 112-118.

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.

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

Hardie, K. R., A. Seydel, I. Guilvout and A. P. Pugsley (1996b). The secretin-specific, chaperone-like protein of the general secretory pathway: separation of proteolytic protection and piloting functions. *Mol. Microbiol.* **22**: 967-976.

He, S. Y., M. Lindeberg, A. K. Chatterjee and A. Collmer (1991a). 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.

He, S. Y., C. Schoedel, A. K. Chatterjee and A. Collmer (1991b). 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.

Henderson, I. R., F. Navarro-Garcia and J. P. Nataro (1998). The great escape: structure and function of the autotransporter proteins. *Trends Microbiol* **6**: 370-378.

Hol, W. G. J., T. K. Sixma and E. A. Merritt (1995). Structure and function of *E. coli* heat-labile enterotoxin and cholera toxin B pentamer. *Bacterial Toxins and Virulence Factors in Disease*. J. Moss, B. Iglewski, M. Vaughan and A. T. Tu. New York, Marcel Dekker, Inc. **8:** 185-223.

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

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

Hueck, C. J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev* **62**: 379-433.

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

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.

Jobling, M. G. and R. K. Holmes (1992). Fusion proteins containing the A2 domain of cholera toxin assemble with B polypeptides of cholera toxin to form immunoreactive and functional holotoxin-like chimeras. *Infect. Immun.* **60**: 4915-4924.

Jose, J., J. Kramer, T. Klauser, J. Pohlner and T. F. Meyer (1996). Absence of periplasmic DsbA oxidoreductase facilitates export of cysteine-containing passenger proteins to the *Escherichia coli* cell surface via the IgA β autotransporter pathway. *Gene* **178**: 107-110.

Kagami, Y., M. Ratliff, M. Surber, A. Martinez and D. N. Nunn (1998). Type II protein secretion by *Pseudomonas aeruginosa*: genetic suppression of a conditional mutation in the pilin-like component XcpT by the cytoplasmic component XcpR. *Mol. Microbiol.* **27**: 221-233.

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.

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

Lazdunski, A., J. Guzzo, A. Filloux, M. Bally and M. Murgier (1990). Secretion of extracellular protein by *Pseudomonas aeruginosa* . *Biochimie* **72**: 147-156.

Letellier, L., S. P. Howard and J. T. Buckley (1997). Studies on the energetics of proaerolysin secretion across the outer membrane of *Aeromonas* species - Evidence for a requirement for both the protonmotive force and ATP. *J. Biol. Chem.* **272**: 11109- 11113.

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

Linderoth, N. A., P. Model and M. Russel (1996). Essential role of a sodium dodecyl sulfate-resistant protein IV multimer in assembly-export of filamentous phage. *J. Bacteriol.* **178**: 1962-1970.

Linderoth, N. A., M. N. Simon and M. Russel (1997). The filamentous phage pIV multimer visualized by scanning transmission electron microscopy. *Science* **278**: 1635-1638.

Lu, H. M. and S. Lory (1996). A specific targeting domain in mature exotoxin A is required for its extracellular secretion from *Pseudomonas aeruginosa*. *EMBO J.* **15**: 429-436.

Lu, H. M., S. T. Motley and S. Lory (1997). Interactions of the components of the general secretion pathway: role of *Pseudomonas aeruginosa* type IV pilin subunits in complex formation and extracellular protein secretion. *Mol. Microbiol.* **25**: 247-259.

Marsh, J. W. and R. K. Taylor (1998). Identification of the *Vibrio cholerae* type 4 prepilin peptidase required for cholera toxin secretion and pilus formation. *Mol. Microbiol.* **29**: 1481-1492.

McVay, C. S. and A. N. Hamood (1995). Toxin A secretion in *Pseudomonas aeruginosa*: the role of the first 30 amino acids of the mature toxin. *Mol. Gen. Genet.* **249**: 515-525.

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

Murata, H., M. Fons, A. Chatterjee, A. Collmer and A. K. Chatterjee (1990). Characterization of transposon insertion Out- mutants of *Erwinia carotovora* subsp. *carotovora* defective in enzyme export and of a DNA segment that complements *out* mutations in *E. carotovora* subsp. *carotovora, E. carotovora* subsp. *atroseptica*, and *Erwinia chrysanthemi. J. Bacteriol.* **172**: 2970-2978.

Nouwen, N., N. Ranson, H. Saibil, B. Wolpensinger, A. Engel, A. Ghazi and A. P. Pugsley (1999). Secretin PulD: Association with pilot PulS, structure, and ion-conducting channel formation. *Proc. Natl. Acad. Sci. USA* **96**: 8173-8177.

Nunn, D. 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. and S. Lory (1993). Cleavage, methylation, and localization of the *Pseudomonas aeruginosa* export proteins XcpT, -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.

Orlandi, P. A. (1997). Protein-disulfide isomerase-mediated reduction of the A subunit of cholera toxin in a human intestinal cell line. *J. Biol. Chem.* **272**: 4591-4599.

Overbye, L. J., M. Sandkvist and M. Bagdasarian (1993). Genes required for extracellular secretion of enterotoxin are clustered in *Vibrio cholerae*. *Gene* **132**: 101-106.

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

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.

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

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

Possot, O. M., L. Letellier and A. P. Pugsley (1997). Energy requirement for pullulanase secretion by the main terminal branch of the general secretory pathway. *Mol. Microbiol.* **24**: 457-464.

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, pilin-like component of the general secretory pathway of *Klebsiella oxytoca. Mol. Microbiol.* **9**: 295-308.

Pugsley, A. P. (1996). Multimers of the precusor of a type IV pilin-like component of the general secretory pathway are unrelated to pili. *Mol. Microbiol.* **20**: 1235-1245.

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., M. G. Kornacker and I. Poquet (1991). The general protein-export pathway is directly required for extracellular pullulanase secretion in *Escherichia coli* K12. *Mol. Microbiol.* **5**: 343-352.

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

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.

Reeves, P. J., P. Douglas and G. P. C. Salmond (1994). Beta-lactamase topology probe analysis of the OutO NMePhe peptidase, and six other Out protein components of the *Erwinia carotovora* general secretion pathway apparatus. *Mol. Microbiol.* **12**: 445-457.

Rietsch, A. and J. Beckwith (1998). The genetics of disulfide bond metabolism. *Annu Rev Genet* **32**: 163-184.

Sandkvist, M. and M. Bagdasarian (1993). Suppression of temperature-sensitive assembly mutants of heat-labile enterotoxin B subunits. *Mol. Microbiol.* **10**: 635-645.

Sandkvist, M., M. Bagdasarian, S. P. Howard and V. J. DiRita (1995). Interaction between the autokinase EpsE and EpsL in the cytoplasmic membrane is required for extracellular secretion in *Vibrio cholerae. EMBO J.* **14**: 1664-1673.

Sandkvist, M., L. O. Michel, L. P. Hough, V. M. Morales, M. Bagdasarian, M. Koomey, V. J. DiRita and M. Bagdasarian (1997). General secretion pathway (*eps*) genes required for toxin secretion and outer membrane biogenesis in *Vibrio cholerae*. *J. Bacteriol.* **179**: 6994-7003.

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.

Sauvonnet, N., I. Poquet and A. P. Pugsley (1995). Extracellular secretion of pullulanase is unaffected by minor sequence changes but is usually prevented by adding reporter proteins to its N- or C-terminal end. *J. Bacteriol.* **177**: 5238-5246.

Sauvonnet, N. and A. P. Pugsley (1996). Identification of two regions of *Klebsiella oxytoca* pullulanase that together are capable of promoting β-lactamase secretion by the general secretory pathway. *Mol. Microbiol.* **22**: 1-7.

Schneider, E. and S. Hunke (1998). ATP-binding-cassette (ABC) transport systems: functional and structural aspects of the ATP-hydrolyzing subunits/domains. *FEMS Microbiol. Rev.* **22**: 1-20.

Shevchik, V. E. and G. Condemine (1998). Functional characterization of the *Erwinia chrysanthemi* OutS protein, an element of a type II secretion system. *Microbiology* **144**: 3219-3228.

Shevchik, V. E., J. Robert-Baudouy and G. Condemine (1997). Specific interaction between OutD, an *Erwinia chrysanthemi* outer membrane protein of the general secretory pathway, and secreted proteins. *EMBO J.* **16**: 3007-3016.

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.

Thomas, J. D., P. J. Reeves and G. P. C. Salmond (1997). The general secretion pathway of *Erwinia carotovora* subsp *carotovora*: Analysis of the membrane topology of OutC and OutF. *Microbiology-Uk* **143**: 713- 720.

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.

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.

CHAPTER 2

PROTEINS INVOLVED IN THE TYPE II SECRETION SYSTEM ENCODED BY THE *EPS* GENES OF *VIBRIO CHOLERAE*

The results presented in this chapter appeared as part of the publication: Sandkvist et. al. 1997. General secretion pathway (eps) genes required for toxin secretion and outer membrane biogenesis in V. cholerae. J. Bacteriol. 179:6994-7003.

Abstract

The general secretion pathway (GSP) of *Vibrio cholerae* is required for the secretion of proteins, including chitinase, enterotoxin, and protease through the outer membrane, while not required for the secretion of DNase or amylase. Proteins produced from several of the previously identified open reading frames in the *eps* gene cluster are identified by the use of the T7 polymerase-promoter system in *Escherichia coli*. One of the proteins, EpsG, was also analyzed in *V. cholerae* and found to migrate as two bands on polyacrylamide gels, suggesting that EpsG might be processed or otherwise modified by prepilin peptidase not found in *E. coli*. It is also shown that the TcpJ prepilin peptidase, required for processing of the TcpA subunit of the toxin-coregulated pilus (TCP), is not involved in this modification. It is thus concluded that a second prepilin peptidase is encoded by the genome of *V. cholerae*.

Introduction

In *Vibrio cholerae* the type II secretion system, or general secretory pathway (GSP) is required for the extracellular secretion of several proteins, including cholera toxin (CT), chitinase, and protease (Overbye *et al.*, 1993; Sandkvist *et al.*, 1993). This pathway is likely to play a significant role in the survival of *V. cholerae* in different environments, as well as in its pathogenicity . Chitinase and protease may allow *Vibrio* to detach from waterborne chitinaceous particles or allow the procurement of nutrients from macromolecular sources in the environment. CT, the major determinant of the diarrheal disease cholera, may also be involved in the dissemination of *Vibrio*.

Human isolates of enterotoxigenic *Escherichia coli* produce a heat labile enterotoxin (LT) that is 81% and 78% identical with the cholera toxin A and B subunits, respectively. These toxins are multimeric proteins consisting of a single A subunit and a pentamer of B subunit polypeptides (Spangler, 1992), which also have a similar biological effect and the ability to be secreted by *V. cholerae* (Neill *et al.*, 1983). The secretion of LT from *V. cholerae* proceeds through a two step mechanism, similar to CT, in which the toxin is first translocated to the periplasm, presumably by a pathway similar to the *sec* system of *E. coli* (Danese and Silhavy, 1998). The signal peptides of the precursor A and B polypeptides are removed and the mature subunits are released into the periplasm where they undergo folding and assembly(Hirst *et al.*, 1984; Hofstra and Witholt, 1984; Hirst and Holmgren, 1987a; Hirst and Holmgren, 1987b; Yu *et al.*, 1992; Sandkvist *et al.*, 1994). The second step of translocation requires the

function of the GSP that is believed to occur by the combined action of 12 or more proteins.

Several of the proteins in *V. cholerae* have been identified through complementation of spontaneous secretion defect mutants and through transposon mutagenesis. These techniques resulted in the identification of the *eps*C, *eps*E, and *eps*M genes, and subsequently resulted in the cloning and sequencing of a 12 kb fragment of *V. cholerae* chromosomal DNA (Overbye *et al.*, 1993; Sandkvist *et al.*, 1993; Sandkvist *et al.*, 1997). Analysis of the sequenced region identified 12 open reading frames (ORFs) that share homology with genes identified in homologous secretion systems in other Gram-negative bacteria (Sandkvist *et al.*, 1997).

In this work, the visualization of polypeptides produced from the putative ORFs is demonstrated. The *in vivo* processing of one of the polypeptides, EpsG, and the requirement for a prepilin peptidase other than the previously described TcpJ prepilin peptidase is demonstrated. Finally, it is shown that mutations in the *eps* genes result in a specific secretion defect and is not a general secretion defect, through the analysis of secreted DNase and protease.

Materials and Methods

Bacterial Strains, plasmids, and culture conditions.

The bacterial strains and plasmids used in this study are listed in Table 2.1. Strains of *Escherichia coli* and *Vibrio cholerae* were grown at 30°C or 37°C as indicated. LB medium, liquid or solidified with 1.5% Bacto-agar (Difco), supplemented with 100 μg/ml thymine was used for subculturing bacteria. M9 medium (Miller, 1972) supplemented with 0.4% glucose, 100 μg/ml thymine, 10 μg/ml thiamine, and 20 μg/ml each of 19 amino acids (all except methionine) was used for the radioactive labeling of proteins. For selection of plasmids, antibiotics were added to the media at the following concentrations: Ap and Km 10 μg/ml, Cm 25 μg/ml. For selection of resistance genes in the chromosome, Km was used at 50 μg/ml.

Expression of genes under bacteriophage T7 ϕ10 promoter control.

Derivatives of pT7-5 or pT7-6 containing different fragments of the eps gene cluster (Figure 2.1) were introduced into *E. coli* MC1061 carrying plasmid pGP1-2. Cells were grown at 30°C in M9 medium for 2 hr. The temperature was then raised to 42°C to induce production of T7 RNA polymerase and 300 μg/ml rifampicin was added 30 min. later. Twenty minutes after adding rifampicin, the temperature was lowered to 37°C and proteins were labeled with 10 μCi/ml L-[^{35}S]-methionine (1,000 Ci/mmol) (NEN Life Science, Boston, MA) for 10 min. The cells were pelleted, resuspended in sample buffer and heated to 95°C for 5 min. Proteins were separated on a 0.1% SDS-15% polyacrylamide gel, which

Table 2.1 Strains and plasmids used in this study.

Strain or Plasmid	Relevant Characteristic(s)	Source or Reference
Strains		
V. cholerae		
TRH7000	El Tor *thy* Hg^R Δ(*ctx*A-*ctx*B)	(Hirst *et al.*, 1984)
J71K-1	O395 *tcp*J::Tn5	(Kaufman *et al.*, 1991)
E. coli		
MC1061	F- *ara*D139 Δ(*ara-leu*)7697 Δ(*lac*)X74 *rps*L *hsd*R2 *mcr*A *mcr*B1	(Casadaban, 1980)
Plasmids		
pGP1-2	Km^R T7 gene 1 under $P_{\lambda L}$ control, cI^{857}	(Tabor and Richardson, 1985)
pT7-5	Ap^R T7 ϕ10 promoter	(Tabor and Richardson, 1985)
pT7-6	Ap^R T7 ϕ10 promoter, MCS in opposite orientation of pT7-5	(Tabor and Richardson, 1985)
pMMB560	pT7-6::*eps*C	(Sandkvist *et al.*, 1997)
pMMB547	pT7-5::*eps*E-N	(Sandkvist *et al.*, 1997)
pMMB551	pT7-5::*eps*E-J	(Sandkvist *et al.*, 1997)
pMMB564	pT7-5::*eps*G	(Sandkvist *et al.*, 1997)
pMMB586	pGP1-2 with Km^R replaced with Cm^R	(Sandkvist *et al.*, 1997)
pMMB608	pT7-5::*eps*E	(Sandkvist *et al.*, 1997)
pMS19	pT7-5::*eps*F-J	(Sandkvist *et al.*, 1997)
pMS37	pT7-5::*eps*H-K	(Sandkvist *et al.*, 1997)
pMMB531	pT7-5::*eps*H-M	(Sandkvist *et al.*, 1997)
pTKK4	pT7-5::*eps*KL	(Sandkvist *et al.*, 1997)
pMS38	pT7-5::*eps*L	(Sandkvist *et al.*, 1997)
pTKK5	pT7-6::*eps*LM	(Sandkvist *et al.*, 1997)
pMS21	pMMB207::*tcp*J	(Sandkvist *et al.*, 1997)

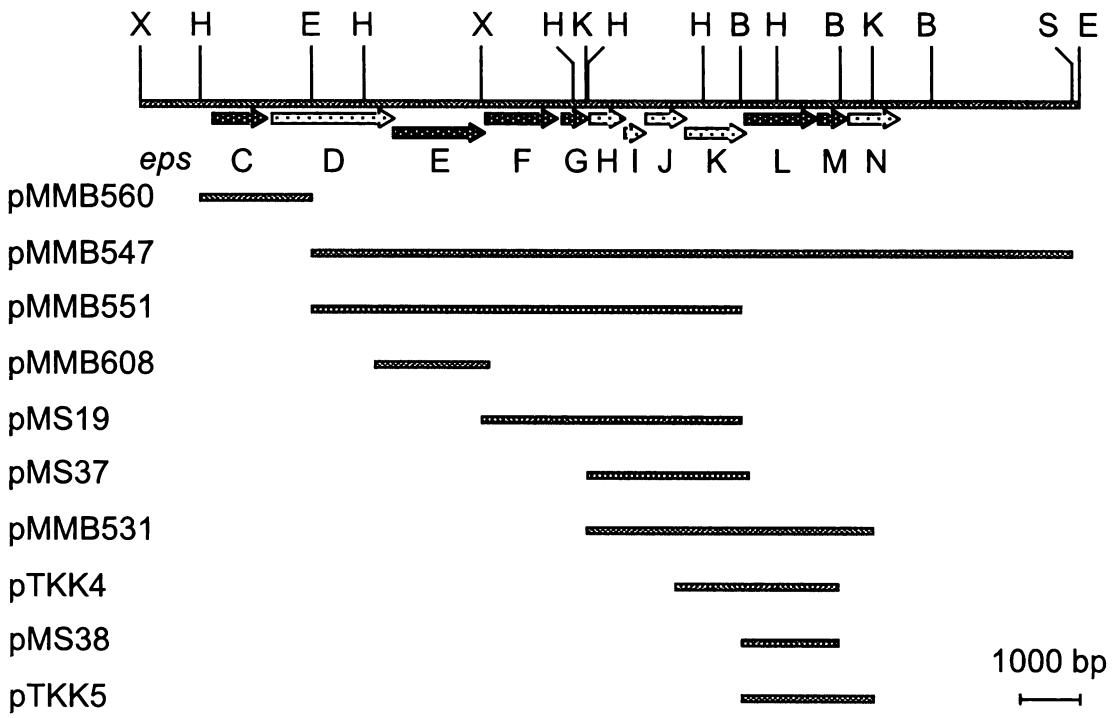


Figure 2.1 Physical and genetic map of the *eps* gene cluster. The extent of DNA fragments inserted into the pT7-5 or pT7-6 vectors used for deletion mapping and visualization of gene products is indicated by horizontal lines. Restriction site abbreviations: B, *Bam*HI; E, *Eco*RI; K, *Kpn*I; S, *Sma*I; X, *Xba*I.

was then stained with Coomassie Brilliant Blue R-250, dried, and subjected to autoradiography.

EpsG was expressed in *V. cholerae* by introducing pMMB586 carrying the gene for T7 RNA polymerase, and pMMB564, carrying the *epsG* gene cloned behind the T7 ϕ10 promoter, into strains TRH7000 and J71K-1. Cells were grown at 30°C overnight in M9 medium. Two hundred microliters of this culture was then pelleted and resuspended in 1 ml of fresh medium, and incubated at 30°C for 2 hr, followed by 30 min. of incubation at 42°C. Rifampicin was added to a final concentration of 300 µg/ml, and the incubation continued for another 20 min., when the temperature was lowered to 37°C for 10 min. The cells were

labeled for 10 min. with 10 μCi/ml (1,000 Ci/mmol) L-[^{35}S]-methionine, pelleted and resuspended in sample buffer, and heated to 95°C for 5 min. Proteins were separated on a 0.1% SDS-15% polyacrylamide gel, which was stained with Coomassie Brilliant Blue R250, dried, and subjected to autoradiography. For pulse-chase labeling of *V. cholerae* J71K-1 [pMMB586 pMMB564], cells from 1 ml of an overnight culture in M9 medium were pelleted and resuspended in 11 ml of fresh medium and grown at 30°C, induced at 42°C, and treated with rifampicin as described above. It was pulsed with 10 μCi/ml L-[^{35}S]-methionine for 30 s and chased with 0.6 mM non-radioactive L-methionine. Two-milliliter samples were withdrawn at 0.5, 1, 2, 5, and 10 min. into 2 ml of ice-cold 10% trichloroacetic acid (TCA). After 15 min. on ice, the samples were centrifuged, washed with acetone, suspended in sample buffer, and heated at 95°C for 10 min. Proteins were separated on a 0.1% SDS-17% polyacrylamide gel, stained with Coomassie Brilliant Blue R-250, dried and subjected to autoradiography.

Enzyme Assays.

Extracellular DNase was determined by combining in a 1.5 ml microfuge tube: Sephadex G-25 filtered culture medium (3.0 to 12.0 μg of protein/ml), 400 μg/ml salmon sperm DNA, and buffer (25 mM HEPES, 4 mM $MgCl_2$, 4 mM $CaCl_2$, pH 7.4). Assay mixtures were incubated for 0, 15, and 30 min. at 37°C, before an equal volume of 6% $HClO_4$ was added to the samples to stop the reaction and precipitate the high molecular weight DNA. After 15 min. on ice, the

samples were centrifuged at 20,000 x g and the OD_{260} was determined. One OD unit was considered equivalent to 50 μg of DNA.

Extracellular protease was determined in the same samples of culture medium by a modification of the method of Julius *et. al.* (Julius *et al.*, 1984). The mixture contained, in 10 μl, 100 mM HEPES adjusted to pH 7.0 with 100 mM Tris base, and 0.1 mM N-*tert*-butoxy-carbonyl-Gln-Ala-Arg-7-amido-4-methylcoumarin (Sigma Chemical Co., St. Louis, MO). The mixture was placed in the microcuvette of the DynaQuant 2000 fluorometer (Amersham Pharmacia-Biotech, Piscataway, NJ) and the signal was recorded every minute for 10 min. at RT.

Results

Expression of the eps genes by the T7 ϕ10 promoter-polymerase system.

DNA fragments inserted into pT7-5 or pT7-6 are shown in Figure 2.1, and the proteins specified by these fragments are shown in Figure 2.2. Only those plasmids in which the DNA fragments were inserted in the orientation presented in Figure 2.1 produced detectable amounts of labeled proteins. Although different genes were expressed at different levels and no polypeptide band could be assigned to some of the ORFs, six, possibly seven, bands were identified as corresponding to certain ORFs on the basis of their M_rs and the expression by the appropriate DNA fragment (Figure 2.1). Thus, the insert in plasmid pMMB560, which contains epsC and the 5' portion of *eps*D, produces two bands, a 32-kDa band, likely the EpsC protein, which has a predicted molecular mass of 33.5 kDa, and a 23-kDa band, presumably the truncated EpsD. The bands of approximately 33 kDa, specified by plasmids pMMB547 (*eps*E to -N), pMMB551 (*eps*E to -J), and pMS19 (*eps*F to -J) (lanes 3, 4, and 5 in), which should not encode *eps*C, may represent an aberrantly migrating EpsF protein which has an expected molecular mass of 44 kDa. The insert in plasmid pMMB547, which contains the 3' portion of *eps*D and the entire *eps*E through *eps*N genes, produced several bands. The band of 52 kDa corresponds to the EpsE protein (Sandkvist *et al.*, 1993). The 37.2-kDa protein is presumably EpsL. The predicted molecular mass of this protein is 45.3 kDa. The question of whether this discrepancy is due to the abnormal migration on the SDS gel or to a possible processing of the EpsL protein has not been resolved yet. The strongly

expressed 17.9-kDa protein is likely EpsG, whose predicted molecular mass is 16.0 kDa. The 16-kDa protein, produced by the cells carrying plasmids pMMB547, pMMB531 (*eps*H to -M), and pTKK5 (*eps*L to -M) (lanes 3, 8, and 11 in Figure 2.2), is most likely EpsM (Overbye *et al.*, 1993). The band running at 13 kDa in lane 10 of Figure 2.2 is presumably a truncated EpsM. In these experiments, EpsG protein was synthesized in large amounts, whereas ORFs downstream of the EpsG ORF, encoding pilin-like proteins EpsH to -J, were not expressed at similar levels. The band of 14 kDa visible in lanes 3, 4, 5, and 8 of Figure 2.2 may be the EpsI protein, whereas the band of 25 kDa in lanes 3, 4, 5, and 8 could be EpsJ. Different intensities of the different pilin-like protein bands may indicate differences in expression due, for example, to translational regulation or weaker ribosome binding sites . Nunn and Lory (Nunn and Lory, 1993) found that in *Pseudomonas aeruginosa* the ratio of amounts of XcpT, -U, -V, and -W (homologs of EpsG, -H, -I, and -J) produced was 16:1:1:4, respectively. If similar differences in expression exist for the *eps* genes, it may explain why EpsG is visible on our gels while, for instance, EpsH is not. The EpsN protein could not be detected in these experiments. However, in another experiment a band with the mobility expected of EpsN was visible.

Requirement of eps functions for secretion.

It has been reported that, in addition to being unable to secrete LT, *eps* mutants are also defective in secretion of protease(s) and chitinase(s), based on

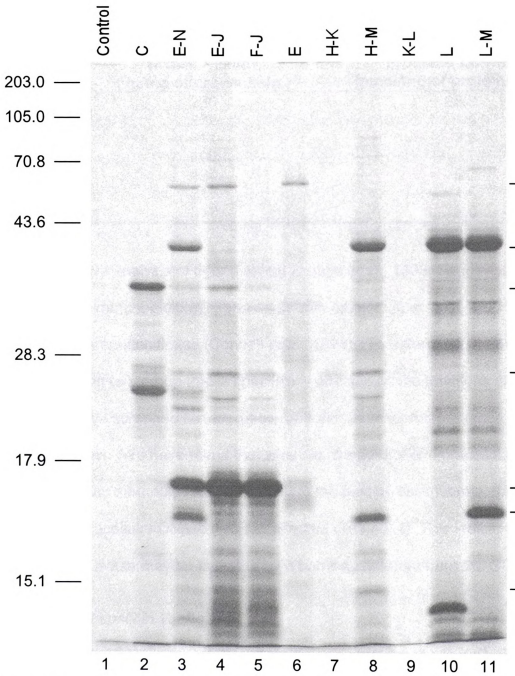


Figure 2.2 Expression of *eps* genes under the control of the bacteriophage T7 ϕ10 promoter. Cells of *E. coli* MC1061 containing recombinant plasmids indicated in Figure 2.1 were labeled, separated by SDS-PAGE, and subjected to autoradiography as described in Materials and Methods. The bands which correspond to the molecular masses expected for the Eps proteins (Table 2.3) encoded by the plasmids are indicated by the appropriate letters. Positions of molecular mass markers (in kDa) are indicated on the left. Lanes: 1, pT7-5 vector (without insert); 2, pMMB560; 3, pMMB547; 4, pMMB551; 5, pMS19; 6, pMMB608; 7, pMS37; 8, pMMB531; 9, pTKK4; 10, pMS38; 11, pTKK5.

Table 2.2 DNase and protease activities in the culture medium of *eps* mutants of *Vibrio cholerae* TRH7000.

Genotype	DNase Activity (μg/mg of protein/min.)	Protease Activity (pmol/mg of protein/min.)
WT	230	26.8
*eps*C	202	1.4
*eps*E	311	3.9
*eps*F	408	4.7
*eps*G	424	8.2
*eps*L	317	1.6
*eps*M	317	8.0

non-quantitative analysis of agar plates (Overbye *et al.*, 1993; Sandkvist *et al.*, 1993) and by immunoblotting experiments with antisera specific to the *V. cholerae* ChiA endochitinase (Connell *et al.*, 1998). To determine whether these proteins belong to a group that is specifically affected by the functions of *eps* genes, we have screened culture media of *V. cholerae* for the presence of other soluble proteins. We have found that whereas the activity of extracellular protease(s) was markedly reduced in culture medium of *eps* mutants, the specific activity of extracellular DNase was not affected (Table 2.2) This indicates that extracellular DNase may be secreted by a pathway different from the GSP.

Processing of epsG in V. cholerae

As stated earlier, the predicted amino acid sequences of EpsG, EpsH, EpsI, and EpsJ proteins contain hydrophobic regions which resemble the signal peptides of type IV pilin subunits. It was also shown by Nunn and Lory (Bally *et al.*, 1992; Nunn and Lory, 1992; Nunn and Lory, 1993) that the G, H, I, and J homologs in *P. aeruginosa* are both cleaved and methylated by the PilD/XcpA prepilin peptidase. However, no coding region with homology to any known

prepilin peptidase has been found immediately upstream or downstream of the *eps* gene cluster. In order to demonstrate that the prepilin-like Eps proteins are also processed in *V. cholerae*, the *eps*G gene was inserted downstream of the T7 ϕ10 promoter in pT7-5 to create pMMB564. This plasmid was introduced into *E. coli* MC1061 and *V. cholerae* TRH7000, both carrying the T7 RNA polymerase gene on pMMB586. The EpsG protein produced by this plasmid was labeled with [^{35}S]-Met and visualized by SDS-PAGE and autoradiography (Figure 2.3A). It was found that in *E. coli*, the EpsG protein was seen as a single band, whereas in *V. cholerae*, an additional band of lower M_r was produced. This suggested that the second band was a processed form of EpsG. The inefficient processing of radiolabeled EpsG in *V. cholerae* was most likely due to the continuous labeling conditions, since in experiments in which proteins were pulse-labeled for only 30 s and then chased with unlabeled methionine, most of the EpsG was present in the processed form at the end of the chase (Figure 2.3B).

It could be imagined that the prepilin peptidase, encoded by the *tcp*J gene of *V. cholerae* (Kaufman *et al.*, 1991) for processing of the TCP, might be involved in the processing of the EpsG, EpsH, EpsI, and EpsJ proteins. This would be analogous to the dual function of the PilD prepilin peptidase in *Pseudomonas*, in which PilD is responsible for the processing of both the type IV prepilin subunits and the pilin-like proteins XcpT to XcpW required for extracellular secretion (Nunn and Lory, 1991; Bally *et al.*, 1992; Nunn and Lory, 1992; Nunn and Lory, 1993). However, the expression of *eps*G in a *tcp*J mutant

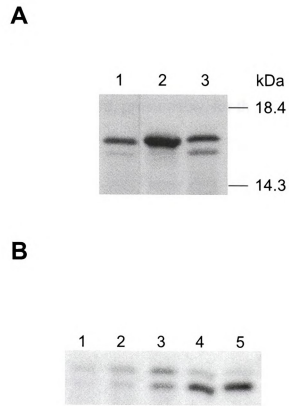


Figure 2.3 Processing of the EpsG protein in *V. cholerae*. (A) Cells of *E. coli* and *V. cholerae* strains containing plasmid pMMB586, expressing the T7 RNA polymerase, and plasmid pMMB564, expressing the *eps*G gene from the T7 ϕ10 promoter, were labeled as indicated in the Materials and Methods. Total cell proteins were subjected to SDS-PAGE and autoradiography. Lanes: 1, *V. cholerae* TRH7000 [pMMB564 pMMB586]; 2, *E. coli* MC1061 [pMMB564 pMMB586]; 3, *V. cholerae* J71K-1 [pMMB564 pMMB586]. (B) Cells of the *tcp*J :: Tn5 mutant, *V. cholerae* J71K-1, containing plasmids pMMB564 and pMMB586 were pulse-labeled and chased as described in the Materials and Methods. Lanes: 1, 0.5 min.; 2, 1 min.; 3, 2 min.; 4, 5 min.; 5, 10 min. after chase.

of *V. cholerae* resulted in a two-band pattern of EpsG similar to that in the WT *V. cholerae* strain (Figure 2.3). We have to conclude, therefore, that the *V. cholerae* genome encodes at least two prepilin peptidases. One, encoded by the *tcp*J gene, functions in the processing of the prepilin TcpA protein, the main component of the TCP type IV pilus. The other, functions in the processing of the pilin-like Eps proteins required, presumably, for the assembly of the type II secretion apparatus.

Discussion

CT is secreted via a two-step pathway that requires a specific set of genes, the *eps* genes, for translocation across the OM of *V. cholerae*. In this study, proteins produced *in vivo* were correlated to putative ORFs identified by sequencing and analysis of a region of the *V. cholerae* chromosome done by Overbye (Overbye, 1994) and shown in Table 2.3.

As mentioned previously, there are significant differences in expression levels of several of the proteins, possibly owing to transcriptional and/or translational regulation. An analysis of the coding sequences of the *eps* genes, demonstrated differences in the codon usage. Table 2.4 presents an analysis of the percentage of *E. coli* low-usage codons used in the *V. cholerae eps* genes. One striking observation made from this analysis was that the coding sequence

Table 2.3 Location and characteristics of predicted Eps reading frames in the sequence of the *eps*C to *eps*N gene cluster.

Protein	ORF[b]	No. of Amino Acid Residues	Predicted MW (kDa)
EpsC	213-1130	305	33,592
EpsD	1176-3200	674	73,337
EpsE[a]	3197-4708	503	56,358
EpsF[a]	4708-5928	406	44,916
EpsG	5969-6409	146	16,063
EpsH	6443-7027	194	21,739
EpsI[a]	7017-7370	117	13,493
EpsJ[a]	7364-8022	210	23,757
EpsK[a]	8012-9022	336	37,599
EpsL[a]	8991-10202	403	45,343
EpsM	10209-10709	166	18,521
EpsN[a]	10711-11466	251	27,322

[a]Initiation codon of ORF overlaps termination codon of preceeding ORF or is separated by less than 2 nucleotides.
[b]Nucleotide numbers given indicate the first nucleotide of the initiation codon and the last nucleotide of the termination codon of the ORF as supplied in record L33796 of the GenBank database.

Table 2.4 Percentage of all condons for selected amino acids in *V. cholerae eps* genes that are low-usage codons[A] in *Escherichia coli*.

Low-Usage Codons[A]	*eps*C	*eps*D	*eps*E	*eps*F	*eps*G	*eps*H	*eps*I	*eps*J	*eps*K	*eps*L	*eps*M	*eps*N
Gly	31	26	14	25	0	31	25	25	69	30	36	20
Arg	27	13	29	7	0	25	20	24	21	24	17	11
Ser	60	67	66	65	0	73	78	57	62	50	80	47
Thr	6	8	4	0	0	14	13	11	5	0	0	20
Leu	21	16	21	14	0	31	20	21	26	15	31	34
Pro	54	57	63	40	75	50	67	75	64	63	63	47

[A]Low-usage codons in *E. coli* used in determining the percentage of codons that are low-usage are those reported by Makrides (1996).
[B]Codons for each amino acid that were considered low-usage: (Gly) GGA, GGG; (Arg) AGA, AGG, CGA, CGG; (Ser) TCA, AGU, UCG, UCC; (Thr) ACA; (Leu)CTA, CTC; (Pro) CCC, CCT, CCA.

of *epsG* is the only *eps* gene that specifies only low-usage codons for 6 of the 8 prolines present in EpsG, and as seen in lanes 3, 4, and 5 of Figure 2.2, EpsG is one of the most abundant Eps proteins.

Additional sequence analysis of the predicted amino acid sequence for EpsG, EpsH, EpsI, and EpsJ contains a hydrophobic region which resembles the signal sequence of Type IV pilin subunits. In at least three other GSP operons, a prepilin peptidase required for processing of the prepilin-like proteins could be located immediately downstream of the last gene in the GSP operon (Pugsley and Reyss, 1990; Lindeberg and Collmer, 1992; Pugsley and Dupuy, 1992; Reeves *et al.*, 1993; Reeves *et al.*, 1994). However, sequence analysis of regions upstream and downstream of the *eps* gene cluster identified no gene encoding a prepilin peptidase. The lack of an adjacent prepilin peptidase demonstrates the similiarity between the *V. cholerae* GSP system and those of *Aeromonas* and *Pseudomonas*, in which the prepilin peptidase is found elsewhere on the chromosome (Pepe *et al.*, 1996; Filloux *et al.*, 1998). However, in contrast to the GSP's of *Pseudomonas* and *Aeromonas*, in which a single prepilin peptidase is required for processing the EpsG through EpsJ homologs and the type IV prepilin subunits (Nunn and Lory, 1991; Bally *et al.*, 1992; Nunn and Lory, 1992; Nunn and Lory, 1993; Pepe *et al.*, 1996), it was demonstrated that in *V. cholerae* a prepilin peptidase different from TcpJ is required to process EpsG. On the other hand the prepilin peptidase of *Neisseria gonorrhoeae*, PilD, was shown to process EpsG (Sandkvist *et al.*, 1997), allowing the conclusion that EpsG is a substrate for a type IV prepilin peptidase and that it is likely that,

similar to the homolog PulG (Pugsley, 1993), processing of EpsG is required for its function. The signal sequence of EpsG more closely resembles that of other type IV prepilin signal sequences than that of the *V. cholerae* TcpA prepilin signal sequence. The shorter signal sequence in EpsG and the presence of Phe instead of Met at position +1 at the predicted cleavage site, may be the reason that TcpJ is unable to process EpsG. Since TcpJ is not involved in the secretion of CT or the processing of EpsG, it is probable that another prepilin peptidase, active in the GSP and with a specificity similar to the prepilin peptidase of *N. gonorrhoeae*, exists in *V. cholerae*. The likelihood for another type IV prepilin peptidase became even more probable when the presence of a second type IV pilus, the mannose-sensitive haemagglutinin pilus in *V. cholerae* was realized (Jonson *et al.*, 1994). Table 2.5 shows the differences between the TcpA signal peptide and those of the Eps prepilin-like proteins and MshA. After completion of this work, two groups (Marsh and Taylor, 1998; Fullner and Mekalanos, 1999), have since cloned the predicted second prepilin peptidase and demonstrated processing of another prepilin-like protein in *V. cholerae*, EpsI, and its requirement in the GSP.

Mutants defective in the genes *eps*C, *eps*E, *eps*F, *eps*G, *eps*L, and *eps*M have been analyzed for their ability to secrete extracellular enzymes. Although *eps* mutants appear to be defective in a number of different functions, the defect in the extracellular secretion of toxin and protease is not simply a secondary result from a general defect in the OM, since the secretion of another protein, DNase, is not affected. In addition, these findings demonstrate that at least two

Table 2.5 Putative prepilin cleavage sites of the *V. cholerae* prepilin-like GSP proteins, EpsG, EpsH, EpsI, EpsJ, and EpsK, compared with the known cleavage sites of two *V. cholerae* type IV prepilin subunits, TcpA and MshA.

Pilin	Prepilin Peptidase Cleavage Site	
TcpA	MQLLKQLFKKKFVKEEHDKKTGQEG	MTLLEVIIVLGIMGVVSAGVVTLAQ
EpsG	MKKMRKQTG	FTLLEVMVVVVILGILASFVVPNLL
EpsH	MTATRG	FTLLEILLVLVLVSASAVAVIATFP
EpsI	MALCVYWLREKVMKSKRG	FTLLEVLVALAIFATAAISVURSVS
EpsJ	MWRTNQVSSRQNMAG	FTLIEVLVAIAIFASLSV.GAYQVL
EpsK	MRAKQRG	VALIVILLLLAVMVSIAATMAERLF
MshA	MVIMKRQGG	FTLIELVVVIVILGILAVTAAPRFL

protease(s) use the GSP, the secretion of DNase occurs by a different, as yet unidentified secretion mechanism. *V. cholerae* is known to secrete two different DNases into the extracellular environment (Newland *et al.*, 1985; Focareta and Manning, 1991). In this assay for DNase activity, it is not possible to distinguish whether one or both DNases are being detected. Nonetheless, these finding indicate that secretion of at least one of the DNases is unaffected by *eps* mutations in *V. cholerae*.

References

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.

Casadaban, M. C. (1980). Analysis of gene control signals by DNA fusion in *Escherichia coli*. *J. Mol. Biol.* **138**: 179-207.

Connell, T. D., D. J. Metzger, J. Lynch and J. P. Folster (1998). Endochitinase is transported to the extracellular milieu by the *eps*-encoded general secretory pathway of *Vibrio cholerae*. *J. Bacteriol.* **180**: 5591-5600.

Danese, P. N. and T. J. Silhavy (1998). Targeting and assembly of periplasmic and outer-membrane proteins in *Escherichia coli*. *Annu Rev Genet* **32**: 59-94.

Filloux, A., G. Michel and M. Bally (1998). GSP-dependent protein secretion in Gram-negative bacteria: the Xcp system of *Pseudomonas aeruginosa*. *FEMS Microbiol. Rev.* **22**: 177-198.

Focareta, T. and P. A. Manning (1991). Distinguishing between the extracellular DNases of *Vibrio cholerae* and development of a transformation system. *Mol. Microbiol.* **5**: 2547-2555.

Fullner, K. J. and J. J. Mekalanos (1999). Genetic characterization of a new type IV-A pilus gene cluster found in both classical and El Tor biotypes of *Vibrio cholerae*. *Infect. Immun.* **67**: 1393-1404.

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

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

Hirst, T. R., J. Sanchez, J. B. Kaper, S. J. S. Hardy and J. Holmgren (1984). Mechanism of toxin secretion by *Vibrio cholerae* investigated in strains harboring plasmids that encode heat-labile enterotoxins of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **81**: 7752-7756.

Hofstra, H. and B. Witholt (1984). Kinetics of synthesis, processing, and membrane transport of heat-labile enterotoxin, a periplasmic protein in *Escherichia coli*. *J. Biol. Chem.* **259**: 15182-15187.

Jonson, G., M. Lebens and J. Holmgren (1994). Cloning and sequencing of *Vibrio cholerae* mannose-sensitive haemagglutinin pilin gene: localization of *msh*A within a cluster of type 4 pilin genes. *Mol. Microbiol.* **13**: 109-118.

Julius, D., A. Brake, L. Blair, R. Kunisawa and J. Thorner (1984). Isolation of the putative structural gene for the lysine-arginine- cleaving endopeptidase required for processing of yeast prepro-alpha- factor. *Cell* **37**: 1075-1089.

Kaufman, M. R., J. M. Seyer and R. K. Taylor (1991). Processing of TCP pilin by TcpJ typifies a common step intrinsic to a newly recognized pathway of extracellular protein secretion by Gram-negative bacteria. *Genes Dev* **5**: 1834-1846.

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.

Makrides, S. C. (1996). Strategies for achieving high-level expression of genes in *Escherichia coli. Microbiol. Rev.* **60**: 512-538.

Marsh, J. W. and R. K. Taylor (1998). Identification of the *Vibrio cholerae* type 4 prepilin peptidase required for cholera toxin secretion and pilus formation. *Mol. Microbiol.* **29**: 1481-1492.

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

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

Newland, J. W., B. A. Green, J. Foulds and R. K. Holmes (1985). Cloning of extracellular DNase and construction of a DNase-negative strain of *Vibrio cholerae. Infect. Immun.* **47**: 691-696.

Nunn, D. 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. and S. Lory (1993). Cleavage, methylation, and localization of the *Pseudomonas aeruginosa* export proteins XcpT, -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.

Overbye, L. J. (1994). Identification and characterization of the general secretion pathway genes in *Vibrio cholerae*. Ph.D. thesis. Michigan State University, East Lansing.

Overbye, L. J., M. Sandkvist and M. Bagdasarian (1993). Genes required for extracellular secretion of enterotoxin are clustered in *Vibrio cholerae*. *Gene* **132**: 101-106.

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

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

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 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.

Reeves, P. J., P. Douglas and G. P. C. Salmond (1994). Beta-lactamase topology probe analysis of the OutO NMePhe peptidase, and six other Out protein components of the *Erwinia carotovora* general secretion pathway apparatus. *Mol. Microbiol.* **12**: 445-457.

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.

Sandkvist, M., L. O. Michel, L. P. Hough, V. M. Morales, M. Bagdasarian, M. Koomey, V. J. DiRita and M. Bagdasarian (1997). General secretion pathway (*eps*) genes required for toxin secretion and outer membrane biogenesis in *Vibrio cholerae*. *J. Bacteriol.* **179**: 6994-7003.

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.

Sandkvist, M., L. J. Overbye, T. K. Sixma, W. G. J. Hol and M. Bagdasarian (1994). Assembly of *Escherichia coli* heat-labile enterotoxin and its secretion from *Vibrio cholerae*. *Molecular Mechanisms of Bacterial Virulence*. C. I. Kado and J. H. Crosa. Netherlands, Kluwer Academic Publishers: 293-309.

Spangler, B. D. (1992). Structure and function of cholera toxin and the related *Escherichia coli* heat-labile enterotoxin. *Microbiol. Rev.* **56**: 622-647.

Tabor, S. and C. C. Richardson (1985). A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci. USA* **82**: 1074-1078.

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.

CHAPTER 3

PURIFICATION AND CHARACTERIZATION OF EPS PROTEINS

The results on the purification of EpsM(His)$_6$, preparation of polyclonal antisera against EpsM(His)$_6$, and the gel filtration analysis of EpsL and EpsM(His)$_6$, presented in this chapter appeared as part of the publication: Sandkvist, M., Hough, L. P., M. M. Bagdasarian, and M. Bagdasarian. 1999. Direct interaction of the EpsL and EpsM proteins of the general secretion apparatus in Vibrio cholerae. J. Bacteriol. 181:3129-3135. The results from the sucrose gradient separation of cytoplasmic and outer membranes of Vibrio cholerae will be submitted to the Journal of Bacteriology as part of a publication with coauthors Maria Sandkvist and Michael Bagdasarian.

Abstract

The general secretion pathway of Gram-negative bacteria is responsible for the extracellular secretion of several proteins including proteases and the cholera toxin. Proteins secreted through this pathway are secreted through a two step mechanism that is mediated with the assistance of at least 13 proteins. Four of these proteins have been purified and characterized in this study. Most of the 13 proteins are predicted to be membrane proteins, and the majority of those are predicted to be localized to the cytoplasmic membrane. Analysis of subcellular localization through selective detergent solubilization of these proteins and homologous proteins of the GSP in other Gram-negative bacteria corroborates this prediction. However, in this study it is demonstrated that several proteins predicted and determined to be localized to the cytoplasmic membrane exhibit a distribution to both membranes after sucrose gradient separation of the membranes. Additionally, it is shown that two proteins, EpsL and EpsM, are present as homodimers in Triton X-100 extracts of *Escherichia coli* membranes.

Introduction

Gram-negative bacteria have evolved several independent mechanisms for the extracellular secretion of proteins (Binet *et al.*, 1997; Hueck, 1998; Russel, 1998). One of these pathways, the type II secretion system or General Secretory Pathway (GSP), supports secretion of proteins across the cytoplasmic membrane (CM) and outer membrane (OM) of Gram-negative bacteria in two steps (Hirst and Holmgren, 1987b). Proteins are translocated across the CM via the *sec* machinery (Pugsley *et al.*, 1991; Danese and Silhavy, 1998). Then after folding, and sometimes assembly into multimeric protein complexes, they cross the OM by a mechanism that requires the products of 14-16 genes depending on the species (Hirst *et al.*, 1983; Hirst and Holmgren, 1987a; Pugsley, 1992; Russel, 1998). The genes encoding these 14-16 proteins have been designated by the letters A through O and S in most systems (for review, see Russel, 1998).

Previous work with the type II secretion system in *Vibrio cholerae* identified and sequenced 12 ORFs from the chromosome that had homology with some of the 14 genes shown to be required for type II protein secretion in other Gram-negative bacteria. Genetic evidence confirmed the requirement of at least 6 of these ORFs for secretion of toxin and protease (Sandkvist *et al.*, 1999). Eleven of the thirteen genes identified thus far in *V. cholerae* are predicted by various computer algorithms to contain at least one transmembrane helix, and to localize to the CM in Gram-negative bacteria (von Heijne, 1992; Rost, 1996; Sandkvist *et al.*, 1997; Marsh and Taylor, 1998; Nakai and Horton, 1999). However, only selective detergent solubilization of a few of these proteins has

been used for localization in *V. cholerae* (Sandkvist *et al.*, 1995; Sandkvist *et al.*, 1999). In order to permit the biochemical dissection of the type II secretion apparatus it was necessary to clone, express, purify, and develop immunoreagents for the detection of some of the Eps proteins.

In this study, the cloning, expression, purification, and the preparation of antisera, of EpsD, EpsF, EpsG, and EpsM is reported. Also reported is the selective solubilization of these proteins from the membranes of WT *V. cholerae*, the gel filtration determination of the apparent molecular mass of solubilized EpsL and EpsM, and the sucrose density gradient separation of the CM and OM fractions of *V. cholerae*.

Materials and Methods

Bacterial Strains, plasmids, and culture conditions.

The bacterial strains and plasmids used in this study are listed in Table 3.1 and Table 3.2. Strains of *Escherichia coli* and *V. cholerae* were grown at 30°C or 37°C as indicated. LB medium, liquid or solidified with 1.5% Bacto-agar (Difco), supplemented with 100 μg/ml thymine was used for subculturing bacteria. M9 medium supplemented with 0.4% glucose, 100 μg/ml thymine, 10 μg/ml thiamine, and 20 μg/ml of 19 amino acids (all except methionine) was used for the radioactive labeling of proteins. When required the antibiotics ampicillin (Ap) and kanamycin (Km) were supplemented into both types of media at 100 μg/ml for the selection of plasmid-encoded resistance genes and at 50 μg/ml for chromosomal-encoded resistance genes.

Recombinant DNA techniques and generation of constructs.

Plasmid pMMB603 was constructed by from pMMB67EH. The P_{tac} was removed by *Bal*31 nuclease digestion and replaced with an *Xho*I linker. The resulting intermediate was digested with XhoI and HindIII and the MCS of pQE70 was inserted as an *Xho*I/*Hin*dIII fragment. The resulting broad host-range, low-copy number, expression vector allows *lacI*^{q} regulated expression from the bacteriophage T5 promoter with tandem lac operators between the promoter and a strong ribosome binding site (RBS). The vector includes a start codon and restriction sites for constructing a fusion with codons for 6 C-terminal His residues, a stop codon, and the *rrn* transcriptional terminator.

Table 3.1 Bacterial strains used in this study.

Strain	Relevant Characteristic(s)	Source or Reference
Vibrio cholerae		
TRH7000	El Tor *thy* Hg^R Δ(*ctx*A-*ctx*B)	(Hirst *et al.*, 1984)
VB9	TRH7000 *eps*M::Tn5	PU3 in (Overbye *et al.*, 1993; Sandkvist *et al.*, 1997)
VB37	TRH7000 *eps*G::Km^R	Mut5 in (Sandkvist *et al.*, 1997)
VB109	TRH7000 *eps*F::Km^R	Mut6 in (Sandkvist *et al.*, 1997)
Escherichia coli		
DH10B	F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lac*ZΔM15 Δ*lac*X74 *end*A1 *rec*A1 *deo*R Δ(*ara-leu*)7697 *ara*D139 *gal*U *gal*K *nup*G *rps*L (Sm^R) λ^-	Life Technologies, Inc.
MC1061	F^- *ara*D139 Δ(*ara-leu*)7697 Δ(*lac*)X74 *rps*L *hsd*R2 *mcr*A *mcr*B1	(Casadaban, 1980)
XL1-Blue MRF'	Δ(*mcr*A)183 Δ(*mcr*CB-*hsd*SMR-*mrr*)173 *end*A1 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 relA1 *lac* [F' *pro*AB *lacI*^{q}ZΔM15 Tn10 (Tc^R)	Stratagene

Table 3.2 Plasmids used in this study.

Plasmid	Relevant Characteristic(s)	Source or Reference
pGP1-2	Km^R T7 gene 1 under $P_{\lambda L}$ control, cI^{857}	(Tabor and Richardson, 1985)
pMMB67EH	Ap^R P_{tac}, *lacI*^{q}, *mob*$^+$	(Fürste *et al.*, 1986)
pMMB587	*eps*M(His)$_6$ in pQE60	This study
pMMB603	Ap^R, $P_{T5laclac}$, *lacI*^{q}, Codons for 6 C-terminal His residues	This study
pMMB606	*eps*M(His)$_6$ in pMMB603	This study
pMMB672	*eps*G(His)$_6$ in pQE70	This study
pMMB674	*eps*G(His)$_6$ in pMMB603	This study
pMMB688	*eps*F(His)$_6$ in pQE60	This study
pMMB690	*eps*F(His)$_6$ in pMMB603	This study
pMMB706	Ap^R T7 ϕ10 promoter Codons for 6 C-terminal His residues	This study
pMMB710	*eps*D(His)$_6$ in pMMB706	This study
pMS44	*eps*L in pMMB67EH	(Sandkvist *et al.*, 1997)
pQE60	Ap^R, $P_{T5laclac}$, Codons for 6 C-terminal His residues	Qiagen, Inc.
pQE70	Ap^R, $P_{T5laclac}$, Codons for 6 C-terminal His residues	Qiagen, Inc.
pT7-5	Ap^R T7 ϕ10 promoter	(Tabor and Richardson, 1985)

Plasmid pMMB706 is a derivative of pT7-5 with the multiple cloning site (MCS) of pQE70 (Qiagen, Inc., Chatsworth, CA) cloned as an *Eco*RI/*Hin*dIII fragment. This vector provides a RBS, a start codon, codons for 6 His residues, and stop codons in all three reading frames under T7 ϕ10 promoter control.

Oligohistidine fusion proteins to the C-terminus of EpsD, EpsF, EpsG and EpsM were constructed by the PCR amplification of fragments of each gene with either *Pwo* DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IA), *Pfu* DNA polymerase (Stratagene, La Jolla, CA) or *Taq* DNA polymerase (Gibco, Rockville, MD) and oligonucleotide primers (Genosys Biotechnologies, The Woodlands, TX) as indicated in Table 3.3. *Taq*-amplified fragments were polished with T4 DNA polymerase. PCR amplified fragments were cloned into plasmids pQE60, pQE70, or pMMB706 as *Sph*I, *Nco*I, or blunt-ended *Sph*I, and *Bgl*II to create C-terminal oligohistidine fusions.

Purification of EpsM.

E. coli TG1 carrying plasmid pMMB587 was grown in 2 L of LB supplemented with 100 µg/ml Ap at 37°C to an OD_{650} of 0.3. IPTG was added to a final concentration of 0.05 mM, and the culture was allowed to grow overnight at 37°C. Cells were harvested by centrifugation, suspended in 44 ml Lysis Buffer (50 mM sodium phosphate buffer [pH 8.0], 300 mM NaCl), and lysed by sonication in the presence of 1 mg/ml lysozyme. DNase I and $MgCl_2$ were added to a final concentration of 10 U/ml and 10 mM, respectively, and incubated at RT for 10 min. The lysate was centrifuged for 45 min. at 30,000 rpm and 4°C in a

Table 3.3 Construction of C-terminal oligohistidine tagged fusion proteins with individual Eps proteins.

Gene Amplified	Primers	DNA Polymerase	PCR Product Cut with:	Vector	Vector Cut with:[B]	Fusion Protein C-terminus[C]	Resulting Plasmid Clone
epsD	EPSD1 & EPSD2	*Pfu*	*Bam*HI	pMMB706	(*Sph*I)/*Bgl*II	KQ**GS(H)$_6$**	pMMB710
epsF	EPSF1 & EPSF2	*Taq*	*Nco*I/*Bgl*II	pQE60	*Nco*I/*Bgl*II	MSR**S(H)$_6$**	pMMB688
epsG	EPSG1 & EPSG2	*Taq*	*Bgl*II	pQE70	(*Sph*I)/*Bgl*II	FQ**RS(H)$_6$**	pMMB672
epsH	EPSH1 & EPSH2	*Taq*	*Bgl*II	pMMB706	(*Sph*I)/*Bgl*II	EE**RS(H)$_6$**	pMMB711
epsI	EPSI1 & EPSI2	*Taq*	*Bgl*II	pMMB706	(*Sph*I)/*Bgl*II	AN**RS(H)$_6$**	pMMB712
epsJ	EPSJ1 & EPSJ2	*Taq*	*Bgl*II	pMMB706	(*Sph*I)/*Bgl*II	AG**RS(H)$_6$**	pMMB713
epsK	EPSK1 & EPSK2	*Taq*	*Sph*I/*Bgl*II	pMMB706	*Sph*I/*Bgl*II	TE**RS(H)$_6$**	pMMB709
epsM	MMB112 & MMB111	*Pwo*	*Bgl*II	pQE60	(*Nco*I)/*Bgl*II	GG**RS(H)$_6$**	pMMB587
epsN	EPSN1 & EPSN2	*Taq*	*Bgl*II	pMMB706	(*Sph*I)/*Bgl*II	QL**RS(H)$_6$**	pMMB727

[A]Sequences of oligonucleotide primers are given in Table 3.4
[B]Restriction enzymes in parenthesis indicate that the resulting DNA ends were blunt-ended with T4 DNA polymerase
[C]Amino acid residues in bold indicate amino acids added to the C-terminus of the WT protein sequence

Table 3.4 Sequences of oligonucleotides used in the construction of oligohisitidine fusion proteins with various Eps proteins.

Oligonucleotide Name	Oligonucleotide Sequence (5'-3')
EPSD1	AGTGAAATATTGGCTGAA
EPSD2	CGCGGATCCTTGCTTGGGTTCCATCTG
EPSF1	TAGCCATGGCCGCGTTTGAATACAA
EPSF2	CTAGATCTACTCATTAAGTTATTCATTT
EPSG1	TATGAAAAAAATGCGTAAACAAACGGG
EPSG2	CTAGATCTTTGAAAATCTTGGATATTCCAGTTAC
EPSH1	TATGACAGCGACACGCGGTTTTAC
EPSH2	CTAGATCTCTCTTCATCACTTTCTCCCGGAGC
EPSI1	GATGAAGAGTAAACGCGGTTTT
EPSI2	CTAGATCTGTTCGCCACATAGCTACGCACC
EPSJ1	TATGTGGCGAACTAACCAAGT
EPSJ2	CTAGATCTGCCCGCATTTTCAACACTCT
EPSK1	CAGCATGCGGGCTAAACAGCGCGG
EPSK2	CTAGATCTCTCAGTCGAACGGTCAGAAA
EPSN1	CATGAAGCGTGCTGTTGGCT
EPSN2	GAAGATCTGAGCTGACCTTGTTGATTG
MMB111	AAGAAGATCTGCCTCCACGCTTCAGTTGCAGACGTTT
MMB112	AAAGAATTATTGGCTCCTGTGCAGGCTTGG

Beckman Type 35 rotor, and the resulting pellet was extracted twice with 50 ml Buffer M1 (50 mM sodium phosphate buffer [pH 8.0], 300 mM NaCl, 60 mM imidazole, 0.5% Triton X-100). Insoluble material was pelleted by centrifugation for 45 min. at 30,000 rpm and 4°C in a Beckman Type 35 rotor after each extraction. Both extractions were combined and centrifuged again for 45 min. at 30,000 rpm and 4°C in a Beckman Type 35 rotor before being applied to a 25 ml POROS 20 MC metal chelate column charged with $NiSO_4$ and equilibrated with Buffer M1. After being washed with Buffer M1, proteins were eluted with a linear gradient of 60 to 700 mM imidazole in Buffer M1. EpsM$(His)_6$ eluted at approximately 300 mM imidazole. Fractions containing EpsM$(His)_6$, identified by Coomasie Brilliant Blue staining of 17% polyacrylamide SDS-PAGE gels, were

pooled, and dialyzed against Buffer M2 (Lysis Buffer supplemented with 1% Triton X-100).

Purification of EpsG.

E. coli DH10B carrying plasmid pMMB672 was inoculated into 1 L of LB supplemented with 100 μg/ml Ap and grown at 37°C to an OD_{650} of 0.3. IPTG was added to a final concentration of 0.1 mM, and the culture was grown at 37°C to an OD_{650} of 1.0. Cells were harvested by centrifugation, resuspended in 40 ml Lysis Buffer, and lysed by sonication in presence of 1 mg/ml lysozyme. DNAse I and $MgCl_2$ were added to a final concentration of 10 U/ml and 10 mM, respectively, and incubated at RT for 15 min. The lysate was centrifuged for 20 min. at 35,000 rpm and 4°C in a Beckman Type 35 rotor, and the resulting pellet was extracted 3 times with 40 ml Buffer G1 (Lysis Buffer supplemented with 10 mM imidazole and 1% Triton X-100). Insoluble material was pelleted by centrifugation for 20 min. at 35,000 rpm and 4°C in a Beckman Type 35 rotor after each extraction. The three 40 ml extractions were combined and centrifuged again for 20 min. at 35,000 rpm and 4°C in a Beckman Type 35 rotor before being applied to a 5 ml POROS 20 MC metal chelate column precharged with $NiSO_4$ and equilibrated with Buffer G1. After being washed with Buffer G1, proteins were eluted from the column with a linear gradient of 10 to 200 mM imidazole in Buffer G1. EpsG$(His)_6$ eluted from the column in two broad peaks at approximately 10 and 100 mM imidazole. Fractions containing EpsG$(His)_6$ were dialyzed against Buffer G2 (Lysis Buffer supplemented with 1% Triton X-100) at 4°C to remove imidazole.

A small scale purification of EpsG(His)6 was performed essentially as described above. Cultures of *E. coli* XL1-Blue MRF' [pMMB6974] and *V. cholerae* VB37 [pMMB674] were grown in LB supplemented with 100 μg/ml thymine and 100 μg/ml Ap at 37°C to an OD_{650} of 0.3. Expression of *eps*G$(His)_6$ was induced with 100 μM IPTG, and the cultures were incubated at 37°C until and OD_{650} of 1.0 was reached. Cells from 50 ml of culture were harvested by centrifugation for 10 min. at 6,000 rpm and 4°C in a Beckman JA-20 rotor, and the cell pellet was resuspened in 1 ml Lysis Buffer. Cells were lysed by sonicatio, using 3 pulses of 10 s at 10 s intervals in an ice bath. Membranes were pelleted by centrifugation for 15 min. at 35,000 rpm and 4°C in a Beckman 70.1.Ti rotor. The membrane pellets were each resuspended in 1 ml Buffer G1, and centrifuged again for 15 min. @ 35,000 rpm and 4°C in a Beckman 70.1.Ti rotor. The membrane proteins solubilized in Buffer G1 was collected and 600 μl was applied to a Ni-NTA Spin Column (Qiagen, Inc.), washed with 600 μl Buffer G1, and eluted with 100 μl of 300 mM imidazole in Buffer G1.

Purification of EpsD.

E. coli XL1-Blue MRF' carrying plasmid pGP1-2 and pMMB710 was grown in 2 L of LB supplemented with 100 μg/ml Ap and 100 μg/ml Km to an OD_{650} of 1.0 at 30°C. Expression of EpsD$(His)_6$ from the T7 ϕ10 promoter was induced by incubating the culture at 42°C for 30 min., and then allowing the culture to continue to grow for 2 hours at 37°C. Cells were harvested by centrifugation, resuspended in 100 ml PBS. The cells were again pelleted and frozen at -20°C.

The cell pellet was thawed and resuspended in 40 ml Lysis Buffer (50 mM $NaPO_4$ Buffer [pH 8.0], 300 mM NaCl). Resuspended cells were lyzed by sonication with four 15 s pulses at 50 MHz and 4°C at 15 s intervals. The crude envelope fraction was pelleted by centrifugation for 30 min. at 35,000 rpm and 4°C in a Beckman Type 35 rotor. The pelleted membranes were resuspended in 40 ml Buffer D1 (Lysis Buffer supplemented with 10 mM imidazole and 1% Triton X-100) and incubated on ice for 30 min. to permit solubilization of CM proteins. Pellet insoluble material by centrifugation for 30 minat 35,000 rpm and 4°C. The supernatant was discarded and the pellet was resuspended in 40 ml Buffer D2 (Lysis Buffer supplemented with 5 mM imidazole and 0.5% SDS). Incubate for 30 min. @ 37°C. SDS insoluble material was removed by centrifugation for 30 min. at 35,000 rpm and 4°C in a Beckman Type 35. The supernatant containing EpsD$(His)_6$ was harvested and loaded onto a 2 ml POROS 20 MC column, charged with $NiSO_4$ and equilibrated with Buffer D2, at 0.5 ml/min. The column was washed extensively with Buffer D2, and EpsD$(His)_6$ was eluted from the column in 1 ml fractions with a 40 ml linear gradient of 5 to 200 mM imidazole at 0.5 ml/min. Elution of EpsD$(His)_6$ was monitored by measuring the OD_{280} and fractions containing EpsD$(His)_6$ were identified by Coomassie Brilliant Blue R-250 staining of 10% polyacrylamide SDS-PAGE gels. Fractions containing EpsD$(His)_6$ were pooled and dialyzed against Buffer D3 (Lysis Buffer supplemented with 0.5% SDS) to remove imidazole.

Purification of EpsF.

E. coli XL1-Blue MRF' carrying plasmid pMMB688 was grown in 4 L of LB supplemented 100 μg/ml of Ap to an OD_{650} of 0.9 at 37°C. IPTG was added to a final concentration of 0.1 mM, and the culture was incubated for an additional 2 h at 37°C. Cells were harvested by centrifugation and resuspended in 100 ml PBS. The cells were again pelleted and frozen at -20°C. The cell pellet was thawed and resuspended in 40 ml 50 mM Lysis Buffer (50 mM $NaPO_4$ Buffer [pH 8.0], 300 mM NaCl). Resuspended cells were lyzed by sonication with four 15 s pulses at 15 second intervals at 80 MHz. Membranes and insoluble material were pelleted by centrifugation for 30 min. at 35,000 rpm and 4°C in a Beckman Type 35 rotor. The supernatant was aspirated and the pellet was extracted with 40 ml Buffer F1 (Lysis Buffer + 1% Triton X-100) and incubated for 15 min. on ice. The Triton insoluble material was pelleted by centrifugation for 30 min. at 35,000 rpm and 4°C. The supernatant was aspirated and the pellet was extracted with 40 ml Buffer F2 (Lysis Buffer + 0.5% SDS). The SDS insoluble material was pelleted by centrifugation for 30 min. at 35,000 rpm and 4°C in a Beckman Type 35 rotor. The supernatant was harvested and applied to a 2 ml metal chelate column at 1 ml/min., POROS 20 MC (PerSeptive Biosystems, Framingham, MA), charged with $NiSO_4$ and equilibrated with Buffer F2. After being washed with Buffer F2, proteins were eluted with a 50 ml linear gradient of 0 to 50 mM imidazole in Buffer F2 at 1 ml/min.. EpsF$(His)_6$ eluted at approximately 25 mM imidazole. The eluted protein was concentrated by centrifugation for 90 min. at 3,000 rpm and 4°C in a Beckman JA-20 rotor

through an Ultrafree-15 centrifugal ultrafiltration device with a Biomax-10 10,000 MWCO membrane (Millipore, Bedford, MA).

Purified EpsF$(His)_6$ was blotted onto ProBlott PVDF membrane (PE Applied Biosystems, Foster City, CA), and the N-terminal amino acid sequence was determined by an automated Edman degradation procedure. Fifteen residues matched exactly the amino acid residues 2 through 16 predicted by the nucleotide sequence of the gene, as determined previously (Sandkvist *et al.*, 1997).

Antibody Production

Polyclonal antisera was raised against EpsD, EpsF, EpsG, and EpsM in New Zealand White rabbits by Genosys Biotechnologies (The Woodlands, TX). For each protein, preimmune serum was collected from a minimum of 4 rabbits, and tested for detection of antigens in whole cell extracts of *E. coli* XL1-Blue MRF', or *V. cholerae* TRH7000 by western blotting at a 1:20,000 dilution of the preimmune serum. Two rabbits exhibiting the lowest responses to *E. coli* and *V. cholerae* antigens were selected for immunization with each protein. Both rabbits were immunized subcutaneously with 200 μg of the purified protein in Freund's complete adjuvant. Booster immunizations with 100 μg of the purified protein in Freund's incomplete adjuvant were administered subcutaneously at 14, 28, 42, 56, and 70 days after immunization. Production bleeds were collected at 49 and 63 days after immunization. Antibody production projects were ended and a final production bleed was collected at 77 days after immunization by exsanguination.

Serum collected 77 days after initial immunization was the source of polyclonal antibodies used in all immunological procedures.

Gel Filtration Analysis of EpsL and EpsM

Approximately 1.6 mg of EpsM$(His)_6$ in 0.5 ml of 50 mM sodium phosphate buffer, 150 mM NaCl, and 30 mM n-octyl-β-D-glucopyranoside (OG) was loaded onto a Superdex 200 HR column 10 mm wide by 30 cm long at a flowrate of 0.25 ml/min. Fractions of 1 ml were collected. A. 0.5 ml of 1% Triton X-100 (in 50 mM sodium phosophate and 150 mM NaCl) extract of *E. coli* MC1061 [pMS44] expressing *eps*L without IPTG induction was applied to a 16 mm by 60 cm Sephacryl S-300 HR column (Amersham Pharmacia Biotech) at a flow rate of 0.5 ml/min. Fractions of 3 ml were collected. Elution of proteins for both separations were monitored by measurement of the OD_{280}. Fractions in which EpsM eluted were identified by SDS-PAGE separation of proteins on 17% polyacrylamide gels stained with Coomassie Brilliant Blue R-250. Fractions in which EpsL eluted were identified by SDS-PAGE and immunoblotting with anti-EpsL$(His)_6$ antibodies. Reference proteins apoferritin (443 kDa), β-amylase (200 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (20 kDa), and myoglobin (17.6 kDa) were analyzed under the same conditions and used to estimate the molecular masses of EpsM$(His)_6$ and EpsL.

Extraction of EpsC, EpsD, EpsG, EpsL, and EpsM by Different Detergents

V. cholerae TRH700 from an overnight culture grown in LB supplemented with 100 μg/ml thymine and 100 U/ml polymyxin B sulfate at 37°C was inoculated

into 200 ml of fresh LB supplemented with 100 μg/ml thymine and grown at 37°C to an OD_{650} of 1.0. Cells from 100 ml of this culture were pelleted and washed once with 10 ml PBS. The cell pellet was resuspended in 10 ml PBS and the cells were lysed by 4 pulses of 10 s at 10 s intervals in an ice bath with a Vibracell sonicator on a setting of 50 MHz. Aliquots of 1 ml of the lysate were centrifuged for 15 min. at 35,000 rpm and 4°C in a Beckman 70.1.Ti rotor to pellet the membranes and insoluble material. Each pellet was then mechanically resuspended in 1 ml of PBS containing 1% Triton X-100 and 10 mM $MgCl_2$, 1% Triton X-100 and 10 mM EDTA, 4% octylpolyoxyethylene (BACHEM, King of Prussia, PA), 1% Thesit (Roche Molecular Biochemicals), 1% sucrose monolaurate (Roche Molecular Biochemicals), 1% lauryldimethylamine oxide (LDAO) (Sigma), 1% Zwittergent 3-12 (Roche Molecular Biochemicals), or 1% SDS. Resuspended membranes were incubated for 15 min. on ice (except 1% SDS which was incubated at RT) to allow proteins to be solubilized. Each sample was then centrifuged for 15 min. at 35,000 rpm and 4°C (24°C for 1% SDS) in a Beckman 70.1.Ti rotor, and the supernatant containing proteins solubilized by the detergents being tested were harvested. The detergent insoluble material was then extracted again with PBS containing 1% SDS by mechanical resuspension of the pellet and incubation at RT for 15 min, followed by another centrifugation for 15 min. at 35,000 rpm and 24°C in a Beckman 70.1.Ti rotor. Proteins were separated by SDS-PAGE after acetone precipitation if necessary, transferred to nitrocellulose by semi-dry electroblotting, and immunodetected with antisera specific for EpsC, EpsD, EpsG, EpsL, or EpsM as

the primary antibody and horseradish peroxidase conjugated goat anti-rabbit IgG as the secondary antibody. Peroxidase activity was detected with SuperSignal chemiluminescent peroxidase substrate (Pierce Chemical Co., IL) and exposure to X-ray film. Proteins that were not detected in the first extraction, but detected in the subsequent extraction with SDS were considered not to be solubilized by the detergent used in the first extraction.. Proteins detectable in both extractions were considered to be partially solubilized by the detergent used in the first extraction. Proteins that were only detected in the first, and not in the subsequent extraction with 1% SDS were considered to be completely solubilized by the detergent used in the first extraction.

Sucrose Gradient Separation of Cytoplasmic and Outer Membrane Fractions

V. cholerae TRH7000 from a 50 ml overnight culture grown in LB supplemented with 100 μg/ml thymine and 100 U/ml polymyxin B sulfate at 37°C was inoculated into a 500 ml of fresh LB supplemented with 100 μg/ml thymine and incubated at 37°C to an OD_{650} of 1.0. Four hundred milliliters of this culture was pelleted by centrifugation for 10 min. at 6,000 rpm and 4°C in a Beckman JA-20 rotor. The pellet was washed once with 50 ml ice-cold PBS (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4), and resuspended in 20 ml PBS supplemented with 200 μl 10 mg/ml PMSF (dissolved in 95% ethanol). The cells were lysed by two passes through an ice-cold French Pressure Cell at 1,000 psi. DNase I (Roche Molecular Biochemicals) was added to a final concentration of 20 U/ml and incubated for 15 min. at RT. Unbroken cells were pelleted by centrifugation for 10 min. at 6,000 rpm and 4°C in a

Beckman JA-20 rotor, and the supernatant was harvested. Envelopes from 10 ml of the crude lysate were partially purified by sedimentation onto a 1 ml 65% sucrose (wt/vol. in 50 mM sodium phosphate buffer [pH 7.4]) cushion overlaid with 1 ml of 20% sucrose by centrifugation for 1 hr at 36,000 rpm and 4°C in a Beckman SW41 rotor. The envelopes were harvested in a minimum volume and resuspended in 10 ml. of PBS. One milliliter of the partially purified envelopes was applied to the top of a sucrose step gradient formed with 0.55 ml 65% and 0.95 ml ea. of 60%, 58%, 56%, 54%, 52%, 50%, 48%, 46%, 44%, 42%, and 40% sucrose (w/v in 50 mM sodium phosphate buffer [pH 7.4]). Membranes were separated by centrifugation for 36 hr at 36,000 rpm and 4°C in a Beckman SW41 rotor. Successive 0.5 ml fractions were collected from the bottom of the gradient and were analyzed. Ten percent of each fraction was used to determine the activity of β-NADH oxidase by the method of Osborne *et. al.* (1972). For determination of the distribution of LPS across the gradient, 0.15 ml of each fraction was brought to a volume of 1 ml in 10% tricholoracetic acid. After incubation for 15 min. on ice, acid-precipitated material was collected by centrifugation for 15 min. at 16,000 rpm and 4°C in a Beckman JA-20 rotor, and washed with one 1 ml volume of distilled water without resuspension to remove residual sucrose. The amount of LPS in the samples was determined essentially as described by Lee and Tsai (1999). Acid-precipitated material was resuspended in 200 µl water and 100 µl 64 mM $NaIO_4$ was added. After incubation for 20 min. at RT, 200 µl 136 mM Purpald (Sigma-Aldrich, St. Louis, MO) in 2N NaOH was added. After further incubation for 20 min. at RT, 200 µl

64 mM $NaIO_4$ was added and incubated for another 20 min. at RT. Eighty microliters of isopropanol was added to eliminate the resulting foam, and the absorbance of each sample was recorded and plotted against a standard curve constructed with *V. cholerae* 569B LPS (Sigma). Fractions were then assayed for the distribution of EpsC, EpsD, EpsG, EpsL, and EpsM by western blotting.

Results

Purification of EpsM(His)$_6$

The *V. cholerae* gene *eps*M was efficiently cloned into the expression vector pQE70, to create a fusion protein with a C-terminal oligohistidine tag. The resulting construct, pMMB587, was transformed into *E. coli* TG1 and expression of the recombinant fusion protein was induced with IPTG. Large amounts of the recombinant fusion protein were produced and were then solubilized from the membrane fraction of the cell with the detergents Triton X-100 and octyl-β-D-glucopyranoside (OG). Since Triton X-100 is known to solublize primarily CM proteins (Schnaitman, 1971b), this suggests that the EpsM(His)$_6$ fusion protein localizes to the CM in *E. coli*. EpsM(His)$_6$ was purified from both 0.5% Triton X-100 and 30 mM OG extracts of *E. coli* membranes as described in the materials and methods. EpsM(His)$_6$ was found to elute from the column at approximately 300 mM imidazole, which suggests that EpsM(His)$_6$ may be a dimer or multimer (Qiagen, 1997), under these conditions (Figure 3.1)

To determine whether the C-terminal oligohistidine tag affected the function of EpsM *in vivo*, the recombinant gene was cloned into the broad-host range, low copy number vector, pMMB603, resulting in construction pMMB606.

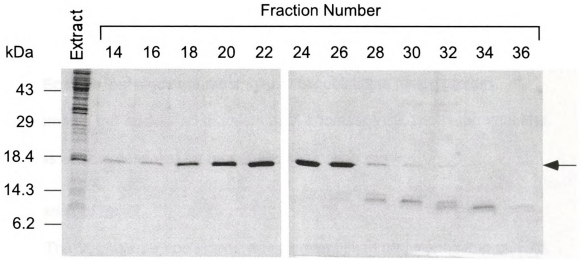


Figure 3.1 Purification of EpsM(His)$_6$. SDS-PAGE analysis of 10 µl samples of protein from the 1% Triton X-100 extract of *E. coli* TG1 [pMMB587], and each fraction after purification by metal chelate affinity chromatography as described in the materials and methods. The arrow indicates the position of EpsM(His)$_6$.

This plasmid was transferred to *V. cholerae* VB9, a derivative of TRH7000 in which *Tn*5 had inserted into the *eps*M gene (Overbye *et al.*, 1993; Sandkvist *et al.*, 1997), and the strain was tested for restoration of protease secretion on Luria agar (LA) plates supplemented with 1% skim milk. Halos formed around colonies carrying either the WT *eps*M gene or the recombinant oligohistidine tagged *eps*M, indicating that the C-terminal fusion did not significantly alter or affect the function of EpsM *in vivo*.

To verify the identity of the purified protein as EpsM the first 15 N-terminal amino acids sequenced by the Edman degradation. The sequence of the first 15 amino acids matched exactly the predicted sequence of EpsM from the nucleotide sequence determined previously (Overbye *et al.*, 1993; Sandkvist *et al.*, 1997). However, there are two tandem Met codons at the beginning of *eps*M. Since the putative ribosome binding site (UAAGGAG) is only separated from the

first Met codon by 2 nucleotides, the second Met codon is predicted to be the start codon for *eps*M. Thus, it is still unknown which Met codon is the start codon of WT EpsM is, but since the recombinant oligohistidine fusion protein complements the *eps*M::*Tn*5 mutation in *V. cholerae* VB9, two N-terminal Met residues are not essential for proper EpsM function *in vivo.*

Purification of EpsG

The *V. cholerae eps*G gene was also amplified and cloned into pQE70. The resulting construct, pMMB671, was transformed in *E. coli* XL1-Blue MRF' and induced with IPTG to express a recombinant fusion protein with a C-terminal polyhisitidine tag. In contrast to the *Klebsiella oxytoca* EpsG homolog, PulG (Pugsley and Possot, 1993), EpsG$(His)_6$ was found to be completely extracted from the membranes of *E. coli* in solutions containing 0.5% Triton X-100 , suggesting a CM location for the recombinant protein. Purification of EpsG was performed as described in the materials and methods. EpsG$(His)_6$ eluted from the column across the range of imidazole concentrations from 10 to 200 mM, with a minor peak at ~30 mM and a major peak at approximately 100 mM imidazole (Figure 3.2). This elution pattern suggests that at least some fraction of EpsG$(His)_6$ molecules may be monomeric under these conditions.

Since it was known at this time that PreEpsG was processed by a signal peptidase in *V. cholerae* (see chapter 2 and Sandkvist *et al.*, 1997), it was decided to see if the C-terminal oligohistidine-tagged protein could complement an *eps*G defect in *V. cholerae*, to see if the recombinant EpsG$(His)_6$ was processed in *V. cholerae*, and to see if processing of EpsG resulted in any

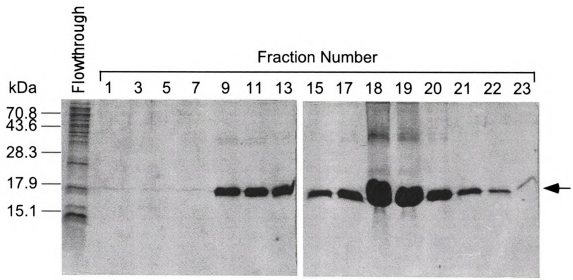


Figure 3.2 Purification of EpsG(His)$_6$. SDS-PAGE analysis of 10 µl samples of protein from the 1% Triton X-100 extract of *E. coli* XL1-MRF' [pMMB670] and each fraction after purification by metal chelate affinity chromatography as described in the materials and methods. The arrow indicates the position of EpsG(His)$_6$.

difference in the detergent solubilization of EpsG(His)$_6$. The recombinant gene for EpsG(His)$_6$ was subcloned into the broad-host range, low-copy number expression vector pMMB603, resulting in construction pMMB674, and transferred by conjugation to *V. cholerae* VB37. *V. cholerae* VB37 is an *eps*G mutant of *V. cholerae* TRH7000 constructed by inserting a gene for Km^R into *eps*G in vitro and introducing it into the chromosome of *V. cholerae* by homologous recombination (Sandkvist *et al.*, 1997). The presence of EpsG(His)$_6$ in VB37 restored secretion of protease evidenced by the formation of halos around colonies growing on LA + 1% skim milk (results not shown).

When expression of EpsG(His)$_6$ was induced by the addition of IPTG in *V. cholerae* VB37, EpsG(His)$_6$ could be purified from both Triton X-100 and OG extracts of the membrane fraction. When the purified EpsG(His)$_6$ was analyzed

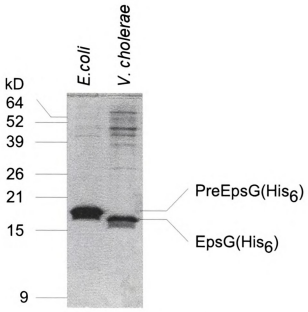


Figure 3.3 Comparison of precusor EpsG(His)$_6$ purified from *E. coli* DH10B [pMMB674] and processed EpsG(His)$_6$ purified from *V. cholerae* VB37 [pMMB674]. A small scale purification was performed as described in the materials and methods.

by SDS-PAGE on an 18% polyacrylamide gel and compared with the EpsG(His)$_6$ purified from *E. coli*, it could be clearly seen that the recombinant fusion protein was processed in *V. cholerae* (Figure 3.3). The bulk of EpsG was present in the Triton X-100 extract. These results indicate that the majority of processed EpsG remains Triton X-100 soluble, suggesting that processing does not affect subcellular localization.

Purification of EpsD

The gene for EpsD was amplified by PCR. The PCR amplified fragment was eventually cloned into the expression vector pMMB706. The expression vector pMMB706 is a derivative of pT7-5 in which the MCS was replaced with the RBS, MCS, C-terminal oligohistidine tag, and stop codon of pQE70. The resulting construction, pMMB710, could be maintained in *E. coli* hosts carrying the T7

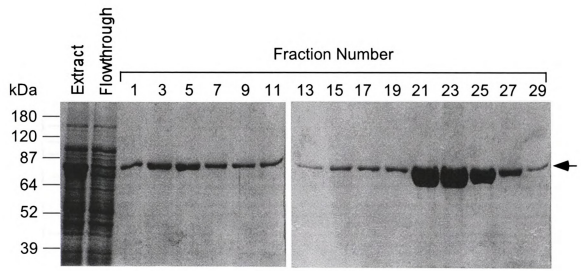


Figure 3.4 Purification of EpsD(His)$_6$. SDS-PAGE analysis of 10 µl samples of protein from the 0.5% SDS extract of *E. coli* MC1061 [pGP1-2 pMMB710] and each fraction after purification by metal chelate affinity chromatography as described in the materials and methods. The arrow indicates the position of EpsD(His)$_6$.

RNA polymerase under stringent control, such as MC1061 [pGP1-2]. The clone of *eps*D could not be maintained in strains such as BL21 (λDE3) without also including the plasmid pLysS to lower the basal level of expression.

EpsD(His)$_6$ expressed in *E. coli* MC1061 was produced as an insoluble protein which was not solubilized in PBS containing 1% Triton X-100 or 1% Triton X-100 supplemented with 10 mM EDTA. EpsD(His)$_6$ was however efficiently extracted from the membrane (insoluble) fraction of *E. coli* with 0.5% SDS. EpsD(His)$_6$ was purified from a 0.5% SDS extract of *E. coli* membranes as described in the materials and methods (Figure 3.4). EpsD(His)$_6$ eluted from the column at less than 50 mM imidazole.

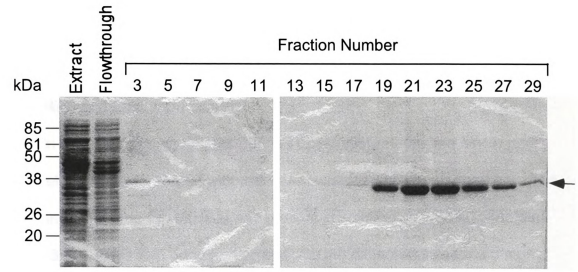


Figure 3.5 Purification of EpsF(His)$_6$. SDS-PAGE analysis of 10 µl samples of protein from the 0.5% SDS extract of *E. coli* XL1-Blue MRF' [pMMB688] and each fraction after purification by metal chelate affinity chromatography as described in the materials and methods. The arrow indicates the position of EpsF(His)$_6$.

Purification of EpsF(His)$_6$

The *eps*F gene was amplified and cloned into pQE70 and transformed into *E. coli* XL1-Blue MRF'. Expression of EpsF(His)$_6$ was induced with IPTG and was found in the insoluble fraction of the cell. Although, similar to EpsD(His)$_6$, EpsF(His)$_6$ was not extractable in 1% Triton X-100 or 1% Triton X-100 supplemented with 10 mM EDTA, but could be solubilized by 0.5% SDS. EpsF(His)$_6$ was purified from a 0.5% SDS extract of *E. coli* insoluble protein by metal chelate affinity chromatography as described in the materials and methods (Figure 3.5). Similar to the purification of EpsD(His)$_6$ but not to quite the same extent, some amount of EpsF(His)$_6$ eluted from the column at the beginning of the gradient. This elution pattern is attributed to the use of the anionic detergent

SDS in the purification, which is known to leach Ni^{+2} from the column (Qiagen, 1997).

EpsM(His)$_6$ and EpsL form dimers.

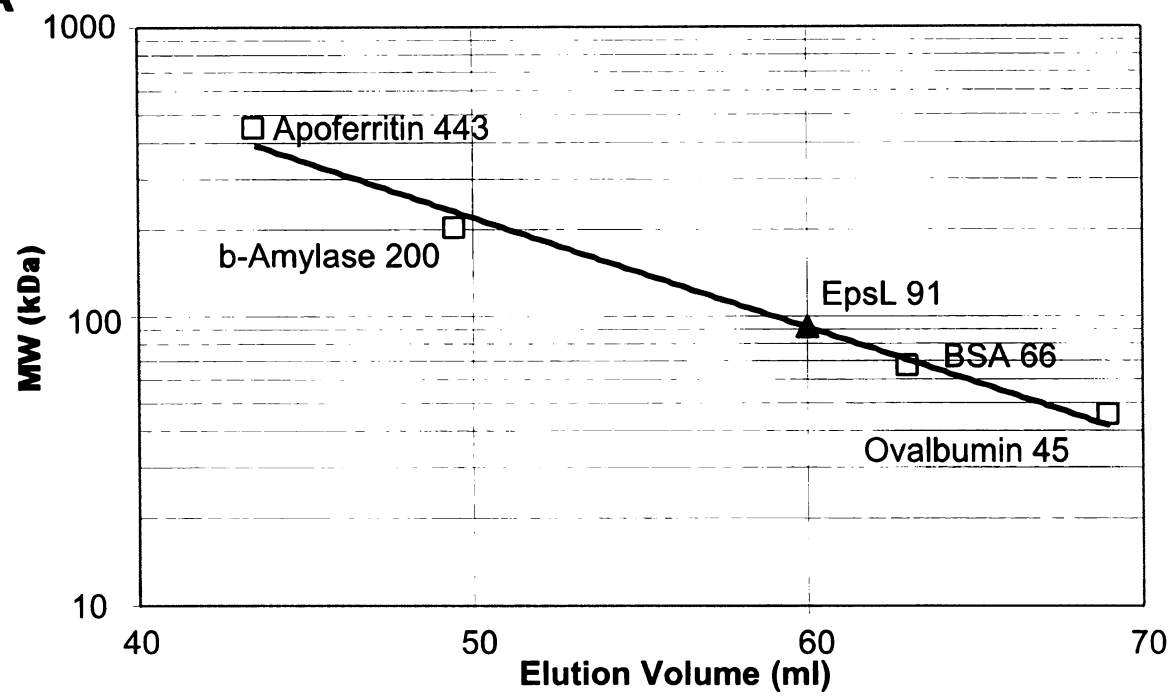
In gel filtration experiments purified EpsM(His)$_6$ eluted in a single peak, and its elution was compared with that of known molecular mass markers. The results indicated that EpsM(His)$_6$ was present in solution as a 35 kDa protein (Figure 3.6A). This is in good agreement with the expected size for a dimer of the 18.5 kDa molecule predicted from the translation of the *eps*M gene sequence (Sandkvist *et al.*, 1997). The molecular size of EpsL in solution could not be determined by analysis of purified material, since EpsL has only been purified under denaturing conditions (Sandkvist *et al.*, 1999). Therefore, gel filtration analysis was performed on EpsL extracted from the membrane of *E. coli* expressing the *eps*L gene from a low-copy number vector. Figure 3.6B shows the result from a gel filtration through Sephacryl S-300 in which the molecular mass of EpsL was 91 kDa, which is in good agreement for the expected size of an EpsL dimer of the 45.4 kDa monomer predicted from the nucleotide sequence of *eps*L (Sandkvist *et al.*, 1997). These results suggest that EpsL and EpsM both form dimers when produced in *E. coli* in the absence of other Eps proteins.

Detergent Solubility of Eps Proteins from V. cholerae *TRH7000.*

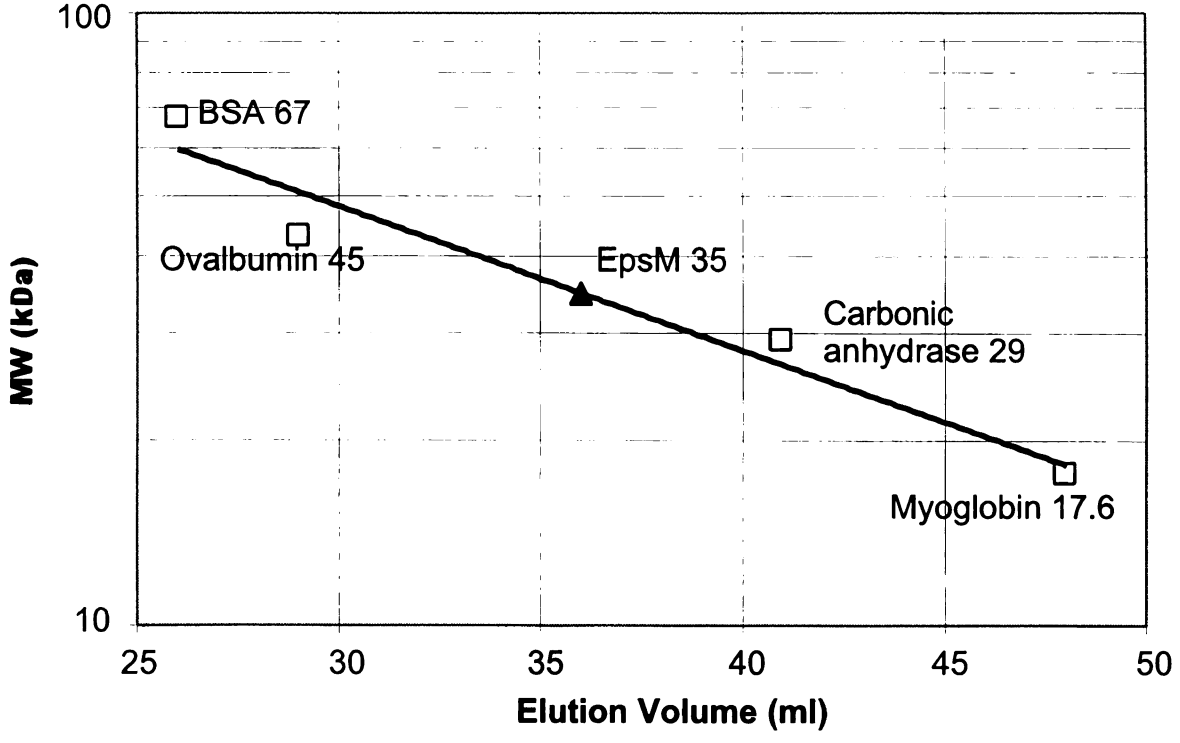
Since the majority of the proteins that comprise the type II secretory apparatus are located in either the CM or the OM, the ability of various detergents and conditions to solublize these proteins was tested. The insoluble fraction of *sonicated V.* cholerae TRH7000 cells was resuspended in a 1%

Figure 3.6 Size determination of EpsM(His)$_6$ and EpsL. Fractionation of purified EpsM(His)$_6$ and EpsL present in a Triton X-100 extract of membranes from an *E. coli* expressing *eps*L was performed on a Sephacryl S-300 HR column (A) and a Superdex 200 HR column (B), respectively. Fractions of 1.0 ml (A) or 3.0 ml (B) were analyzed by OD_{280} as well as SDS-PAGE and Coomassie brilliant blue staining or immunoblotting with anti-EpsL(His)$_6$ antibodies. Elution of EpsM(His)$_6$ and EpsL was compared with the elution of standard proteins of known molecular mass. The volume of eluent at which the individual standard proteins emerged from the column was plotted against the logarithm of their molecular mass and fit by linear regression. The elution peak of EpsM(His)$_6$ and EpsL is indicated by a triangle, and the apparent molecular mass for these proteins was calculated from this data.

A


1000
100
10
MW (kDa)
Apoferritin 443
b-Amylase 200
EpsL 91
BSA 66
Ovalbumin 45
40
50
60
70
Elution Volume (ml)

B


100
10
MW (kDa)
BSA 67
Ovalbumin 45
EpsM 35
Carbonic
anhydrase 29
Myoglobin 17.6
25
30
35
40
45
50
Elution Volume (ml)

solution of each of the detergents and allowed to sit for 15 min.at RT. The results presented in Table 3.5 indicate whether the protein was completely, partially, or not extracted in relation to amount of protein that could be detected in a subsequent extraction with 1% SDS. As expected EpsD, putatively an OM protein, was not extracted by 1% Triton X-100 supplemented with 10 mM $MgCl_2$, but was efficiently extracted from the membranes of *V. cholerae* by 1% Triton X-100 supplemented with10 mM EDTA. Also as expected, the proteins EpsC, EpsG, EpsL, and EpsM, predicted to be CM protein were all extracted in 1% Triton X-100 supplemented with 10 mM $MgCl_2$ suggesting that these proteins are indeed CM proteins. However, the extraction of EpsG in 1% Triton X-100 with 10 mM $MgCl_2$ is in contrast with the solubilization of the *K. oxytoca* PulG, an EpsG homolog, which was only partially released from the membrane by 1% Triton X-100 and 5 mM EDTA (Pugsley and Possot, 1993). Also interesting is the lack of solubilization of EpsD by the detergent octylpolyoxyethylene, which effectively

Table 3.5 Detergent solubility of EpsC, EpsD, EpsG, EpsL, and EpsM from the membranes of *V. cholerae* TRH7000. Legend: (++) protein is completely extracted by the detergent, (+) protein is partially extracted by the detergent, (-) protein is not extracted by indicated detergent.

Detergent	EpsC	EpsD	EpsG	EpsL	EpsM
Non-Ionic Detergents					
4% Octylpolyoxyethylene	++	-	++	++	?
1% Triton X-100/10 mM $MgCl_2$	++	-	++	++	++
1% Triton X-100/10 mM EDTA	++	++	++	++	++
1% Thesit	+	-	+	+	++
1% Sucrose Monolaurate	++	?	++	++	++
Zwitterionic Detergents					
1% Lauryl Dimethylamine Oxide	++	++	++	++	++
1% Zwittergent 3-12	++	++	+	++	++
Ionic Detergents					
1% SDS	++	++	++	++	++

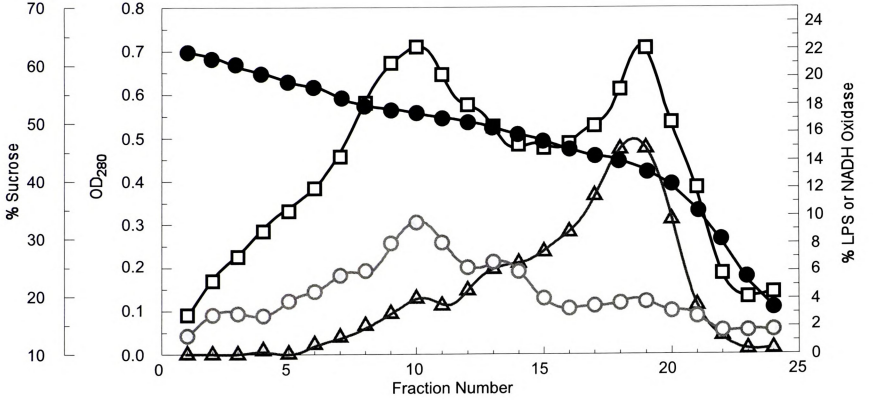
solubilized the EpsD homolog,the M13 bacteriophage pIV from the OM of *E. coli* (Linderoth *et al.*, 1997).

Density Gradient Separation of V. cholerae Membranes.

In order to confirm the specificity of selective detergent solubilization of CM and OM proteins in *V. cholerae* the membranes were separated by density gradient centrifugation through a sucrose step gradient. The results presented in Figure 3.7 shows the sedimentation profile of EpsC, EpsD, EpsG, EpsL, and EpsM. The results demonstrate that EpsD is firmly associated with the OM, and that it appears to fractionate with the more dense fragments of the OM since the peak for EpsD was lower in the gradient than the peak for LPS and protein. EpsG demonstrate a nearly homogeneous distribution between the two membranes, which was also seen in the distribution of the *Pseudomonas aeruginosa* G homolog (Nunn and Lory, 1993). EpsC and EpsM both demonstrate a bimodal distribution between the two membranes with the majority of each protein localized to the CM. A similar distribution of the *K. oxytoca* homolog of EpsC, PulC was recently demonstrated (Possot *et al.*, 1999). However, there has been no report of such a distribution for EpsM or any of its homologs. Finally, the results show that EpsL is primarily located in the CM but some traces of EpsL can be detected fractionating with the OM. This could be contamination of the OM fractions with fragments of the CM, or could be an indication of a weak association with the OM, EpsD, or another OM component of the secretion apparatus. Taken together these results suggest that there is an

association between the CM components, either directly or indirectly, and the OM component(s) of the secretion apparatus.

Figure 3.7 Sucrose density gradient separation of cytoplasmic and outer membrane proteins of *V. cholerae*. Legend (filled circles) % sucrose determined by refractive index; (open squares) OD_{280}; (open triangles) % NADH Oxidase activity; (open circles) LPS determined by Purpald Assay as described in the materials and methods. Samples from fractions 5 to 20 were precipitated with TCA separated by SDS-Page and immunoblotted with anti-sera specfic for the protein indicated. The blots correlate with the fractions represented in the graph.


% Sucrose
OD_{280}
% LPS or NADH Oxidase
Fraction Number
EpsC
EpsD
EpsG
EpsL
EpsM

Discussion

Each of the proteins discussed in this study are components involved in the transport of some extracellular proteins through the type II secretion mechanism. This pathway is required for the extracellular secretion of CT and other putative virulence factors. In this study, the purification and characterization of several proteins comprising the type II secretory apparatus is reported. The purification of each of these proteins has been critical in the development of methods and reagents to explore the underlying organization of the proteins in the putative secretory apparatus and to further characterize the individual components of the proteins.

Initially much of the information about the localization of each of these proteins, has come from analysis of the deduced amino acid sequence or from comparison with characterization of homologous proteins in other Gram negative bacteria. Based on the amino acid sequence, analysis of each of the 13 proteins with computer programs that predict transmembrane topology (Cai and Wolk, 1990; von Heijne, 1992; Cserzo *et al.*, 1997; Sonnhammer *et al.*, 1998; Nakai and Horton, 1999), or subcellular location (Nakai and Horton, 1999), resulted in the prediction of CM localization for all but two of the proteins known to be involved in the GSP. EpsD was predicted to be localized to the OM, because it's C-terminus is predicted to share structural elements found in OM porins. EpsE was predicted to be localized to the cytoplasm because of a lack of a signal sequence and hydrophobic transmembrane domains. In fact, EpsE has been shown to exist as a soluble cytoplasmic protein in the absence of any other Eps

proteins (Sandkvist *et al.*, 1995). The remaining proteins were predicted to be integral CM proteins, most spanning the membrane only once, with either large cytoplasmic or periplasmic domains. Indeed, subcellular localization experiments using the selective solubilization of certain detergents (Bally *et al.*, 1992; Howard *et al.*, 1993; Nunn and Lory, 1993; Sandkvist *et al.*, 1995; Sandkvist *et al.*, 1999), and localization experiments based on topological analysis using genetic fusions to *pho*A or *bla*M fusions (Reeves *et al.*, 1994; Bleves *et al.*, 1996; Thomas *et al.*, 1997), have been used to demonstrate CM localization for several components of the type II secretion system.

Purification of EpsM, and the antisera raised against EpsM, has revealed a predominantly CM localization of EpsM. EpsM is completely extracted from the membranes of *E. coli* and *V. cholerae* by the detergent Triton X-100, known to selectively solubilize CM proteins (Schnaitman, 1971b). This suggests that EpsM properly localizes to the membrane in the absence of other Eps proteins. However, sucrose gradient fractionation reveals a more details about the subcellular localization of EpsM in *V. cholerae* than demonstrated by the detergent extraction data. Sucrose gradient separation of EpsM demonstrates distribution between both membranes, peaking at fractions containing the majority of the CM or OM proteins. This suggests that the detection of EpsM in the OM is not the result of contamination of the OM fractions with CM fragments, but may more likely be due to a direct or indirect interaction with the OM or OM components. Furthermore, complementation of a defect in EpsM by the C-terminal hexahistidine tagged EpsM indicates that this C-terminal modification

does not affect the function of EpsM, and may therefore not be involved in its functions or interactions. Finally, both the elution from metal chelate affinity supports and the gel filitration data suggest that EpsM may be a dimer. EpsM$(His)_6$ elutes from metal chelate affinity supports charged with Ni^{+2} at the unusually high concentration of 300 mM imidazole, while most oligohistidine-tagged monomers elute between 80-100 mM imidazole (Qiagen, 1997). EpsM$(His)_6$ also elutes from gel filtration columns with a molecular mass almost exactly twice the predicted monomer mass of 18.5 kDa.

EpsD is one of the two proteins not predicted to localize to the CM. Studies with the homologous proteins from M13 bacteriophage (pIV), *K. oxytoca* (PulD), and *P. aeruginosa* (PilQ and XcpQ) indicate that the D proteins are multimeric OM proteins consisting of 12-16 subunits that are resistant to denaturation by boiling in SDS (Linderoth *et al.*, 1997; Bitter *et al.*, 1998; Nouwen *et al.*, 1999). However, expression of the *eps*D gene in *E. coli* resulted in the production and purification of only monomeric proteins. This may be an indication for the requirement of another protein, as yet unidentified in *V. cholerae*, similar to the *Neisseria gonorrhoeae* PilP protein which is required for the efficient multimerization of the *N. gonorrhoeae* D homolog, PilQ (Drake *et al.*, 1997).

After EpsD specific antisera was raised, multimers of EpsD could be detected in *V. cholerae* (results not shown), and EpsD could then be solubilized from the membrane under conditions reported to permit the solubilization of OM

proteins (Schnaitman, 1971a). The localization of EpsD to the OM in *V. cholerae* is further confirmed by the sucrose gradient separation of the membranes.

EpsG, one of the 4 prepillin-like, putative CM proteins encoded by the *eps* gene cluster, was purified from Triton X-100 extracts of the membranes of both *E. coli* and *V. cholerae*. This suggests that like EpsM, EpsG may localize to the CM, in the absence of any other Eps proteins in *E. coli*. However, EpsG may not localize to the CM in *V. cholerae*. Sucrose gradient fractionation and detergent extraction of membranes from an *E. coli,* in which the GSP of the K. oxytoca had been reconstituted, suggested that the EpsG homolog was localized to the OM (Pugsley, 1993; Pugsley and Possot, 1993). Analysis of fractions of a sucrose gradient separation of the CM and OM from *V. cholerae* indicate however, that EpsG is found associated with both membranes. Taken together with detergent extraction this suggests that EpsG is likely anchored to the CM and interacting with the OM or OM components, either directly or indirectly, in a manner that is distrupted by Triton X-100. However, no distinction can be made between whether or not there is a difference between the localization of Pre-EpsG and processed EpsG on the data presented.

Analysis of EpsL by various prediction methods indicates that EpsL is most likely a CM protein with a single TM helix (Rost, 1996; Rost *et al.*, 1996; Nakai and Horton, 1999) with the N-terminus of the protein in the cytoplasm. Recent purification of EpsL and the subsequent development of polyclonal antisera has allowed for a confirmation of the predictions. EpsL is extracted from

Table 3.6 Status of different Eps proteins.

Protein	Purification	Immunodetection reagents	Reference
EpsC	Native	Polyclonal	(Lee and Bagdasarian, unpublished)
EpsD	Denatured	Polyclonal	This study
EpsE	Native	Polyclonal	(Sandkvist *et al.*, 1995)
EpsF	Denatured	In preparation	This study
EpsG	Native	Polyclonal	This study
EpsH	No	No	
EpsI	No	No	
EpsJ	No	No	
EpsK	No	No	
EpsL	Denatured	Polyclonal	(Sandkvist *et al.*, 1999)
EpsM	Native	Polyclonal	This study
EpsN	No	No	
VcpD	No	No	

the membrane of *V. cholerae* by Triton X-100, which initially suggested that EpsL was a CM protein.

EpsL is also extracted from the membranes of *E. coli* expressing *eps*L in the absence of IPTG induction by Triton X-100. Gel filtration analysis of this extract reveals that EpsL elutes at a molecular mass approximately twice that expected for the monomer size of 45.4 kDa. After completion of this work a yeast two hybrid study of the *Erwinia chrysanthemi* homolog of EpsL, OutL, demonstrated that the L protein can form homomultimers in yeast (Py *et al.*, 1999). However, there is no evidence for multimerization of EpsL in the presence of other GSP proteins or in the context of a functional secretory apparatus.

Sucrose gradient centrifugation also corroborates the prediction and detergent extraction indications of a CM location for EpsL. EpsL predominantly

fractionates with the CM, although some EpsL can be detected in the OM fractions. Detection of EpsL in the OM fractions is likely due to the contamination of the OM vesicles with CM fragments, but may be due to a weak association with OM components in the OM.

References

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.

Binet, R., S. Létoffé, J. M. Ghigo, P. Delepelaire and C. Wandersman (1997). Protein secretion by Gram-negative bacterial ABC exporters--a review. *Gene* **192**: 7-11.

Bitter, W., M. Koster, M. Latijnhouwers, H. de Cock and J. Tommassen (1998). Formation of oligomeric rings by XcpQ and PilQ, which are involved in protein transport across the outer membrane of *Pseudomonas aeruginosa*. *Mol. Microbiol.* **27**: 209-219.

Bleves, S., A. Lazdunski and A. Filloux (1996). Membrane topology of three Xcp proteins involved in exoprotein transport by *Pseudomonas aeruginosa*. *J. Bacteriol.* **178**: 4297-4300.

Cai, Y. and C. P. Wolk (1990). Use of a conditionally lethal gene in *Anabaena* sp. Strain PCC7120 to select for double recombinants and to entrap insertion sequences. *J. Bacteriol.* **172**: 3138-3145.

Casadaban, M. C. (1980). Analysis of gene control signals by DNA fusion in *Escherichia coli*. *J. Mol. Biol.* **138**: 179-207.

Cserzo, M., E. Wallin, I. Simon, G. von Heijne and A. Elofsson (1997). Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: the dense alignment surface method. *Protein Eng.* **10**: 673-676.

Danese, P. N. and T. J. Silhavy (1998). Targeting and assembly of periplasmic and outer-membrane proteins in *Escherichia coli*. *Annu Rev Genet* **32**: 59-94.

Drake, S. L., S. A. Sandstedt and M. Koomey (1997). PilP, a pilus biogenesis lipoprotein in *Neisseria gonorrhoeae*, affects expression of PilQ as a high-molecular-mass multimer. *Mol. Microbiol.* **23**: 657-668.

Fürste, 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.

Hirst, T. R., S. J. S. Hardy and L. L. Randall (1983). Assembly *in vivo* of enterotoxin from *Escherichia coli*: formation of the B subunit oligomer. *J. Bacteriol.* **153**: 21-26.

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

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

Hirst, T. R., J. Sanchez, J. B. Kaper, S. J. S. Hardy and J. Holmgren (1984). Mechanism of toxin secretion by *Vibrio cholerae* investigated in strains harboring plasmids that encode heat-labile enterotoxins of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **81**: 7752-7756.

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.

Hueck, C. J. (1998). Type III protein secretion systems in bacterial pathogens of animals and plants. *Microbiol Mol Biol Rev* **62**: 379-433.

Lee, C. H. and C. M. Tsai (1999). Quantification of bacterial lipopolysaccharides by the purpald assay: measuring formaldehyde generated from 2-keto-3-deoxyoctonate and heptose at the inner core by periodate oxidation. *Anal Biochem* **267**: 161-168.

Linderoth, N. A., M. N. Simon and M. Russel (1997). The filamentous phage pIV multimer visualized by scanning transmission electron microscopy. *Science* **278**: 1635-1638.

Marsh, J. W. and R. K. Taylor (1998). Identification of the *Vibrio cholerae* type 4 prepilin peptidase required for cholera toxin secretion and pilus formation. *Mol. Microbiol.* **29**: 1481-1492.

Nakai, K. and P. Horton (1999). PSORT: a program for detecting sorting signals in proteins and predicting their subcellular localization. *Trends Biochem. Sci.* **24**: 34-36.

Nouwen, N., N. Ranson, H. Saibil, B. Wolpensinger, A. Engel, A. Ghazi and A. P. Pugsley (1999). Secretin PulD: Association with pilot PulS, structure, and ion-conducting channel formation. *Proc. Natl. Acad. Sci. USA* **96**: 8173-8177.

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

Osborn, M. J., J. E. Gander, E. Parisi and J. Carson (1972). Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation an characterization of cytoplasmic and outer membrane. *J. Biol. Chem.* **247**: 3962-3972.

Overbye, L. J., M. Sandkvist and M. Bagdasarian (1993). Genes required for extracellular secretion of enterotoxin are clustered in *Vibrio cholerae*. *Gene* **132**: 101-106.

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

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

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

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

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

Py, B., L. Loiseau and F. Barras (1999). Assembly of the type II secretion machinery of *Erwinia chrysanthemi*: direct interaction and associated conformational change between OutE, the putative ATP-binding component and the membrane protein OutL. *J. Mol. Biol.* **289**: 659-670.

Qiagen, I. (1997). The QIAexpressionist. Chatsworth, CA., Qiagen, Inc.

Reeves, P. J., P. Douglas and G. P. C. Salmond (1994). Beta-lactamase topology probe analysis of the OutO NMePhe peptidase, and six other Out protein components of the *Erwinia carotovora* general secretion pathway apparatus. *Mol. Microbiol.* **12**: 445-457.

Rost, B. (1996). PHD: predicting one-dimensional protein structure by profile-based neural networks. *Methods Enzymol* **266**: 525-539.

Rost, B., P. Fariselli and R. Casadio (1996). Topology prediction for helical transmembrane proteins at 86% accuracy. *Protein Sci* **5**: 1704-1718.

Russel, M. (1998). Macromolecular assembly and secretion across the bacterial cell envelope: Type II protein secretion systems. *J. Mol. Biol.* **279**: 485-499.

Sandkvist, M., M. Bagdasarian, S. P. Howard and V. J. DiRita (1995). Interaction between the autokinase EpsE and EpsL in the cytoplasmic membrane is required for extracellular secretion in *Vibrio cholerae*. *EMBO J.* **14**: 1664-1673.

Sandkvist, M., L. P. Hough, M. M. Bagdasarian and M. Bagdasarian (1999). Direct interaction of the EpsL and EpsM proteins of the general secretion apparatus in *Vibrio cholerae*. *J. Bacteriol.* **181**.

Sandkvist, M., L. O. Michel, L. P. Hough, V. M. Morales, M. Bagdasarian, M. Koomey, V. J. DiRita and M. Bagdasarian (1997). General secretion pathway (*eps*) genes required for toxin secretion and outer membrane biogenesis in *Vibrio cholerae*. *J. Bacteriol.* **179**: 6994-7003.

Schnaitman, C. A. (1971a). Effect of ethylenediaminetetraacetic acid, Triton X-100, and lysozyme on the morphology and chemical composition of isolate cell walls of *Escherichia coli*. *J. Bacteriol.* **108**: 553-563.

Schnaitman, C. A. (1971b). Solubilization of the cytoplasmic membrane of Escherichia coli by Triton X-100. *J. Bacteriol.* **108**: 545-552.

Sonnhammer, E. L., G. von Heijne and A. Krogh (1998). A hidden Markov model for predicting transmembrane helices in protein sequences. *Ismb* **6**: 175-182.

Tabor, S. and C. C. Richardson (1985). A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci. USA* **82**: 1074-1078.

Thomas, J. D., P. J. Reeves and G. P. C. Salmond (1997). The general secretion pathway of *Erwinia carotovora* subsp *carotovora*: Analysis of the membrane topology of OutC and OutF. *Microbiology-Uk* **143**: 713- 720.

von Heijne, G. (1992). Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. *J. Mol. Biol.* **225**: 487-494.

CHAPTER 4

INTERACTIONS BETWEEN *EPS* PROTEINS IN THE TYPE II SECRETION APPARATUS

Results presented in this chapter will be submitted to the Journal of Bacteriology as part of a paper with coauthors Maria Sandkvist and Michael Bagdasarian.

Abstract

The general secretion pathway of Gram-negative bacteria is responsible for the extracellular secretion of several proteins including proteases, toxins, and other putative virulence factors. Secretion through this pathway in *Vibrio cholerae* is mediated by the combined efforts of at least 13 proteins, the 12 Eps proteins and the VcpD prepilin peptidase. Coimmunoprecipitation, and a combination of *in vivo* crosslinking and coimmunoprecipitation, are used to demonstrate an association of a complex that contains EpsC and EpsD, and a complex that contains EpsD and EpsL. Additionally, an indication is provided that the association between EpsC and EpsD is through a direct interaction by demonstrating a proteolytic protection conferred upon EpsD by EpsC in the absence of any other Eps protein. Evidence is also presented that at least 5 Eps proteins solubilized from the membranes of *V. cholerae* cofractionate upon separation by gel filtration. These results provide evidence for the interaction of cytoplasmic membrane proteins with the outer membrane components of the type II secretion system, and provide an indication that they may form a multiprotein complex responsible for the translocation of proteins across the outer membrane.

Introduction

In Gram-negative bacteria the Type II secretion pathway involves two steps (Russel, 1998). In the first step proteins containing an N-terminal signal peptide are translocated across the cytoplasmic membrane (CM) via the *sec* machinery. Then, after folding (and assembly) in the periplasm, they cross the outer membrane (OM) in a separate step. The second step requires between 13 and 16 genes, depending on the species, and the homologous genes and gene products in most Gram-negative species have been designated by letters A through O, and S (reviewed in Pugsley, 1993a; Russel, 1998).

In *Vibrio cholerae* proteins such as cholera toxin, protease, neuraminidase and lipase are secreted by the Type II mechanism. This process requires the function of 12 genes, *eps*C through N, that are clustered on the *Vibrio* chromosome (Sandkvist *et al.*, 1997). An unlinked gene, *vcp*D, encoding a specific prepilin peptidase that processes some of the proteins encoded by the *eps* cluster, was predicted (Sandkvist *et al.*, 1997) and recently isolated *from V. cholerae* strains and shown to be essential for Type II secretion (Marsh and Taylor, 1998).

Although the mechanism by which proteins cross the OM is still poorly understood, information on the function of several gene products involved in this process has been gathered. Homologues of the D protein are present in the OM and form large oligomeric rings of 12-18 subunits, which have been visualized by electron microscopy in *Pseudomonas* (Bitter *et al.*, 1998). These proteins, also called secretins, are thought to form the actual pore through which secreted

proteins are translocated (Kazmierczak *et al.*, 1994; Linderoth *et al.*, 1996; Linderoth *et al.*, 1997; Bitter *et al.*, 1998). The E protein is located on the cytoplasmic side of the CM and might act as a kinase that regulates the secretion process or supplies energy required for OM translocation or for the assembly of the secretion apparatus (Sandkvist *et al.*, 1995). The homologues of the O protein are responsible for the N-terminal processing and methylation of at least four other secretion proteins, G, H, I, and J (Bally *et al.*, 1992; Nunn and Lory, 1992; Pugsley and Dupuy, 1992; Nunn and Lory, 1993; Pugsley, 1993b; Marsh and Taylor, 1998). Finally, the S protein, which is a lipoprotein and might be specific to *Klebsiella* and *Erwinia*, appears to stabilize and assist protein D in its OM localization (Hardie *et al.*, 1996a; Hardie *et al.*, 1996b; Daefler *et al.*, 1997a; Daefler *et al.*, 1997b; Shevchik and Condemine, 1998).

The function of 12 to 14 proteins is essential for the type II secretion pathway. These proteins must be present in stoichiometric amounts for the pathway to function. For example, overexpression of the E protein (Possot and Pugsley, 1994; Sandkvist *et al.*, 1995), or the G protein (Pugsley, 1993b, and our unpublished observations), in a host with a functional secretion system results in the inhibition of secretion, most likely through titration of another protein in the secretion complex. This suggests that proteins participating in the type II secretory pathway are assembled into a multiprotein complex. However, no evidence for such a complex has been previously reported. Several interactions between proteins in the CM and between proteins of the OM have been characterized. These interactions include an interaction between EpsE, a

cytoplasmic autophosphorylase, and EpsL, such that EpsE becomes membrane associated (Sandkvist *et al.*, 1995). EpsL, an integral CM protein, has in turn been shown to interact with another protein, EpsM, which was also predicted to be in the CM. Temperature sensitive mutations in the G protein have been complemented by suppressor mutations in the E protein, suggesting an interaction between these two, even though the *V. cholerae* G homolog was shown to separate with both the CM and OM fractions (see chapter 3), and the *K. oxytoca* G protein fractionated with the OM fractions (Pugsley, 1993b; Pugsley and Possot, 1993). The prepilin-like GSP proteins H, I, and J, have been shown by copurification to be associated with the G protein, but there was no demonstration of any direct interactions (Lu *et al.*, 1997). Finally, the S protein was shown to act as a pilot and putatively like a chaperone with the D protein (Hardie *et al.*, 1996a; Hardie *et al.*, 1996b).

Recently several indications that the CM C protein may interact with the OM localized D protein were obtained (Lindeberg *et al.*, 1996; Bleves *et al.*, 1999; Possot *et al.*, 1999). In this study, stronger evidence for an interaction between the C and D proteins and indications for a multiprotein complex is presented.

Materials and Methods

Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed in Table 4.1. All cultures were used at an OD_{650} of 1.0, grown at 37°C in LB.

Gel Filtration.

V. cholerae TRH7000 was grown in LB supplemented with 100 μg/ml thymine to an OD_{650} of 1.0 at 37°C. Cells from 50 ml of culture were pelleted by centrifugation for 10 min. at 6,000 rpm and 4°C in a Beckman JA-20 rotor. The cell pellet was washed with 50 ml PBS, and resuspended in 2 ml PBS. The resuspended cells were sonicated, in 1 ml fractions, 4 times for 10 s at 10 s intervals in an ice bath with a setting of 50 on a Vibracell sonicator (Sonics and Materials, Danbury, CT). After sonication the lysed cells were centrifuged for 15 min. at 35,000 rpm and 4°C in a Beckman 70.1Ti rotor to pellet the cell envelopes. The pelleted material was resuspended in 1 ml Buffer IP (PBS, 1% Triton X-100, 10 mM EDTA) and incubated on ice for 15 min. then centrifuged for 15 min. at 35,000 rpm and 4°C to remove insoluble material. Five hundred microliters of the supernatant were passed through a Sephacryl S-300 HR 16/60 gel filtration column (Amersham Pharmacia Biotech), equilibrated with Buffer IP, at a flow rate of 0.5 ml/min. with 120 ml of Buffer IP. Elution of proteins was monitored by measurement of OD_{280} and fractions of 3 ml were collected. Six volumes of ice-cold acetone was added to 0.2 ml of each fraction and incubated

Table 4.1 Strains and Plasmids used in this study.

Strain	Relevant characteristics	Source or Reference
Vibrio cholerae		
TRH7000	El Tor *thy* Hg^R Δ(*ctx*A-*ctx*B)	(Hirst *et al.*, 1984)
VB12	TRH7000 *eps*C::Tn5 (Km^R)	PU6 in (Overbye *et al.*, 1993; Sandkvist *et al.*, 1997)
VB106	TRH7000 *eps*L :: Km^R	Mut8 in (Sandkvist *et al.*, 1995)
Escherichia coli		
DH5α-MCR	F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *end*A1 *rec*A1 *deo*R *thi*-1 *pho*A *sup*E44 λ^- *gyr*A96 *rel*A1	Life Technologies, Inc. (Bethesda, MD)
XL1-Blue MRF'	Δ(*mcr*A)183 Δ(*mcr*CB-*hsd*SMR-*mrr*)173 endA1 *sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 *lac* [F' *pro*AB $lacI^qZ\Delta M15$ Tn10 (Tc^R)	Stratagene, Inc. (La Jolla, CA)
MC1061	F^- *ara*D139 Δ(*ara-leu*)7697 Δ(*lac*)X74 *rps*L (Sm^R) *hsd*R2 *mcr*A *mcr*B1	(Casadaban, 1980)
Plasmids		
pGP1-2	T7 RNA polymerase	(Tabor and Richardson, 1985)
pMMB207	Cm^R P_{TAC} mob^+	(Morales *et al.*, 1991)
pMMB503EH	Sm^R P_{TAC} mob^+	(Michel *et al.*, 1995)
pMMB699	pT7-5 :: *eps*C-N	This study
pMMB733	pT7-6 :: *eps*C	This study
pMMB734	pT7-6 :: N-terminal fragment of *eps*D	This study
pMMB741	pMMB503EH :: *eps*C	This study
pMMB748	pT7-5 :: *eps*D	This study
pMMB759	pT7-5 :: *eps*CD	This study
pMMB771	pMMB207 :: *eps*D	This study

for 30-60 min. at -20°C to precipitate protein. Precipitated protein was collected by centrifugation for 20 min. at 16,000 rpm and 4°C in a Beckman JA-20 rotor. Protein pellets were washed with 3 volumes ice-cold 6:1 acetone:water and resuspended in 20 µl 4% SDS. Samples were incubated for 15 min. at 95°C, before adding 20 µl 2X TGS-β sample buffer (126 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 0.0025% Bromophenol blue, 5% β-mercaptoethanol), incubating for an additional 15 min. at 95°C. Twenty microliter samples were analyzed by SDS-PAGE on 12%, 14%, or 3-10% gradient polyacrylamide gels and immunoblotted with polyclonal rabbit anti-EpsC(His_6), anti-EpsD(His_6), anti-EpsG(His_6), anti-EpsL(His_6), or anti-EpsM(His_6) and Horseradish peroxidase conjugated Goat Anti-Rabbit IgG (Life Technologies, Bethesda, MD).

Expression of EpsC and EpsD in Escherichia coli under bacteriophage T7 ϕ10 promoter control.

Two hundred microliters of *E. coli* MC1061 harboring plasmids pGP1-2 and pMMB733, pMMB734, pMMB748, or pMMB759 grown overnight at 30°C in M9 minimal medium supplemented with 0.1 mg/ml thymine, 10 µg/ml thiamine, 0.4% glucose, and 19 amino acids (all except methionine) was collected by centrifugation for 5 min. at 4,000 rpm and 4°C in a Beckman JA-20 rotor, resuspended in 1 ml of fresh media, and incubated for 2 hr. at 30°C. Expression of T7 RNA polymerase and genes cloned behind the bacteriophage T7 ϕ10 promoter in pT7-5 or pT7-6 was induced by incubation of the culture(s) for 30 min. at 42°C. Transcription by the host RNA polymerase was inhibited by the

addition of 300 μg/ml rifampin and continued incubation for 20 min. at 42°C. The culture(s) were then incubated for 20 min. at 37°C before the addition of 10 μCi of L-[^{35}S]-methionine (NEN Life Science) The culture was incubated for 10 min. at 37°C, after the addition of radiolabeled Met, and labeling was chased by the addition of 18 μl unlabeled 0.5% L-Methionine and continued incubation for 1 min. at 37°C. Cells were collected by centrifugation for 15 s at top speed in a tabletop microfuge, and immediately resuspended in 20 μl of water and 20 μl 2X TGS-β sample buffer. Samples were incubated for 5 min. at 95°C and separated by SDS-PAGE on a 12% polyacrylamide gel, which was stained with Coomasie Blue R-250, destained, and dried before subjected to autoradiography.

Expression of unlabelled proteins from the same strains was performed by incubating a culture of each strain in LB supplemented with 100 μg/ml ampicillin and 100 μg/ml kanamycin to an OD_{650} = 0.7 at 30°C. T7 RNA polymerase was induced by incubating the culture at 42°C for 30 min., and proteins were allowed to be expressed at 37°C until ea. culture reached an OD_{650} of 1.0. One milliliter of each culture was harvested and resuspended in 100 μl water and 100 μl 2X TGS-β sample buffer. Proteins from the equivalent of 2.5 μl or 100 μl of an 1.0 OD_{650} culture were analyzed by SDS-PAGE on a 12% polyacrylamide gel and immunoblotting with polyclonal rabbit anti-EpsD(His_6), and horseradish peroxidase conjugated Goat anti-rabbit IgG antisera.

Purification and biotinylation of anti-EpsC, anti-EpsD, anti-EpsG, anti-EpsL, and anti-EpsM IgG.

Three milliliters of antisera raised against EpsC(His_6), EpsD(His_6), EpsG(His_6), EpsL(His_6) or EpsM(His_6) was dialyzed against Buffer I (20 mM sodium phosphate buffer [pH 7.0], 300 mM NaCl) overnight at 4°C. Three milliliters of dialyzed serum was applied to a 1 ml Protein G-Sepharose Fast Flow column (Amersham Pharmacia Biotech, Piscataway, NJ), equilibrated with Buffer I, at a flow rate of 0.5 ml/min. After washing with 10 column volumes of Buffer I at a flowrate of 1 ml/min., immunoglobulin G (IgG) was eluted with 10 column volumes of Buffer II (0.1 M glycine [pH 2.9], 300 mM NaCl) at a flow rate of 0.5 ml/min. as 1 ml fractions into tubes containing 0.1 ml 1M sodium phosphate buffer (pH 7.0). Fractions containing IgG, as determined by measuring the OD_{280} were then dialyzed against PBS and then concentrated to 1 ml by centrifugation in an Ultrafree-10 ultrafiltration device with a 10,000 MWCO (Millipore, Bedford, MA). A 20-fold molar excess of Sulfo-NHS-LC-Biotin (Pierce Chemical Co, Rockford, IL) was added. After incubation at 4°C for 2 hr., the samples were dialyzed against PBS.

Preparation of Triton X-100/EDTA Extracts of crosslinked and non-crosslinked V. cholerae or E. coli for Immunoprecipitation

E. coli XL1-Blue MRF' [pMMB741] or *V. cholerae* TRH7000, VB12 [pMMB771], or VB106 were grown in LB (supplemented with 100 µg/ml thymine for *V. cholerae*) supplemented with 30 µg/ml chloramphenicol or 100 µg/ml streptomycin at 37°C to an OD_{650} of 0.7. Production of EpsC and EpsD in *E. coli*

and *V. cholerae* VB12, respectively, was induced with 2 μM IPTG. All cultures were grown at 37°C until a final OD_{650} of 1.0 was reached. Cells from 10 ml of culture were centrifuged for 10 min. at 6,000 rpm and 4°C in a Beckman JA-20 rotor to pellet the cells. The cell pellet was washed with 10 ml PBS. If cells were to be crosslinked *in vivo*, the cells were resuspended in 10 ml PBS, and 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 mM dithiobis(succinimidyl propionate) (DSP) (Sigma Chemical Co, St. Louis, MO) was added and incubated at RT for 30 min. After incubation, the crosslinking reaction was quenched with a final concentration of 0.1 M Tris pH 7.4. Cells were pelleted by centrifugation for 10 min. at 6,000 rpm and 4°C in a Beckman JA-20 rotor. The cell pellet was resuspended in 1 ml PBS and sonicated 4 times for 10 s at 10 s intervals, 4°C, and a setting of 50 on a Vibracell sonicator (Sonics & Materials, Danbury, CT). The sonicated material was centrifuged for 15 min. at 35,000 rpm and 4°C in a Beckman 70.1Ti rotor to pellet the membranes and insoluble material. The pellet was resuspended in 1 ml Buffer IP and incubated on ice for 15 min. Solubilized proteins were separated from insoluble material by centrifugation for 15 min. at 35,000 rpm and 4°C in a Beckman 70.1Ti rotor..

Immunoprecipitation of Eps proteins

One hundred microliters of a 50% suspension of preswollen Protein G-Sepharose (Amersham Pharmacia Biotech), equilibrated with PBS, was incubated with 50 μl of antisera raised against EpsC(His_6), EpsD(His_6), EpsG(His_6), EpsL(His_6) or EpsM(His_6) for 4 hr or overnight at 4°C in PBS. After

extensive washing with PBS, 40 µl of a 50% suspension of antisera/protein G-sepharose was mixed with 200 µl of protein solubilized from the membranes of *V. cholerae* TRH7000 in Buffer IP and 260 µl Buffer IP. After incubation on a rocker for 2 hr. at 4°C, the beads were collected by centrifugation for 15 s at 10,000 x g in a tabletop microfuge, and washed three times with 1 ml volumes of Buffer IP. Twenty microliters of 4% sodium dodecyl sulfate (SDS) was added and the samples were incubated for 15 min. at 95°C. Twenty microliters of 2X TGS-β Sample buffer (126 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 0.0025% Bromophenol blue, 5% β-mercaptoethanol) was added and the samples were incubated for an additional 15 min. at 95°C. After incubation the sepaharose beads were pelleted by centrifugation for 15 s at 10,000 x g. Twenty microliters of the supernatants were analyzed by SDS-PAGE on 12%, 14%, or 3-10% gradient polyacrylamide gels and immunoblotting with biotinylated anti-EpsC(His_6), anti-EpsD(His_6), or anti-EpsL(His_6) IgG, and horseradish peroxidase conjugated strepavidin (Pierce Chemical Co.).

Immunoblotting

Proteins were transferred to BA-S 83 nitrocellulose (Schleicher and Schuell, Keene, NH) by discontinuous semi-dry electroblotting with the TransBlot SD apparatus (Bio-Rad, Hercules, CA), by placing the gel on top of the nitrocellulose and 3 sheets of Whatman 3MM paper soaked in Anode Buffer (12 mM Tris Base, 96 mM glycine, 20% methanol) and placing 3 additional sheets of Whatman 3MM paper soaked in Cathode Buffer (12 mM Tris Base, 96 mM glycine, 0.1% SDS) on top of the gel. Gels were transferred for 1 hour at ~2

mA/cm^2. Membranes were blocked with 5% skim milk in 1X PBST (PBS + 0.5% Tween 20) for 30 min. at RT or overnight at 4°C. Primary and secondary immunoreagents were incubated at appropriate dilutions in 5% skim milk in PBST for 1 hour at RT. Immunoblots were washed, after incubation with primary and secondary immunoreagents, five times with PBST. Peroxidase activity was visualized with SuperSignal chemiluminescent substrate (Pierce Chemical Co.).

Results

EpsC is immunoprecipitated by Anti-EpsD Antibodies.

Antibodies raised against EpsC(His_6), EpsD(His_6), EpsG(His_6), EpsL(His_6), and EpsM(His_6) were used in immunoprecipitations of proteins from a 1% Triton X-100/10 mM EDTA extract of *V. cholerae* TRH7000 membranes. Figure 4.1A shows the immunoprecipitation of EpsC by EpsC and EpsD-specific antisera, while antisera specific for EpsG, EpsL, and EpsM show no immunoprecipitation of EpsC. To verify that EpsD antibodies do not cross-react with EpsC, EpsC was produced in *E. coli* DH5α-MCR from plasmid pMMB741, and immunoprecipitated with Anti-EpsC and Anti-EpsD antisera, as shown in Figure 4.1B, lanes 1 and 2, respectively.

EpsC-specific antisera inefficiently immunoprecipitates EpsD.

Shown in Figure 4.1A, when the same immunoprecipitations were screened with Anti-EpsD antibodies, multimeric EpsD could be detected in immunoprecipitations with EpsC, EpsD, and EpsL-specific antisera, while EpsG, and EpsM-specific did not appear to immunoprecipitate any EpsD.

EpsC-specific Antisera efficiently immunoprecipitates EpsD after in vivo crosslinking with DSP.

Since the recipirocal immunoprecipitation of EpsD by EpsC-specific antisera was rather inefficient, it was decided to determine if the efficiency of immunoprecipitation could be improved by a limited *in vivo* crosslinking. Crosslinking of *V. cholerae in vivo* with DSP resulted in the formation of several

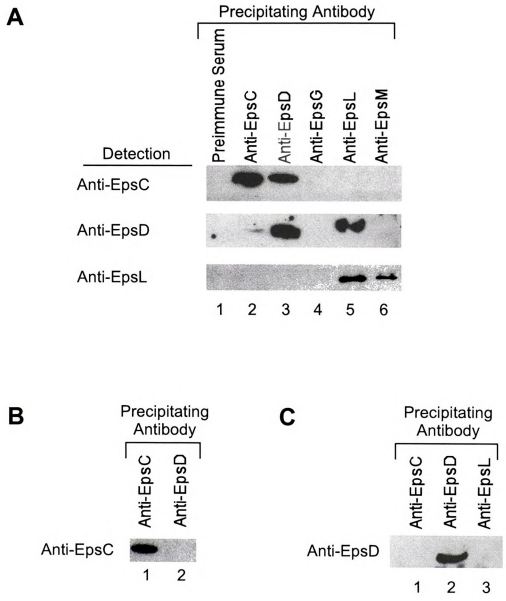


Figure 4.1 Coimmunoprecipitation of EpsC, EpsD, or EpsL proteins with EpsC, EpsD, EpsG, EpsL, or EpsM. Membrane Extracts of *V. cholerae* or *E. coli* strains were prepared in 1% Triton X-100/10 mM EDTA as described in the materials and methods. Immunoprecipitations were performed with the precipitating antibody indicated, separated by SDS-PAGE, and immunoblotted using biotinylated Anti-EpsC IgG, Anti-EpsD IgG, or Anti-EpsL IgG as described in the materials and methods. (A) Membrane proteins of *V. cholerae* TRH7000. (B) Control immunoprecipitations with membrane proteins of *E. coli* XL1-Blue MRF' [pMMB781] induced to express EpsC with 0.1 mM IPTG. (C) Control immunoprecipitations of *V. cholerae* VB12 (*epsC*$^-$) [pMMB771 *epsD*$^+$] induced to express *epsD* with 2 μM IPTG.

crosslinking products that were detected with EpsC-specific antisera (Figure 4.2). One fragment at ~85 kDa is presumed to be a dimer of 37 kDa EpsC monomers because of the size and strength of immunodetection. There are a pair of bands, between 85 and 118 kDa, either of which could potentially be a crosslinked EpsC-EpsD species. The 0 mM and 0.05 mM DSP crosslinked samples represented in Figure 4.2 were used in immunoprecipitations with polyclonal Anti-EpsC-specific antisera. Immunoprecipitation of the crosslinked material with polyclonal Anti-EpsC antiserum results in the efficient coimmunoprecipitation of multimers of EpsD (Figure 4.3A). This suggests that the relative inefficiency of coimmunoprecipitation of EpsD multimers with EpsC by polyclonal Anti-EpsC antiserum (Figure 4.1A, middle panel) is a result of the destabilization of the EpsC-EpsD interaction by the antiserum.

EpsD Coprecipitates with EpsL.

Also shown in Figure 4.1A (Lane 5), when the immunoprecipitations of EpsC, EpsD, EpsG, EpsL, and EpsM were screened with Anti-EpsD antibodies, multimers of EpsD were detected. However, no coimmunoprecipitation of EpsL could be detected with antisera specific for EpsD (Figure 4.1A, Lane 3).

EpsL Coprecipitates with EpsD after in vivo Crosslinking

Under the hypothesis that, like EpsC, antisera specific for EpsD was altering the conformation of EpsD or sequestering EpsD in such a way that the L protein could no longer remain in a complex with EpsD, an attempt was made to stabilize such a complex by crosslinking the proteins *in vivo*. Similar, to the results obtained with crosslinked EpsC and EpsD, after crosslinking EpsL could

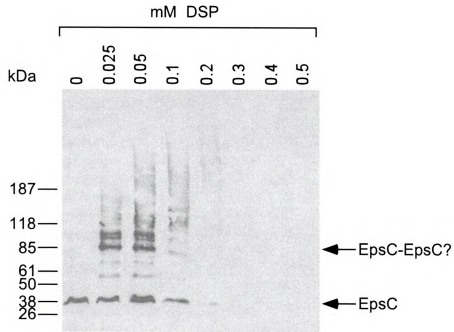
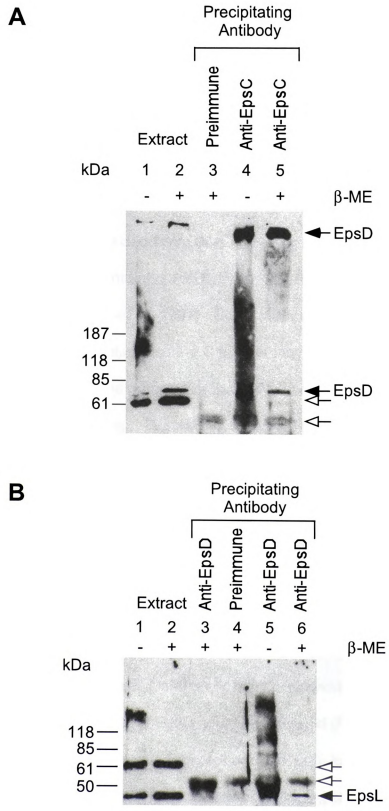


Figure 4.2 *In vivo* crosslinking of *V. cholerae* detected with Anti-EpsC antibodies. *V. cholerae* TRH7000 cells were treated with the indicated concentrations of the homobifunctional, cleavable crosslinker DSP for 30 min. at RT. Membrane proteins were solubilized in 1% Triton X-100/10 mM EDTA as described in the materials and methods. Proteins were separated on a 3-10% gradient gel, transferred to nitrocellulose and immunoblotted with Anti-EpsC specific antisera.

Figure 4.3 Coimmunoprecipitation of EpsD with EpsC and EpsL with EpsD after *in vivo* crosslinking of *V. cholerae*. Membrane proteins of *V. cholerae*, crosslinked *in vivo* with 0 mM or 0.05 mM DSP, were solubilized in 1% Triton X-100/10 mM EDTA. (**A**) Detection of EpsD coimmunoprecipitating. with EpsC crosslinking *in vivo*. (Lanes 1 and 2) 0.05 mM DSP crosslinked extract, (Lane 3) Control immunoprecipitation of crosslinked membrane proteins with preimmune serum , (Lanes 4 and 5) Immunoprecipitation of crosslinked membrane proteins with Anti-EpsC specific antiserum. (**B**) Detection of EpsL coimmunoprecipitating with EpsD after crosslinking *in vivo*. (Lanes 1 and 2) 0.05 mM DSP crosslinked extract, (Lane 3) Immunoprecipitation of non-crosslinked membrane proteins with Anti-EpsD specific antiserum for comparison, (Lane 4) Control immunoprecipitation of crosslinked membrane proteins with preimmune serum, (Lanes 5 and 6) Immunoprecipitation of crosslinked membrane proteins with Anti-EpsD specific antiserum. The locations of EpsD and EpsL are indicated. The top open arrow in both panels indicates the location a biotinylated protein of *V. cholerae* detected directly by Avidin-HRP (results not shown), and the lower open arrow indicates the location of the heavy chain of IgG. The addition of β-mercaptoethanol (β-ME) to the sample buffer, to sever the thiol-cleavable crosslinks introduced by DSP, is indicated at the top of each lane by a '+' or '-'.


A
Precipitating Antibody
Preimmune
Anti-EpsC
Anti-EpsC
Extract
kDa
1 2 3 4 5
- + + - +
β-ME
EpsD
187
118
85
61
EpsD
B
Precipitating Antibody
Anti-EpsD
Preimmune
Anti-EpsD
Anti-EpsD
Extract
1 2 3 4 5 6
- + + + - +
β-ME
kDa
118
85
61
50
EpsL

be detected coimmunoprecipitating with EpsD (Figure 4.3B, Lanes 4 and 5).

EpsC Protects EpsD from Proteolytic Degradation in E. coli.

During the course of experiments by Lee *et al.* (unpublished results) to purify recombinant EpsC from *E. coli*, several specific degradation products could be detected. However, in previous experiments in which *eps*C was expressed, these degradation products were not detected (Sandkvist *et al.*, 1997). In the expression experiment by Sandkvist *et al.*, *eps*C was expressed from plasmid, pMMB560, which also contained *eps*D*, producing the N-terminal 190 amino acids of EpsD (Sandkvist *et al.*, 1997). Since there was some indication provided by Shevchik *et al.* suggesting that the C protein may interact with the D protein (Shevchik *et al.*, 1997), the potential mutual stabilization of EpsC and EpsD in *E. coli* was investigated further. Figure 4.4A demonstrates the detection of full length, truncations, and degradation products of EpsD by immunoblotting. Whole cell samples of *E. coli* expressing *eps*D* or *eps*D had to be loaded on the gel at a concentration of 40 times that of samples of *E. coli* expressing *eps*C and *eps*D* or *eps*D together, to permit comparable detection of EpsD. As shown in Figure 4.4A, when *eps*D (Lane 5), or the 5' portion of *eps*D from pMMB560 (Lane 4) was expressed without coexpressing *eps*C, barely any EpsD, EpsD*, or degradation products of EpsD could be detected. However, when *eps*C was coexpressed, large amounts of EpsD could be detected (Figure 4.4A, Lanes 2, 6 and 7). The detection of degradation products of EpsD, in samples in which *eps*C was coexpressed is attributed to the large amount of EpsD that accumulated during the course of expression. To ensure that full length products

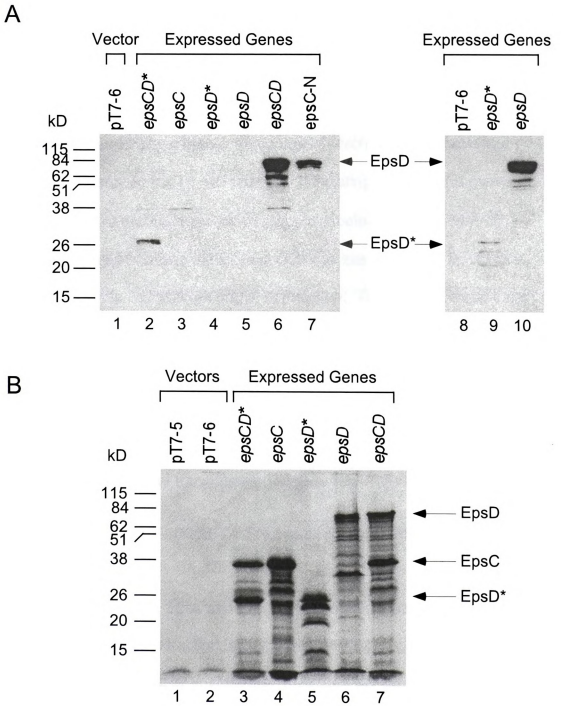


Figure 4.4 *In vivo* stabilization of EpsD and EpsD* by EpsC in *E. coli.* (A) Stabilization of EpsD and EpsD* evidenced by detection with Anti-EpsD antibodies. Forty-fold more sample was loaded into lanes 8, 9, and 10 to permit detection of EpsD and EpsD* in the absence of EpsC. (B) Stabilization of EpsD and EpsD* as evidenced by [^{35}S]-Met labeling of genes expressed using the T7 polymerase/promoter system.

of EpsD and EpsD* were being produced, a similar experiment was undertaken in which *eps*D, *eps*D*, and *eps*C were expressed together and separately, and detected by [^{35}S]-Met labeling using the T7 RNA polymerase/promoter system (Tabor and Richardson, 1985). The results of this labeling are shown in Figure 4.4B. Lanes 1 and 2 show the vector control in which no genes have been cloned downstream of the T7 ϕ10 promoter, indicating that only the genes cloned behind the T7 ϕ10 promoter produced proteins labeled with [^{35}S]-Met. The western blot analysis shown in Figure 4.4A visualizes EpsD and EpsD* that accumulated in the cell after ~2 hrs. of expression. The labeling experiment on the other hand, shows the result of a 10 min. pulse. Since both EpsD and EpsD* are clearly visible in the autoradiograph of the gel (Figure 4.4B, Lanes 5 and 6) it is clear that both proteins had been produced at the 10 min. time point. These results strongly suggest that EpsC exerts protection from degradation rather than enhanced transcription or translation of *eps*D or *eps*D* genes.

Gel Filtration of Triton X-100/EDTA Solubilized Proteins from V. cholerae Membranes

The CM proteins, EpsL and EpsM of *V. cholerae*, have been well characterized for their extraction from the membranes of *E. coli*, expressing the heterologous genes, and in *V. cholerae*. However, the EpsD protein, which fractionates with the OM by sucrose gradient sedimentation (see Chapter 3, Figure 3.9), was not solubilized by Triton X-100, consistent with an OM location for this protein. When the crude envelopes of *V. cholerae* TRH7000 were extracted with 1% Triton X-100 and 10 mM EDTA, which has been reported to

solubilize OM proteins in addition to CM proteins (Schnaitman, 1971), it was determined that the EpsC, EpsD, EpsG, EpsL and EpsM proteins could be solubilized (results not shown). To see if perhaps a macromolecular structure, containing each of these proteins could be detected, a extract of *V. cholerae* membranes was passed through a Sephacryl S-300 Gel Filtration column and fractions were analyzed for the presence of each of the five proteins by western blotting. Figure 4.5 shows the elution of protein from the column, indicating that the majority of solubilized proteins could be detected in three major peaks, with EpsC, EpsG, EpsL, and EpsM present in the second peak, and EpsD present in both the first and the second peak.

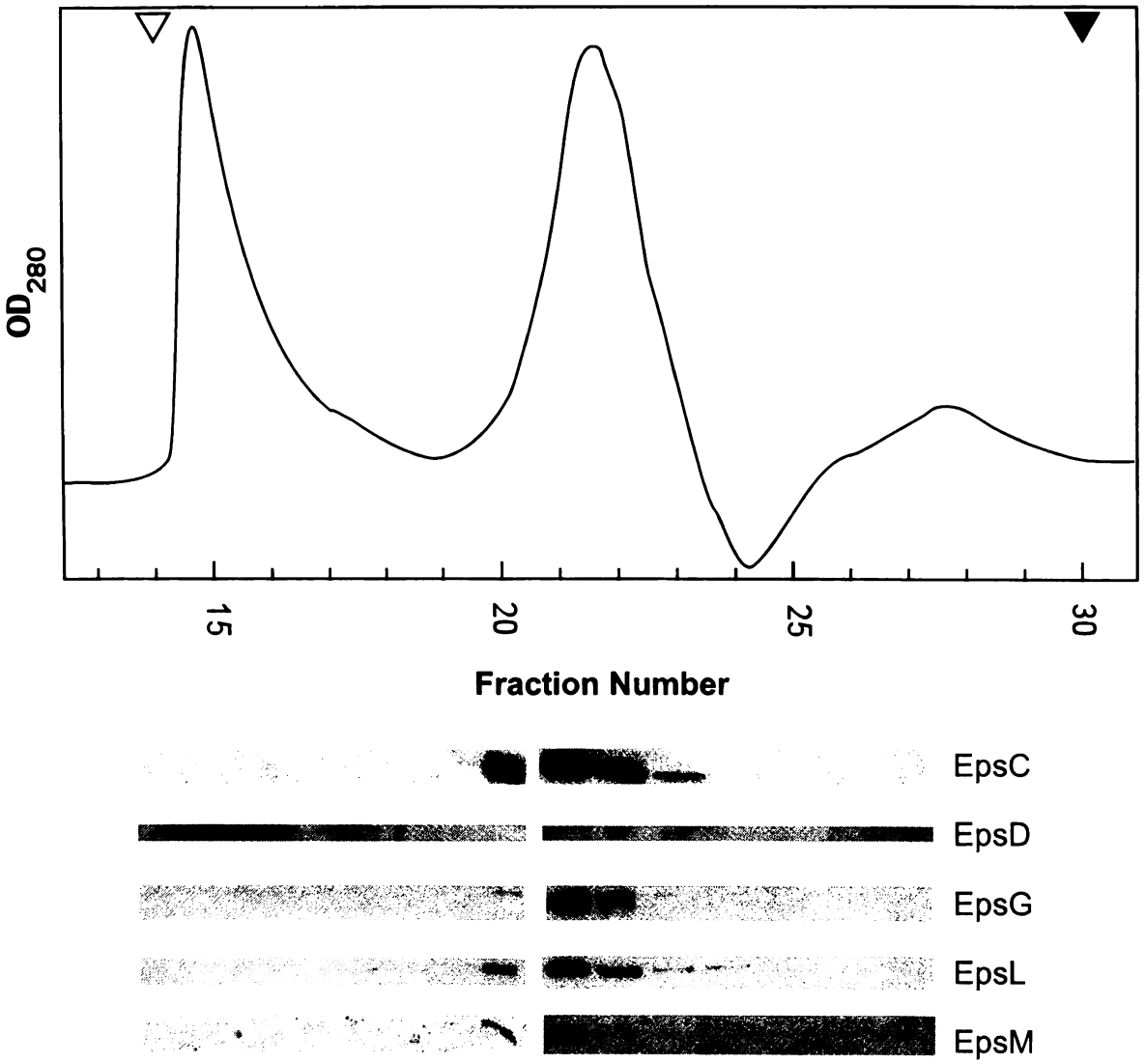


Figure 4.5 Gel filtration fractionation of proteins solubilized from *V. cholerae* membranes in 1% Triton X-100 and 10 mM EDTA. Fractions were acetone precipitated, separated by SDS-PAGE, and immunoblotted with Anti-EpsC, Anti-EpsD, Anti-EpsG, Anti-EpsL, or Anti-EpsM as indicated.

Discussion

Several indications for a potential interaction between the C protein and the D protein have been published. Shevchik *et. al.* proposed that since the presence of the D protein alone is insufficient for secretion of proteins from *E. coli* that one of the CM proteins with a large periplasmic domain, like the C, K, or M protein might interact with the D to allow for opening and closing of the pore (Shevchik *et al.*, 1997). Lindberg *et al.* noted that *out*C and *out*D, the *Erwinia chrysanthemi* genes for the C and D proteins, were the only non-interchangeable components of the *E. carotovora* in the *E. chrysanthemi* GSP secretion of PelB, suggesting that the C protein may interact directly with the D protein (Lindeberg *et al.*, 1996). However, it is just as likely that C protein fails to interact with another protein in the presumed multiprotein complex, that in turn fails to interact with the D protein. Another interesting finding of this study was the determination that complementation of a D mutant required both the C and D genes. Therefore the authors suggested that the requirement for D to be expressed with C may be limited to *cis*-acting regulatory elements that prevent synthesis of the D protein above or below required levels (Lindeberg *et al.*, 1996). This result is interesting because the a *Tn*5 insertion in *eps*C of *V. cholerae* is unable to be complemented with the *eps*C gene alone, and has therefore only been complemented with fragments containing the entire *eps*C-N operon (Sandkvist *et al.*, 1997). The requirement for *eps*C-N to complement the *eps*C::*Tn*5 insertion may simply be the effect of a polar mutation, however, expression of several

downstream proteins at significantly reduced levels has been observed (results not shown).

Bleves *et al.* more recently suggested that there is an interaction between the C (XcpP) and D (XcpQ) proteins of *P. aeruginosa*, on the basis that the C protein was unstable in a strain of *P. aeruginosa* lacking the D protein (Bleves *et al.*, 1999). However, since the remaining 11 Xcp proteins known to function in the GSP in *Pseudomonas* are still present, an indirect interaction between the C protein and the D protein cannot be ruled out. In this study it is demonstrated that EpsC exerts a stabilizing effect on EpsD when coexpressed in *E. coli* from the same transcript (Figure 4.4A, compare lanes 6 and 7). This stabilizing effect of EpsC is also seen on EpsD*, a C-terminal truncation of EpsD that consists of the first 221 amino acids of EpsD and a 15 amino acid extension derived from vector sequences (Figure 4.4A, compare lanes 3 and 5 and lanes 6 and 7). This suggests that a potential site of interaction between EpsC and EpsD would be through the N-terminus, as has been proposed by other investigators (Russel, 1993; Shevchik *et al.*, 1997; Bleves *et al.*, 1999). However, the results presented in Figure 4.4A show that there is essentially no EpsD or EpsD* produced in the absence of EpsC. These results could be explained by a regulatory effect by the *cis*-regulatory elements in the upstream C gene that were proposed to be responsible for the lack of complementation of *out*D mutants by *out*D alone (Lindeberg *et al.*, 1996). To address this, the stability of EpsD and EpsD* in the presence or absence of EpsC was assessed by ^{35}S-Met labeling of EpsC and EpsD in *E. coli* using the T7 polymerase/promoter system. Figure 4.4B shows

that indeed proteins are produced by expression of both the complete and truncated forms of *eps*D. Furthermore, since the radiolabeling permits one to visualize the effect of synthesis and degradation of EpsC and EpsD over only 10 min., and reasonably equivalent amounts of labeled material is seen in each lane where equal amounts of sample were loaded. The results of protection provide strong evidence for a direct interaction between the C protein and the D protein, through the N-terminus of EpsD, because other Eps proteins are absent in *E. coli.*

Other methods were explored to corroborate evidence for such an interaction. Initially, the localization of EpsC to both membranes (see Chapter 3) could have been another indicator that EpsC might interact with EpsD. However, Possot *et al.* demonstrated that a similar dual membrane localization for PulC was independent of the D protein, or any other GSP protein, and was therefore likely to be the result of a direct interaction between the C protein and other OM components (Possot *et al.*, 1999).

Since, EpsC, EpsD, and three other Eps proteins to which antibodies are available, are all efficiently solubilized from the membranes of *V. cholerae* in 1% Triton X-100/10 mM EDTA (see Chapter 3), coimmunoprecipitation from such an extract was deemed the next best alternative. This method has recently been used to successfully demonstrate an interaction between EpsL and EpsM (Sandkvist *et al.*, 1999). Extracts of *V. cholerae* TRH7000 were immunoprecipitated with antibodies specific for EpsC, EpsD, EpsG, EpsL, and EpsM. The results demonstrate that EpsD immunoprecipitates EpsC, and EpsC

immunoprecipitates EpsD, albeit inefficiently. The immunoprecipitation of EpsC by EpsD, and EpsD by EpsC is specific since anti-EpsC antibodies are unable to immunoprecipitate EpsD, when EpsD is overexpressed in a *V. cholerae epsC* mutant, and anti-EpsD antibodies are unable to immunoprecipitate EpsC expressed in *E. coli*. Thus the reciprocal coimmunoprecipitation of EpsC and EpsD is not the result of cross reactivity between the antibodies and the coimmunoprecipitated protein. This provides the first conclusive evidence that EpsC and EpsD are located together in a complex, and provides an indication that EpsC and EpsD may interact directly. However this does not eliminate the possibility of an indirect association of EpsC and EpsD through a complex consisting of any number of other Eps proteins.

Since the efficiency of the reciprocal coimmunoprecipitations was not equal, it was hypothesized that antibodies present in the polyclonal anti-EpsC antiserum interfered with the interaction between EpsC and EpsD, either direct or indirect through a complex, by altering the conformation of EpsC or by sequestering EpsC from the complex through high-affinity antigen-antibody interactions. If this were the case, then it was proposed that *in vivo* crosslinking could stabilize direct or indirect interactions between EpsC and EpsD, and the reciprocal coimmunoprecipitation of EpsD by EpsC should achieve a similar efficiency. Crosslinking of proteins in *V. cholerae* was performed with the homobifunctional, cleavable crosslinker DSP at different dilutions of crosslinker to identify conditions at which the extent of crosslinking was limited to prevent detection of artifacts. Immunodetection of EpsC in crosslinked *V.* cholerae

revealed the formation of 5 different crosslinking products that appeared under the lowest levels of crosslinking, and the formation of a smear indicating crosslinking of EpsC into higher and higher MW products as the concentration of crosslinker increased. Because of the amount of uncrosslinked EpsC detected, the products produced under the lowest levels of crosslinking are assumed to contain one or two crosslinks to another protein(s). Under this assumption the major crosslinking product at ~90 kDa is presumed to be an aberrantly migrating dimer of EpsC because of the strength of the signal, but it could also be an abundant crosslinking product with another large protein like EpsF, EpsL, or EpsK. One of the two larger crosslinking products may possibly be EpsC crosslinked to monomers of EpsD. Possot *et al.* also find that the *K. oxytoca* C protein, PulC, can be crosslinked *in vivo* to form a species of the appropriate MW to be a C protein – D protein crosslinked product that does not form without the D protein. However, they were unable to detect any D protein in that crosslinked product and therefore suggest that this product may be a trimer of the C protein that requires the D protein to form.. Similarly, results of the immunodetection of EpsD in these samples were inconclusive because of limited disruption of multimers of EpsD by boiling in SDS (results not shown).

Returning to the primary reason for *in vivo* crosslinking, when crosslinked samples were then immunoprecipitated with anti-EpsC antiserum, EpsD was efficiently coprecipitated. This conclusively indicates that EpsC is associated with EpsD, directly or indirectly in a larger complex.

While performing the immunoprecipitations of EpsC and EpsD it was noticed that serum specific for EpsL was capable of coprecipitating multimers of EpsD. However, a similar situation as the EpsC and EpsD coprecipitations was encountered, in which immunoprecipitation by Anti-EpsD antiserum failed to coprecipitate any detectable EpsL. Again it was hypothesized that like antibodies to EpsC, antibodies in the polyclonal serum were interfering with the direct or indirect interactions between EpsD and EpsL by altering the conformation of EpsD or sequestering EpsD from a complex that contained EpsL. This hypothesis was again tested by immunoprecipitating EpsD from samples exposed to limited amounts of the cleavable crosslinker DSP, and checking for EpsL as a coprecipitate. As was the case with EpsC, once interactions with EpsL were stabilized, antibodies specific for EpsD were capable of coprecipitating EpsL, providing convincing evidence that EpsL and EpsD interact, possibly directly, but at least as components in the same multiprotein complex. Interestingly, and perhaps providing additional support to the notion of a direct interaction between EpsD and EpsL, antibodies specific for EpsM, which have been demonstrated to coprecipitate EpsL (Sandkvist *et al.*, 1999, and Figure 4.1A, Lane 6), do not also coprecipitate multimers of EpsD (Figure 4.1A, Lane 3).

The available information of the structure of the C, D, and L proteins is limited to an analysis of the membrane topology (Reeves *et al.*, 1994; Bleves *et al.*, 1996; Thomas *et al.*, 1997). Therefore, it is not possible to estimate at present whether the size of EpsC, EpsD, or EpsL molecules would permit the interactions for which evidence has been provided. However, computerized

predictions of the membrane topology of both EpsC and EpsL conform to results observed for the membrane topology of homologous proteins in other Gram-negative bacteria (Reeves *et al.*, 1994; Bleves *et al.*, 1996; Thomas *et al.*, 1997). Thus, approximately 270 residues of EpsC, and 115 residues of EpsL, are expected to be periplasmic. In comparison to the CM anchored TonB, in which 137 periplasmic residues were required to permit an interaction with an OM target (Larsen *et al.*, 1997), it would seem possible that a similar conformation could also be adopted by EpsC and EpsL to allow interaction with the OM located EpsD. Linderoth *et al.* have found the best evidence suggesting that such interactions are possible. Using scanning transmission electron microscopy of the M13 bacteriophage homolog of EpsD, pIV, the authors concluded that that a single mulitmer ~70 Å high could span the ~25 Å thick OM and still have a substantial periplasmic domain (Linderoth *et al.*, 1997). While it is possible that the confomation of EpsC or EpsL anchored in the CM may permit a direct interaction with EpsD in the OM, the results presented here still cannot exclude the possibility that an indirect interaction occurs between these proteins.

Finally, in this study we present a gel filtration separation of proteins from the Triton X-100/EDTA extraction of *V. cholerae* membranes. It was previously known that each of the five proteins to which specific antisera was available were extracted from the membranes under these conditions (see chapter 3). Therefore, to test the hypothesis that these proteins are assembled into a multiprotein complex that spans both the cytoplasmic and outer membranes, this extract was fractionated on a Sephacryl S-300 HR gel filtration column which

separates proteins in the range of 1 x 10^4 to 1.5 x 10^6 daltons (Amersham Pharmacia Biotech). The major peak of EpsD eluted as multimers from the column very close to the void volume of the column. This is not surprising considering that homologs of EpsD, the M13 bacteriophage pIV, and *P. aeruginosa* XcpQ and PilQ, have all been shown to consist of approximately 12-14 monomer subunits of ~75 kDa (Linderoth *et al.*, 1997; Bitter *et al.*, 1998). What was surprising was that EpsC, monomers of EpsD, EpsG, EpsL, and EpsM were eluted from the column together in a second peak. While this is in conflict with observations made by coimmunoprecipitation and crosslinking, it is possible that these results could suggest that there exists a Triton X-100 stable multiprotein complex in the CM and in the OM, and interaction between these two complexes is dependent on multiple dynamic low affinity contacts between more than one protein. Thus, in the membrane where the complexes are anchored in close proximity, the CM proteins can find appropriate contacts in the OM complex. Solubilized in Triton X-100 these proteins may be concentrated enough to find each other often enough to be coprecipitated, but as fractionation separates the OM components from the CM components the CM proteins prefer to associate with each other.

It will be interesting to see, if additional proteins can be included in complexes already identified by crosslinking and immunoprecipitation, and if the larger secreton, or secretion machinery, can be stabilized and cofractionated by combining the techniques of *in vivo* crosslinking, detergent extraction, and gel filtration.

References

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.

Bitter, W., M. Koster, M. Latijnhouwers, H. de Cock and J. Tommassen (1998). Formation of oligomeric rings by XcpQ and PilQ, which are involved in protein transport across the outer membrane of *Pseudomonas aeruginosa*. *Mol. Microbiol.* **27**: 209-219.

Bleves, S., M. Gérard-Vincent, A. Lazdunski and A. Filloux (1999). Structure-function analysis of XcpP, a component involved in general secretory pathway-dependent protein secretion in *Pseudomonas aeruginosa*. *J. Bacteriol.* **181**: 4012-4019.

Bleves, S., A. Lazdunski and A. Filloux (1996). Membrane topology of three Xcp proteins involved in exoprotein transport by *Pseudomonas aeruginosa*. *J. Bacteriol.* **178**: 4297-4300.

Casadaban, M. C. (1980). Analysis of gene control signals by DNA fusion in *Escherichia coli*. *J. Mol. Biol.* **138**: 179-207.

Daefler, S., I. Guilvout, K. R. Hardie, A. P. Pugsley and M. Russel (1997a). The C-terminal domain of the secretin PulD contains the binding site for its cognate chaperone, PulS, and confers PulS dependence on pIV^{f1} function. *Mol. Microbiol.* **24**: 465-475.

Daefler, S., M. Russel and P. Model (1997b). Module swaps between related translocator proteins pIV(f1), pIV(IKe) and PulD: identification of a specificity domain. *J. Mol. Biol.* **266**: 978-992.

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

Hardie, K. R., A. Seydel, I. Guilvout and A. P. Pugsley (1996b). The secretin-specific, chaperone-like protein of the general secretory pathway: separation of proteolytic protection and piloting functions. *Mol. Microbiol.* **22**: 967-976.

Hirst, T. R., J. Sanchez, J. B. Kaper, S. J. S. Hardy and J. Holmgren (1984). Mechanism of toxin secretion by *Vibrio cholerae* investigated in strains harboring plasmids that encode heat-labile enterotoxins of *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **81**: 7752-7756.

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.

Larsen, R. A., D. Foster-Hartnett, M. A. McIntosh and K. Postle (1997). Regions of *Escherichia coli* TonB and FepA proteins essential for *in vivo* physical interactions. *J. Bacteriol.* **179**: 3213-3221.

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

Linderoth, N. A., P. Model and M. Russel (1996). Essential role of a sodium dodecyl sulfate-resistant protein IV multimer in assembly-export of filamentous phage. *J. Bacteriol.* **178**: 1962-1970.

Linderoth, N. A., M. N. Simon and M. Russel (1997). The filamentous phage pIV multimer visualized by scanning transmission electron microscopy. *Science* **278**: 1635-1638.

Lu, H. M., S. T. Motley and S. Lory (1997). Interactions of the components of the general secretion pathway: role of *Pseudomonas aeruginosa* type IV pilin subunits in complex formation and extracellular protein secretion. *Mol. Microbiol.* **25**: 247-259.

Marsh, J. W. and R. K. Taylor (1998). Identification of the *Vibrio cholerae* type 4 prepilin peptidase required for cholera toxin secretion and pilus formation. *Mol. Microbiol.* **29**: 1481-1492.

Michel, L. O., M. Sandkvist and M. Bagdasarian (1995). Specificity of the protein secretory apparatus: Secretion of the heat-labile enterotoxin B subunit pentamers by different species of Gram$^-$ bacteria. *Gene* **152**: 41-45.

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

Nunn, D. and S. Lory (1993). Cleavage, methylation, and localization of the *Pseudomonas aeruginosa* export proteins XcpT, -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.

Overbye, L. J., M. Sandkvist and M. Bagdasarian (1993). Genes required for extracellular secretion of enterotoxin are clustered in *Vibrio cholerae*. *Gene* **132**: 101-106.

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

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

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, pilin-like component of the general secretory pathway of *Klebsiella oxytoca*. *Mol. Microbiol.* **9**: 295-308.

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 O. Possot (1993). The general secretory pathway of *Klebsiella oxytoca:* no evidence for relocalization or assembly of pilin-like PulG protein into multiprotein complex. *Mol. Microbiol.* **10**: 665-674.

Reeves, P. J., P. Douglas and G. P. C. Salmond (1994). Beta-lactamase topology probe analysis of the OutO NMePhe peptidase, and six other Out protein components of the *Erwinia carotovora* general secretion pathway apparatus. *Mol. Microbiol.* **12**: 445-457.

Russel, M. (1993). Protein-protein interactions during filamentous phage assembly. *J. Mol. Biol.* **231**: 689-697.

Russel, M. (1998). Macromolecular assembly and secretion across the bacterial cell envelope: Type II protein secretion systems. *J. Mol. Biol.* **279**: 485-499.

Sandkvist, M., M. Bagdasarian, S. P. Howard and V. J. DiRita (1995). Interaction between the autokinase EpsE and EpsL in the cytoplasmic membrane is required for extracellular secretion in *Vibrio cholerae*. *EMBO J.* **14**: 1664-1673.

Sandkvist, M., L. P. Hough, M. M. Bagdasarian and M. Bagdasarian (1999). Direct interaction of the EpsL and EpsM proteins of the general secretion apparatus in *Vibrio cholerae*. *J. Bacteriol.* **181**.

Sandkvist, M., L. O. Michel, L. P. Hough, V. M. Morales, M. Bagdasarian, M. Koomey, V. J. DiRita and M. Bagdasarian (1997). General secretion pathway (*eps*) genes required for toxin secretion and outer membrane biogenesis in *Vibrio cholerae*. *J. Bacteriol.* **179**: 6994-7003.

Schnaitman, C. A. (1971). Effect of ethylenediaminetetraacetic acid, Triton X-100, and lysozyme on the morphology and chemical composition of isolate cell walls of *Escherichia coli*. *J. Bacteriol.* **108**: 553-563.

Shevchik, V. E. and G. Condemine (1998). Functional characterization of the *Erwinia chrysanthemi* OutS protein, an element of a type II secretion system. *Microbiology* **144**: 3219-3228.

Shevchik, V. E., J. Robert-Baudouy and G. Condemine (1997). Specific interaction between OutD, an *Erwinia chrysanthemi* outer membrane protein of the general secretory pathway, and secreted proteins. *EMBO J.* **16**: 3007-3016.

Tabor, S. and C. C. Richardson (1985). A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc. Natl. Acad. Sci. USA* **82**: 1074-1078.

Thomas, J. D., P. J. Reeves and G. P. C. Salmond (1997). The general secretion pathway of *Erwinia carotovora* subsp *carotovora*: Analysis of the membrane topology of OutC and OutF. *Microbiology-Uk* **143**: 713- 720.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

Previous work in this group had identified and sequenced a 12 kb fragment of the *V. cholerae* chromosome containing 12 genes essential for secretion of cholera toxin (CT) and the closely related heat-labile enterotoxin (LT) of *Escherichia coli*. The *eps* genes were putatively identified, based upon homology to the genes involved in the General Secretion Pathway (GSP) of other Gram-negative bacteria and the analysis of mutants.

In this study, the desire was to begin the analysis of the GSP, now called the type II secretion pathway, at the level of the proteins by examining some of the protein-protein interactions that were hypothesized to occur. The knowledge of the architecture of the Eps secretion system was limited to a rudimentary determination of the subcellular location of the EpsE, EpsL, and EpsM proteins determined by the selective solubilization of certain membrane protein by detergents (Overbye *et al.*, 1993; Sandkvist *et al.*, 1993; Sandkvist *et al.*, 1995), and most often from the heterologous expression of cloned genes in *E. coli*.

The first objective of this study was to try to visualize and correlate a particular polypeptide to each one of the putative ORF's identified previously. This objective was accomplished and presented in Chapter 2. In addition, to visualizing and correlating several polypeptides with particular genes, it was possible to demonstrate that at least one of the prepilin-like proteins, EpsG, was processed in *V. cholerae* and not processed in *E. coli*. It was also demonstrated that this processing activity was dependent on a prepilin peptidase in *V. cholerae* that had not been previously identified (Chapter 2 and Sandkvist *et al.*, 1997). The gene for that prepilin peptidase was subsequently cloned by Marsh and

Taylor (1998), and shown to process another prepilin-like protein, EpsI that has a cleavage site very similar to EpsG (see chapter 2, Table 2.5).

After visualizing several proteins, the task at hand was to methodically clone each gene so that the corresponding polypeptide could be purified. Each of the genes has since been cloned into an appropriate expression vector, that fused an oligohistidine-tag to the C-terminus of each. During the course of this study, 5 of the Eps proteins have been purified in this manner to bring the number of purified proteins to 6 (Chapter 2; Sandkvist *et al.*, 1995; Sandkvist *et al.*, 1999, and Lee and Bagdasarian, unpublished results). The purification of 4 of the proteins, EpsD, EpsG, EpsF, and EpsM is presented here.

Purification of these proteins allowed the development of immunological reagents. Antisera specific for EpsD, EpG, and EpsM has allowed the determination of the subcellular location of each of these proteins in *V. cholerae*. Notably, it was shown that EpsD, a member of the secretin family of membrane transporters, was located exclusively in the outer membrane (OM). EpsG, one of the prepilin-like proteins, along with EpsC and EpsM, were shown to localize predominantly with the cytoplasmic membrane (CM), but to also cofractionate with vesicles of the OM. EpsL, fractionates with the CM however traces of EpsL can be detected in OM fractions (Chapter 2).

The EpsL and EpsM proteins were further characterized by gel filtration and determined to exist in a complex consistent with dimers *in vitro*.

The most important findings of this study, were the demonstration that EpsC and EpsD, and EpsD and EpsL associate in one or more complexes in the

membranes of *V. cholerae*. This finding provides the first conclusive evidence that there is an association between the majority of the Eps proteins located in the CM and the putative pore, EpsD, which was shown to be exclusively in the OM. The evidence presented in this study does not exclude the possibility that the interactions between EpsC and EpsD and between EpsD and EpsL are indirect. However, since EpsC confers a protease stabilization on EpsD (Chapter 4) it is likely that EpsC and EpsD do interact directly.

Furthermore, evidence of a complex that can be solubilized is provided by a cofractionation of Eps proteins extracted from the CM in 1% Triton X-100 which solubilizes only the CM proteins and in 1% Triton X-100/10 mM EDTA which solubilizes both CM and OM proteins (Chapter 3 and Chapter 4). In Figure 5.1, I present an updated model for type II protein secretion that reflects these and other recent indications for protein-protein interactions and function.

The methodical analysis of the architecture can only lead to a greater understanding of the entire system. The continued purification of the remaining proteins will allow more antisera to be raised, and subsequently used for the dissection of the type II secretion system. Antisera specific for EpsF, one of two polytopic membrane proteins in the Eps system, is forthcoming. It is expected that this antisera, will allow the first determination of the subcellular location of the F protein by detergent extraction or sucrose gradient fractionation. It will permit an analysis of the oligomeric state of EpsF, if any, and it should permit further dissection of the Eps system through immunoprecipitation and *in vivo* crosslinking. The advantage of employing these techniques allows the system to

be tested without perturbing the system, by overexpressing individual components, or by removing them from their natural environment. However, no technique will be truly useful until methods become available to generate multiple mutations in *eps* genes in the chromosome of *V. cholerae*, or to reconstitute the system outside of *V. cholerae*, perhaps in *E. coli*.

With the extensive similarities that are quickly being identified between such diverse processes as proteins secretion, pilus biogenesis, filamentous phage biogenesis, flagellar export, DNA uptake, and DNA transfer, an understanding of protein translocation across membranes will be an important advantage in understanding the microbial world around us.

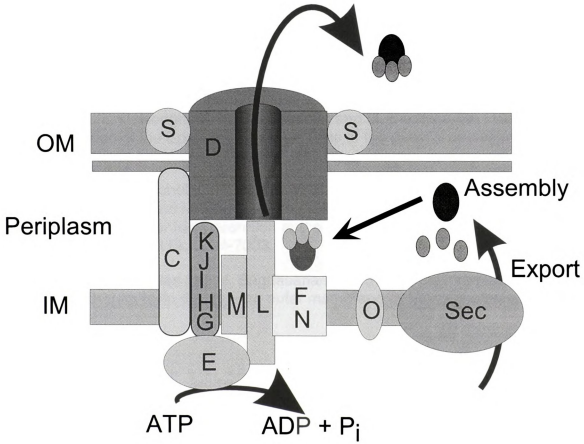


Figure 5.1 An updated model for secretion of proteins through the type II secretion pathway. Reflecting evidence that the CM proteins C and L interact with the OM D protein through their periplasmic domains.

References

Marsh, J. W. and R. K. Taylor (1998). Identification of the *Vibrio cholerae* type 4 prepilin peptidase required for cholera toxin secretion and pilus formation. *Mol. Microbiol.* **29**: 1481-1492.

Overbye, L. J., M. Sandkvist and M. Bagdasarian (1993). Genes required for extracellular secretion of enterotoxin are clustered in *Vibrio cholerae*. *Gene* **132**: 101-106.

Sandkvist, M., M. Bagdasarian, S. P. Howard and V. J. DiRita (1995). Interaction between the autokinase EpsE and EpsL in the cytoplasmic membrane is required for extracellular secretion in *Vibrio cholerae*. *EMBO J.* **14**: 1664-1673.

Sandkvist, M., L. P. Hough, M. M. Bagdasarian and M. Bagdasarian (1999). Direct interaction of the EpsL and EpsM proteins of the general secretion apparatus in *Vibrio cholerae*. *J. Bacteriol.* **181**.

Sandkvist, M., L. O. Michel, L. P. Hough, V. M. Morales, M. Bagdasarian, M. Koomey, V. J. DiRita and M. Bagdasarian (1997). General secretion pathway (*eps*) genes required for toxin secretion and outer membrane biogenesis in *Vibrio cholerae*. *J. Bacteriol.* **179**: 6994-7003.

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.